The Change of Glycolytic Intermediates in *myo*-Inositol-Deficient *Saccharomyces carlsbergensis*

**TAKAKO TOMITA, TATSUO OZAWA AND ISAO TOMITA**

*Shizuoka College of Pharmaceutical Sciences*

160 Oshika, Shizuoka (Post No. 420)

[Received January 10, 1969]

The changes of metabolites in the culture medium, the intracellular concentrations of glycolytic intermediates in the actively fermenting yeast cells and the related enzyme activities were studied to establish a possible mechanism of acetoin accumulation in *Saccharomyces carlsbergensis* due to *myo*-inositol deficiency.

During the cultivation of the yeast for five days, the amounts of acetaldehyde and pyruvic acid were found to be low compared with that of acetoin but significantly large amount of acetaldehyde was detected in the *myo*-inositol deficient culture medium.

Specific activity of alcohol dehydrogenase extracted from the *myo*-inositol deficient yeasts cultured for 24 hours, was higher than that from the normal ones and more ethanol was found in the deficient cell extract when glucose was used as the substrate. Though the activity of pyruvate decarboxylase of the deficient yeasts was low, they produced 2-3 times as much acetaldehyde and 10-18 times as much acetoin as the normal yeasts by fermentation of glucose for 15 or 30 minutes. The accumulation of acetaldehyde is considered to be a main cause for the abnormal acetoin production in the deficient *Saccharomyces carlsbergensis*.

In relation with the abnormal accumulation of acetoin in the *myo*-inositol depleted culture medium of *Saccharomyces carlsbergensis* 4228 (ATCC 9080), the following observations have already been reported (1, 2). (a) myo-Inositol deficiency in the yeast led to the increased concentrations of acetaldehyde and glycerol besides acetoin in 214 or 428-hour culture medium (3). (b) The deficient yeast consumed medium glucose in the slower rate and seemed to receive larger respiratory inhibition than the normal one (4). (c) The specific activity of pyruvate decarboxylase (EC 4.1.1.1) (PDC) extracted from the deficient yeast was very low. The activity was found to increase proportionally as the increase of the amount of inositol added to the medium (5).

---

1 The Role of *myo*-Inositol in Metabolic Control. V.

2 窪田多嘉子, 小沢樹夫, 窪 田.
The present paper deals with the changes of the metabolites in the culture medium, the intracellular concentrations of the glycolytic intermediates and the related enzyme activities in the actively fermenting yeast cells due to myo-inositol deficiency. A possible mechanism of abnormal acetoin formation in the deficient yeast was discussed.

METHODS AND MATERIALS

1. Organisms

The maintenance and the inoculation of *Saccharomyces carlsbergensis* 4228 (ATCC 9080) and the composition of the medium employed were as described earlier (6).

2. Measurement of Glycolytic Intermediates in the Culture Medium

The amounts of pyruvate and acetaldehyde in the culture medium during aerobic cultivation of the yeast were determined enzymatically by lactic dehydrogenase (LDH) (7) or alcohol dehydrogenase (ADH) (8) and acetoin was measured by the method of Westerfeld (9). Aliquots of samples were withdrawn aseptically from the cultures for the analysis.

3. PDC and ADH Activities in the Normal and Deficient Yeasts

The yeast cells harvested in 24 hours were dried in vacuum and used for enzyme extraction. PDC was extracted with 0.5 *M* phosphate buffer (pH 7.2) at 37° for 3 hours according to the method of Aoshima (10). Upon the removal of cells, the supernatants were again centrifuged at 8,000 rpm for 15 minutes. The PDC activity of the supernatant was determined manometrically by incubation with 1 × 10⁻³*M* MgSO₄ and 2 × 10⁻³*M* thiamine pyrophosphate in 0.1 *M* citrate buffer (pH 6.0) for 15 minutes, followed by the addition of sodium pyruvate (1/12 *M*) (11). It was also determined by the amounts of acetoin formation (12).

ADH was extracted by incubating the dried cells in 1/15 *M* Na₂HPO₄ at 37° for 3 hours (13). The cell free extracts were heated at 50° for 10 minutes. ADH activity of the supernatant fraction was determined by the average increase in absorbancy of NADH at 340 mµ per minute (14). The reading of absorbancy was taken at 15, 30 and 45 sec. after 0.2 ml of enzyme preparation was added to the reaction mixture of 2.0 ml of 0.001 M phosphate buffer (pH 8.8), 0.5 ml of 2 *M* ethanol and 0.5 ml of 2.5 × 10⁻³*M* NAD at 25°.

4. The Extraction of Glycolytic Intermediates in the Actively Fermenting Yeast Cells

The yeast cells cultivated in complete or the depleted medium for 24 hours were starved by incubating with 3 volumes of 0.1 *M* phosphate buffer (pH 5.0) for 1 hour, followed by reincubation with another portion of the buffer solution for 2 hours at 30° with shaking. The cells were collected by centrifugation. Fermentation was started upon addition of 0.5 ml of *M* glucose solution to the starved yeast cells (70 mg dry cell weight per 2 ml of the buffer) in glass centrifugation tube and carried out at 30° with shaking. In completion of experimental period (0, 15 and 30 min) 5 ml of 7.5% perchloric acid was added to each tube to stop the reaction. The acidified samples were allowed to stand in the incubator with shaking for 30 minutes to ensure complete inactivation of the enzymes which might become
reactivated on neutralization and also to ensure complete extraction of the intermediates. After incubation in the acid, the samples were centrifuged in the cold and the supernatant fluids were neutralized in the cold with a modified solution of trihydroxylamine (TRA) buffer by Estabrook and Maitra (15) (a mixture of 0.5 ml of M TRA and 0.5 ml of 7 M KOH). The supernatants were made up to 10 ml with water after the removal of precipitated potassium perchlorate by centrifugation.

5. Analysis of the Extracts

Glucose in the extracts was determined by the glucose oxidase method of Huggett and Nixon (16). The amount of glucose consumed was figured out by subtracting the amount of glucose in the fermented cell extract from that in the extract at 0 time. The amounts of acetoin, acetaldehyde and pyruvate in the extracts were measured as mentioned above. Alcohol was enzymatically determined (17).

6. CO\textsubscript{2} Evolution and O\textsubscript{2} Uptake

Both O\textsubscript{2} uptake and CO\textsubscript{2} production were measured by the conventional Warburg apparatus at 30°. The cells (1 mg dry cell weight) were suspended in 2 ml of 0.1 M phosphate buffer (pH 5.0) and was incubated with 0.5 ml of M glucose solution as substrate. O\textsubscript{2} uptake was also measured by Yanagimoto Oxygen Consumption Recorder (Model PO-100).

7. Materials

Chemicals were obtained from the following manufacturers: Glucomesser for glucose analysis from Teikoku Zoki Co. Ltd(Tokyo), LDH and ADH from Boehringer, and NAD and NADH from Sigma Chemicals.

RESULTS

1. The Changes in the Composition of the Culture Medium

The results are shown in Fig. 1. In this experimental condition the maximum level of acetoin was observed at 12 hours with the normal (0.12 and 0.10 \(\mu\)mole acetoin per mg of dry cell weight) and at 48 hours with the deficient culture (7.95 \(\mu\)moles acetoin per mg of dry cell weight). The level of acetaldehyde was maximum in the 12 and 48 hour normal culture (0.30 and 0.19 \(\mu\)mole acetaldehyde per mg of dry cell weight) and in the 24 hour deficient culture (1.53 \(\mu\)moles acetaldehyde per mg of dry cell weight), while the level of pyruvate was maximum at 12 hours with the normal (0.50 \(\mu\)mole pyruvic acid per mg of dry cell weight) and at 48 hours with the deficient (0.45 \(\mu\)mole pyruvic acid per mg

FIG. 1 The Changes in Compositions of the Culture Media of Normal and myo-Inositol-Deficient Saccharomyces carlsbergensis during Aerobic Fermentation

Dashed lines show the amounts of the metabolites in the normal culture medium and broken lines the amounts in the deficient culture medium.

\(\bullet\) = \(\bullet\), acetoin; \(\times\) = \(\times\), acetaldehyde; \(\triangle\) = \(\triangle\), pyruvic acid.
of dry cell weight). Though the concentrations of both intermediates were very low as compared with the amount of acetoin in the deficient culture medium, the patterns of acetoin level were similar to that of acetaldehyde in the normal culture and to that of pyruvate in the deficient culture.

2. The Intracellular Concentrations of Glycolytic Intermediates in the Fermenting Yeasts and the Related Enzyme Activities

As given in Table 1, the deficient yeasts consumed slightly more glucose than the normal by 1.48 μmoles/mg per hr. This seemed to result in higher ethanol production of the former (2.81 μmoles ethanol/mg per hr more) so that they supposedly compensated the decreased energy production by 56% decrease of O2 uptake due to the deficiency.

In coincidence with the abnormal accumulation of acetoin, the amount of acetoin in the extract of the deficient yeast cells was 10-18 times that in the normal ones. The concentration of a key metabolite, pyruvate, was quite low in the deficient (1/4-1/5 of that in the normal), but the concentration of the decarboxylated product, acetaldehyde, in the former cells was 2-3 times that in the latter, which would be a main cause for the abnormal production of acetoin as discussed later.

The activity of ADH from the deficient cells was found to be two times that of the normal cells. The activity of PDC responsible for acetoin formation was remarkably decreased by the deficiency. The change of PDC activity of the yeast as a function of myo-inositol concentration in the medium was reported elsewhere (5).

| TABLE 1 | Dissimilation of Glucose by the Normal and the myo-Inositol-Deficient Cell Suspensions and the Activities of the Related Enzymes |
|-----------------|-----------------|-----------------|
| Reaction time (min) | Normal | 15 | 30 | Deficient | 15 | 30 |
| Glucose | μmoles/mg of dry cell weight | -2.45 | -4.80 | -2.59 | -6.00 |
| Pyruvic acid | 0.002 | 0.020 | 0.002 | 0.004 |
| Acetaldehyde | 0.095 | 0.112 | 0.297 | 0.197 |
| Acetoin | 0.002 | 0.003 | 0.024 | 0.054 |
| Ethanol | 2.67 | 5.67 | 3.27 | 7.28 |
| CO2 | 4.68 | 10.46 | 5.06 | 11.12 |
| O2 | 0.45 | -0.9 | -0.2 | -0.4 |

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Normal</th>
<th>0.110</th>
<th>0.221</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDC (μmoles acetoin/mg protein per hr)</td>
<td>1.452</td>
<td>0.480</td>
<td></td>
</tr>
</tbody>
</table>

*Casamino acid R 69 was used.*

**DISCUSSION**

The mechanism of abnormal acetoin formation by the deficient *Saccharomyces carlsbergensis* has not been elucidated sufficiently. The results obtained from the extracts of the actively fermenting yeasts (Table 1) indicate that acetaldehyde was unusually accumulated (besides acetoin) in the deficiency. Assuming that water content
of the packed cells after centrifugation is 47% (18) so that 2.36 μl of water per mg of dry cell weight of the yeast exists, the concentration of pyruvate in the cells would be estimated to be $5.93 \times 10^{-3} M$ (mean value at 15 and 30 min) in the normal cells and $1.27 \times 10^{-3} M$ in the deficient ones, while that of acetaldehyde $4.38 \times 10^{-2} M$ and $10.46 \times 10^{-2} M$ respectively. The acetaldehyde-pyruvic acid ratio is 7.4 in the former and 82.5 in the latter.

It is well established that free acetaldehyde accelerates the rate of pyruvate decarboxylation of pyruvate catabolized by pyruvate dehydrogenase (PDH) (19), serving as a good acceptor of active acetaldehyde for acetoin formation (20). It may thus be assumed that the abnormal accumulation of acetaldehyde is a direct cause for acetoin formation. Holzer et al. (21) have reported that maceration juice of yeast oxidatively decarboxylates pyruvate to acetate via acetaldehyde. The enzyme does not require thiamine pyrophosphate as a cofactor. Yeast mitochondria, on the other hand, contain PDH which requires thiamine pyrophosphate and behaves in the same way as the known animal and bacterial pyruvate dehydrogenase. Acetaldehyde and hydroxyethylthiamine, the intermediate for the above enzyme reaction, would accumulate if respiratory activity was inhibited in the deficient yeast (Note the difference of O₂ uptake between the deficient and normal yeasts).

![Scheme 1](chart.png)

The deficient yeast can therefore produce more acetoin with accumulated acetaldehyde than the normal one though PDC activity is low in the former.

**ACKNOWLEDGEMENT**

It is a pleasure to acknowledge the helpful advice of Dr. C. Kawasaki of Osaka University and Dr. E. Hayashi of this college throughout this study.

**REFERENCES**