Enzymatic Conversion of D-Glucose to D-Fructose

Part VIII. Propagation of *Streptomyces phaeochromogenus* in the Presence of Cobaltous Ion*

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Propagation of *Streptomyces phaeochromogenus* by both shaking and submerged cultivations is described. The addition of Co^{++} to medium remarkably improved the activity of the cell to convert glucose into fructose. The cell grown with Co^{++} isomerized glucose at pH 9.0 and 7.5 similarly, whereas the cells grown without Co^{++} reacted with glucose at pH 9.3 as the optimum. In submerged cultivation, the mode of pH change during cultivation period varied depending on the kind of strain, carbon source and the presence of Co^{++} .

In the propagation of the strain SK, carbon and nitrogen sources were rather rapidly consumed resulting in cell growth, when pH value of the medium was falling. On the other hand, however, growth hardly developed at the pH values lower than 6.0.

Therefore, it was necessary to adjust pH with alkali in order to get active cell yield.

The authors previously reported on the enzyme from *Streptomyces phaeochromogenus*, Strain SK, which catalyzes the conversion of D-glucose to D-fructose.^{1,2)}

In the course of studies on the cultivation method of the strain, it was found that the enzyme of the cells grown in the presence of cobaltous ion had a stronger heat stability and a wider pH range for activity than the enzyme produced without added cobaltous ion. Yamanaka³⁾ already described the supplemental effect of cobaltous ion to manganese in the production of glucose-isomerizing enzyme from heterolactic acid bacteria. In the case of the author's microorganism, cobaltous ion showed a supplemental effect to magnesium. In this paper, the propagation of *Streptomyces phaeochromogenus*, as the enzyme

source, in the presence of cobaltous ion, by shaking cultivation in flask and by aeration with agitation in a jar fermenter is described.

METHODS

Strain, Medium and Cultivation Methods

Strain. *Streptomyces phaeochromogenus*, strain SK and strain No. 3105**. The strain SK was mainly used unless otherwise mentioned.

Basal constituents. Xylose, 1%; peptone, 1%; meat extract, 0.5%; yeast extract, 0.25%; NaCl, 0.5%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; in water.

Shaking cultivation. The cells from potato agar slant were inoculated into 100 ml of the basal medium in 500-ml Sakaguchi flask and incubated for 24 hours with shaking at $28^\circ\sim 30^\circ\text{C}$. Then 2 ml of the broth was added as the seed to 100 ml of the medium containing the basal constituents plus CoCl_2 at 10^{-3} M/l. After 24 hours of shaking, cells were harvested by a refrigerated centrifuge and washed.

Cultivation in jar fermenter. Cells grown in the basal medium for 24 hours with shaking were used as the seed and the amount of inoculum was

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1) N. Tsumura and T. Sato, This Journal, **29**, 1129 (1965).

2) N. Tsumura, E. Sakakura, M. Ishikawa and T. Sato, *J. Ferm. Assoc., Japan*, **23**, 32 (1965).

3) K. Yamanaka, This Journal, **27**, 265 (1963).

** Given by the courtesy of the Institute for Fermentation, Osaka, Japan.

4% of main cultivation medium. The composition of main cultivation medium was identical to the basal medium except xylose was partly replaced with glucose and CoCl_2 at 10^{-3} mols/l was added. After cultivation with controlled aeration and agitation at $28^\circ\sim 30^\circ\text{C}$, the broth was cooled to $4\sim 10^\circ\text{C}$ with ice and then spun down by continuous centrifuge at about 12,000 r.p.m. The time required to separate 15 l of the broth was about 1 hour.

Assay Method

Cell yield. Dried matter of cell suspension was measured similarly as in the previous paper²⁾ and cell yield was expressed by the dry substance from 100 ml of the broth.

Activity. Enzyme activity of the cell was followed by the determination of fructose formed in the reaction mixture. The mixture consisted of 0.8 M glucose, 0.01 M MgSO_4 , 0.05 M buffer (phosphate buffer, pH 7.5, or ammonium buffer, pH 9.0), 12.5 mg/ml of cell (as dry matter) in final concentration and was incubated for 3 hours at 60°C . Enzyme activity was expressed as the amount of fructose (mg/ml of the reaction mixture) produced under the above conditions.

Xylose and nitrogen. Xylose and nitrogen contents in the medium were measured by the Bial reaction⁴⁾ and by Kjeldahl's method, respectively.

pH. pH value of cultivation medium was determined with glass-electrode pH meter.

RESULTS AND DISCUSSION

I. Shaking Cultivation

Effect of Co^{++} addition. It was found from the results of preliminary experiments that higher concentrations of Co^{++} in the medium up to 10^{-3} M brought stronger activation effect. The addition of 10^{-3} M Co^{++} exhibited a remarkable

activating effect and that of 5×10^{-5} M Co^{++} showed little effect on the activity of the cell. On the other hand, cell growth was strongly prevented in the presence of 2×10^{-3} M Co^{++} . Table I shows the activity and yield of the cells grown with or without the addition of 10^{-3} M Co^{++} . The cells grown with Co^{++} showed far more activity than the cells grown without Co^{++} and this tendency appeared stronger in longer reaction time. Presumably this was due to the intensified heat tolerance of the enzyme produced under the presence of Co^{++} , referring to the effect of Co^{++} on the heat stability as reported previously.¹⁾ The effect of Co^{++} addition was also observed on active pH range. The cells grown with Co^{++} showed similar activity at both pH 9.0 and 7.5, whereas the control cells showed higher activity at pH 9.0 than at 7.5.

In early experiments, cell growth under the presence of 10^{-3} M Co^{++} was partly inhibited. However, this obstacle was overcome by an adaptive cultivation on potato agar slant containing Co^{++} (5×10^{-4} M) and thus the cells could be harvested with similar yield as the control.

Time of Co^{++} addition. The effect of Co^{++} was influenced by the time of addition. Addition within 6 hours from the start of cultivation intensified the production of the enzyme similarly, but, after 12 hours, the effect decreased gradually. The results are shown in Table II.

TABLE I. EFFECT OF Co^{++} ADDITION

Co ⁺⁺ Addition	Final pH of medium	Cell yield*	Activity**			
			Reaction, 3 hrs.		Reaction, 18 hrs.	
			pH 9.0	pH 7.5	pH 9.0	pH 7.5
0	7.8	530	100	76	100	80
10^{-3} M	7.0	280	180	180	215	211

* mg dry wt./100 ml broth

** Figures are expressed as per cent of the activity at pH 9.0 of the cell grown without Co^{++} .

4) W. R. Fernell and H. K. King, *Analyst*, **78**, 80 (1953).

TABLE II. EFFECT OF THE TIME OF Co^{++} ADDITION

Time of Co^{++} addition	Cell yield mg/ml	Activity, mg Fru/ml	
		pH 9.0	pH 7.5
0 hr	580	39.7	37.7
6	480	39.7	37.7
12	530	32.9	31.1
18	610	32.3	30.9

Cultivation period: 24 hours

FIG. 1. Effect of Glucose Addition.

□ Activity at pH 9.0
 ■ " pH 7.5
 ▨ Cell yield

Effect of glucose. When xylose in the medium was partly replaced with glucose, acceleration of enzyme production was observed, though cell yield was decreased gradually corresponding with the increase of glucose amount. As shown in Fig. 1, replacement of 30% of xylose with glucose was most effective and the activity of the cell was 1.5 times as strong as the control. A half amount of xylose could be substituted by glucose still with the improving effect on the activity per cell weight, but the total enzyme production decreased as a result of the diminution of cell yield. When intact cells are used as the enzyme source in the isomerization of glucose, it may be more favorable to diminish the amount of cell addition by using the cells of higher activity. Replacement of xylose with starch also showed similar effect, but the

efficiency was smaller than with glucose.

II. Cultivation in Jar Fermenter

From the results of above experiments done with shaking cultivation, it was demonstrated that the enzyme production of the strain SK was remarkably stimulated by the addition of Co^{++} and by a partial replacement of xylose as carbon source with glucose.

Generally it has been known that the results obtained from the shaking cultivation do not always coincide with the one from submerged cultivation with aeration and agitation. Therefore the experiments with shaking method were succeeded with following propagation experiments in jar fermenter.

Variation of pH during cultivation. It was revealed that the pH value of the media varies remarkably depending on the kind of the carbohydrate source. Aeration rate and addition of Co^{++} also influenced the pH variation. Fig. 2 shows the variation of pH value of media containing xylose, glucose or starch as a sole carbon source, respectively. The pH value of the xylose medium shifted gradually to the alkaline side during the growth period, whereas the pH values of glucose and starch media showed a small rise at the first half period of growth and then lowered to the acid side, rather rapidly in glucose medium.

FIG. 2. Effect of Carbon Source on pH.

Medium; 2 l in 5 l, fermenter

1; C source, glucose 1%

2; " starch "

3; " xylose "

4; " xylose "

1~3; agitation 200 r.p.m., aeration 6 l/min.

4; " 300 " " 10 "

FIG. 3. pH Change in Xylose-Glucose Medium.

Medium; 12l in 30l fermenter

C source; Xylose 0.7%+Glucose 0.3%

Aeration; 30 l/min, Agitation; 100 r.p.m.

It is also seen that the strong aeration results in the high pH value of xylose medium.

From the results of shaking cultivation, it was found that the partial replacement of xylose in the medium with glucose stimulated the enzyme production. In Fig. 3 the change of pH value of the medium containing 0.7% of xylose and 0.3% of glucose as carbon source is shown. The pH value changed similarly as in the case of glucose medium shown in Fig. 2 and, after 24 hours of cultivation period, the final pH was 4.7. The cell yield was only 20 g per 12l of medium. The growth of this strain was suppressed at pH below 6.0. Therefore, it is apparently necessary to adjust pH during the growth period. Fig. 3 also shows the effect of the Co^{++} addition on pH value. In the absence of added Co^{++} , the pH value did not exhibit notable falling, conversely it showed gradual rise.

Relation among pH value, cell growth and activity. On the basis of the results mentioned above experiments of cultivation were carried out adjusting the pH value with an alkaline solution in order to prevent the pH falling below 6.0. In order to investigate the correlation among the pH value, growth and activity of the cell, three examples of cultivation, A, B, and C, with which the modes of pH change were different from each other, were selected from many replicate cultivations. The results are shown in Fig. 4, 5 and 6.

FIG. 4. pH Change during Cultivation.

Medium; 15l in 30-l fermenter

C source; xylose 0.7%+glucose 0.3%

Aeration; 30 l/min. with A

20 " with B and C

Agitation; 200 r.p.m. with A

100 " with B and C

Different batches of seed-culture were used as inocula for A, B, and C.

* Start of pH adjustment.

FIG. 5. Decrease in the Amounts of Nutrients in the Media.

A, B, and C; see the legend to Fig. 4.

Although the culture conditions for B and C were the same, different batches of seed-culture were used in these experiments, thus causing a considerable difference in the resulting cell growth, pH values, and enzyme activities. In A a rise of pH value at the early stage of growth period took a rather

FIG. 6. Cell Yield and Activity.

long time and it was about 20 hours after the starting time to reach pH 6.0. On the other hand, the value in B went to pH 6.0 within 8 hours through a small rise, and the behavior of pH in C was in the middle between A and B (Fig. 4). The decrease of nutrient in these cultivation media was corresponding with the lowering of pH value as shown in Fig. 5. That is, the consumption of nitrogen and carbon sources occurred rather rapidly during the period of pH adjustment after the termination of pH rise in the early stage, and so faster in the order of B, C and A. Accordingly, the time required to reach to some level of cell growth was shortest in the case of B and followed by C. In the case of A, on the other hand, it took a rather long time to reach, a similar cell growth level to that of B or C, and moreover, the cell harvested after prolonged cultivation showed a declining enzyme activity (Fig. 6). Usually, in shaking cultivation with the medium containing a mixture of glucose and xylose as carbon source and Co^{++} , the final pH value after 24 hrs of shaking was found to be 6.0~7.0 through a drop in the course of cultivation, even without the adjustment. In submerged cultivation, however, it was necessary to

adjust pH during cultivation period and the mode of pH change often varied as described above. These variations may partly depend on the rate of aeration and the mechanical effect of agitation, and also may be due to the subtle difference of the seed culture. The definite factors are not yet known. However, the above experiments suggest that the consumption of nutrients and hence the cell growth and enzyme production would occur efficiently at the period in which the pH value of medium shows declination to acid side. This was observed also in the experiment which compared the cultivations of two strains. Starting with similar conditions, the pH value of the medium inoculated with the strain No. 3105 did not show any significant drop but conversely exhibited a gradual rise whereas, in the cultivation of the strain SK, it needed to adjust it by alkali addition to prevent the lowering of pH value. Xylose consumption in each medium was fairly different, i.e., after 18 hours of cultivation the amounts of xylose consumed were 76% in the medium with the strain SK and 22% with the strain No. 3105 for instance. Accordingly, the rises of cell growth and enzyme production with the strain No. 3105 considerably delayed, although a prolonged cultivation of the strain No. 3105 did not deteriorate the enzyme activity and produced similar levels of enzyme activity and cell growth, after 32 hrs of cultivation, to the levels of the strain

TABLE III. ISOMERIZATION IN A CONCENTRATED GLUCOSE SOLUTION

Cell added, % (as dry matter)	Fructose, % in total sugar	
	24 hr	48 hr
C1 {	0.5	21.1
	1.0	28.2
C2 {	0.5	37.2
	1.0	41.6

Reaction mixture: Glucose, 50% (w/w); phosphate buffer pH 7.5, 0.05 M; MgSO_4 , 0.02 M and resting cell. The amounts of cells added are per cent to glucose weight.

C1: Cells grown on xylose

C2: Cells grown on xylose-glucose (7:3) mixture

SK obtained by the cultivation of 24 hrs.

Isomerization with concentrated glucose solution. As demonstrated previously,²¹ the enzyme from the strain SK was not inhibited by the substrate glucose even in a concentrated solution such as 4 moles/l. The isomerization rate of 50%(w/w) glucose solution by

the cell grown in the presence of Co^{++} is shown in Table III. About a half amount of glucose was converted into fructose within 48 hours at 60°C by the addition of the cell in an amount of 0.5% (as dry matter) of the glucose amount.