Formate Production by *Streptococcus thermophilus* Cultures

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**ABSTRACT**

Formate production of three strains of *Streptococcus thermophilus* was studied in whole milk and 1.1.1 medium at 37 and 42°C. Results showed that formate production was dependent on strain, culture medium, and temperature. The kinetics of formate production suggest that production takes place during the late exponential and early stationary growth phases both in milk and 1.1.1 medium.

(Key words: formate, yogurt, *Streptococcus thermophilus*, fermented milks)

**INTRODUCTION**

Formate production by *Streptococcus thermophilus* has been reported nearly 20 yr ago (3, 18). However, this matter has received little attention in spite of its potential importance in the improvement of starter performance. To our knowledge, only data from lactose-limited chemostat cultures (16) and from a whey permeate medium (2) are available.

Recently, preliminary information about formate production in milk and medium cultures of *S. thermophilus* has been presented (9), and the results show that the formate concentration achieved depends on the medium used to grow the cells.

In *S. thermophilus*, sugar metabolism is mainly homolactic (6, 16). However, a small portion of pyruvate is metabolized to formate rather than to lactate. The key enzyme for conversion of pyruvate into formate in *Streptococcus mutans* and *Streptococcus faecium* is pyruvate formate lyase (1, 7, 12), and some authors suggest that this enzyme may be involved in heterolactic fermentation of *Streptococcus lactis* (17) and *Lactobacillus bulgaricus* (11).

Control of pyruvate-formate lyase activity is exerted by intracellular products such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate that strongly inhibit this enzyme (12). Composition of culture medium might then modify intracellular triose phosphates indirectly affecting formate production.

The symbiotic relationship between *S. thermophilus* and *L. bulgaricus* is very important for yogurt and cheese production because it reduces fermentation time (10, 13, 14). Stimulation of lactobacilli is caused mainly by the production of formate by the streptococci, although CO₂ and pyruvate possess stimulatory effects (4, 10, 13). For this reason, the selection of *S. thermophilus* strains on the basis of formate production in milk could be useful to improve starters.

Provided that milk is the natural environment for *S. thermophilus* and taking into account the lack of information for this medium, we have studied formate production in milk and compared it with data corresponding to another culture medium.

**MATERIALS AND METHODS**

Reagents

Tryptone, yeast extract, and agar were obtained from Difco (Detroit, MI). Formate dehydrogenase and β-NAD were from Sigma (St. Louis, MO) and TCA, KHCO₃, and KH₂PO₄ from Mallinkrodt Chemical Works (New York, NY).

Microorganisms and Culture Conditions

*Streptococcus thermophilus* ATCC 19258 and CRL 410 were obtained from Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina), and the CP4 strain was isolated in our laboratory.

Stock cultures maintained in skim milk at 4°C were reactivated overnight at 37 or 42°C.
in whole milk sterilized by UHT process and supplemented with .1% (wt/vol) yeast extract or in 1.1.1 medium (basal medium containing tryptone 1% (wt/vol) and yeast extract 1% (wt/vol), pH = 7, in which a filtered 10% (wt/vol) lactose solution was added to a final concentration of 1% (wt/vol).

After reactivation, strains were inoculated in the supplemented whole milk or in 1.1.1 medium. Incubations were at 37 or 42°C. Viable counts were performed by plating serial dilutions of the cultures (tryptone, .1% wt/vol) in 1.1.1 medium with 1.5% (wt/vol) of agar and incubating them at 37°C during 24 h. For 1.1.1 medium cultures, absorbances at 550 nm were measured in addition to viable counts.

Formate Determinations

Milk samples for formate determinations were deproteinized with 4.5% (wt/vol) TCA and neutralized with solid KHC03 (5, 9). Samples were centrifuged at 1500 × g for 15 min and formate determined in supernatants without deproteinization.

Analyses were performed with formate dehydrogenase (EC 1.2.1.2) according to the method of Hopner and Knappe (5). Composition of reaction mixtures was 20 μl of β-NAD (0.5 M); 160 μl of sample; 800 μl of phosphate buffer (KH2PO4 .5 M, pH = 7), and 20 μl of formate dehydrogenase (20 U/ml). Reactions were made in a quartz cuvette with a 1-cm light path (25°C).

After enzyme addition, absorbance at 340 nm was measured in a Shimadzu Double Beam Spectrophotometer UV-150-02 (Shimadzu Seisakusho Ltd., Kyoto, Jpn.) following the changes through time in a Sargent Recorder Model SRG (Sargent-Welch Scientific Company, Skokie, IL). Maximal absorbance at 340 nm was computed and corrected by subtracting blanks obtained without the enzyme.

Statistical Analysis

Data were arranged in a 3 × 4 factorial design, and analysis of variance was performed. Differences between means were determined using t tests (19).

RESULTS AND DISCUSSION

Figure 1 shows the formate concentrations produced by different strains of S. thermophilus grown under different conditions. The three strains produced more formate in milk than in 1.1.1 medium after 16 h of incubation. In spite of the initial concentration of cells, approximately 2 × 10^6 cfu/ml in 1.1.1 medium and 3 × 10^7 cfu/ml in milk, all cultures were in stationary or death phase after 16 h.

When grown in 1.1.1 medium, the three strains produced more formate at 37 than at 42°C. However, these differences were not significant in milk (P < .01).

The strain ATCC 19258 has the lowest production under all conditions. No formate was detected when this strain was incubated in 1.1.1 medium at 42°C, and average concentrations were 22 μM in the same medium at 37°C. These values are at least 10 times lower than those obtained with CRL 410 and CP4 strains. However, in milk, the ATCC 19258 strain produced only 1.7 times less formate than the others (Figure 1). In 1.1.1 medium, this strain reached lactate concentrations similar to or higher than those with strains CRL 410 and CP4 (data not shown). These results indicated a strong dependence of the formate production pathways with the culture medium. This dependence could be due to some substance needed for the action of the enzyme that catalyzes formate production from pyruvate (pyruvate-formate lyase). Such a substance might be present or produced in milk but not in 1.1.1 medium. It can be also assumed that in 1.1.1 medium, substances inhibiting pyruvate-formate lyase, such as triose phosphates, were not present in milk.
produced or that the synthesis of the enzyme was suppressed (12).

In order to study the yield of formate (micromoles of formate per colony-forming unit), kinetics of growth and formate production were studied for the CP4 strain. In these experiments, the same starting concentration of bacteria was used for all conditions.

Figures 2A and 3A show that, for cultures in milk, the stationary phase was reached after 7 h at 37°C and 4 h at 42°C, and the viable counts remained constant during the next 10 to 12 h. However, for cultures in 1.1.1 medium (Figures 2B and 3B), the stationary phase started after 4 to 6 h, and a decrease in viable counts was observed around 10 h later. After 6 h, cultures in 1.1.1 medium were at the death phase. We found that death of the cells was not due to low pH conditions per se, provided that pH values were lower in milk than in 1.1.1 cultures (Figure 4). After 24 h of incubation, pH in milk cultures was 4.52 ± .01, whereas for the same incubation period in 1.1.1 medium pH was 4.72 ± .01.

However, pH decreased .60 units in 1.1.1 cultures between 7 and 24 h of incubation. For milk cultures, the pH decrease in the same period was only .14 units, so the cells in 1.1.1 medium were subjected to a major stress after exponential phase than those grown in milk. In addition, the sensitivity of cells to suboptimal conditions was not the same in different media. Neutralization of cultures in 1.1.1 medium did not significantly increase cell counts (data not shown). In spite of the viability of the cultures, formate levels in this medium reached the maximal value after 6 h, and maximal formate was maintained until the end of incubation (16 h).
TABLE 1. Yields of formate at different stages of growth of *S. thermophilus* CP4 in 1.1.1 medium or supplemented whole milk at 37 and 42°C.

<table>
<thead>
<tr>
<th>Time (h:min)</th>
<th>Yield (μmol of formate per cfu) × 10⁻¹⁰</th>
<th>1.1.1 Medium</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>...</td>
<td>11.5</td>
<td>6.4</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6:20</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>7</td>
<td>9.0</td>
<td>1.1</td>
<td>...</td>
</tr>
<tr>
<td>7:40</td>
<td>...</td>
<td>14.6</td>
<td>5.8</td>
</tr>
<tr>
<td>8:35</td>
<td>258.0</td>
<td>111</td>
<td>400.0</td>
</tr>
<tr>
<td>16</td>
<td>872.0</td>
<td>812</td>
<td>300.0</td>
</tr>
<tr>
<td>22:40</td>
<td>872.0</td>
<td>812</td>
<td>300.0</td>
</tr>
<tr>
<td>23:50</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

1 When no formate was detected, yields were considered to be zero.

At 37°C, the maximum increase in formate concentration for CP4 strain was 586 μM in 8 h in milk and 88 μM in 1.1.1 medium. For cultures at 42°C, the values were similar, 672 μM in milk and 95 μM in 1.1.1 medium.

Yields obtained for cultures of *S. thermophilus* CP4 are shown in Table 1. For milk cultures, at 37 and 42°C, the micromoles of formate per colony-forming unit reaches values of around 9 × 10⁻¹⁰ after which it remains constant. In contrast, yields increase for cultures in 1.1.1 medium, probably because some cells are dying. Yields at the maximal number of viable counts showed no differences between cultures in 1.1.1 medium at 37 and 42°C. Therefore, the high formate concentrations in milk could be attributed to the large number of cells. Recently, Audet et al. (2) showed that *S. thermophilus* grown in a whey permeate medium with agitation reaches viable counts near 10¹⁰ cfu/ml and formate concentrations around 12 times higher than those we found for milk cultures. The yield obtained by these authors for cultures at the maximal concentration of viable cells is approximately 11 × 10⁻¹⁰ μmol/cfu. This value is comparable with those shown in the present paper for CP4 strain grown in milk and 1.1.1 medium (Table 1).

The comparison of the kinetics of growth with that of formate production showed that the lag phase of the former was three times lower than the second. In addition, production starts when the culture are in exponential phase.

It must be taken into account that the reaction that produces formate from pyruvate...
PFL

pyruvate + CoA \rightarrow acetyl CoA + formate

(where PFL = pyruvate-formate lyase and CoA = coenzyme A) can be used for ATP generation via acetyl CoA synthesis of compounds that require acetyl groups or generation of C1 fragments for synthesis (8, 15).

Therefore, a possible cause of variation of formate production is assumed to be the requirement of acetyl groups or C1 fragments that might vary from one medium to another. Moreover, it can also change at different stages of bacterial growth in the same medium. Furthermore, formate levels detected in S. thermophilus cultures might be dependent upon the extent of liberation of this metabolite to the culture medium.

CONCLUSIONS

Formate production by S. thermophilus is an extremely variable feature that depends on strain, culture medium, and temperature. Maximal formate concentration is detected during the late period of exponential growth and early stationary phase. Provided that lag phase for growth is shorter than lag phase for formate production, some metabolic adaptation appears to be necessary.

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REFERENCES