Kor. J. Appl. Microbiol. Bioeng.
Vol. 17, No. 1, 29—34 (1989)

Intracellular Accumulation of Cadmium by Intact Cadmium Tolerant Yeast Cells

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카드뮴 내성 효모의 Intact Cells에 의한 카드뮴의 세포내 축적

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An intracellular accumulation of cadmium by the intact cell of an extremely cadmium tolerant yeast, *Hansenula anomala* B-7, was investigated in the presence of Triton X-100.

The uptake of cadmium by the intact cell was efficiently enhanced up to approximately 40% or more by 0.1% of Triton X-100 and Aerosol OT, respectively. The Michaelis constant, K_m, done by Lineweaver-Burk plot of accumulation velocity of cadmium *vs.* cadmium concentration was calculated to be 0.247 mM. The optimal conditions of pH and the temperature for the effective cadmium uptake were from neutrality to alkali and 40°C, respectively.

The accumulation of cadmium was increased approximately 3 times under the shaking incubation, with no correlation to shaking rate. By zinc the cadmium accumulation was decreased.

Recently a few studies have focussed on the isolation and tolerance of cadmium-tolerant microorganisms (1-6).

Heldwein (7) observed that cadmium was about 100 times more toxic than cobalt to *Saccharomyces cerevisiae,* and that growth was inhibited immediately after adding cadmium into the culture. Because cadmium is very toxic to eukaryotic cells, only a few studies of cadmium-tolerant eukaryotes such as yeast (7-9), mould (10) and algae (11) have been reported. Furthermore, *Saccharomyces cerevisiae* studied by Heldwein (7) and by Norris (8) was a sensitive strain. Tohoyama and Murayama (9) obtained cadmium-resistant yeast strains by repeated training of the parent strain with medium containing a definite concentration of cadmium. The cadmium-resistant yeast showed an increase in the activity of cadmium absorption.

In cadmium resistant *Saccharomyces cerevisiae* (9, 12) and *staphylococcus aureus* (13-15) one of the mechanism of cadmium resistance might seem to be the decreased permeability of the resistant cell membrane towards cadmium.

However, Laddaga and Silver (16) reported that cadmium uptake occurred by an active transport system in *Escherichia coli.*

In previous papers, authors isolated an extreme cadmium tolerant yeast from the sludge of zinc mining district and identified it as *Hansenula anomala* B-7. The yeast grew fairly well at 2,700 μg/ml of cadmium by density gradient agar plate method and accumulated 34.17 mg of cadmium per gram of dry cell (17, 18) and cadmium was accumulated up to approximately 40% when they yeast was grown in aqueous medium containing 0.1% of Triton X-100 at 30°C for 24 hours with shaking

Key words: *Hansenula anomala* B-7, Cadmium accumulation, Triton X-100
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cultivation (19).

In the present study, we described the effect of surfactants on intracellular accumulation of cad mium in intact cell of an extreme cadmium tolerant yeast. *Hansenula anomala* B-7 cells. It was demonstrated that the cadmium accumulation was greatly affected by the surfactants.

Materials and Methods

Chemicals

Cadmium nitrate and the standard solution of cadmium (1,000 ppm) were purchased from Hayashi Chemicals Co., Osaka. Triton X-100 and Aerosol OT were obtained from Wako Pure Chemicals Co., Tokyo. Silicone KM-70, Tween 80, and cetyltrimethylammonium bromide were purchased from Shinetsu Chemicals Co., Osaka, Junsei Chemicals Co., Tokyo, and Tokyo Kasei Co., Tokyo, respectively. Polypeptone and yeast extract used were obtained from Kyokuto Pharmacia Co., Tokyo and Sigma Chemicals Co., St. Louis, Missouri, respectively. And the other reagents used were guaranteed ones.

Microorganisms and cultivation

Hansenula anomala B-7 that had already been isolated from the activated sludge of a zinc mining area by the present authors (17, 18) as an extreme cadmium tolerant yeast was used throughout this work. The strain is able to grow in the solid medium containing cadmium up to the concentration of 2,700 ug/m*l* of cadmium and to grow well in a aqueous medium containing 1,000 ug/m*l* of cadmium.

The basal medium contained 10g of glucose, 10g of polypeptone, 5g of yeast extract, 1g of NaCl, 0.3g of $MgSO_4 \cdot 7H_2O$, and 0.1g of KH_2PO_4 in 1,000 m*l* of deionized water, and pH was adjusted to 6.0. The yeast cells were grown in a 500 m*l* Sakaguchi shaking flask containing 100 m*l* of medium at 25 °C for 24 hours on a reciprocal shaker (120 strokes/min., amplitude 7 cm). The cultivated cells were harvested by centrifugation (3,000 × g, 10 min.) and were washed 3 times with deionized water, and the washed cells were used as a intact cell. The intact cells were suspended in 0.8% NaCl solution. These suspended cells were put into a 250 m*l* stoppered centrifuge bottle containing 100 m*l* of NaCl solution.

Concentration of cadmium and surfactants were adjusted to 100 μg/m*l* and 0.2%, respectively, and further incubated on a reciprocal shaker under above mentioned conditions except in the experiment of time course.

The growth was determined by measuring the optical density of the broth at 660 nm On the other hand, the treated cells were dried at 104 °C for 8 hr after washing 3 times with deionized water and then the dried cell weight(g) was measured.

Analysis of cadmium

The accumulated cadmium content in the cells was determined by atomic absorption method using atomic absorption spectrophotometer (Shimadzu, AA-646) with 228.8nm wave length as shown in the previous paper (19).

Results and Discussion

Effect of surfactants in accumulation of cadmium by intact cells

In order to elucidate the role of surface active agents on intracellular accumulation of cadmium by the cells of *Hansenula anomala* B-7, the intact cells were incubated in saline solution containing 100 ug/m*l* of cadmium and 0.2% of different surfactants for 24 hr, and then the cadmium accumulated in the cells was determined.

As shwon in Table 1, the intracellular accumula-

Table 1. Effect of surfactants on the accumulation of cadmium by the intact cells.

Surfactant (0.2%)	Total accumulated cadmium/cell mass (mg/g dry cell)
None	4.03 (100)*
Tween 80	3.95 (98)
Triton X-100	6.21 (154)
CTAB**	2.95 (73)
Aerosol OT	5.85 (145)
Silicone KM-70	3.95 (98)

*; Relative values in parentheses.
**; Cetyltrimethylammonium bromide.
Incubation of the tolerant yeast was carried out in 100 m*l* of the saline solution containing 100 ug/m*l* of cadmium and 0.2% of various surfactants with shaking at 25 °C for 24 hours. And then, accumulated cadmium was assayed.

tion of cadmium was accelerated up to 54% compared to surfactant-free suspension by Triton X-100, a non-ionic surface active agent. And anionic surface active agent such as Aerosol OT also efficiently enhanced a cadmium accumulation into the intact cells.

Kimura *et al.* (20) reported that in the yeast cell surface some functional changes might occur by the treatment of Triton X-100. And Iimura *et al.* (21) observed that the pellicle formation of *Hansenula anomala* I-2-1 was efficiently repressed by Triton X-100, and then illustrated that the change from non-film to film stage was due to a change in cell surface from hydrophilic to hydrophobic. From the above results, they inferred that Tirton X-100 treatment might provide a useful method for enhanced uptake by intact yeast cells of various substances.

Not only growth but also an intracellular accumulation of cadmium of *Hansenula anomala* B-7 was inhibited by cetyltrimethylammonium bromide (CTAB) which is a cationic surface active agent (19). Some film strains of *Saccharomyces* had strongly repressed growth by CTAB (21). Furthermore, in this study CTAB strongly inhibited intracellular accumulation of cadmium. The intracellular accumulation of cadmium by intact cells was in proportion to the concentration of Triton X-100 up to 0.1%, thereafter cadmium accumulation rate was decreased with increased addition of Triton X-100. In the presence of 0.1% of Triton X-100, cadmium accumulation was found up to 90% compared with 0.3% of Triton X-100 (data not shown). Therefore, concentration of Triton X-100 as surface active agent was adjusted to 0.1% for the ensuing experiments.

In the present work the enhancement of cadmium accumulation by surface active agents such as Triton X-100 and Aerosol OT indicates that these furfactants caused increased permeability of the cell membrane. It is interesting that cadmium accumulation by Aerosol OT, which is used as detergent, is nearly equal to that of Triton X-100.

Effect of cell mass and shaking

In order to investigate the relationship between an intact cell mass and intracellular accumulation of cadmium, various cell mass of the cell was incubated in the saline solution containing 100 ug/ml of cadmium and 0.1% of Triton X-100 at 25 °C for 24 hours with shaking, and then the amount of accumulated cadmium was assayed. About 0.506 mg of cadmium was accumulated in 0.11 g of intact dry cells of *Hansenula anomala* B-7 (data not shown).

Table 2. Comparison of cadmium accumulation by the intact cells between static and shaking incubation.

Incubation	Cell mass* (g)	Accumulated cadmium in cells: Total cadmium (mg)	Accumulated cadmium in cells: Total cadmium/cell mass (mg/g dry cell)
Static	0.4574	0.27 (100)**	0.60 (100)**
Shaking	0.4370	0.79 (293)	1.81 (302)

*; Cell mass after incubation for 24 hours.
**; Relative values in parentheses.
The tolerant yeast was incubated in the saline solution containing of 100 μg/ml of cadmium and 0.1% of Triton X-100 at 25 °C for 24 hours with static or shaking.

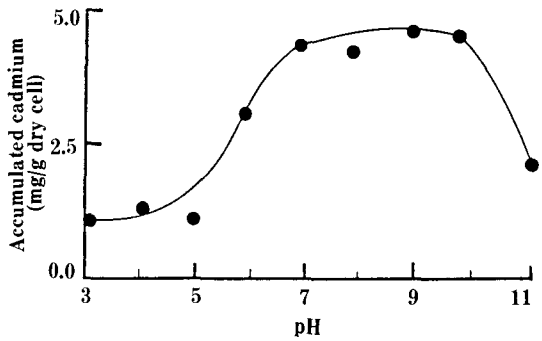

Fig. 1. Effects of pH on the accumulation of cadmium by the intact cells.
The intact yeast cells were incubated in saline solution containing 0.1% of Triton X-100 and 100 μg/ml of cadmium for 24 hours at 25 °C and different pH with shaking.

Effects of shaking on intracellular accumulation of cadmium by the intact cells were determined. As shown in Table 2, cadmium was accumulated approximately 3 times more in shaking incubation than in the static incubation. And it was found that no significant differences was observed at any shaking rate (data not shown). From the above results it was probable that metabolic rate of cadmium into the cell was related to an aerobic condition.

Optimal pH and temperature on the accumulation of cadmium by intact cells

In order to examine the effect of pH on the ac-

Table 3. Effect of incubation temperature on the accumulation of cadmium by the intact cells.

Incubation temp. (°C)	Cell mass* (g)	Accumulated cadmium in calls: Total cadmium (mg)	Accumulated cadmium in calls: Total cadmium/cell mass (mg/g dry cell)
20	0.2004	0.32 (52)**	1.60 (32)**
30	0.1695	0.52 (85)	3.07 (61)
40	0.1206	0.61 (100)	5.06 (100)
50	0.1081	0.32 (52)	2.96 (58)

*; Cell mass after incubation for 24 hours.
**; Relative values in parentheses.
Incubated conditions were the same as in the footnote of Table 1 except temperature as indicated in the table.

Table 4. Effect of metals on the accumulation of cadmium by the intact cells.

Metal ion (1 mM)	Total accumulated cadmium/cell mass (mg/g dry cell)
None	4.91 (100)*
Fe^{2+}	4.70 (96)
Ca^{2+}	4.73 (96)
Mn^{2+}	5.30 (108)
Zn^{2+}	4.17 (85)

*; Relative values in parentheses.
The tolerant yeast was incubated in 100 m*l* of the saline solution containing 100 ug/m*l* of cadmium and 0.1% of Triton X-100 and 1 mM of various metals at 25 °C for 24 hours with shaking.

cumulation of cadmium by the cells, the intact cell suspension was adjusted to each pH at the ranges of 3.0 to 11.0 by using McIlvaine (pH 3.0 to 8.0) and Michaelis (pH 8.0 to 11.0) buffer.

As shwon in Fig. 1, a cadmium was accumulated much less over the range of pH 3.0 to 5.0. At pH 6.0 accumulation was 60% of the maximum accumulation of cadmium, which accumulated for intact cells at pH 7.0 to pH 10.0, but at pH 11.0 the cadmium accumulation was strongly inhibited. During growth of this strain the maximum accumulation of cadmium had been found at pH 5.0 to 6.0 (17, 19) but by the incubation of the intact cells a cadmium was shown to accumulate at more alkaline ranges than pH 6.0.

The results suggested that the variation of cadmium accumulation related to pH depended on the alteration of membrane permeability. Total intracellular accumulated cadmium by intact cells reached a maximum at an incubation temperature of 40 °C, but the tolerant yeast grew very well at 20 °C (18). At 20 °C total accumulated cadmium was inhibited up to approximately 50% of the maximum cadmium accumulation, as shwon in Table 3. Yu *et al.* (18) reported that *Hansenula anomala* B-7 grew very well at 20 to 22 °C and the range of pH 5.0 to 8.0, but during growth the optimal pH for intracellular accumulation of cadmium was pH 6.0. However, in the intact cells, cadmium was accumulated under conditions that were not good for growth; pH 7.0 to 10.0 and incubation temperature of 40 °C.

From the results, it was considered that increase of cadmium accumulation was related to the change of membrane permeability which accelerated the penetration of cadmium into the cells with the rise of temeprature up to 40 °C and by the addition of surface active agent, Tirton X-100 to the intact cell of *Hansenula anomala* B-7.

In order to determine an accumulation rate of cadmium into the intact cells the time course was determined under the above optimum conditions in the saline solution containing 100 μg/m*l* of cadmium and 0.1% Triton X-100. By shaking cultivation of this strain B-7, the intracellular accumulation of cadmium was rapidly increased during the second half of the stationary phase and the maximum was found to require longer than was required for maximum cell growth (22). The accumulation of cadmium into the intact cells of *Hansenula anomala* B-7 was abruptly increased with incubation time. About ninety percent of cadmium was accumulated at 5 hours after incubation (data not shown).

Effect of other metals on accumulation of cadmium by intact cells

The effect of other metals on intracellular accumulation of cadmium by intact cells was investigated.

Intact cells of *Hansenula anomala* B-7 were incubated in saline solution containing 100 μg/m*l* of cadmium and 1 mM of various metals for 24 hours at 25 °C with shaking, and then cadmium accumulation in the cells was determined.

Generally, a physicochemical properties of cad-

Table 5. Effect of zinc concentration on the accumulation of cadmium by the intact cells.

Zinc ion conc. (mM)	Relative accumulated cadmium (%)
None	100
0.25	89
0.50	88
1.00	83
2.00	78
3.00	70

Incubated conditions were the same in the footnote of Table 4 except the various concentration of zinc as indicated in the table.

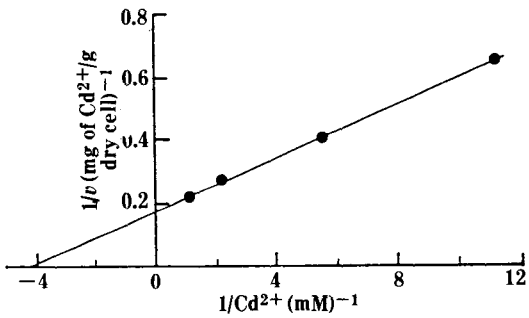

Fig. 2. Double reciprocal plot of accumulation velocity of cadmium *vs.* cadmium concentration by the intact cells.

The intact yeast cells were incubated in saline solution containing 0.1% of Triton X-100 and various concentration of cadmium for 24 hours at 25°C on the reciprocal shaker. Velocity (v) was expressed in total intracellular accumulated cadmium (mg) per dry cell (g), and cadmium ion concentration (Cd^{2+}) was in mM.

mium are similar to that of zinc; zinc is involved in a wide range of cellular activities and is known to be an essential trace element for normal growth in microorganisms. Zinc is an enzyme cofactor of many enzymes, such as carboxypeptidase A and dehydrogenase (23), alkaline phosphatase (24) and is vital to protein synthesis (25, 26). Moreover, zinc is known to stabilize biomembranes and prevent the breakdown of unsaturated lipids during lipid peroxidation (27).

However, Nakano *et al.* (11) reported that cadmium uptake was greatly antagonized by the addition of zinc in the cultivation of *Euglena gracilis,* and RNA synthesis was inhibited by zinc, copper, lead and cadmium ions at 100 uM in mouse liver nuclei (28). As shown in Table 4, cadmium accumulation was not affected by the addition of metal ions such as iron, calcium and manganese, but it was slightly inhibited by 1 mM of zinc. And a cadmium accumulation was gradually decreased with the increase of zince concentration in the cell suspension, as shwon in Table 5.

Hence, our present results agree to those of Nakano *et al.* (11) and Yu *et al.* (19).

Effect of cadmium concentration on accumulation of cadmium by intact cell

The relationship between intracellular accumulation of cadmium and cadmium concentration in the medium was investigated. The accumulation of cadmium increased in proportion to the concentration of cadmium. Fig. 2 shows a double reciprocal plot of accumulation velocity of cadmium by intact cells of *Hansenula anomala* B-7 in various concentrations of cadmium ion.

The cadmium accumulation followed the Michaelis Menten kinetics with a K_m of 0.247 mM of cadmium and a V_{max} 5.71 mg of cadmium per gram of dry cell weight.

요 약

고도 카드뮴 내성 효모, *Hansenula anomala* B-7의 intact cell에 의한 카드뮴의 세포내 축적에 미치는 계면활성제의 영향를 검토하고, Triton X-100의 존재하에서의 카드뮴 축적에 미치는 제인자 등을 검토했다. 카드뮴의 축적은 0.1% Triton X-100과 Aerosol OT에 의하여 약 40% 이상 증가되었다. 카드뮴 이온에 대한 K_m값은 0.247 mM로 계산되었다. 카드뮴의 축적 최적 pH는 pH 7.0에서 pH 10.0 사이였으며, 최적온도는 40℃였다. 카드뮴의 최적 축적은 정치보다 진탕하므로 약 3배 증가되었으며, 진탕속도는 영향을 미치지 않았다. 카드뮴과 아연 이온을 공존시키므로 카드뮴의 축적이 아연 이온에 의하여 저해되었다.

Acknowledgement

This investigation was supported in part by a research grant from the Korea Science and Engineering Foundation (1986-1987).

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(Received November 1, 1988)