Removal of Cadmium from Scallop Hepatopancreas by Microbial Processes

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A microbial process for removing cadmium from a homogenate of hepatopancreas, a waste of scallop processing, was devised to use this waste for value-added protein resources. Microorganisms were screened on the basis of the ability to remove cadmium from a medium with the initial concentration of 10 mg/l of cadmium. One soil isolate, identified as Xanthomonas sp. UR No. 2 by its taxonomical characteristics, removed 98% of the cadmium in the medium in 2 d. During cultivation of this strain in the homogenates of hepatopancreas digested by endopeptidases, 90% of cadmium was removed, while this strain had little effect on the simple non-digested homogenate. The mass balance of cadmium during homogenizations of the hepatopancreas tissues and cultivations in the protease-treated homogenate were examined. The content of crude proteins of culture supernatant treated by Xanthomonas sp. UR No. 2 was equivalent to those of various feedstuffs on the market.

Key words: microbial removal of cadmium; scallop hepatopancreas; Xanthomonas sp.; waste tissue; protease

The scallop, Patinopecten yessoensis, a bivalves shellfish, is extensively aquacultured in the northern part of Japan. The catch of scallops is approximately half a million tons per year and is in the first place in marine products in Hokkaido, Japan. Its processing is one of the major industries in Hokkaido. On the other hand, the amounts of scallop wastes, consisting mainly of hepatopancreas, the mid-gut gland of scallops, which are the residue after removing the edible parts such as adductor muscles from the scallops, reaches about fifty thousand tons per year. It has become difficult in recent years to ensure the disposal of scallop wastes. Scallop wastes give off a bad smell in a short time. Furthermore, dumping of hepatopancreas has become a serious problem in Hokkaido, which is associated with environmental pollution due to unlawful dumping in landfill sites. Hepatopancreas contains a large amount of cadmium, which is a toxic heavy metal bad for human health and to various ecosystems. Cadmium concentrations in the hepatopancreas are approximately 20-40 ppm on a wet weight basis and exceed 100 ppm on a dry basis. Therefore, its removal is also required from the viewpoint of environmental protection. Furthermore, hepatopancreas with the cadmium removed has the potential to be used as a feedstock, since it is rich in protein.

It has been reported that cadmium in hepatopancreas can be removed by treating it with sulfuric acid. However, sulfuric acid treatment has intrinsic problems of corrosion of equipment and loss of protein by acid hydrolysis. Microbial processes have the potential to overcome some of these problems. Recently, the removal of cadmium in the extract of hepatopancreas by sulfur-reducing bacteria has been reported. However, this process also has a problem of corrosion due to H₂S produced by these bacteria and has some practical problems due to the anaerobic process.

From this background, an attempt was made in this study to obtain aerobes capable of removing cadmium from scallop hepatopancreas (homogenate of the tissue). Materials and Methods

Medium and isolation of microorganisms. Several microorganisms were isolated on the basis of the ability to grow in the cadmium-medium consisting of Bacto-Tryptone (Difco Laboratories, Detroit, USA) 5.0, yeast extract (Oriental Yeast Co. Ltd., Tokyo, Japan) 2.0, NaCl 5.0, Cd(NO₃)₂·4H₂O 0.14 (g per liter, pH 7.0). The concentration of cadmium in the medium corresponds to approximately 50 mg/l (=50 ppm). Soil samples were collected from marine wastes-contaminated and ordinary sites in Hokkaido. Supernatants of soil samples suspended in physiological saline were inoculated in the medium and incubated with shaking. After 3 to 5 d of cultivation at 30°C, 0.3 ml of the broth culture was inoculated into 10 ml of a fresh medium. This enrichment process was repeated eleven times and then the cultures were isolated as single colonies on the screening medium containing 2% agar.

Screening for aerobes capable of removing cadmium in a model system. Isolates obtained by this enrichment method were screened for their capability of removing cadmium from a culture medium. The test medium was the same as the cadmium medium except the final concentration of cadmium was set at 10 ppm. Seed cultures

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were prepared by growing in 10 ml of the test medium in 50-ml Erlenmeyer flasks at 30°C for 1 d on a reciprocal shaker. Ten ml of seed cultures were inoculated into 100 ml of the fresh medium in 500-ml shaking flasks and incubated at 30°C on a reciprocal shaker. Samples were withdrawn at appropriate times and their cadmium contents were measured as described in Analytical methods.

Cadmium-removing tests in a practical system. Hepatopancreas tissues excised during food processing were obtained from scallops harvested in the Funka Bay of Hokkaido. These were homogenized in 5 volumes of deionized water with a blender-type homogenizer (TAITEC Co. Ltd., Model HG-92G) at 10,000 rpm for 1 min x 10 times. Then it was boiled for 4 h by stirring and centrifuged at 1,870 x g for 1 h. The supernatant was used as the simple homogenate (non-digested) of hepatopancreas. In another run, hepatopancreas tissues were digested (solubilized) by protease (endopeptidase Protin PC10 (Daiwa Kasei Co., Ltd., 10^4 PU/g) for 4 h by stirring at 45-50°C. The weight ratio of proteinase, hepatopancreas, and water was 1:10:30 (pH not adjusted). The resulting solution was boiled for 15 min for deactivation of proteinase, and centrifuged at 1,870 x g, 4°C for 1 h to obtain the protease-treated homogenate. To examine the ability to remove cadmium from the simple homogenate and the protease-treated homogenate, seed cultures (10 ml) prepared as shown in the model systems above were inoculated into 100 ml of the homogenate solutions and cultivated. The amounts of cadmium remaining in the culture supernatants were measured as described in below.

Analytical methods. Growth of bacteria in the cadmium-medium was measured by the optical density at 660 nm. Viable cells were counted during cultivation in hepatopancreas homogenates by dilution in physiological saline and plating on nutrient agar. The amounts of cadmium remaining in the culture supernatants were measured as follows. Samples were periodically withdrawn from the cultures, and were centrifuged at 4,530 x g, 4°C for 30 min. The supernatants were digested by incubation at 80°C in the conc. HNO₃ and 30% H₂O₂ (wet ashing), then dried, and diluted with deionized water. These samples were analyzed by the atomic absorption spectrophotometer (Hitachi, Model Z-8000) operated in the flame mode. The crude proteins in the hepatopancreas homogenates with cadmium removed were measured by the Kjeldahl method. The base sequence of 16S rDNA of the bacteria was analyzed with the Vista SYSTEMS Thermo Sequenase core sequencing kit with 7-deaza-dGTP reaction mixtures (Pharmacia) for automated sequencing with an A.L.F. Sequencer (Pharmacia). The primers used were 5F(5'-AGTTTGATCCTGGCTC OH-3') corresponding to the 10 th to 15 th base of E. coli 16S rRNA and 1540R(5'-AAGGAGGTGATCCAGCC OH-3') corresponding to 1541 th to 1525 th base of E. coli 16S rRNA. According to comparison with data base available through the internet (http://www.dna.affrc.go.jp/), the strain was characterized.

Results and Discussion

Removal of cadmium from the medium

Enrichment culture from soil yielded seven different bacteria. We termed these bacteria the UR No. series. As shown in Table 1, the isolates UR No. 1–7 showed various degrees of capabilities of removing cadmium. Among strains obtained, UR No. 2 showed the best capability of removing cadmium and removed 98% of cadmium by culturing for 2 d and UR No. 4 removed 54% of cadmium. Both strains grew well in the test medium and the growth (OD₅60) reached maximum after cultivation for 12 h, as shown in Fig. 1. In the case of UR No. 4 bacteria, the increase of cadmium concentration after 24 h appears to be due to the release of cadmium that had once adsorbed on the bacterial cells to the cul-

Table 1. Tests of Cadmium Removal from the Medium by the Isolated Bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cd in supernatant (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Control</td>
<td>10.90</td>
</tr>
<tr>
<td>UR No. 1</td>
<td>9.98</td>
</tr>
<tr>
<td>UR No. 2</td>
<td>10.15</td>
</tr>
<tr>
<td>UR No. 3</td>
<td>9.76</td>
</tr>
<tr>
<td>UR No. 4</td>
<td>9.78</td>
</tr>
<tr>
<td>UR No. 5</td>
<td>9.83</td>
</tr>
<tr>
<td>UR No. 6</td>
<td>9.88</td>
</tr>
<tr>
<td>UR No. 7</td>
<td>9.62</td>
</tr>
</tbody>
</table>

* Isolated bacteria were cultivated in medium containing cadmium (=10 mg/l), and amounts of cadmium in culture supernatants were measured as described in Materials and Methods.

Fig. 1. Courses of Cadmium Removal from the Medium by UR No. 2 and UR No. 4 Bacteria.
Both bacteria were cultivated in medium containing cadmium (=10 mg/l), and amounts of cadmium in culture supernatants were measured as described in Materials and Methods. Growth was measured by the optical density at 660 nm of culture broth. Cadmium concentrations: ○ Control; △ UR No. 4; ● UR No. 2; OD₅60; ● UR No. 2; ○ UR No. 4
ture medium. Additional experiments showed that the amount of cadmium in cells separated from the culture by centrifugation after cultivation of UR No. 2 strain for 1 d was 4.75 µg (42 nmol)/mg-dry cell. Dry weight of the cell pellet from 100 ml of the culture was 232 mg. These results indicate that an uptake and/or adsorption of cadmium in the culture medium on or into the bacterial cells undoubtedly occurs. In cultivation in the medium containing 20 mg/l of cadmium, the courses of cadmium removal by both bacteria closely resembled those in Fig. 1. Namely, UR No. 2 removed more than 98% of cadmium within 2 d and UR No. 4 removed 68% of cadmium (data not shown). These data clearly indicated that cadmium in the medium can be removed without difficulty by this aerobe (UR No. 2) and two strains, UR No. 2 and UR No. 4 were selected for further studies.

Taxonomic characteristics of the strain UR No. 2

The strain UR No. 2, which showed the most potent cadmium-removing capability among the isolates, was a Gram-negative rod, aerobic. Cell size was 0.4-0.6 µm wide × 0.8-1.0 µm long. Colonies are characteristically yellow and smooth. The strain was catalase positive and oxidase negative and did produce acid from glucose, mannose, fructose, lactose, and maltose. The search for the 16S rDNA base sequence found for the strain UR No. 2 on the data bank showed that the strain had a similarity of 97.5% to Xanthomonas sp. On the basis of these characteristics and structural analysis, the strain UR No. 2 was named Xanthomonas sp. UR No. 2 and the detailed data on base sequence will be published elsewhere.

There are several reports concerning the biosorption of cadmium by microbial biomass from the viewpoint of environmental purification and metal recovery.4,6 Most of the cadmium-sorbing microorganisms belong to algae, fungi, yeasts, bacteria (Bacillus sp., E. coli, Pseudomonas sp., Paracoccus sp., Streptomyces sp.). However, there has been no report on biosorption of cadmium by Xanthomonas sp. and this is the first report on the microbial sorption of cadmium by a bacterium belong to this genus. The UR No. 2 strain grew well in media containing 50 ppm and 100 ppm (0.45 mm and 0.9 mm, respectively) cadmium, while in medium with 200 ppm (1.8 mm) cadmium, no growth was observed. By the way, it has been reported that a cadmium-sensitive E. coli C600 was capable of growing in medium containing 0.4 mm Cd²⁺ but not growing in that with 1.5 mm Cd²⁺.3

Removal of cadmium from homogenates of hepatopancreas

The effects of bacteria on cadmium removal from the homogenates of scallop hepatopancreas were examined. During cultivations of the strains UR No. 2 and UR No. 4, the courses of cadmium removal from the simple homogenate (non-digested) are shown in Fig. 2. The cadmium content in the homogenate was 0.5 mg/l and was low. There were a slight decrease in cadmium concentration because of the presence of bacteria. The strain UR No. 2 grew to some extent in the homogenate, as indicat-

![Fig. 2. Courses of Cadmium Removal from the Simple Homogenates (Non-digested) of Hepatopancreas by UR No. 2 and UR No. 4 Bacteria.](image)

Both bacteria were cultivated in the above sample, and amounts of cadmium in culture supernatants and number of viable cells were measured as described in Materials and Methods. Cell numbers: □ URNo.2, Cadmium concentrations; ○ Control; △ UR No. 4; ● UR No. 2

ed in viabilities in Fig. 2. The amount of cadmium transferred from the raw material (hepatopancreas tissue) to the homogenate solution by the simple homogenization was estimated. As described in the next section (Table 2), the homogenization could release only a small amount of cadmium present in hepatopancreas tissue into solution. Although the structural form of cadmium present in scallop hepatopancreas tissue is not known in detail, it has been known that most of cadmium and zinc present in various organs such as gonad and kidney of scallops are tightly bound to protein complexes of high molecular weight. Accordingly, it appears that simple homogenization cannot extricate most of the cadmium present in hepatopancreas tissue, and that the form of cadmium present in this homogenate is recalcitrant to removal by the strain UR No. 2.

Next, with a view to transforming protein-bound cadmium in the tissue to a free state, hepatopancreas were digested by protease. Using the proteinase-treated homogenate, removal of cadmium by UR No. 2 and UR No. 4 were examined. As shown in Fig. 3, cadmium contents in the control solution were 4.0-4.5 mg/l. The strain UR No. 2 removed much cadmium from the homogenates, and the cadmium content decreased to below 0.5 ppm in 2 d of cultivation. During incubation for 3 d, the proportion of cadmium remaining in the culture supernatant was 10%. The strain UR No. 2 grew well in the homogenate, as indicated in the viabilities in Fig. 3. The pH (=5.7 at 0 h) of the culture solution increased to 8.1 after 3 d with cultivation, due to the production of ammonia. The strain UR No. 4 could not remove cadmium as effective as strain UR No. 2. As the pH (=5.9 at 0 h) of the culture for UR No. 4 increased to 8.2 by culturing for 3 d, this strain certainly grew in the
homogenate. With cultivation in another homogenate of hepatopancreas (prepared by the digestion due to 2.0% proteinase), the strain UR No. 2 decreased the cadmium content of 2 mg/l to 0.17 mg/l within 3 days (not shown). That is, this organism is effective for the removal of cadmium from the proteinase treated homogenate of hepatopancreas. Treatment of hepatopancreas tissue with the endopeptidase increased the transformation of the tightly protein-bound cadmium to a free state or a susceptible organic cadmium complex, resulting in promotion of cadmium removal by UR No. 2. UR No. 4 had no effect on the removal of cadmium in the homogenate, while this strain removed to some extent the free Cd²⁺ in the cadmium medium as indicated in Fig. 1. From these results, we may deduce that the proteinase-treated homogenate contains a higher concentration of the soluble organic cadmium complex than free Cd²⁺, and that such a organic cadmium is hard to remove for UR No. 4. The elucidation of the type of cadmium species in this homogenate is the subject of a further study.

**Mass balance of cadmium**

The distribution of cadmium in the soluble (homogenate solution) and the residue fractions after the homogenization treatments of hepatopancreas tissue were examined as follows. Hepatopancreas tissues were simply homogenized or treated with protease and centrifuged. The amounts of cadmium in supernatants and residues were measured by the atomic absorption method after wetashing of those samples, as shown in Table 2. In the simple homogenization, only 9% of the cadmium present originally in the tissue moved to the homogenate, and the greater part of cadmium remained in the tissue residue. On the other hand, the proportion of amount of cadmium in the tissue transferred to the homogenate by protease treatment increased to 54%. By the way, yields of protein extraction from the organ in the simple homogenization and the protease treatment were about 20% and 60%, respectively. It can be said that the protease treatment is effective for the solubilization of hepatopancreas tissue and the release of cadmium in the tissue to the homogenate. In both cases, the amount of cadmium in the raw material well balanced with the total amount of those of each fraction after homogenization of the tissue, as seen from Table 2.

Table 2. Distribution of Cadmium* in the Supernatant and the Residue Fractions after Homogenization of Hepatopancreas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supernatant</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td>2.81 (100)</td>
<td>5.65 (100)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.253 (9%)</td>
<td>3.06 (54%)</td>
</tr>
<tr>
<td>Residue</td>
<td>2.50 (88%)</td>
<td>2.45 (43%)</td>
</tr>
<tr>
<td>Total</td>
<td>2.753 (98%)</td>
<td>5.51 (97%)</td>
</tr>
</tbody>
</table>

* The unit is mg of cadmium present in each fraction. The values in brackets are expressed as percentage of those.
** Hepatopancreas were simply homogenized, boiled and centrifuged, and amounts of cadmium in supernatant and residue were measured as described in Results and Discussion. The weights of the starting material, supernatant, and residue were 100 g, 511 ml, and 55 g, respectively.
*** Hepatopancreas were treated with proteinase and centrifuged, and amounts of cadmium in supernatants and residues were measured as described in Results and Discussion. The weights of the starting material, supernatant, and residue were 201.3 g, 683 ml and 91.3 g, respectively.

Table 3. Distribution of Cadmium* in the Supernatant and the Residue Fractions after Cultivation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 h Culture</th>
<th>24 h Culture</th>
<th>48 h Culture</th>
<th>72 h Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0.428 (97)</td>
<td>0.343 (78)</td>
<td>0.051 (12)</td>
<td>0.040 (9)</td>
</tr>
<tr>
<td>Residue</td>
<td>0.04 (9)</td>
<td>0.102 (23)</td>
<td>0.386 (88)</td>
<td>0.404 (92)</td>
</tr>
<tr>
<td>Total</td>
<td>0.468 (106)</td>
<td>0.445 (101)</td>
<td>0.437 (100)</td>
<td>0.444 (101)</td>
</tr>
</tbody>
</table>

* The unit is mg of cadmium present in each fraction. The values in brackets are expressed as percentage of those. The amount of cadmium in the starting homogenate (100 ml) treated with proteinase was 0.439 mg (100%).
** UR No. 2 strain was cultivated in a 500 ml shaking flask containing 100 ml of the hepatopancreas homogenate treated with proteinase. After centrifugation, amounts of cadmium in supernatants and residues were measured by the atomic absorption method after acid digestion.
scallop hepatopancreas.

**Analysis of hepatopancreas homogenates with cadmium removed**

Contents of cadmium-removed hepatopancreas homogenates were analyzed and compared with feeds on the current market. In general, feed stuffs are required to have the content of crude protein over 50%. As shown in Table 4, the homogenate treated by UR No. 2 contains good amounts of crude proteins, fats, and ashes comparable to those on the current market. In addition, little of the content of crude protein was lost during this procedure. The content of cadmium in this homogenate sample (dry stuff) is also shown in Table 4. According to an official notice from the Ministry of Agriculture, Forestry, and Fisheries of Japan, the controlled values for cadmium in feedstuffs are 5 mg/kg-dry stuff for the manure, and 2.5 mg/kg-dry stuff for fish powder. Although the value of cadmium in the homogenate treated by the strain UR No. 2 was slightly over the controlled value of those for fish powder, it may be changed for the better by further studies. On the other hand, the cadmium concentration of the UR No. 2-treated homogenate comes up to the standard for use as manure. There is some possibility that this hepatopancreas homogenate can be used in feeds.

In conclusion, we proposed a bioprocess scheme for manufacturing the protein resources from scallop hepatopancreas by treatments with protease and the strain UR No. 2, as summarized in Fig. 4. First, hepatopancreas tissues are digested by protease and are solubilized. A bacterium (UR No. 2) capable of removing cadmium was cultivated in the solution of hepatopancreas. Cells were separated from the solution by centrifugation, resulting in the removal of cadmium from the solution. Since this solution contain abundant organic matter like protein, it appears to be effectively used as feedstuff after drying. In this paper, we described the screening of bacteria removing cadmium from the homogenates of hepatopancreas and the validity of our method. It seems highly probable that application of this process to various marine wastes containing toxic heavy metals may remove these metals, producing value-added protein resources. Further studies are in progress on the removal of cadmium from the homogenates of hepatopancreas under various conditions.

**Tables**

<table>
<thead>
<tr>
<th>Composition (% dry free)</th>
<th>Proteins</th>
<th>Fats</th>
<th>Ashes</th>
<th>Cd****</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate*</td>
<td>79.4</td>
<td>3.3</td>
<td>16.0</td>
<td>27.3</td>
</tr>
<tr>
<td>UR No. 2-treated-</td>
<td>75.8</td>
<td>4.4</td>
<td>18.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Homogenate**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish powder***</td>
<td>70.5</td>
<td>6.6</td>
<td>21.9</td>
<td>0.20</td>
</tr>
<tr>
<td>Fish soluble adsorbate***</td>
<td>60.8</td>
<td>11.2</td>
<td>12.9</td>
<td>0.48</td>
</tr>
<tr>
<td>Meat-bone meal***</td>
<td>53.4</td>
<td>11.2</td>
<td>32.6</td>
<td>N.T.</td>
</tr>
</tbody>
</table>

* Hepatopancreas homogenate treated with protease. Incubated for 3 d at 30°C.
** Hepatopancreas homogenate treated with proteinase and the strain UR No. 2. Cultivated for 3 d at 30°C.
*** Feed stuffs on the market
Fish powder (Abasiri Marine Product Co-operative Association)
Fish soluble adsorbate (Nippon Chemical Feed Co., Ltd)
Meat-bone meal (Hakodate Kasei Co., Ltd)
**** The unit is (mg-cadmium/kg-dry sample). N.T., not tested.

**Fig. 4.** Scheme for Manufacturing the Feedstuffs from Hepatopancreas of Scallops by a Microbial Process.

**References**


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