THE PREPARATION OF GLYCOLYTICALLY ACTIVE
WASHED CELLS OF LACTOBACILLI

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Received for publication March 7, 1947

Lactobacillus casei is a useful organism for microbiologists and biochemists because it can be used in certain microbiological assays. Extensive investigations have been conducted and considerable information has been obtained concerning the nutritional requirements of this homolactic organism by measuring its growth response to various substances. On the other hand, investigations with washed cells of this organism have been surprisingly few in spite of the fact that experimental conditions with such cell preparations can be more readily defined and controlled than in studies conducted with growing organisms in culture media.

That the optimum growth conditions and nutritive requirements for the production of cells of a particular organism vary with the enzyme system to be studied has been well established. Thus, Gale (1940) found that cells of Streptococcus faecalis harvested from a trypsin-digested casein medium with a terminal culture pH below 5.0 possessed an active tyrosine decarboxylase system. Wood and Gunsalus (1942) found that cells of Streptococcus mastitidis grown in a tryptone, yeast extract, low glucose medium harvested before the culture pH had dropped below 6.8 displayed excellent glucose dehydrogenase properties. Bellamy and Gunsalus (1944) subsequently confirmed and extended Gale’s findings, describing a medium, synthetic except for the incorporation of acid-hydrolyzed gelatin and a folic acid concentrate, which supported cells demonstrating good decarboxylation properties. Wood and his associates (1945) used Lactobacillus casei cells grown in a glucose, yeast extract medium to degrade glycogen hydrolyzates to lactic acid. Reithel (1946) prepared a cell-free extract of Streptococcus thermophilus, which fermented lactose and glucose to lactic acid in the presence of adenosine triphosphate (ATP), from cells grown on a medium containing Difco broth, lactose, and peptonized milk. The present study is an attempt to devise a medium which could be used to prepare fairly large amounts of washed cells of lactobacilli (which could be lyophilized or used in the preparation of cell-free extracts) which would actively ferment glucose to lactic acid. No attempt was made to obtain an intimate knowledge of the chemical composition of such a medium.

EXPERIMENTAL PROCEDURES

Organisms. Lactobacillus casei 7469, which is used in our laboratories for routine riboflavin assays, and Lactobacillus arabinosus 17-5, for nicotinic acid assays, were used in these studies.

1 With the technical assistance of Arlene M. Larson.

719
Preparation of cell suspensions. Media of varying composition were generally made up in 600-ml quantities in 1-liter Erlenmeyer flasks; 1.0 per cent inocula were used and the cultures were incubated for 16 to 20 hours at 37 C. After the cells were harvested by centrifugation, they were washed twice with about one-third the culture volume of chilled 0.5 per cent salt solution. The composition of the stock salt solution (used in our riboflavin assays), from which the chilled 0.5 per cent salt solution was prepared, was

\[
\begin{align*}
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 4.0 \text{ per cent} \\
\text{NaCl} & \quad 0.2 \text{ per cent} \\
\text{FeSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.2 \text{ per cent} \\
\text{MnSO}_4 \cdot \text{H}_2\text{O} & \quad 0.2 \text{ per cent}
\end{align*}
\]

After the second washing, the cells were transferred to graduated centrifuge tubes and compacted by centrifugation. The volumes of the packed cells were read and chilled salt solution was added to make 1.0 per cent (by volume) cell suspensions. Kjeldahl N values were determined on aliquots of the 1.0 per cent suspensions.

Tests for glycolytic activity. The activities of cell suspensions from various media were measured in a Warburg apparatus by the usual methods. The flask concentrations and other pertinent experimental conditions were as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.01 M</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.02 M</td>
</tr>
<tr>
<td>Adenosine triphosphate (ATP)</td>
<td>0.01 M</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1.0 per cent of the stock salt solution</td>
</tr>
<tr>
<td>Cells</td>
<td>0.15 per cent by volume</td>
</tr>
<tr>
<td>Total volume</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>CO₂</td>
</tr>
<tr>
<td>pH</td>
<td>ca. 6.2</td>
</tr>
<tr>
<td>Temperature</td>
<td>ca. 37 C ± 0.01 C</td>
</tr>
</tbody>
</table>

Because Gunsalus and Niven (1942) have shown that at pH values above 6.5 homolactic streptococci produce formic acid, acetic acid, and ethanol instead of lactic acid from glucose, the pH of the reaction mixture was kept below this point. The gas evolution was followed for 180 minutes. Under these experimental conditions, the lactic acid produced is measured as carbon dioxide liberated. Preliminary experiments showed that the addition of both ATP and the salt mixture was necessary for the maximum production of lactic acid. From the Kjeldahl N values obtained on aliquots of the 1.0 per cent cell suspensions and the amount of carbon dioxide evolved in the Warburg apparatus, the amount of gas evolved per 0.1 mg N (cells) in 180 minutes was calculated. In this manner the effect of the variations between the densities of the various cell suspensions was obviated.

RESULTS

The medium of Wood and Gunsalus for the "production of active cells of streptococci" was selected as a guide because the streptococci and the lactobacilli are
GLYCOLYTICALLY ACTIVE WASHED CELLS

metabolically related, both being homolactic organisms. Their medium, made up with Difco tryptone, yeast extract, glucose, and K₂HPO₄, was first used in an attempt to grow glycolytically active L. casei cells. The medium supported heavy growths of organisms which displayed excellent dehydrogenase properties. Table 1 shows some of their dehydrogenase properties.

The tube concentrations were: cells (1:300 by volume); methylene blue (2.5 × 10⁻⁴ M); substrate (0.01 M); pH 7.3, K₂HPO₄-NaOH buffer (0.025 M); 0.5 per cent salt solution; and the total volume was 10 ml. The tests were run at 37°C with a stream of nitrogen (purified of oxygen by running it over heated copper) constantly bubbling through the reaction mixture to ensure anaerobic conditions and continued agitation so the cells would not settle out. Under these conditions, the cells were capable of using ethanol, glucose, pyruvic acid, lactic acid, and glycerol, but not formic and succinic acids, as hydrogen donors and methylene blue as the hydrogen acceptors.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>METHYLENE BLUE REDUCTION TIME IN MINUTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>9</td>
</tr>
<tr>
<td>Glucose</td>
<td>39</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>43</td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>74</td>
</tr>
<tr>
<td>Glycerol</td>
<td>90</td>
</tr>
<tr>
<td>Sodium succinate</td>
<td>210</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>235</td>
</tr>
<tr>
<td>Water (control)</td>
<td>200</td>
</tr>
</tbody>
</table>

When these cells were tested in the Warburg apparatus, however, they were only slightly active in the breakdown of glucose to lactic acid. Therefore this medium was modified in attempts to devise one which could be used to grow glycolytically active cells.

*Potassium dibasic phosphate.* The effect of K₂HPO₄ in concentrations of 0.25, and 0.5 per cent was determined in a basal medium containing tryptone (1.0 per cent), yeast extract (1.0 per cent), and glucose (0.1 per cent). The initial pH of the cultures was adjusted to approximately 7.0 as measured by the glass electrode. The results are given in figure 1. As can be seen, the glycolytic properties of the cells were considerably increased by the removal of K₂HPO₄ from the medium. In subsequent experiments, K₂HPO₄ was not added to the medium.

In order to localize this peculiar effect of K₂HPO₄ to either the potassium or phosphate ion, L. casei was grown in a basal medium of tryptone (0.5 per cent), yeast extract (1.0 per cent), and glucose (0.5 per cent); the basal medium plus K₂HPO₄ (0.5 per cent); basal medium plus Na₂HPO₄·12H₂O (1.03 per cent), so that the phosphate concentration would be the same as the medium with the
potassium salt; and basal medium plus KCl (0.43 per cent), so that the potassium concentration would be the same as in the medium with K$_2$HPO$_4$. The initial pH values of the media were adjusted to approximately 7.0. The results are given in figure 2.

As can be seen, both the K$_2$HPO$_4$ and Na$_2$HPO$_4$ adversely affected the glycolytic activity of the cells harvested from media to which they were added. These results were also obtained to an even more marked degree in experiments in which the basal medium was the same as that used for the K$_2$HPO$_4$ concentration effect. It should be noted and emphasized that neither K$_2$HPO$_4$ nor Na$_2$HPO$_4$ affect adversely the degree of growth of the cells in media to which they are added. As a matter of fact, the cell harvests from media with K$_2$HPO$_4$ were sometimes greater than those from the basal medium.

It was thought that possibly this effect might be due to the phosphate-buffering effect. Since the terminal pH values of the cultures containing K$_2$HPO$_4$ and Na$_2$HPO$_4$ were 4.3 as compared to 4.0 and 4.1 for the KCl and the basal medium, respectively, there existed the possibility that lower pH values favored the development of glycolytically active cells, or, conversely, the higher pH conditions adversely affected the production of the glycolytic enzymes. Therefore cells were grown and harvested from media containing tryptone (0.5 per cent), yeast extract (1.0 per cent), and glucose (0.1 per cent), initially adjusted to pH 5.0, 5.5, 6.0, 6.5, and 7.0. The terminal pH values of these cultures were 4.7, 4.6, 4.6, 4.7, and 4.8, respectively. The glucose concentration of 0.1 per cent was used instead of 0.5 per cent because, if lower pH values do favor the development of glycolytic enzymes, there was the possibility that the higher sugar concentration, and thus greater acid production, might cover up the effect of adjusting the initial pH of the cultures to the higher values by rapidly lowering the pH. However, the results shown in figure 3 show that such was not the case. The lower the initial pH of the cultures, the lower were the activities and also the cell harvests.
At present we are unable to account for this phosphate effect. It will be noted that K₃HPO₄ is missing from the media which Wood and his associates used to produce *L. casei* cells to degrade glycogen hydrolyzates to lactic acid.
Glucose. The effect of glucose in 0, 0.1, 0.5, 1.0, and 2.0 per cent concentration on the activity of *L. casei* cells was determined in a basal medium of tryptone (1.0 per cent) and yeast extract (1.0 per cent). The terminal pH values were 5.4, 4.2, 4.0, and 4.0, respectively. The media were initially adjusted to pH 6.8 to 7.1. The results are given in figure 4. Apparently the presence of glucose or the breakdown products of this hexose in the medium is essential for the development of at least one of the enzymes involved in the glycolysis of glucose by
this organism. This might be taken as evidence of the "adaptive" nature of certain of the mechanisms playing roles in glycolysis.

Yeast extract. The effect of 0, 0.5, 1.0, and 2.0 per cent yeast extract on the activity of *L. casei* cells was measured in a basal medium of glucose (0.5 per cent) and tryptone (1.0 per cent). The terminal pH values were 5.4, 4.2, 4.0, and 4.0, respectively. The media were initially adjusted to pH 6.8 to 7.1. The results are given in figure 5. Cells grown and harvested from 0.5 per cent yeast extract media were as active as those obtained from the 2.0 per cent media. However, the cell harvest was greater from media containing at least 1.0 per cent yeast extract so this was considered to be the optimum amount.

Tryptone. The effect on *L. casei* cells of 0, 0.5, 1.0, and 2.0 per cent tryptone was measured in basal medium containing glucose (0.5 per cent) and yeast extract (1.0 per cent). The terminal pH values of the culture were 3.9, 4.0, 4.1, and 4.1, respectively. Initially the media were adjusted to pH 6.8 to 6.9. The results are given in figure 6. As will be noted, the glycolytic activity and the cell harvest curves are practically mirror images of each other. The higher the concentration of tryptone, the greater is the cell harvest but weaker the glycolytic activity. Apparently the glycolytic enzymes are "diluted out" by the larger number of cells. In terms of the maximum amount of glycolytic enzymes per 1.0 liter of medium, 0.5 per cent concentration of tryptone seems to be the most satisfactory. However, medium containing 2.0 per cent tryptone might also be satisfactory depending upon the purposes for which the cells are grown.

*Lactobacillus arabinosus*. The strain of *Lactobacillus arabinosus* 17-5, used routinely in our laboratory for the microbiological assay of nicotinic acid, was also grown in medium containing glucose (0.5 per cent), yeast extract (1.0 per cent...
cent), and tryptone (0.5 per cent), with an initial pH of 7.0. Although the growth was not so luxuriant as that of Lactobacillus casei, fairly active cells were obtained. From 1.0 liter of culture the cell harvest was cells with a Kjeldahl N value of 10.7 mg (as compared to values of approximately 32 mg with L. casei). At the end of 3 hours, the gas evolution ranged from approximately 210 to 250 microliters of carbon dioxide.

Lyophilization. Cells of L. casei harvested from media containing glucose 0.5 per cent), yeast extract (1.0 per cent), and tryptone (0.5 and 2.0 per cent) and dried from the frozen state displayed fair glycolytic properties. The gas evolution per 1.0 mg N of bacterial cells (0.5 ml of a 1.0 per cent lyophilized cell suspension added to a Warburg flask containing a reaction mixture with a total volume of 3.3 ml) in 3 hours varied from 350 to 550 microliters depending on the conditions.

DISCUSSION

The Warburg method of determining the glycolytic activity of lactobacilli may not be so absolutely accurate as the direct estimation of lactic acid. It will be noted that Reithel found some discrepancies between the amount of lactic acid measured directly and indirectly by the manometric technique. However, the method does give a reliable measure of the relative glycolytic activities of homolactic organisms. The ease with which the activities of a number of cell preparations can be estimated is its principal recommendation.

The fact that cells which display excellent dehydrogenase properties do not necessarily possess comparably good glycolytic activity has been demonstrated. Wood and Gunsalus (1942) were not unaware of this possibility, for they were aware of the criticisms offered by Barron and Jacobs (1938), who warned of certain inherent weaknesses of depending solely on the use of the Thunberg method for measuring activity of cells. The evidence presented here does not contradict the work of Wood and Gunsalus. L. casei cells grown in their medium do display excellent dehydrogenase properties. The evidence does seem to indicate, however, that conditions under which cells with good dehydrogenase activity are produced are not the best, at least with some homolactic organisms, for the production of glycolytically active cells.

The effect of K₃HPO₄ was unexpected. As yet, we have no explanation for this peculiar phenomenon.

CONCLUSIONS

Under the experimental conditions described, the data seem to justify the conclusions which follow:

Washed cell suspensions of Lactobacillus casei and Lactobacillus arabinosus which are glycolytically active can be prepared from a medium containing glucose (0.5 per cent), yeast extract (1.0 per cent), and tryptone (0.5 to 2.0 per cent).

Despite the fact that the terminal pH values of the cultures drop as low as 3.9 or 4.0, the cells are still active.
GLYCOLYTICALLY ACTIVE WASHED CELLS

K$_2$HPO$_4$ and Na$_2$HPO$_4$ at 0.5 per cent concentration do not inhibit the growth of the organisms in the culture but do affect adversely the activity of the cells harvested from the phosphate-containing media. The cells harvested from the medium described and dried from the frozen state display fair glycolytic activity.

REFERENCES


