Effect of Thiamine on the L-Malate Fermentation by *Schizophyllum commune*¹

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It was found that *Schizophyllum commune* which produced L-malate through CO₂-fixation process with a phosphoenolpyruvate carboxytransphosphorylase-like enzyme required thiamine. Upon the addition of oxythiamine at a concentration of 100 µg/100 ml, both the L-malate production and the glucose consumption were inhibited about 50 %, while the conversion of glucose to L-malate was inhibited only 10 %. The optimum concentration of thiamine to be added in the medium for the L-malate fermentation was 0.5 µg/100 ml. Upon the addition of 100 µg/100 ml thiamine, both the yield of L-malate and the conversion degree of glucose to L-malate were reduced about 50 %, while the glucose consumption was not affected. One of the effects of thiamine appears to be a stimulative action on pyruvate decarboxylation. The optimum amount of thiamine to be added for the growth of the mold seemed to be more than 10 µg/100 ml.

It was previously reported that *Schizophyllum commune* IFO 4928, a Basidiomycete, produced L-malate as much as over 90 % of glucose consumed through CO₂-fixing process (1, 2).

The effect of thiamine on the L-malate fermentation was investigated, since a certain amount of oxythiamine inhibited the growth of the mold. It was proved that there existed an optimum concentration of thiamine to be added in the medium for the L-malate fermentation, which was much lower than the optimum concentration for the growth of the mold.

EXPERIMENTAL

1. Microorganism

Two strains, *Schizophyllum commune* IFO 4928 and IFO 6138 were used. *Schizophyllum commune* IFO 4928 was found to produce mainly L-malate through reductive carboxylation of phosphoenolpyruvate coupled with glyceraldehyde dehydrogenase (3). The main product of the strain IFO 6138 was pyruvate (4). The stock cultures of the strain were maintained on the slants of malt extract agar, or of synthetic medium composed of glucose 3 g, peptone 0.2 g, NH₄NO₃ 0.1 g KH₂PO₄ 0.1 g,

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Abbreviation: PEP, phosphoenolpyruvate; Pi, orthophosphate; OAA, Oxaloacetate; PPI, inorganic pyrophosphate; GAP, glyceraldehyde 3-phosphate; 1, 3-dipGA, 1, 3-diphosphoglycerate.

MgSO₄·7H₂O 0.05 g, KCl 0.05 g, agar 1.5 g and tap water 100 ml.

2. Chemicals

Thiamine-HCl was purchased from Wako Junyaku Co., Ltd. Oxythiamine was a generous gift from Dr. S. Fukui, Kyoto University. Other reagents were of analytical grade.

3. Preparation of Seed Culture

The mold was grown on a solid culture consisting of wheat bran, pine wood-powder and water (1:1:2) for about two weeks at 30°.

4. Fermentation Procedure

The basal medium used was composed of glucose 5 g, NH₄NO₃ 0.1 g, KH₂PO₄ 0.1 g, MgSO₄·7H₂O 0.05 g, KCl 0.05 g, MnSO₄ 0.15 mg, CaCO₃ 5 g and tap water 100 ml. Sixty milliliters of this medium in a 500 ml shaking flask was inoculated with about 0.3 g of the solid culture or three loopfuls of the slant culture, and then cultured on a reciprocal shaker at 28–30°.

5. Analysis

L-Malic acid was determined manometrically using the malic enzyme of *Lactobacillus arabinosus* (5). Pyruvate was measured colorimetrically according to the method of Friedemann and Haugen (6). Residual glucose was determined by the modified method of Bertrand (7). Mycelia were washed, and dried for 60 minutes at 105°.

RESULTS

1. Effect of Oxythiamine

Oxythiamine was employed as an antagonist of thiamine, because it was assumed that the solid culture composed of wheat bran and pine wood-powder should contain an adequate amount of thiamine which might affect on the L-malate fermentation of the strain IFO 4928. The effect of oxythiamine was studied using the basal medium containing the graded amounts of 10–500 µg/100ml of the compound to which the solid seed culture of IFO 4928 strain was inoculated. The inhibitory effect of oxythiamine on the glucose consumption by this strain was so remarkable that the addition of 200 µg/100 ml

TABLE 1
The effect of oxythiamine on the L-malate fermentation by *Schizophyllum commune* IFO 4928^a

Oxythia- mine added	L-Malate produced	per cent ratio	Glucose consumed	per cent ratio	Conver- sion	per cent ratio
µg/100ml	mg/100ml		mg/100ml		per cent	
0	2067 ± 283	100	3562 ± 237	100	57.8	100
50	1405 ± 80	68	2907 ± 242	82	48.4	84
100	1046 ± 36	51	1960 ± 160	55	53.7	93
200	944 ± 19	47	1820 ± 20	51	51.9	90
400	746 ± 162	36	1511 ± 469	42	50.9	88
500	582 ± 80	28	1120 ± 120	31	51.8	89

^a Cultured for 7 days at 30°, using the solid seed culture

of the antagonist resulted in a 50 % suppression, as shown in Table 1. Nevertheless, the conversion degree of glucose to L-malic acid was not so inhibited, suggesting that the L-malate forming process was not affected by the addition.

2. Effect of Thiamine on L-Malate Fermentation

When a few loopfuls of the slant culture was used as a seed in place of the solid seed culture, the growth of the strain was very poor. Both the growth and L-malate fermentation were stimulated by the addition of thiamine in amounts of 10–400 µg/100 ml as shown in Table 2, whereas the conversion rate to L-malate of glucose was somewhat lower than that obtained with the solid seed as shown in Table 1. The addition of much lower concentrations of thiamine was tested in order to increase the yield

TABLE 2
The effect of thiamine on the L-malate fermentation by *Schizophyllum commune* IFO 4928^a

Thiamine added	L-Malate prodced	per cent ratio	Glucose consumed	per cent ratio	Conver- sion	per cent ratio
µg/100ml	mg/100ml		mg/100ml		per cent	
0	38	20	558	13.7	6.8	14
10	1941	100	4090	100	47.5	100
60	1600	83	3652	89	43.9	93
100	1610	83	3754	92	42.8	91
200	1380	71	3630	89	37.9	80
400	1400	72	3620	88	38.8	82

Values are the average of the results obtained with three experiments.

^a Cultured for 8 days at 28°–30°, using the slant seed culture

TABLE 3

The optimum concentration of thiamine added in the medium for L-malate fermentation of *Schizophyllum commune* IFO 4928^a

Thiamine added	L-Malate produced	Glucose consumed	Conversion	per cent ratio
$\mu\text{g}/100\text{ml}$	$\text{mg}/100\text{ml}$	$\text{mg}/100\text{ml}$	per cent	
0	40.1 $\pm$ 27.7	441 $\pm$ 9	9.1	13.1
0.1	2257 $\pm$ 107	3772 $\pm$ 281	59.9	86.5
0.5	2902 $\pm$ 117	4192 $\pm$ 74	69.3	100
1.0	2035 $\pm$ 330	3894 $\pm$ 86	52.1	75.1
10.0	1908 $\pm$ 310	4564 $\pm$ 17	41.7	60.1
50.0	1805 $\pm$ 195	4650 $\pm$ 175	39.5	57.0

^a Cultured for 8 days at 28°–30°, using the slant seed culture

TABLE 4

The effect of thiamine on the pyruvate production by *Schizophyllum commune* IFO 6138^a

Thiamine added	L-Malate produced	Glucose consumed	Conversion	Pyruvate produced	Conversion
$\mu\text{g}/100\text{ml}$	$\text{mg}/100\text{ml}$	$\text{mg}/100\text{ml}$	per cent	$\text{mg}/100\text{ml}$	per cent
0.1	112 $\pm$ 54	1965 $\pm$ 228	5.7	775 $\pm$ 55	39.9
0.5	343 $\pm$ 9	3763 $\pm$ 44	9.1	890 $\pm$ 50	23.6
1.0	310 $\pm$ 39	3479 $\pm$ 149	8.9	715 $\pm$ 25	20.6
10.0	217 $\pm$ 76	4012 $\pm$ 614	5.4	73 $\pm$ 7	1.82
50.0	70	4505	1.5	70	1.55

^a Cultured for 10 days at 28°–30°, using the slant seed culture

of L-malate. As shown in Table 3, the optimum concentration of thiamine to be added for the enhancement of L-malate yield was found to be as little as 0.5 $\mu\text{g}/100\text{ml}$, while a larger amount of thiamine was preferable for the glucose consumption of this strain. It was, therefore, assumed that the stimulative effect of thiamine on the glucose consumption would be attributable to its stimulative effect on the oxidative decarboxylation of pyruvate. Accordingly, *Schizophyllum commune* IFO 6138 which produced much more pyruvate rather than L-malate was employed to clarify the function of thiamine. As shown in Table 4, the amount of pyruvate was markedly decreased upon the addition of 10 $\mu\text{g}/100\text{ml}$ of thiamine, while the L-malate production was not so reduced. Consequently, it was suggested that the pyruvate dehydrogenation would be accelerated by the addition of thiamine, causing the stimulation of glucose consumption.

TABLE 5

The effect of thiamine on the growth of *Schizophyllum commune* IFO 4928

Thiamine added	pH	Mycelial weight	Glucose consumed	Conversion	L-Malate produced
$\mu\text{g}/100\text{ml}$		$\text{mg}/100\text{ml}$	$\text{mg}/100\text{ml}$	per cent	
0	3.9	62	—	—	trace
0.5	4.1	195 $\pm$ 6	501 $\pm$ 20	38.8	trace
10.0	4.1	245 $\pm$ 14	608 $\pm$ 86	40.9	trace
50.0	3.9	250 $\pm$ 3	811 $\pm$ 50	30.4	trace
0	2.2	17	—	—	trace
50	2.3	139 $\pm$ 9	915 $\pm$ 54	15.2	trace
500	2.3	158 $\pm$ 48	1482 $\pm$ 308	10.6	trace
1000	2.4	117 $\pm$ 10	1173 $\pm$ 250	10.2	trace

^A Cultured for 7 days at 28°–30°, using the slant seed culture

^B Cultured for 8 days at 28°–30°, using the slant seed culture

3. Effect of Thiamine on Growth

By using a CaCO_3 -free medium, it was shown that the effect of thiamine on the growth of *Schizophyllum commune* IFO 4928 was not comparable to the effect on the L-malate production, as shown in Table 5. The optimum amount, 0.5 $\mu\text{g}/100\text{ml}$ for L-malate production would not be sufficient for the optimum growth, which may require more than 50 $\mu\text{g}/100\text{ml}$. Moreover, it was proved that no significant inhibition of growth was observed upon the addition of as much as 1 $\text{mg}/100\text{ml}$ thiamine.

DISCUSSION

In our preceding papers (2, 3), the main process of the L-malate fermentation of *Schizophyllum commune* was proposed as follows:

Equation (1) is catalyzed by a phosphoenolpyruvate carboxytransphosphorylase-like enzyme. Equation (2) shows a malic dehydrogenase reaction coupled with glyceraldehyde dehydrogenase linked by NAD. The larger part of the amount of L-malate which was produced under the addition of 0.5 $\mu\text{g}/100\text{ml}$ thiamine would be formed from phosphoenolpyruvate via the above-mentioned two reactions, while the smaller part of the amount which was diminished under the

addition of 1 μg /100 ml thiamine would be formed from pyruvate by a pyruvate carboxylase.

Moreover, it seemed reasonable to assume that the more decrease of L-malate production upon the addition of over 10 μg /100 ml thiamine would be attributed to the more acceleration of pyruvate dehydrogenation which might cause a stimulation of pyruvate kinase followed by an impediment of phosphoenolpyruvate carboxylation.

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