Amylolytic lactic acid bacteria in fish silage

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Received 17 January 1983 and accepted 12 October 1983


An α-amylase activity has been observed in lactic acid bacteria occurring initially in fermented fish silage. The organisms belong to the genus Leuconostoc. The main fraction of the amylolytic enzyme produced by one of the isolated bacteria is cell-bound and is released into the medium at a late stage of growth. Treating cells with ultrasound or Triton X-100 increases enzyme activity in the culture filtrate. The pH range for enzyme activity is 5.0-7.0, with an optimum at pH 6.0. The enzyme is extremely labile at pH 8.0 and is inactivated at temperatures above 50°C at pH 5.8. Two enzyme fractions were found by isoelectric focusing, the main one at pH 5.00 and another at pH 4.5. Chromatography on DEAE cellulose gave two active peaks.

The ability of lactic acid bacteria to ferment starch was first reported by Sherman (1937). He found that some streptococci produced acid on starch substrates, a characteristic common in streptococci isolated from the alimentary tracts of ruminants (Mann et al. 1954; Seeley & Dain 1960). The starch-hydrolysing enzymes found among the streptococci may be cell-bound or extracellular α-amylases (Walker 1965; Boyer & Hartman 1971).

In Bergey's Manual (Buchanan & Gibbons 1974), lactic acid bacteria of the genera Pediococcus, Lactobacillus and Leuconostoc are reported as not utilizing starch. Langston & Bouma (1960a; b) as well as Steinböck (1962), however, isolated starch-fermenting leuconostocs and lactobacilli. Wirahadikusumah (1971) studied the succession of lactic acid bacteria in fermented fish silage prepared according to the description of Nilsson & Rydin (1965) and found that a group of oval cocci appeared at the beginning of the fermentation process. He also found that some organisms were able to utilize starch. Two different strains of starch-hydrolysing lactobacilli were recently observed in fermented swine and cattle waste-corn (Nakamura & Cromwell 1979; Nakamura 1981).

The present paper describes the isolation and identification of starch-hydrolysing lactic acid bacteria in fermented silage, and the production, partial purification and some properties of their amylases.

Materials and Methods

Isolation of Bacteria

Starch-hydrolysing lactic acid bacteria were selected from one-day-old fish silage. Single colonies were isolated at 28°C in modified MRS agar without dextrose and ammonium citrate but with 10 g/l soluble starch (De Mann et al. 1960). The fish silage was prepared from minced herring, wheat flour and brewer's malt (80:16:4) as recommended by Nilsson & Rydin (1965). The organisms isolated are kept as lyophilized samples at this Department with collection numbers 10135-10143/DMAC.

Morphological and Physiological Characteristics

The selected lactic acid bacteria were characterized according to routines recommended by Sharpe (1980). Production of L(+) lactic acid,
\( \text{d(-)-lactic acid and acetic acid was analysed enzymatically (Boehringer-Mannheim) (Anon. 1980).} \)

**Amylase Production**

Strain 10137/DMAC was grown for amylase production in a 10 l fermenter (EL-110, Biotec) or in 300 ml flasks containing 200 ml substrate. Modified MRS broth with 20 g/l soluble starch (Merck) or commercial wheat flour was used as the growth medium. The inoculum (1 ml/l) was an 18 h culture grown in the starch medium. The flask culture was gently shaken during fermentation. The fermenter culture was stirred continuously at 28°C in an atmosphere of nitrogen and carbon dioxide (9:1). The pH was buffered automatically to 5.8 with potassium monohydrogen phosphate (0.2 mol/l). It was found, however, that it was necessary to allow the pH to fall to 5.2 before adding buffer or fermentation ceased.

The cultures to be assayed for enzymatic activity were prepared in the following manner. A 100 ml culture was centrifuged in a MSE centrifuge at 3000 g for 30 min. The cells were washed in 100 ml NaCl (9 g/l). After centrifugation the cells were suspended in 25 ml of 0.1 mol/l phosphate buffer (pH 6.0). Cell-bound amylases were released either by shaking the cells in 0.1 mol/l sodium phosphate buffer (pH 6.0) containing 0.1 g/l Triton X-100 or by mild sonication in 0.1 mol/l sodium phosphate buffer (MSE ultrasonicator) at 20°C for 1 min. Cells and cell debris were removed by centrifugation at 3000 g. The supernatant fluid was dialyzed against tap water for 24 h at 2°C and concentrated four times with a Diaflo membrane XM-10 having 10000 dalton cut-off (Amicon). Filtration was carried out at 5°C with gaseous nitrogen at a pressure of 206 kPa.

**Amylase Assay**

Production of reducing sugars from soluble starch was used as a criterion of amylase activity. One ml of the enzyme solution obtained as described above or as described below was mixed with 3 ml starch solution (20 g/l) buffered to pH 6-0 with 0-1 mol/l phosphate buffer. After incubation at 39°C for 90 min, 2 ml of the reaction mixture were added to 3 ml of 3-5 dinitrosalicylic reagent (Miller 1959) in a stoppered glass tube. The tube was heated in a boiling water-bath for 15 min. The colour was measured in a spectrophotometer (Zeiss) at 640 nm. The zero time sample was obtained after heating the enzyme solution before mixing with the starch solution. Activity (EU) was calculated from a standard curve, and expressed as reducing capacity equal to 1 mg/ml maltose during 90 min.

The ratio of D-glucose and maltose liberated by amylase activity on soluble starch was analysed. D-glucose was assayed enzymatically with the Boehringer-Mannheim test method (Anon. 1980). Maltose content was assayed as D-glucose, after hydrolysing the maltose with a-glucosidase (Sigma) in 0-1 mol/l phosphate buffer pH 6-6. Both sugars were determined from a standard curve with known amounts of D-glucose.

**Preparation of Partly Purified Enzyme**

**Enzyme extraction**

The following procedure was used to study and further characterize the amylase. To obtain cell lysis, the pH was adjusted to 3-5 with HCl (370 g/l, w/v) in 5 day old bacterial culture grown in MRS-minimal broth supplemented with 20 g/l soluble starch. The culture was further treated with Triton X-100 (0-1 g/l) and shaken vigorously for 2 h at 5°C. The pH was then adjusted to 5-0 with sodium hydroxide (400 g/l, w/v). Cell debris and undissolved material were removed by centrifugation in a Sharples continuous centrifuge. The supernatant fluid was treated with cold (\(-20°C\)) ethanol to a final concentration of 300 g/l (v/v). The precipitate was discarded. A further quantity of cold ethanol was then added to a concentration of 700 g/l (v/v). The resulting precipitate, which contained the enzyme fraction, was dissolved in 0.05 mol/l phosphate buffer, pH 6-0. The enzyme solution was dialysed against tap water at 5°C for 48 h and freeze-dried.

**Ultrafiltration**

Five grams of the freeze-dried enzyme preparation were dissolved in 150 ml 0-02 mol/l sodium phosphate buffer, pH 6-0, and centrifuged at 10100 g for 2 h in a MSE centrifuge.
The supernatant fluid was filtered through a Diaflo membrane XM-10 with 10000 dalton cut-off (Amicon), with six volumes of the above buffer and concentrated to 50 ml. Filtration was carried out at 5°C, with gaseous nitrogen at a pressure of 206 kPa. The concentrated enzyme was filtered through a Diaflo membrane UM-50 with 50000 dalton cut-off, with six volumes of 0.02 mol/l Tris-HCl buffer pH 7.2 and further concentrated to 5 ml.

**Ion-exchange chromatography on DEAE-cellulose**

Microgranular pre-swollen DEAE cellulose (DE-52) (Reeve Angel) was prepared as recommended by the manufacturer. The exchanger was stirred into an equal volume of 1.0 mol/l Tris-HCl buffer (pH 7.2). The packing was settled to a depth of 44 cm in a column (0.9 x 60 cm, Pharmacia AB, Uppsala, Sweden) with 1 litre 0.02 mol/l Tris-HCl buffer (pH 7.2). A volume of 2.2 ml of the concentrated enzyme solution (144 mg protein) was applied to the top of the column. The column was eluted with increasing concentrations of Tris buffer (pH 7.2), the gradient being linear between 0.02 and 0.2 mol/l. The eluate was collected and the adsorption at a wavelength of 280 nm was read in fractions of 4.5 ml.

**Isoelectric focusing of the amylase produced**

A volume of 4.0 ml of the concentrated enzyme (250 mg protein) was mixed with an ampholyte solution (pH 3-10, LKB, Sweden). The mixture was applied to a 110-ml I.P. focusing column (LKB, Sweden). Electrophoresis was carried out for 48 h at a current of 150 V between pH 3.0 and 10.0 at 4°C. Fractions of 2 ml were collected and analysed for protein absorption, amylase activity and pH measurements.

**Enzyme Characterization**

The effect of pH on enzyme activity was determined in a phosphate-citrate buffer (0.1 mol/l) in the pH range 4.5-7.5. Except for the variation in buffer composition, the activity was assayed under standard conditions. The pH stability of the α-amylase was assessed by incubating the enzyme at various pH for 2 h and for 24 h at 30°C. After incubation, each enzyme solution was adjusted to pH 5.8 and the residual activity was measured by the standard method. From pH 3.0 to 7.0 citrate phosphate buffer (0.1 mol/l) was used; between 7.0 and 8.5, phosphate buffer (0.1 mol/l); and between 8.5 and 9.0, Tris buffer (0.1 mol/l).

The temperature activity profile was determined by incubating the enzyme in 0.1 mol/l phosphate buffer at pH 5.8 with 20 g/l starch as substrate at various temperatures.

**CEREALS AS A SILAGE ADDITIVE**

Addition of 180 g/kg of cereals and 20 g/kg of malt initiates a proper fermentation of fish. Replacement of the malt with cereals causes a fermentation failure. When the material is supplemented with extra cereals (up to 280 g/kg of total mixture), lactic acid production is initiated (Fig. 1).

**CHARACTERISTICS OF AMYLOLYTIC BACTERIA**

Nine amylolytic organisms were isolated from fish silage during the first day of fermentation in MRS-minimal medium supplemented with starch. All isolates were Gram positive catalase-negative oval cocci with a heterofermentative glucose metabolism yielding lactic acid, acetic acid and carbon dioxide. The physiological characteristics of the isolated bacteria are shown in Table 1.

**CULTURE CONDITIONS FOR AMYLASE PRODUCTION**

Strain 10137/DMAC was employed for amylase production. The highest yield was obtained in wheat-flour medium (Table 2). Organisms were cultured in a nitrogen-carbon dioxide atmosphere. If the carbon dioxide was eliminated, and only nitrogen used, fermentation ceased. In order to raise the yield of enzyme and to prevent the lysis of cells because of their acid production, various buffers were evaluated during continuous addition. It was found necessary to allow the pH of the culture to drop to 5.2 before raising it to 5.8 by adding 0.2 mol/l potassium hydroxide.
Fig. 1. Effect of added cereal flour and brewer’s malt on the production of organic acids and glucose in fish silage.
(a) flour : fish, 20 : 80; (b) flour : fish, 28 : 72; (c) flour : malt : fish, 18 : 2 : 80. (a) ●, acetic; —, lactic. (b) ●, acetic; ■, lactic. (c) ●, acetic; ▲, glucose.

Table 1. Characteristics of starch-hydrolysing lactic acid bacteria isolated from fermented fish silage

<table>
<thead>
<tr>
<th>Organism/DMAC*</th>
<th>Test 10135</th>
<th>10136</th>
<th>10138</th>
<th>10139</th>
<th>10140</th>
<th>10142</th>
<th>10137</th>
<th>10141</th>
<th>10143</th>
</tr>
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<tbody>
<tr>
<td>Catalase reaction</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NH₃ from arginine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Lactic acid isomer</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Acid production from starch (10 g/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactic acid (g/l)</td>
<td>3·5</td>
<td>3·5</td>
<td>3·6</td>
<td>3·6</td>
<td>3·5</td>
<td>3·4</td>
<td>3·9</td>
<td>4·0</td>
<td>3·9</td>
</tr>
<tr>
<td>acetic acid (g/l)</td>
<td>0·8</td>
<td>0·7</td>
<td>0·8</td>
<td>0·7</td>
<td>0·7</td>
<td>0·8</td>
<td>0·8</td>
<td>0·9</td>
<td>0·7</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 15°C</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Growth at 45°C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dextran from sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from</td>
<td>ribose, cellobiose,</td>
<td>fructose, galactose,</td>
<td>maltose, melibiose,</td>
<td>raffinose, sucrose,</td>
<td>trehalose, xylose,</td>
<td>mannosone, starch</td>
<td>lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>salicin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
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<tr>
<td>mannitol</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>arabinose, melezitose,</td>
<td>rhamnose, sorbitol,</td>
<td>sorbose.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Organisms deposited at Collection of freeze-dried cultures in the Department of Microbiology, University of Agricultural Sciences, Uppsala, Sweden.
+ , Positive reaction.
— , Negative reaction.
± , Slight reaction.
Table 2. Amylolytic activity in culture filtrate and on cells of a starch-hydrolysing lactic acid bacterium (10137/DMAC)

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Starch source</th>
<th>Growth period (h)</th>
<th>Viable count/ml</th>
<th>Triton-treated cells</th>
<th>Sonicated cells</th>
<th>Culture supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentor culture</td>
<td>Wheat flour</td>
<td>0</td>
<td>10⁶</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Wheat flour</td>
<td>24</td>
<td>3 x 10⁹</td>
<td>0.17</td>
<td>0.18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Wheat flour</td>
<td>48</td>
<td>4 x 10⁹</td>
<td>0.53</td>
<td>0.61</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Zylkowsky starch</td>
<td>72</td>
<td>3 x 10⁸</td>
<td>0</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Flask culture</td>
<td>Wheat flour</td>
<td>72</td>
<td>7 x 10⁸</td>
<td>--</td>
<td>0.27</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Zylkowsky starch</td>
<td>72</td>
<td>7 x 10⁸</td>
<td>--</td>
<td>0.11</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* The enzyme activity in each preparation was calculated on the basis of a 1 ml culture sample.

CELL ASSOCIATION

During the first day of growth in wheat-flour medium, only low amylase activity was observed in the cell-free filtrate. The main activity was localized in fractions isolated from cell deposits (Table 2). During the phase of decline the amylase activity in the culture filtrate increased.

PARTIAL PURIFICATION OF THE AMYLASE

The extracted amylase did not pass a membrane with a cut-off at 50 000 daltons. Chromatography of the enzyme preparation on DEAE cellulose gave two peaks, one containing the main part of the activity and one appearing at a late stage of elution (Fig. 2). The amylase fractions were localized by the isoelectric

Fig. 2. Separation of the amylolytic enzyme from strain 10137/DMAC on DEAE-cellulose (DE-52) after elution with increasing concentrations of Tris buffer. ○, Tris-HCl concentration; ——, protein; ——, amylase activity.
Fig. 3. Isoelectric focusing of the amylolytic enzyme from strain 10137/DMAC. ---, protein; -- , amylase activity.

Fig. 4. Effect of pH on the activity of the α-amylase from strain 10137/DMAC. △, glucose; ×, maltose.

Fig. 5. Effect of temperature on the activity of the α-amylase from strain 10137/DMAC in 0.1 mol/l phosphate buffer at pH 5.8.

ENZYME CHARACTERISTICS

The effect of pH on the enzyme activities at 39°C is shown in Fig. 4. The optimum pH was around 6.0, giving maltose and glucose in a ratio of 5:1. Enzyme activity was assayed at various temperatures at pH 6.0 (Fig. 5). The optimum temperature was 40°C (±1°C), and the enzyme was extremely labile during storage below pH 4.0 and above pH 8.0 (Fig. 6).

Dialysis of the enzyme against ethylenediaminetetraacetic acid (EDTA, 1 g/l)
Fig. 6. Effect of pH on stability of the α-amylase from strain 10137/DMAC. Residual activity of the enzyme kept at various pH levels for 0, 2 h and 24 h at 30°C.

reduced enzyme activity by about one-fifth. The addition of 0.1 g/l of NaCl did not stimulate enzyme activity.

Discussion

In fish silage fermented according to the methods recommended by Nilsson & Rydin (1965) the minced fish is supplemented with cereals and brewer's malt. The cereals provide the fish with a suitable inoculum of lactic acid bacteria and enrich it with carbohydrates. The malt contains amylolytic enzymes which degrade the cereal starch to fermentable sugars. Microbial amylases have also been used for this purpose (Stanton & Quee Lan Yeoh 1977). Increasing the amount of cereals in relation to fish induces a lactic acid fermentation. During the initial fermentation period, amylolytic lactic acid bacteria belonging to the genus Leuconostoc can be isolated. The fermentation of trehalose, the absence of ammonia production from arginine and the formation of D(-)-lactic acid from glucose are properties characteristic of the genus Leuconostoc and distinguish it from the heterofermentative betabacterium group within the genus Lactobacillus (Sharpe 1980). The organisms isolated resemble Leuc. dextranicum, because they produce dextran from sucrose and do not ferment arabinose. This observation confirms the findings by Langston & Bouma (1960a, b), who isolated starch-fermenting Leuconostoc from grass silage.

The highest yield of amylolytic enzymes was obtained in a wheat-flour medium during culture at pH 5.8 in a nitrogen-carbon dioxide atmosphere. If the carbon dioxide was eliminated, and only nitrogen used, fermentation ceased. Potassium hydroxide was added to prevent the cells from lysing. If buffering started at pH 5.8, the fermentation ceased for an inexplicable reason. It was found necessary to allow the pH of the culture to drop to 5.2 before raising it to 5.8. Similar observations were made by Boyer & Hartman (1971) for the production of α-amylase and transglucosylase by Streptococcus equinus.

The amylolytic enzyme produced maltose and glucose in a ratio between 5:1 and 6:1. A similar ratio is observed for the non-random attack of Bacillus subtilis amylase on amylose (Greenwood & Milne 1968).

Dialysis against EDTA, known to inactivate microbial α-amylases (Fisher & Stein 1960), did not inhibit the enzyme completely. The enzyme activity was reduced by one-fifth. Although chloride may be required for the activity of amylases from Strep. equinus (Boyer & Hartman 1971), the addition of NaCl had no stimulating effect on enzyme activity. Optimal values of pH and temperature are identical with values found for Strep. hovis and the inability of chloride ions to activate amylase has also been reported for that organism (Walker 1965).

No proteolytic activity was demonstrated in the enzyme fraction when tested on undeveloped photographic film by the method of Jonsson & Martin (1964).

Whether the reported amylolytic activity is due to one or two enzymes is not clear. During fermentation the main activity is found with the cells. During cell lysis the activity in the culture filtrate increases, probably as a result of release of the cell-bound amylase. The existence of one extracellular and one cell-bound α-amylase has also been reported for some starch-hydrolysing streptococci (Walker 1968). Separation on DEAE cellulose and isoelectric focusing gives two peaks. No attempt was made to determine whether these peaks correspond to the extracellular and cell-bound enzymes, respectively.
It is unlikely that the starch-hydrolysing *Leu-
conostoc* would benefit fermented products as an
inoculant. Gas production by the bacterium
causes a volume decrease (CO₂ production),
which is unacceptable during large-scale fish fer-
mentation (Raa 1981).

The technical assistance given by Mr Sven
Tegelström in the pilot-plant fermentation and
by Mrs Ulla Uhlin in the taxonomic work is
greatly appreciated.

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