[Chem. Pharm. Bull. 17(11)2217—2222(1969)]

UDC 576.8.095.3 : 547.593.09

The Role of Myoinositol in Metabolic Control. II. The Effects of Medium Constituents and Other Factors on Acetoin Accumulation in *Saccharomyces carlsbergensis* 4228 (ATCC 9080)[1]

Tatsuo Ozawa, Isao Tomita
and Takako Tomita

Shizuoka College of Pharmaceutical Sciences[2]

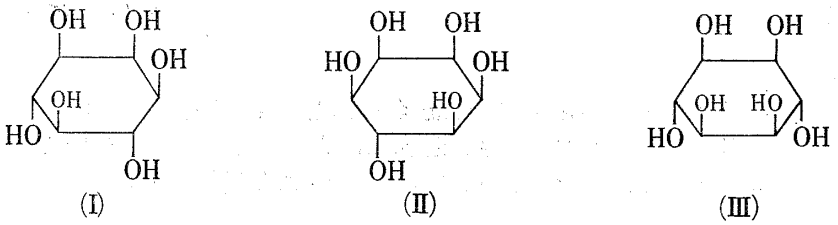
(Received November 21, 1968)

The accumulation of acetoin in the culture medium of *Saccharomyces carlsbergensis* was specific with m-I deficiency. Its concentration in 48 hour culture medium reached to 8 μmoles/mg of dry cell and the ratio of acetoin production by the m-I deficient yeast to that by the normal yeast (m-I supplemented) was found to be 100—1000 in 48 hour cultivation in one of four media tested. Casein hydrolysate used as nitrogen source in the medium, changed this ratio greatly by their lots. pH during the cultivation changed slightly (between 4.5 and 5.0) and it gave no significant effects on acetoin accumulation. Addition of increased amount of ammonium sulfate to the medium gave an adverse effect on the growth of m-I deficient yeast and accelerated the acetoin accumulation. While the addition of cofactors for pyruvate oxidation such as lipoic acid, had no reducing effect for acetoin production, the addition of thiamine was critical for it in m-I deficient *Saccharomyces carlsbergensis*.

The biochemical functions of *myo*-inositol (m-I) (I) are still obscure though it is known as an essential factor for the growth of microorganisms,[3] some higher animals[4] and for human cells in tissue culture.[5] Lewin has reported that m-I deficiency in *Saccharomyces carlsbergensis* 4228 (ATCC 9080) caused abnormal accumulation of acetoin, acetaldehyde and glycerol in culture medium after incubation for 214 hours[6] and the increase of triglycerides and the decrease of the m-I containing phospholipids in the same organism.[7] Though this yeast requires the addition of m-I to the medium for its normal growth, it does synthesize m-I to a certain amount gradually so that the growth of the deficient yeast recovers to the normal level as the cultivation proceeds. The respiratory inhibition due to m-I deficiency[8,9] has thus been observed with 14, 17 and 30 hour grown cells, and the less inhibition has been noted with 48 hour grown cells. The effect of m-I deficiency in this yeast should naturally be more clearly revealed in the experiment of shorter period than that of longer period. In our experimental condition using culture medium 4 (See experimental part) the growth of m-I deficient yeast was depressed to 1/3 in 48 hour culture and a dramatic accumulation of acetoin was observed.

Since this acetoin formation was found to be very much dependent on the deficiency of m-I and the other stereoisomers such as *epi*- (II) or *muco*-inositol (III), could not recover the abnormal phenomenon, a study of the mechanism of acetoin formation in this yeast would be helpful to reveal the biochemical function of m-I.

1) Presented at The Tokai Branch Meeting of Pharmaceutical Society of Japan, Shizuoka, September, 1967.
2) Location: *160 Oshika, Shizuoka, 420, Japan.*
3) E.V. Eastcott, *J. Phys. Chem.*, **32**, 1094 (1928).
4) D.W. Wooley, *J. Biol. Chem.*, **139**, 29 (1941).
5) H. Eagle, V.I. Oyama, M. Levy and A.E. Freeman, *J. Biol. Chem.*, **826**, 191 (1957).
6) L.M. Lewin, *J. Gen. Microbiol.*, **41**, 215 (1965).
7) T. Shafai and L.M. Lewin, *Biochim. Biophys. Acta.*, **152**, 787 (1968).
8) G.J. Ridgway and H.C. Douglas, *J. Bacteriol.*, **76**, 163 (1958).
9) A. Ghosh and S.N. Bhattacharyya, *Biochim. Biophys. Acta.*, **136**, 19 (1967).



(I) *myo*-inositol (II) *epi*-inositol (III) *muco*-inositol

Chart 1

In the present paper, the effects of the composition of basal medium, thiamine and other several cofactors for pyruvate decarboxylase (PDC) (E.C. 4.1.1.1.) or pyruvate dehydrogenase (PDH) (E.C. 1.2.4.1.) on the acetoin formation are presented.

Experimental

Organism——*Saccharomyces carlsbergensis* strain 4228 (ATCC 9080) was maintained on Nissui (Tokyo) malt agar slant at 4° and cultivated at 30° for 20 hours before use.

Medium——The composition of four liquid media designated medium 1,[10] Medium 2,[11] medium 3,[12] and medium 4,[13] are shown in Table I. 2.5 mg of m-I/100 ml of medium was added for m-I supplemented medium and no m-I for m-I depleted medium. pHs of these media were adjusted to 5.0 except in the experiment of testing pH dependency of acetoin formation. An appropriate amount of vitamins, ammonium sulfate or other cofactors were added to medium before medium was sterilized by autoclaving at 10 lb for 10 minutes.

Table I. The Composition of the Media (Double Strength)

Medium No.	1	2	3	4
Glucose, g	4.0	10.0	10.0	10.0
Potassium citrate, g	1.0	1.0	1.0	1.0
Citric acid, g	0.2	0.2	0.2	0.2
Casein hydrolysate, g	0.8	0.8	0.8	1.0
Ammonium sulfate, g	0.38	—	—	0.75
KH_2PO_4, mg	120	110	100	100
KCl, mg	80	86	80	80
$MgSO_4$, mg	25	26	25	26
$FeCl_3$, mg	0.5	0.5	5	0.5
$MnSO_4$, mg	0.5	0.5	5	0.5
L-Tryptophan, mg	1.46	—	(DL)8	—
$CaCl_2 \cdot 2H_2O$, mg	—	26	25	26
Calcium pantothenate, mg	0.7	0.5	0.5	0.5
Pyridoxine, mg	0.05	0.05	0.1	0.05
Thiamine, mg	0.05	0.05	0.05	0.05
Biotin, mg	0.006	0.0016	0.0016	0.005
Nicotinic acid, mg	—	0.5	—	0.12
m-Inositol, mg	±5.0	±5.0	±5.0	±5.0

Growth Conditions——0.5 ml suspension of cells (Turbidity at 610 mμ was adjusted to 1.0 in Shimazu Spectronic 20) which was cultivated statically in the complete medium at 30° for 20 hours was used to inoculate each 100 ml of medium. Following the inoculation, 50 or 100 ml Erlenmyer flasks containing 20 or 40 ml medium were incubated at 30° with shaking by TAIYO Incubator K-II and the reaction was stopped by placing flasks in ice water at appropriate time.

10) A. Ghosh, F. Charalampous, Y. Sison and R. Borer, *J. Biol. Chem.*, 235, 2522 (1960).
11) L. Atkin *et al.*, *Ind. Eng. Chem.*, 15, 141 (1943).
12) Difco Microbiological Assay of Vitamins and Amino Acids (U.S.A.) p. 36.
13) L. Atkin, W.L. Williams, A.S. Schultz and C.N. Frey, Unpublished Method (1944).

Analytical Method——The growth rate of the yeast was followed turbidimetrically at 610 mμ (by using Spectronic 20) and the concentration of acetoin in culture medium was determined by the method of Westerfeld.[14] 500 ml Erlenmeyer flasks containing 250 ml of medium were used for incubation of longer period and samples were periodically taken from cultures for analysis.

Materials: m-I and other coenzymes were purchased from Wako Pure Chemicals. Vitamin free casamino acid was from Nissui Seiyaku Co. Ltd. *epi-* and *muco-* inositol were kindly donated by professor T. Suami of Keio University.

Results

The Effects of Medium Composition

Fig. 1 shows the growth response curves of *Saccharomyces carlsbergensis* incubated for 5 days in four different media either with or without m-I. The growth of the yeast in medium 1 (m-I supplemented) was approximately a half of that in the three others, where the yeast grew at almost the same rate. The low growth of the yeast in medium 1 will be ascribed to its low glucose concentration (2%) in comparison to the three others (5%). The deprivation of m-I from medium resulted in the growth inhibition with different rates; about 2/3, 1/7, 1/3 and 1/5 of normal growths in medium 1, 2, 3 and 4 respectively in 24 hour cultivation. The depressed growth in the absence of m-I was recovered gradually in medium 2 and 3 (almost half of the normal growth was achieved in 48 hours)while no growth recovery was observed after 24 hours in medium 4 where the growth inhibition by m-I deficiency was the strongest.

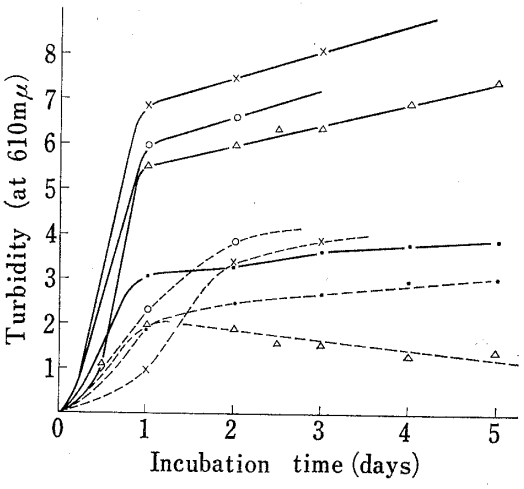


Fig. 1. Growth Response Curves of *Saccharomyces carlsbergensis* on Various Media with or without *m*-Inositol Supplementation

Dashed lines indicate growth on complete media and dotted lines indicate growth on m-I depleted media.
●-----●, ●——●: growth on medium 1
×-----×, ×——×: growth on medium 2
○-----○, ○——○: growth on medium 3
△-----△, △——△: growth on medium 4
The composition of the above media is shown in Table I.

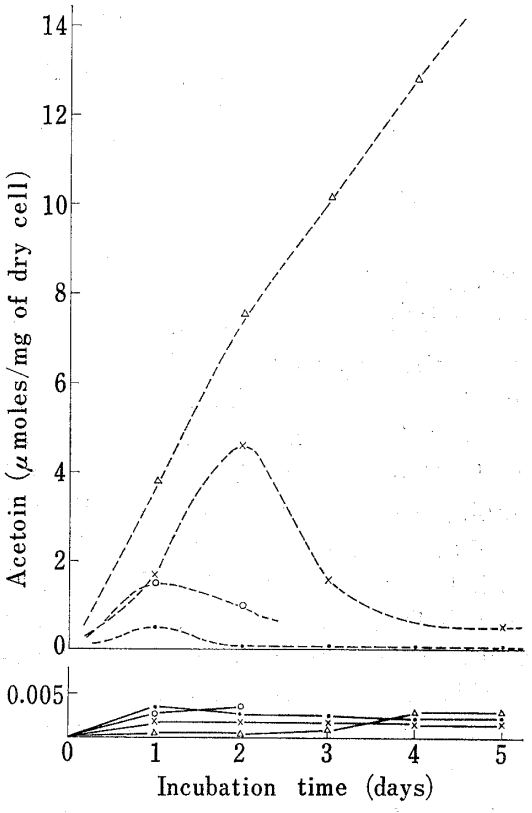


Fig. 2. Acetoin Levels in Various Culture Media during Incubation

Dashed lines indicate the amounts of acetoin found in complete culture media and dotted lines indicate those in m-I depleted culture media.
●-----●, ●——●: acetoin in medium 1
×-----×, ×——×: acetoin in medium 2
○-----○, ○——○: acetoin in medium 3
△-----△, △——△: acetoin in medium 4

14) W.W. Westerfeld, *J. Biol. Chem.*, **161**, 495 (1945).

The deprivation of m-I gave a most remarkable change in the concentration of acetoin in the medium. The level of acetoin was normally in the range of 0.004—0.08 μmole/mg of dry cell in 48 hour cultivation but an astonishing rise of its level was seen in the absence of m-I. The various patterns of acetoin accumulation observed in the four culture media are shown in Fig. 2. The lowest acetoin accumulation was observed in medium 1 and it may be related to its lowest growth inhibition. In medium 2 and 3, a maximum acetoin level (1.540 μmoles and 4.16 μmoles/mg of dry cell) appeared after 24 and 48 hours respectively. In medium 4, on the contrary, the level of acetoin increased steadily, and 3.82 and 8.26 μmoles acetoin/mg of dry cell were detected after 24 and 48 hours respectively. It must be noted that the concentration of ammonium sulfate in medium 4 is the main difference from the composition of medium 2.

The maximum ratios of aceotin accumulation in m-I deficient to m-I supplemented culture medium were 12.7 (medium 1), 242.6 (medium 2), 45.3 (medium 3) and 1032.5 (medium 4 in 48 hours) respectively and the growth inhibition was also the highest in medium 4.

Table II. The Effect of Medium pH on Acetoin Accumulation by *Saccharomyces carlsbergensis*

Medium pH	Complete medium		m-I depleted medium		Acetoin ratio (depleted/complete)
	Growth (turbidity at 610 mμ)	Acetoin (μmoles/mg of dry cell)	Growth (turbidity at 610 mμ)	Acetoin (μmoles/mg of dry cell)	
4.0	5.22	0.017	1.20	8.15	478
4.5	5.10	0.016	1.11	6.94	434
5.0	5.40	0.005	1.18	7.75	1552
5.5	5.34	0.005	1.59	6.15	1230
6.0	5.10	0.005	1.38	7.00	1400

The Effect of pH

The adaptive formation of enzyme system converting pyruvic acid to acetoin is known to be strongly influenced by the change of pH in *Bacillus cereus*.[15] To see the effect of pH on the growth and the amount of acetoin, pH of the medium was adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 with NaOH or H_3PO_4 before inoculation. As it is shown in Table II, both the growth of the yeast and the acetoin level were not significantly affected by this range of pH change. The maximum ratio of acetoin accumulation was 1552 at pH 5.0.

The Effects of Various Factors

Ammonium Sulfate——The effect of ammonium sulfate on acetoin production was suggested by the higher acetoin accumulation in medium 4 than in medium 2. The addition of ammonium sulfate (375 mg/100 ml medium) to m-I depleted medium 2 which

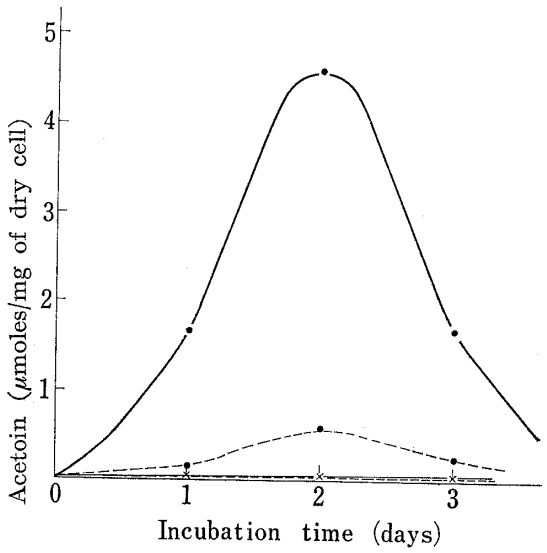


Fig. 3. The Effect of Thiamine on Acetoin Accumulation

Dashed lines indicate acetoin levels in thiamine supplemented culture media and dotted lines indicate those in culture media without thiamine supplementation.

×-----×, ×——× : acetoin in culture media with m-I

●-----●, ●——● : acetoin in culture media without m-I

15) L.A. Kominek, and H.O. Halvorson, *J. Bacteriol.*, **90**, 1251 (1965).

made the concentration of ammonium sulfate in medium 2 to the same in medium 4, in fact accelerated the growth inhibition and acetoin accumulation (3.3 times of that found in original medium 2).

Thiamine——Deprivation of thiamine from medium did not cause any effect both on the growth of the yeast and on the acetoin level in the culture medium during 72 hour cultivation as far as m-I was present. Under the absence of m-I in medium, however, its deprivation brought the acetoin level down to the same level as in m-I supplemented medium (Fig. 3). It is interesting to note that the addition of thiamine to m-I depleted medium depressed the yeast growth (22.5% at 48 hours and 30.2% at 72 hours) as shown in Table III.

Table III. The Effect of Thiamine on Growth and Acetoin Accumulation

Incubation time (days)	Type of medium m-I	Thiamine supplementation: None Growth[a]	None Acetoin[b]	Thiamine 25 μg/100 ml Growth[a]	Thiamine 25 μg/100 ml Acetoin[b]
1	supplemented	6.72	0.01	6.88	0.02
	depleted	1.03	0.13	1.01	1.70
	acetoin ratio		13		85
2	supplemented	7.68	0.02	7.45	0.02
	depleted	4.38	0.63	3.40	4.61
	acetoin ratio		32		231
3	supplemented	8.03	0.02	8.16	0.02
	depleted	5.58	0.29	3.90	1.70
	acetoin ratio		15		85

a) turbidity at 610 mμ *b*) μmoles/mg of dry cell

Lipoic Acid——The addition of lipoic acid to m-I depleted medium (500 μg/100 ml) resulted in 14% increase in growth and 24.2% decrease in acetoin level (Table IV).

Table IV. The Effect of Lipoic Acid on Acetoin Accumulation by *Saccharomyces carlsbergensis*

Lipoic acid supplemented		m-I Supplemented	m-I Depleted	Acetoin ratio
None	growth (turbidity at 610 mμ)	5.93	2.37	
	acetoin (μmoles/mg of dry cell)	0.03	3.56	116
500 μg/100 ml	growth (turbidity at 610 mμ)	4.95	2.70	
	acetoin (μmoles/mg of dry cell)	0.04	2.70	69

(48 hr incubation)

Discussion

Homotype lactic acid bacteria such as *Streptococcus liquefaciencs* or *Streptococcus faecalis* are known to produce acetoin besides their fermentative product, lactic acid. The amount of acetoin produced by these bacteria are influenced largely by the conditions of cultivation and as high as 40% of glucose in the medium could be converted to acetoin in aerobic cultivation with mechanical stirring.[16] The abnormal accumulation of acetoin by *Saccharomyces carlsbergensis* mentioned above, would probably be shown as the result of "disturbed" fermentation due to m-I deficiency, so that the examination of fundamental technical methods was necessary to establish the reproducibility of this abnormality.

Among four inoculums taken at different stages of cultivation, 18 hour growth in m-I supplemented medium gave the highest acetoin formation and as to the methods of cultivation, stirring the yeast aerobically in Erlenmeyer flasks was most effective. The amount

16) A. Oubayashi, A. Oui and K. Kitahara, *J. Agr. Biochem.*, **34**, 272 (1960).

and cell concentration of inoculumn scarcely affected the production of acetoin, while the composition of basal media (1—4) exerted a large effect on it. As it can be seen in Fig. 2, maximum accumulation of acetoin was observed in 24 hours (in medium 1 and 3) and 48 hours (in medium 2) cultivation. The presence of maximum suggests that acetoin accumulated in the culture medium began to be consumed and new production of acetoin decreased presumably by the appearance of m-I synthesized gradually in the yeast cells. The continuous accumulation of acetoin in medium 4, on the other hand, means the difficulty of m-I synthesis by the yeast in this medium and hence it would be suitable for studying the abnormal metabolism of the yeast due to m-I deficiency. The constitutional difference between medium 2 and 4 is the presence of ammonium sulfate in the latter. The importance of ammonium sulfate is evident by the fact that the addition of ammonium sulfate to medium apparently depressed the growth of the yeast and increased acetoin level.

It has been reported[17] that the specific activity of PDC was as much as four times of the originals' upon ammonium sulfate addition to the medium in *Saccharomyces cerevisiae*. The presence of ammonium sulfate in medium 4 might have stimulated the synthesis of PDC and thus increased acetoin level.

Acid hydrolyzed casamino acid used as nitrogen source at 1% concentration of medium resulted in greatly different responses to acetoin accumulation by their lots. Though the variation in growth is very little, acetoin levels varied between 0.008—0.231 μmoles/mg of dry cell in m-I supplemented media and between 2.5—9.7 μmoles/mg of dry cell in m-I depleted media. Among six lots tested, casamino acid NR 1219 gave the lowest ratio of acetoin accumulation (10.7), while R 69 gave the highest value (1000). Preliminary experiment showed that PDC activity of yeast cells was also very much dependent on casamino acid used and the yeast which was cultivated with NR 1219 showed lower activity than that with R 69.[18,19]

The effects of coenzymes, especially thiamine upon the yeast growth and acetoin accumulation must be noted. Though the growth was not significantly affected by thiamine in the presence of m-I, it was apparently inhibited by thiamine in the absence of m-I. A similar growth inhibition by thiamine has been reported to occur in the same yeast when the amount of pantothenic acid was limited in the medium,[20] and in *Saccharomyces cerevisiae* when sorbic acid was present in the medium.[21] The growth inhibition by thiamine in either m-I or pantothenic acid deficiency would be related each other since CoA (therefore pantothenic acid) contents of m-I deficient yeast was reported to be low compared to normal yeast.[8]

As to the formation of acetoin, the addition of thiamine to medium is essential (See Fig. 3). The necessity of thiamine would be explained by assuming that thiamine synthesized *in vivo* by the yeast is only enough for the normal action of PDC or PDH, namely acetaldehyde or acetyl Co A production, and extra thiamine may be required for carboligase action of this enzyme. It can alternatively explained by the assumption that excess thiamine may help the reduction of CoA level by the inhibition of biosynthesis of CoA from pantothenic acid.[21]

The limited contents of NAD in m-I deficient yeast[8] would also lead to acetaldehyde accumulation and assist the abnormal acetoin synthesis though the external addition of NAD to m-I depleted medium gave almost negligible effects upon both growth of the yeast and acetoin production.

Acknowledgement The authors express their gratitude to Dr C. Kawasaki of Osaka University for his encouragement and helpful suggestion and to Dr T. Suami of Keio University for his kind supply of m-I isomers.

17) I. Witt and L. Heilmeyer, *Biochim. Biophys. Research. Comm.*, **25**, 340 (1966).
18) T. Ozawa, I. Tomita and T. Tomita, Presented at The 88th Annual Meeting of Japan Pharmaceutical Society at Tokyo (1968).
19) T. Tomita, T. Ozawa and I. Tomita, *J. Biochem.*, **65**, 829 (1969).
20) K. Oshiba and H. Kawakita, *Vitamins*, **33**, 47 (1966).
21) K. Harada, K. Higuchi and I. Utsumi, *Vitamins*, **38**, 453 (1968).