Preparation and Preservation of Freeze-dried Cells of Acetic Acid Bacteria with Aldehyde Oxidase Activity

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Freeze-dried cells of acetic acid bacteria were prepared to use as an additive for manufacturing and processing foods. When the freeze-dried cells were stored for 1 week at 5°C, however, more than 50% of the original activity of aldehyde oxidase (AOX) was lost.

It was found that this decrease in AOX was caused by damage to both the membrane-bound aldehyde dehydrogenase and terminal oxidase activities involved in the aldehyde oxidase electron transport system of acetic acid bacteria. The addition of 30% sucrose to the cell suspension prevented AOX damage in a McIlvaine buffer (pH 6) before lyophilization. This was found to be effective for preventing the decrease in AOX activity. Cells freeze-dried in this way lost no AOX activity at all during first 3 weeks of storage at 5°C and, even after 9 weeks, 80% of the original activity remained.

Key words: acetic acid bacteria; Acetobacter acetii; aldehyde oxidase; aldehyde dehydrogenase; terminal oxidase

In the preceding papers,1,2 we have reported a new method for reducing the off-flavor of aldehydes as hexanal and for preventing coloration by the amino-carbonyl reaction, in which wet cells of acetic acid bacteria were added to foodstuff during manufacturing and processing that successfully removed aldehydes by oxidizing them to the corresponding acids with the aldehyde oxidizing system of the bacterium. However, it was considered that if freeze-dried cells of the acetic acid bacteria could be obtained, they would be more convenient than wet cells in view of preservation, handling and so on. Moreover, freeze-dried cells could be readily used not only in manufacturing foods but also in cooking at home.

The aldehyde oxidizing system is one of the electron transport systems working in the cytoplasmic membrane of acetic acid bacteria, and seems to comprise primary dehydrogenase, ubiquinone, and cytochrome o-type or a1-type ubiquinol oxidase.3,4 Therefore, in order to use freeze-dried cells of acetic acid bacteria for food processing, it is important to maintain the total aldehyde oxidase (AOX) activity during their preparation and subsequent storage. For this purpose, it is necessary for all the components involved in the aldehyde oxidase electron transport system to be protected from inactivation.

There have been a number of investigations on protection from denaturation by heating, freezing, and drying a variety of enzymes and proteins that can prevent such denaturation. Yasumoto et al.5 have reported that non-reducing sugars such as trehalose, raffinose and sucrose had a protective effect on the freeze-drying of lyszymes, pyruvate kinase or intact cells of Escherichia coli. Moreover, Lippert et al.6 have shown the protective effect of polyols, sugars, amino acids, betaines and eicosines when heating, freezing and drying two sensitive enzymes, lactic dehydrogenase and phosphofructokinase.

In the present study, we examine the stability of AOX activity during the preparation and storage of freeze-dried cells of acetic acid bacteria.

Materials and Methods

Microorganisms. Acetobacter acetii. IFO 3284 was used throughout this study. The medium and cultivation conditions for the bacteria were the same as those described in the previous paper.7 Cells were harvested by centrifugation at 12,000 x g for 20 min and washed with cold water. Wet cells (35 g) were suspended in 250 ml of distilled water, and the solution was lyophilized to make a freeze-dried powder.

Conditions for storage. Each sample of freeze-dried cells of the acetic acid bacteria was incubated at 5°C, and the changes in activity of membrane-bound aldehyde dehydrogenase (ALDH), terminal oxidase, and AOX were measured. Ten milligrams of the freeze-dried cells were periodically removed and suspended in 5 ml of distilled water to use as an enzyme preparation.

Measurement of ALDH activity. ALDH activity was assayed by the method of Wood et al.,8 using potassium ferricyanide as an electron acceptor. The reaction mixture contained 10 μmol of potassium ferricyanide, 0.45 ml of a McIlvaine buffer (pH 5.0), 20 μmol of acetaldehyde, and the freeze-dried cell suspension in a total volume of 1.0 ml. The reaction was started by adding potassium ferricyanide, carried out at 30°C for 20 min and stopped by adding 0.5 ml of the ferric-Dupanol reagent. Water (3.5 ml) was then added to the reaction mix-
ture and well mixed. After standing for 20 min at room temperature, the resulting stabilized Prussian blue color was measured spectrophotometrically at 660 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of acetaldheyde per min under these assay conditions.

Measurement of oxidase activity. All enzyme assays were conducted at 25°C by monitoring the decrease in dissolved oxygen concentration with an oxygen analyzer (Horiba Works, Kyoto, Japan). The initial oxygen concentration in the reaction mixture at 25°C was assumed to be 258 μM, and one unit of enzyme activity is defined as the enzyme amount that causes the consumption of 1 μmol of dissolved oxygen per min under the assay conditions. The reaction mixture for AOX activity contained 120 μmol of acetaldheyde, 4.3 ml of the McIlvaine buffer (pH 5.0), and 0.5 ml of the freeze-dried cell suspension in a total volume of 6.0 ml. The reaction was started by adding the substrate. Terminal oxidase activity was also measured polarographically by using ascorbic acid and N,N,N',N'-tetramethyl-p-phenylenediamine (AsA-TMPD) as electron donors. The reaction mixture contained 10.8 μmol of TMPD, 30 μmol of AsA, 1.2 ml of a 165 mm potassium phosphate buffer (pH 6.5), 1.2 ml of distilled water, and 1.2 ml of the freeze-dried cell suspension in a total volume of 6.0 ml.

Protein determination. Protein content was determined according to the method of Lowry et al., using a bovine serum albumin as the standard.

Measurement of the viability of the freeze-dried cells. The culture medium was composed of 1% yeast extract, 1% polypeptone, 2% glycerol, 0.5% d-glucose, 0.5% calcium carbonate, 0.5% ethyl alcohol, 0.25% acetic acid, and 1.5% agar in 1000 ml of a potato extract. The freeze-dried cells that had been stored at 5°C were suspended in 10 ml of sterilized water. This cell suspension was diluted, mixed with 9 volumes of the culture medium just described, and then solidified in a petri dish. After cultivating at 30°C for 48 hr, the number of viable cells was calculated.

Results and Discussion

Change of aldehyde oxidase activity in the freeze-dried cells during storage

Harvested cells were suspended in distilled water, and the suspension was lyophilized to make freeze-dried cells. When the enzyme activities were measured with both intact and freeze-dried cells, no significant difference could be seen, as shown in Table I, indicating that lyophilization treatment had no effect on either ALDH, terminal oxidase, or AOX activity. Furthermore, these enzyme activities were detected with the cell suspension in an almost similar manner with the cell-free extract, although the ALDH and terminal oxidase activities were relatively low in the cell suspension (Table I). Next, the stability of these enzyme activities related to the aldehyde oxidase system was investigated during the storage of the resulting freeze-dried cells at 5°C. As shown in

![Fig. 1. Changes in the Enzyme Activities of Freeze-dried Cells of Acetic Acid Bacteria during Storage at 5°C.](image)

**Table I. Effect of Lyophilization on the Dehydrogenase and Oxidase Activities**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lyophilization</th>
<th>Activity (unit/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALDH</td>
</tr>
<tr>
<td>Intact cells</td>
<td>before</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>29</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td>before</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>85</td>
</tr>
</tbody>
</table>

Intact cells and freeze-dried cells were suspended in distilled water, before each cell suspension was treated with an ultrasonic oscillator for 5 min. After removing the cell debris by centrifugation, the resulting supernatant was used as the cell-free extract. Enzyme activities were assayed with the cell suspension and the cell-free extract of intact cells (before lyophilization), and also with those of the freeze-dried cells (after lyophilization). Activity is expressed as nmol of acetaldheyde oxidized per min per mg of protein for ALDH, and as nmol of O2 consumed per min per mg of protein for terminal oxidase and AOX.

Fig. 1, more than 50% of the original activity of AOX had been lost after storing at 5°C for 1 week, and it decreased gradually with further storage time. Since both the ALDH and terminal oxidase activities also decreased at the same time, the decrease in AOX activity seems to have been due to the activity decrease of these enzymes which constitute the aldehyde oxidase system.

Effect of pH on the freeze-dried cell preparation

Since the pH of the culture medium reached about 3 at the end of cultivation, the pH of the medium might have affected the pH of the cell suspension in distilled water, and thus the stability of AOX activity. Consequently, wet cells were washed 5 times and suspended in a McIlvaine buffer of different pH values, and then lyophilized to make freeze-dried cells. The resulting freeze-dried cells prepared at different pH values were stored at 5°C for 6 weeks, and the enzyme activities of ALDH, terminal oxidase, and AOX were meas-
Fig. 2. Changes in the Enzyme Activities of Cells Freeze-dried and Stored at Different pH values.

Wet cells were washed well and suspended in a McIlvaine buffer with different pH values, and the suspension was lyophilized to make freeze-dried cells. The resulting freeze-dried cells were stored at 5°C.

△ pH 3; □ pH 4; ■ pH 5; ○ pH 6; ● pH 7

As shown in Fig. 2, it is clear that the stability of the enzyme activities depended on the pH of the cell suspension before lyophilization. All three enzyme activities were more stable at pH 6.0 and 7.0 than at pH 3.0, 4.0 and 5.0. The best enzyme activities were obtained at pH 6.0.

Fig. 3. Effect of Sugars and Amino Acids on the Aldehyde Oxidase Activity of Freeze-dried Cells.

Protective agents such as sugars and amino acids were individually added at a final concentration of 10% or 0.5%, respectively, the pH value of the suspension being adjusted to 6. Each suspension was lyophilized and stored at 30°C for 1 week.

Residual AOX activity (%)  
0.5% Pro  
0.5% Gly  
0.5% Glu  
0.5% Asp  
10% Starch  
10% Dextrin  
10% Raffinose  
10% Sucrose  
10% Fructose  
10% Galactose  
None

Effect of protective agents such as sugars and amino acids on the aldehyde oxidase activity of the freeze-dried cells

We investigated whether protective agents such as sugars and amino acids would prevent a decrease in AOX activity when these agents were added to freeze-dried cells adjusted to pH 6. The remaining AOX activity in freeze-dried cells prepared in the presence of sugars or amino acids were measured after storing at 30°C for 1 week and are presented in Fig. 3. The addition of several different disaccharides such as sucrose, lactose or maltose, and of monosaccharides such as glucose, galactose or fructose was effective, but no polysaccharides were. In contrast, hardly any of the amino acids had an effect as a protective agent, only L-glutamic acid and L-aspartic acid showing a weak effect.

Comparison among disaccharides as a protective agent against the loss of aldehyde oxidase activity

We checked whether or not 10% maltose, lactose, or
sucrese would protect the loss of AOX activity of the freeze-dried cells during storage at 5°C for 7 weeks (Fig. 4). Sucrose showed the strongest protective effect against the loss of AOX activity.

Next, to establish the optimum concentration of sucrose as a protective agent, freeze-dried cells containing various concentrations up to 30% of sucrose were stored at 5°C for 9 weeks (Fig. 5). It was found that the higher concentrations of sucrose were more effective. Thus, in the presence of 30% sucrose, the decrease in AOX activity was only about 20%, even after 9 weeks.

The viability of freeze-dried cells of acetic acid bacteria was also investigated in the presence and absence of 30% sucrose as the protective agent during storage at 5°C. As shown in Fig. 6, it was found that the cell viability was one order higher when the freeze-dried cells had been prepared in the presence of 30% sucrose, but was not changed during storage of the freeze-dried cells. Thus, it can be concluded that the decrease in AOX activity of the freeze-dried cells prepared without sucroses was not due to any adverse effect on cell viability, but to the aldehyde oxidase electron transport system in the freeze-dried cells of acetic acid bacteria.

Judging from the data obtained, it was concluded that preparing the freeze-dried cells at pH 6 in the presence of 30% sucrose was the most effective for preventing a loss of AOX activity during storage.

**References**