Enzymatic Studies on the Conversion of Erythritol Fermentation to D-Mannitol Fermentation in *n*-Alkane-grown Yeast *Candida zeylanoides*

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In the polyol fermentation by *Candida zeylanoides* KY6166, which occurred preferentially by keeping the pH of medium at acidic side (below 4.0), phosphate ion played a precise role in the conversion of erythritol fermentation to D-mannitol fermentation. Enzymatic studies on the conversion mechanism provided the following evidences.

The enzymes involved in pentosephosphate cycle were considerably depressed in polyol production phase in which intracellular pH ranged from 5.5 to 5.7. Particularly transaldolase responsible for the synthesis of erythrose 4-phosphate and fructose 6-phosphate from glyceraldehyde 3-phosphate plus D-sedoheptulose 7-phosphate was significantly depressed at pH 5.5. Besides, transketolase which participated directly in the formation of erythrose 4-phosphate from fructose 6-phosphate was significantly inhibited by phosphate ion. Glucose 6-phosphate dehydrogenase was slightly inhibited by phosphate ion.

From these results, the alteration from erythritol fermentation to mannitol fermentation by phosphate ion was explained as the result of the change in the level of erythrose 4-phosphate and fructose 6-phosphate which was caused by the inhibition of transketolase.

In the enzymatic study on polyol production by citric acid producer,⁴⁾ *Candida zeylanoides* KY6166, the primary cause of the conversion of citric acid fermentation to erythritol fermentation was explained by the depression citrate synthase and the activation of phosphoenolpyruvate carboxykinase by the change of intracellular pH from 6.7 to 5.5 according to the change of the pH of the medium from 5.5 to 3.5.

On the other hand, under the established condition for polyol production, the shift of phosphate ion concentration in the culture medium led to the conversion of erythritol production to mannitol production.²⁾

This phenomenon has not been observed in the production of polyols from carbohydrate sources. Thus, it was of great interest to investigate the enzymatic relations underlying the conversion mechanism.

This paper deals with the effect of pH and phosphate ion on the activities of enzymes involved in pentosephosphate cycle which

closely related with polyol interconversion.

MATERIALS AND METHODS

Microorganisms. *Candida zeylanoides* KY6166 was employed throughout this study. Other strains were also employed partly to compare with above organism. All the strains were kindly supplied by Mr. Takayama *et al.* in this laboratory.

Media and culture conditions. The composition of the medium was as follows; *n*-alkane mixture (C-12 to C-15), 10% (w/v); KH_2PO_4 , 0.03%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; ammonium sulfate, 0.5%; thiamine-HCl, 1mg/liter; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg/liter; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg/liter; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg /liter; in tap water. The pH was adjusted to 5.0 before sterilizing. The yeast was grown in a 5-liter fermentors containing 3 liters of the culture medium with agitation at 600 rpm and aeration of 3 liters per minute at 30°C. In each run, the inoculum (300 ml) was cultured in the seed medium for 30 hr at 30°C on a rotary shaker (220 rpm), which was composed of *n*-alkane mixture (C-12 to C-15), 5% (w/v); KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; ammonium sulfate, 0.5%; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2mg/liter; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg/liter; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg/liter; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg /liter; thiamine-HCl 1 mg/

liter; and tap water. During the fermentation, the medium-pH was automatically controlled with 28% ammonia water.

Analytical methods. Citric acid and *d*-isocitric acid were assayed by the method described in the previous report.²⁾ Polyol was also analyzed with the aqueous phase obtained by centrifugation of the culture broth by the method described in the previous report.¹⁾ Determination of phosphate was performed by the method of Chen *et al.*³⁾

Measurement of intracellular pH of yeast cells. Intracellular pH of yeast cells were determined by the method of Conway and Downey described in the previous report.⁵⁾

Preparation of cell free extracts. All the operations were carried out at 0 to 4°C. The cultured broth was centrifuged at $3,000 \times g$ for 20 min. The cells existed both in the precipitate and in the residual *n*-alkane phase were collected on a filter paper to remove the residual *n*-alkane as much as possible and then washed three times with 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM of mercaptoethanol. Washed cells were disintegrated by a ultrasonic generator KMS-250 (Kubota Seisakusho, Japan) at 0°C for 30 min at 10 kc. Cell homogenate was ultracentrifuged at $25,000 \times g$ for 40 min at 0°C. The clear middle phase was collected carefully with a syringe. The cell free extracts thus obtained were used as the enzyme sources without further purification.

Enzyme assays. All spectrophotometric determinations were performed at room temperature in quartz cuvettes of 1-cm light path with a Hitachi spectrophotometer (139 type). The reduction of D-erythrose or D-fructose was measured by the method described in the previous report.⁵⁾ The determination of transaldolase activity was performed by measuring the formation of glyceraldehyde 3-phosphate from fructose 6-phosphate and erythrose 4-phosphate. Glyceraldehyde 3-phosphate is determined by the combined action of triose phosphate isomerase and α -glycerophosphate dehydrogenase.⁶⁾ The reaction mixture contained in a total volume of 1 ml: 25 μ moles of Tris-maleate buffer (pH 7.0); 0.5 μ mole of fructose 6-phosphate; 0.5 μ mole of erythrose 4-phosphate; 0.12 μ mole of NADH; 20 μ g of mixed crystals of triosephosphate isomerase and α -glycerophosphate dehydrogenase and the enzyme to be tested. One unit of transaldolase is defined as the enzyme amount which catalyzes the decrease of 1 μ mole of substrate per minute. Phosphogluconate dehydrogenase activity and glucose 6-phosphate dehydrogenase activity were assayed according to the methods of Shimamura *et al.*⁷⁾ and, of Brown and Wittenberger,⁸⁾ respectively. One unit of

the enzymes was defined as the enzyme amount which catalyzes the increase in optical density (A_{340}) of 0.0 per minute. Ribulosephosphate 3-epimerase activity was assayed by the method of Huritz and Horecker.⁹⁾ One unit of the enzyme activity was defined as the enzyme amount which catalyzes the formation of 1 μ mole of ribulose 5-phosphate. Ribosephosphate isomerase activity was measured according to the method of Volk.¹⁰⁾ One unit of enzyme was defined as the conversion of 1 μ mole of D-ribose 5-phosphate to D-ribulose 5-phosphate in 1 min.

Glucosephosphate isomerase activity was estimated by measuring the formation of the fructose 6-phosphate from glucose 6-phosphate according to the method of Reithel.¹¹⁾ One unit of enzyme was defined as the amount of the enzyme which catalyzes the formation of 1.0 μ mole of fructose 6-phosphate per minute. Transketolase activity was measured by the method of Datt and Racker.¹²⁾ The assay mixture was made up as follows; 50 μ moles of Tris-HCl buffer (pH 7.5), 2.5 μ moles of xylulose 5-phosphate, 3.5 μ moles of ribose 5-phosphate, 0.2 μ mole of thiamine pyrophosphate, 2.0 μ moles of $MgCl_2$ and enzyme preparation (0.1 ml) to a final volume of 1.0 ml. After incubation at 30°C for 10 min, 0.1 ml of 50% trichloroacetic acid solution was added to stop the reaction. Sedoheptulose 7-phosphate was assayed with 0.1 ml of the supernatant according to the method of Dische.¹³⁾

One unit of enzyme was defined as the amount of enzyme which catalyzes the formation of 1.0 μ mole of sedoheptulose 7-phosphate per 10 min. Specific activities of the all enzymes were expressed as units per milligram of enzyme.

Determination of protein. Protein was determined by the method of Lowry *et al.*¹⁴⁾

Chemicals. D-Erythrose 4-phosphate, D-xylulose 5-phosphate, D-glucose 6-phosphate, D-erythrose, D-fructose, D-sedoheptulose 7-phosphate, disodium 6-phosphogluconate, NAD, NADP, NADH and NADPH were all purchased from Sigma Chemical Corp., U.S.A. All other chemicals were of analytical reagent grade.

RESULTS AND DISCUSSION

Identification of reduction products of D-erythrose and D-fructose by cell-free extracts

In order to ascertain that erythritol or D-mannitol is formed by the reduction of erythrose or fructose, the following reactions were carried out. The reaction mixture containing sugars (20 μ moles); Tris-HCl buffer (200 μ moles) pH 7.5; NADPH (20 μ moles); and 0.2 ml of enzyme preparation in a total volume of 2.0 ml was incubated for 24 hr at

30°C.

For the identification of the product, the reaction mixture was deprotenized by the addition of 10% of zinc sulfate, adjusted to pH 7.5 with 0.6 N barium hydroxide, and then centrifuged. The supernatant solution was applied to paper chromatography as described in the previous report.¹¹ Reduction product of D-erythrose was clearly detected by spraying periodate-benzidine or alkaline silver nitrate, but was not reactive with anisidine.¹⁶ *Rf*-value of this spot in various solvent systems was identical with authentic *meso*-erythritol. Moreover, the reduction product of D-fructose was also consistent with authentic D-mannitol in *Rf*-value, color reaction and in optical rotation.

These results showed that *Candida zeylanoides* KY 6166 had potent enzyme activities to produce *meso*-erythritol and D-mannitol from the corresponding tetrose and hexose. However, as far as the above results were concerned, the specificity of dehydrogenase for the substrate seemed to be broad. Suzuki and Onishi,¹⁶ Verachtert and Doods¹⁷ reported similar findings on the production of polyol

from glucose by osmophilic yeast.

Effect of pH on the activity of pentosephosphate cycle enzymes

In the previous paper,⁵ we showed that the intracellular pH in polyol-producing phase was about 5.5~5.7, but that in citric acid-producing phase was 6.5~6.7. Thus, the change in intracellular pH in the above range resulted in the interconversion between citric acid fermentation and polyol fermentation owing to the remarked changes of the enzyme activities related to the biosynthesis of citric acid and polyols.

Therefore, the activities of enzymes involved in pentosephosphate cycle which played also a possible part in polyol synthesis were compared at various pH and the results are summarized in Table I. The activities of all enzymes tested were inhibited, more or less, at pH 5.5. Particularly transaldolase activity was affected most distinguishably. This result suggested that transaldolase could not take a part in polyol production which occurred preferentially at acidic pH (5.5 to 5.7). Venkalaraman and Racker also showed that transaldolase

TABLE I. EFFECT OF pH ON THE ENZYME ACTIVITY RELATING TO PENTOSEPHOSPHATE CYCLE IN *Candida zeylanoides* KY 6166

The right side column represented the optimal pH for individual enzyme activity.

Enzyme	pH					pH opt
	5.0~6.0	6.0~7.0	7.0~9.0	buffer system		
				acetate buffer		
				Tris-maleate buffer		
				Tris-HCl buffer		
Specific activity (units/mg protein)						
	5.0	5.5	pH 6.0	6.5	7.0	
Transaldolase	0.00	0.02	0.13	0.53	0.95	8.0
Transketolase	0.28	0.32	0.38	0.43	0.69	7.7
Glucose 6-phosphate dehydrogenase	15	23	32	41	55	8.2
Glucosephosphate isomerase	0.39	0.50	0.72	0.85	1.30	8.0
6-Phosphogluconate dehydrogenase	8.0	18.1	21.1	27.0	35.5	8.3
Ribulosephosphate isomerase	0.70	1.08	1.50	1.81	2.10	6.8
Ribulosephosphate 3-epimerase	15	42	63	84	115	7.2

The composition of the reaction mixture was described in MATERIALS AND METHODS except that buffer solutions of different pH were used.

FIG. 1. Effect of Phosphate Ion on the Enzyme Activity in *Candida zeylanoides* KY 6166.

The composition of the reaction mixture was described in the text, except that phosphate ion was added as shown in the figure.

○, transketolase; △, glucose 6-phosphate dehydrogenase; ▲, glucosephosphate isomerase.

FIG. 2. Effect of Phosphate Ion on the Reducing Activity of D-Erythrose and D-Fructose in *Candida zeylanoides* KY 6166.

The composition of the reaction mixture was described in MATERIALS AND METHODS except that phosphate ion was added as shown in the figure.

●, reducing activity of D-erythrose; ▲, reducing activity of D-fructose.

activity in bakers' yeast was depressed at acidic pH (6.0~6.5).⁶⁾ Of the biosynthesis of erythritol from glucose, Spencer and Gorin demonstrated that the most possible reaction was implicated in transaldolase and trans-

FIG. 3. Effect of Phosphate Ion on the Activity of Transketolase.

The composition of the reaction mixture was described in the text except that phosphate ion was added as shown in the figure.

○, *Candida zeylanoides* KY 6166; ×, *Candida lipolytica* KY 6141; ▲, *Candida zeylanoides* KY 6171.

ketolase via Emden-Meyerhof pathway.¹⁸⁾

On the other hand, the optimum pH of acid phosphatase catalyzing dephosphorylation of erythrose 4-phosphate and fructose 6-phosphate was 5.5 to 6.5. Furthermore, the reduction activity of D-erythrose and D-fructose did not vary in the pH from 5.5 to 6.5.⁵⁾

Effect of phosphate ion on the enzyme activity relating to pentosephosphate cycle

In the previous paper,²⁾ we reported that the production ratio between erythritol and mannitol was remarkably affected by the concentration of phosphate in the medium. Keeping its concentration at high level (0.04 to 0.20 mg/ml as KH_2PO_4), erythritol production was almost entirely converted to mannitol production. As described in the preceeding section, the alternation between erythritol fermentation and mannitol fermentation was assumed to depend on the synthetic rate between erythrose 4-phosphate and fructose 6-phosphate.

Thus the effect of phosphate ion on the activities of glucosephosphate isomerase, glucose 6-phosphate dehydrogenase and trans-

ketolase, which mainly participated in the formation of erythrose 4-phosphate and fructose 6-phosphate were examined. The results are shown in Fig. 1. Activity of glucose-phosphate isomerase was not affected by phosphate ion in the wide range of its concentration, but glucose 6-phosphate dehydrogenase and transketolase decrease with the increase in phosphate ion concentration. Especially transketolase activity, which participated directly in the conversion of erythrose 4-phosphate and fructose 6-phosphate was reduced to about 50% with 1 mM of phosphate ion. However, the inhibition of glucose 6-phosphate dehydrogenase by phosphate ion was weak, as compared with the case of transketolase. On the other hand, the reduction activity of erythrose and fructose was not affected as shown in Fig. 2. Boyer *et al.* reported also that the transketolase activity in bakers' yeast was significantly depressed by phosphate ion.¹⁹⁾ From all above results, it was reasonably concluded that the alternation between erythritol production and mannitol production by phosphate ion was caused by controlling the level of erythrose 4-phosphate and fructose 6-phosphate by the change of transketolase activity.

In order to clarify the effect of phosphate ion on the transketolase activity further, enzyme activity of other strains such as *Candida lipolytica* KY 6141 and *Candida zeylanoides* KY 6171, was measured as shown in Fig. 3. KY 6141 produced a large amount of mannitol associating with a small amount of erythritol under standard medium, but KY 6171 produced predominantly erythritol concomitantly with a small amount of mannitol. The transketolase activity in *Candida lipolytica* KY 6141 was significantly low level compared to that in

Candida zeylanoides KY 6171 under the same phosphate level. These results further supported that the conversion of erythritol and mannitol production was regulated by the activity of transketolase which was changed by the concentration of phosphate ion.

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