Studies on Chitin-decomposing Bacteria Present in the Digestive Tract of Aquatic Animals—III. Formation of Organic Acids

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In the previous paper1), it has been suggested that the accumulation of organic acids by a marine chitin-decomposing bacterium, Vibrio gerris, isolated from digestive tracts of fish were butyric, acetic, pyruvic, formic, lactic, glycolic and an unidentified acids.

This paper describes experiments comparing the patterns of organic acids accumulation by eight strains of marine bacteria2) and effects of three organic acids on growth and activity of the chitinolytic enzyme by bacteria.

Materials and Methods

Organisms. The organisms used were chitin-decomposing bacteria isolated from digestive tracts of fish, Lateolabrax japonicus2). They are presented below.

Vibrio gerris var. nongelatoliquefaciens K3.
V. orphus K6.
Aeromonas skiaina K4.
Alginomonas channe K10.

Culture. Bacterial basal medium was the following final composition: 0.5% peptone, 0.1% yeast extract, and 2.5% sodium chloride in tap water. The culture (200 ml/300 ml flask) were grown at pH 7.0 and 25°C for 4 days.

Determination of organic acids. After centrifugation of the cultures, the supernatant was adjusted to pH 1–2 by adding a small amount of H₂SO₄, then the solution was subjected to continuous extraction with ethyl ether for 96 hours in the same manner as described by Neish3). Then an aliquot of this ether extract containing organic acids was chromatographed on a silica gel column according to the technique described by Mueller4), and individual organic acid was eluated by addition of a series of n-butyl alcohol-chloroform solvents. The eluates were titrated with 2-N/100 NaOH using phenol red as an indicator.

Enzyme preparation. After centrifugation of the cultures, the precipitate was discarded, and finely ground ammonium sulfate was added to the supernatant fluid to give a 0 to 80% saturated fraction. The precipitate separated by centrifugation

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Fig. 1. Amount of organic acids in culture filtrates of chitin-decomposing bacteria.
was dissolved in water. This solution was used as the enzyme preparation. Enzyme protein was determined by the method of FOLIN et al\(^5\).

**Enzyme substrate.** The chitin used in these tests is insoluble precipitated chitin (from HCl). Native chitin is a product of Tokyo Kasei Kogyo Co., Tokyo.

**Enzyme assay.** One milliliter of enzyme solution was mixed with 2.0 ml of chitin suspension, 2.0 ml of McILVAIN buffer (pH 7.0) and 0.2 ml of toluene, and then incubated for 6 hours at 25°C. After the incubation the mixture was analyzed for N-acetylamino sugar (monomer) liberated by the method of REISSIG et al\(^6\).

### Results and Discussion

Studies on the formation of organic acids by *V. gerris* have shown butyric, acetic, pyruvic, formic, lactic, glycolic and an unidentified acids in the growing culture\(^1\).

As can be seen from Fig. 1, *V. gerris var. nongelatoliquefaciens* K3 accumulated butyric, acetic, pyruvic, formic, lactic and glycolic acids in the growing culture. These results are consistent with our previous observation on *V. gerris*\(^1\). It is found that the rate of butyric acid accumulation is relatively large in the strains of *V. orphus* K6 and *Al. channe* K10. *A. chitinophthora* K5, K7 and K9 are found to differ distinctly from other strains tested in no accumulation of detectable amounts of lactic acid.

It seems that these organic acids accumulated in the growing culture are the intermediates in the metabolism of bacterial life. So, there may be differences in organic acid accumulations in various organisms. As shown in Fig. 1, no detectable accumulation of formic acid was observed in the cultures of *V. orphus* K6, *A. chitinophthora* K7, K8 and K9, and *Al. channe* K10. In view of the observations of OGINSKY and UMBREIT\(^7\), formic acid produced in bacterial cultures is degraded to CO\(_2\) and H\(_2\) by hydrogenase or oxidized to CO\(_2\) by formic dehydrogenase. Our failure to detect formic acid in five strains above mentioned may merely reflect the fact that in the presence of hydrogenase or formic dehydrogenase it is readily metabolized and does not accumulate.

In the the present experiments, the questions as to which substrate or substrates are served to organic acids accumulation have not yet been answered.

The growth of chitin-decomposing bacteria has been compared with three organic acids, acetic, lactic and succinic acids. Succinate was selected for study, because it has been suggested that rumen bacteria produces very high yields of cellulase when grown on succinate\(^8\).

As shown in Table 1, acetic acid stimulated growth of *V. orphus* K6 and *Al. channe* K10. On the contrary, lactic acid stimulated *V. orphus* K6, *A. chitinophthora* K5, K8 and K9, and *Al. channe* K10. Succinic acid stimulated growth of *V. gerris var. nongelatoliquefaciens* K3, *V. orphus* K6, *A. chitinophthora* K8 and K9, and *Al. channe* K10. None of three organic acids tested stimulated growth of *A. chitinophthora*
**Table 1.** Effect of acetate, lactate and succinate on the growth of chitin-decomposing bacteria.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Organic acids</th>
<th>Final pH of the culture</th>
<th>Dry weight of cells/200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. brio gerris var. nongelatoliquefaciens</em> K3</td>
<td>Acetate</td>
<td>8.2</td>
<td>153.8 mg</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>8.5</td>
<td>149.3</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>7.8</td>
<td>191.6</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>8.6</td>
<td>126.2</td>
</tr>
<tr>
<td><em>V. orphus</em> K6</td>
<td>Acetate</td>
<td>8.6</td>
<td>215.7</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>8.9</td>
<td>150.1</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>8.8</td>
<td>150.0</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>8.2</td>
<td>108.7</td>
</tr>
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<td><em>Aeromonas skiaina</em> K4</td>
<td>Acetate</td>
<td>7.8</td>
<td>42.0</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>7.6</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>7.4</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>7.8</td>
<td>45.8</td>
</tr>
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<td><em>A. chitinophthora</em> K5</td>
<td>Acetate</td>
<td>8.0</td>
<td>57.7</td>
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<tr>
<td></td>
<td>Lactate</td>
<td>8.6</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>8.0</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>8.2</td>
<td>54.6</td>
</tr>
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<td><em>A. chitinophthora</em> K7</td>
<td>Acetate</td>
<td>8.0</td>
<td>68.6</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>8.4</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>8.3</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>8.4</td>
<td>63.2</td>
</tr>
<tr>
<td><em>A. chitinophthora</em> K8</td>
<td>Acetate</td>
<td>8.4</td>
<td>86.7</td>
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<td></td>
<td>Lactate</td>
<td>8.6</td>
<td>127.3</td>
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<td></td>
<td>Succinate</td>
<td>8.5</td>
<td>118.6</td>
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<td>Not added</td>
<td>8.2</td>
<td>74.2</td>
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<tr>
<td><em>A. chitinophthora</em> K9</td>
<td>Acetate</td>
<td>8.4</td>
<td>81.6</td>
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<tr>
<td></td>
<td>Lactate</td>
<td>8.5</td>
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<td></td>
<td>Succinate</td>
<td>8.2</td>
<td>91.1</td>
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<tr>
<td></td>
<td>Not added</td>
<td>8.2</td>
<td>67.8</td>
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<tr>
<td><em>Alginonomonas channe</em> K10</td>
<td>Acetate</td>
<td>8.6</td>
<td>584.9</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>8.8</td>
<td>306.9</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>8.6</td>
<td>316.8</td>
</tr>
<tr>
<td></td>
<td>Not added</td>
<td>8.2</td>
<td>161.2</td>
</tr>
</tbody>
</table>

Bacteria were cultured at initial pH 7.0 and 25°C for 4 days in the media containing sodium acetate, lactate or succinate.

K7 and *A. skiaina* K4. Growth of *V. orphus* K6 and *Al. channe* K10 were stimulated by each of three organic acids.

It was observed that acetic acid stimulated significantly growth of *V. orphus* K6 and *Al. channe* K10. None of the strains tested were inhibited the growth by these
three organic acids. Two of the sensitive strains to organic acids tested were selected for enzyme study, V. orphus K6 and Al. channe K10.

As can be seen from Table 2, a detectable inhibition of enzyme activity took place even at concentration of the organic acid that stimulated growth.

It is interesting that the production of an enzyme is influenced by the organic acids. Growth on succinate lead to the good production of an enzyme in these two species. The addition of lactate did not affect the rate of activity.

Organic acids may be affecting the build-up of chitinolytic enzyme, and in tern may determine the level of enzyme produced.

More data are required to re olve this problem.

Table 2. Effect of acetate, lactate and succinate on the growth and enzyme activity of chitin-decomposing bacteria.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Organic acids*</th>
<th>Chitinolytic activity** (A)</th>
<th>Enzyme protein*** O. D. at 660 mµ (B)</th>
<th>Specific activity (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. orphus K6</td>
<td>Not added</td>
<td>18.5</td>
<td>1.290</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>4.9</td>
<td>0.775</td>
<td>6.3</td>
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<td></td>
<td>Lactate</td>
<td>11.6</td>
<td>0.731</td>
<td>15.9</td>
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<td></td>
<td>Succinate</td>
<td>23.2</td>
<td>0.986</td>
<td>23.5</td>
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<tr>
<td>Alginomonas channe K10</td>
<td>Not added</td>
<td>9.2</td>
<td>0.292</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>2.2</td>
<td>0.186</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>4.5</td>
<td>0.127</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>7.1</td>
<td>0.171</td>
<td>41.6</td>
</tr>
</tbody>
</table>

* Bacteria were cultured at pH 7.0 and 25°C for 4 days in the media containing 0.5% sodium acetate, lactate or succinate.

** Activity = µg N-acetylamino sugar/6 hours/ml of enzyme solution.

*** Colour development by Folin’s method5).

Summary

It was observed that growth of V. orphus K6 and Al. channe K10, marine chitin-decomposing bacteria, were stimulated by acetate, lactate and succinate. On the contrary, among organic acids which were added to the bacterial cultures only succinate had the stimulatory effect on the activity of the chitinolytic enzyme produced. Acetate had inhibitory and locate had no effect on enzyme activity.

Acknowledgment

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References

1) K. Okutani and H. Kitada: *This Bull.*, 34, 88~92 (1968).
8) H. Takahashi: *Unpublished data.*