Studies on Sugar-isomerizing Enzyme

Production and Utilization of Glucose Isomerase from *Streptomyces* sp.

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A glucose isomerase which reversibly catalyzes the reaction between D-glucose and D-fructose was demonstrated in the cell-free extracts of a strain of *Streptomyces* sp. isolated from soil. The enzyme was produced when the strain was grown in the medium containing xylan or xylan-containing material such as wheat bran. A medium which consists of 3% of wheat bran, 2% of corn steep liquor and 0.024% of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ is recommendable for the production of the glucose isomerase enzyme with the strain. With the enzyme, some conditions for the conversion of D-glucose to D-fructose were also studied. The method is very useful for the production of invert sugar from D-glucose and is now on the way to be applied to the practical use.

INTRODUCTION

Numerous studies on glucose isomerase, which converts D-glucose to D-fructose, have hitherto been demonstrated by several workers.¹⁻⁵⁾ However, problems still remain for applying the enzyme to the practical use. Of these problems, the most serious one is that D-xylose is considerably expensive, which has to be in most cases used for producing the enzyme in the cultivation of the organisms.¹⁻⁴⁾ In order to solve this problem, experiments were carried out for obtaining an organism which may have abilities to assimilate xylan which is much less expensive than D-xylose, and to produce the glucose isomerase.

It has been found that certain strain of *Streptomyces* sp. grows well in the medium containing xylan and produces the glucose isomerase. Further, in the present method,

it is unnecessary to use pure xylan for the formation of the glucose isomerase, and any available materials such as wheat bran, corn cob and corn hull can be employed, and yet, the yield of the enzyme is found satisfactory. Accordingly, it would be far economical if xylan is assimilated with organism which forms the enzyme than use of medium containing D-xylose. The method is now on the way to be applied to the practical use.

This paper describes the method of the cultivation for producing the glucose isomerase of a strain of *Streptomyces* sp. isolated from soil and some conditions for isomerizing D-glucose to D-fructose by using the enzyme.

EXPERIMENTAL

Organism used. *Streptomyces* sp. strain YT-No. 5 which was isolated from soil in Chiba prefecture was used in this paper. This strain belongs to the *cinereus* series of *Streptomyces*.

Cultivation Method and Preparation of Enzyme Solution. *Streptomyces* sp. strain YT-No. 5 was inoculated from a slant into 80 ml of a medium containing of 1% of Polypepton, 0.3% of K_2HPO_4 , 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1% of D-xylose in Erlen-

1) R. O. Marshall and E. R. Kooi, *Science*, **125**, 648 (1957).
2) N. Tsumura and T. Sato, *This Journal*, **25**, 616, 620 (1961), **29**, 1123, 1129 (1965).

3) K. Yamanaka, *This Journal*, **27**, 265, 271 (1963).

4) M. Nataka and S. Yoshimura, *This Journal*, **27**, 342 (1963), **28**, 505, 510 (1964).

5) Y. Takasaki and O. Tanabe, *J. Agr. Chem. Soc. Japan*, **36**, 1010, 1013 (1962), **37**, 89, 93 (1963); *This Journal*, **28**, 740 (1964), **30**, 220 (1966).

meyer flask of 200 ml capacity, and cultivated at 30°C for 20~30 hours on a rotary shaker (200 r.p.m.). Then 2 ml of this seed culture was added to 50 ml of a medium (pH 7.0) containing 3% of wheat bran, 2% of corn steep liquor (dry base), 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.024% of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in the flask of 200 ml capacity, and cultivation was carried out at 30°C on the rotary shaker (200 r.p.m.). After 20~30 hours cultivation, cells were harvested by centrifugation, washed twice with distilled water and suspended in distilled water. The cell suspension was then sonicated at a frequency of 10 KC for 10 minutes.

In order to remove cells debris the sonicate was centrifuged at 7,000 r.p.m. for 5 minutes and the supernatant was used as the enzyme solution unless otherwise described.

Assay of Glucose Isomerase. The assay of glucose isomerase was carried out as follows: The reaction mixture was prepared by adding 0.5 ml of 0.2 M phosphate buffer solution (pH 7.2), 0.2 ml of 1 M D-glucose solution and 0.1 ml of 0.1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution to 0.2 ml of the enzyme solution, and final volume was made up to 2 ml with distilled water. After incubation for one hour at 70°C, the reaction was stopped by the addition of 2 ml of 0.5 M perchloric acid and D-fructose produced was determined by the cysteine-carbazole method.⁶⁾

A unit of the enzyme was defined as the amount of the enzyme that produced one mg of D-fructose under the assay conditions shown above.

Determination of Protein. Protein was determined by the method of Lowry et al.,⁷⁾ by using bovine albumin as the standard.

Determination of Xylan. Xylan in wheat bran and in the medium containing wheat bran was determined by the general method to determine pentosan.⁸⁾ In the case of the medium containing pure xylan, a known volume of supernatant of broth was heated with sulfuric acid (final concentration, 4%) for 2.5 hours, and xylan content was determined by measuring reducing power formed by the modified Somogyi's method.

Materials. Xylan was purchased from Pfansthiehl Chemical Co. (USA), Enzyme Digest of Casein and

Acid Digest of Casein from Shieffield Chemical (USA), Enzyme Digest of Soy Protein from Amber Laboratories Inc. (USA), Beef Extract and Yeast Extract from Difco (USA) and Polypepton from Daigo Eiyo Kagaku Co. (Japan).

RESULTS AND DISCUSSION

I. Cultural Conditions

Effect of Carbon Source in Medium. It is well-known fact that the carbon source in medium

TABLE I. EFFECT OF CARBON SOURCE IN MEDIUM

Carbon Source	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
None	0.38	4.26	0.09
Xylan*	21.92	4.54	4.83
D-Xylose*	25.52	5.12	4.98
D-Glucose	1.75	5.44	0.32
D-Fructose	0.50	5.44	0.09
D-Sorbitol	0.38	3.98	0.10
D-Galactose	0.13	4.40	0.03
D-Mannose	0.00	5.98	0.00

Basic medium: Enzyme digest of casein 2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%, K_2HPO_4 0.3%, Carbohydrate 1%

Culture: 41 hours (* 24 hours)

TABLE II. EFFECT OF CONCENTRATIONS OF XYLAN AND D-XYLOSE IN MEDIUM

Xylan Concentration (%)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
None	2.26	3.06	0.74
0.25	14.56	3.40	4.28
0.50	20.30	3.98	5.10
1.00	20.30	3.96	5.13
1.50	17.36	4.26	4.08
D-Xylose Concentration (%)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
0.25	13.50	4.02	3.36
0.50	22.76	4.24	5.37
1.00	16.76	4.12	4.06
1.50	16.90	3.98	5.25
2.00	15.25	3.70	4.12

Basic medium: Corn steep liquor 2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%

Culture: 24 hours

6) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

7) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. T. Randall, *ibid.*, **193**, 265 (1951).

8) "Experiment of Agricultural Chemistry", Tokyo Univ. Press, p. 134.

has a remarkable effect on the yield of glucose isomerase. The formation of the glucose isomerase of *Streptomyces* sp. strain YT-No. 5 was greatly influenced by the carbon source in the growth medium. The results obtained with several carbohydrates are shown in Tables I and II.

Xylan as well as D-xylose is found to be effective carbon source for the formation of the enzyme. On the contrary, D-glucose, D-fructose, D-sorbitol, D-galactose, D-mannose etc. were ineffective or less effective.

The time course of the cultivation of the strain which was grown in medium containing xylan is illustrated in Fig. 1.

FIG. 1. Time Course of Culture in Xylan Medium.

The strain was grown at 30°C in 50 ml of medium which consisted of corn steep liquor 2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024% and xylan 0.5%, in Erlenmeyer flask of 200 ml capacity.

x—x pH ○—○ Enzyme
 ■—■ Xylan □—□ Reducing Sugar (as D-Xylose)
 ●—● Cell Growth (as Protein)

The cell growth reached to maximum at around twenty hours cultivation, and, in the early stage of the cultivation, reducing sugar which is due to hydrolysis of xylan was detected, and the maximum yield of the glucose isomerase could be observed in twenty to twenty-five hours cultivation. Thereafter, the yield of the enzyme gradually decreased.

Effect of Xylan-containing Materials as Xylan Source. The effect of xylan-containing materials, such as wheat bran (xylan content, about 21%), corn cob (xylan content about 35%), corn hull (xylan content, about 35%) etc., instead of pure xylan, was investigated.

As the results shown in Table III, it was found that wheat bran, corn cob and corn hull also can be employed as the inducer of the enzyme formation.

Fig. 2 shows the typical time course of the cultivation of the strain which was grown in the medium containing wheat bran as the xylan source. The maximum yield of the

TABLE III. EFFECT OF XYLAN-CONTAINING MATERIAL

Kind of Xylan containing Material	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
None	3.16	3.64	0.99
Wheat bran 1%	9.38	5.40	1.73
2	19.90	5.84	3.41
3	30.00	6.04	4.97
4	22.24	5.88	3.78
Corn hull 1%	13.60	5.68	2.39
2	21.50	5.50	3.91
3	22.80	5.04	4.52
4	5.50	3.98	2.31
Corn cob 1%	15.50	5.46	2.75
2	20.84	5.70	3.66
3	20.50	5.16	3.97
4	7.74	3.98	1.49

Basic medium: Corn steep liquor 2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%

Culture: 24 hours

Wheat bran, corn hull and cob were ground to about 40 mesh.

FIG. 2. Time Course of Culture in Wheat Bran Medium.

The strain was grown at 30°C in 10l of medium which consisted of wheat bran 3%, corn steep liquor 2%, and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%, in a stainless steel fermenter of 20l capacity. Air flow 5l/min, Agitation 200 r.p.m.

x—x pH ○—○ Enzyme
 ■—■ Xylan ●—● Cell Growth (as Protein)

glucose isomerase was given at the cultivation of twenty~twenty-five hours.

Effect of Various Organic Nitrogens. The effect of natural nutrients, such as corn steep liquor, meat extract, Polypepton, yeast extract etc., on the formation of the glucose isomerase was studied. The results obtained are shown in Tables IV and V

Corn steep liquor, enzyme digest of casein and acid digest of casein were found to be

TABLE IV. EFFECT OF ORGANIC NITROGEN SOURCE

Kind of Nitrogen Source		Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
None		4.16	3.74	1.11
Corn Steep Liquor	1%	14.50	5.76	2.52
	2%	21.92	6.26	3.50
Acid Digest of Casein	1%	19.60	5.56	3.53
	2%	20.88	5.38	3.88
Enzyme Digest of Casein	1%	13.76	3.76	3.66
	2%	21.68	4.34	5.00
Yeast Extract	1%	9.68	3.84	2.52
	2%	12.60	4.32	2.92
Polypepton	1%	5.52	3.98	1.39
	2%	9.40	4.46	2.11
Beef Extract	1%	6.60	3.88	1.70
	2%	8.80	4.16	2.12
Enzyme Digest of Soy Protein	1%	7.94	5.38	1.48
	2%	11.28	6.26	1.80

Basic medium: Wheat bran 3%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%

Culture: 24 hours

TABLE V. EFFECT OF CONCENTRATION OF CORN STEEP LIQUOR

Concentration (%)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
0	5.40	5.72	0.94
0.5	8.94	6.10	1.47
1.0	16.80	6.40	2.63
2.0	19.40	5.76	3.37
3.0	17.90	6.96	2.57
4.0	5.90	6.32	0.94

Basic medium: Wheat bran 3%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%

Culture: 24 hours

effective nitrogen sources for the formation of the enzyme.

Effect of Metal Ions. The effect of the addition of metal ion to the culture medium containing corn steep liquor as a nitrogen source was examined. The results obtained are shown in Tables VI, VII and VIII.

The effect of Mg^{++} was not observed because corn steep liquor contained sufficient amount of magnesium (Table VI), however, the addition of Co^{++} remarkably stimulated the formation of the glucose isomerase, and the optimum concentration was found to be $1 \times 10^{-3} \text{ M}$ (Table VII). The other metal ions tested such as Fe^{++} , Fe^{+++} , Ca^{++} , Mn^{++} , Ni^{++} etc. did not show the effect for the formation of the enzyme.

TABLE VI. EFFECT OF MAGNESIUM CONCENTRATION IN MEDIUM

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ Added (%)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
0	18.50	5.50	3.36
0.05	18.04	5.70	3.16
0.10	18.80	5.54	3.39
0.20	18.90	6.00	3.15
0.30	18.66	5.42	3.44
0.50	19.80	5.26	3.76

Basic medium: Wheat bran 3%, Corn steep liquor 2%, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024%

Culture: 24 hours

TABLE VII. EFFECT OF COBALT CONCENTRATION IN MEDIUM

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ Added (M)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
0	12.60	5.34	2.36
5×10^{-4}	15.60	5.26	2.97
1×10^{-3}	19.80	5.50	3.60
2×10^{-3}	18.66	5.34	3.49
3×10^{-3}	20.80	5.84	3.56
5×10^{-3}	10.14	5.00	2.03

Basic medium: Wheat bran 3%, Corn steep liquor 2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%

Culture: 24 hours

TABLE VIII. EFFECT OF METAL ION
IN MEDIUM

Kind of Metal ($1 \times 10^{-3}M$)	Enzyme Activity (unit/ml broth)	Protein (mg/ml broth)	Specific Activity (unit/mg protein)
None	17.80	5.60	3.18
MnSO ₄	14.20	6.34	2.24
FeSO ₄	12.60	5.56	2.27
Fe ₂ (SO ₄) ₃	11.52	5.60	2.06
CaCl ₂	11.52	5.12	2.25
LiCl ₂	15.76	5.36	2.94
NiSO ₄	12.40	5.64	2.20
ZnCl ₂	7.76	3.70	2.10

Basic medium: Wheat bran 3%. Corn steep liquor 2%,
MgSO₄·7H₂O 0.1%, CoCl₂·6H₂O 0.024%
Culture: 24 hours

II. Conditions for Conversion of D-Glucose to D-Fructose

Effect of pH. The glucose isomerase activity at various pH values was examined. The results obtained are shown in Fig. 3. Chemical isomerization of D-glucose to D-fructose takes place under alkaline pH, so that, the corrected values are presented in the figure. Optimum pH of the reaction lies around seven.

FIG. 3. Effect of pH.

The composition of the reaction mixture: 25 ml of 0.2M phosphate buffer solution of various pH values, 5 ml of 0.1M MgSO₄·7H₂O solution, 1 ml of 0.1M CoCl₂·6H₂O solution, 18 g of D-glucose and the enzyme solution (260 units). After pH of the mixture was adjusted to 6.0, 6.5, 7.0, 7.5 or 8.0, the total volume was made up to 100 ml with distilled water, and the mixture was incubated at 65°C. pH was adjusted with NaOH at every six hours interval.

×—× 5 hr ●—● 20 hr ○—○ 50 hr

Effect of Temperature. The effect of temperature on the glucose isomerase reaction was investigated. The results obtained are shown in Fig. 4. Although the initial velocity increased steadily as the temperature was raised, the rapid inactivation of the enzyme occurred at the reaction temperature of over 70°C. Moreover, the reaction at high temperature induced on intensive color due to heat decomposition of keto-sugar. From these findings it may be stated that the optimum reaction temperature range for the practical use is 65° to 70°C.

Effect of D-Glucose Concentration. Fig. 5 shows the results of an experiment with various concentrations of D-glucose. It was found that the present reaction was progressed without inhibition even if at the high concentration of D-glucose solution, such as almost saturated solution of D-glucose.

FIG. 4. Effect of Temperature.

The composition of the reaction mixture: 25 ml of 0.2M phosphate buffer solution (pH 7.2), 5 ml of 0.1M MgSO₄·7H₂O solution, 1 ml of 0.1M CoCl₂·6H₂O solution, 50 g of D-glucose and the enzyme solution (433 units). The total volume of the mixture was made up to 100 ml with distilled water and incubated at the various temperatures, such as at 60°, 65°, 70°, 75° and 80°C under neutral pH condition as 6.8~7.2. pH was adjusted with NaOH at every twelve hours interval.

●—● 60°C ×—× 65°C ○—○ 70°C
△—△ 75°C □—□ 80°C

FIG. 5. Effect of D-Glucose Concentration.

The Composition of the reaction mixture: 20 ml of 0.2M phosphate buffer solution (pH 7.2), 5 ml of 0.1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution, 18 g, 36 g, 50 g, 60 g or 70 g of D-glucose and the enzyme solution (307 units). The total volume of the mixture was made up to 100 ml with distilled water and incubated at 70°C. pH of the mixture was adjusted to 6.8~7.2 with NaOH at every twelve hours interval.

●—● 18% ×—× 36% ○—○ 50%
 △—△ 60% □—□ 70%

TABLE IX. EFFECT OF METAL IONS

Metal Ion (5×10^{-3} M)	Relative Activity (%)
None	100
Mg^{++} (SO_4^{--})	366
Co^{++} (Cl_2^{--})	258
Mn^{++} (SO_4^{--})	52
Cu^{++} (SO_4^{--})	86
Ni^{++} (SO_4^{--})	17
Fe^{+++} (SO_4^{--})	52
Ca^{++} (Cl_2^{--})	72

This experiment was carried out by using the enzyme preparation which was fractionated (45~67%) from the cell-free extracts with acetone and then dialyzed overnight against distilled water.

The reaction mixture was prepared by mixing 0.5 ml of 0.2M phosphate buffer solution (pH 7.2), 0.2 ml of 1M D-glucose, 0.1 ml of 0.1M metal solution and 0.2 ml of the enzyme solution (27 unit/ml). The total volume was made up to 2.0 ml with distilled water and incubated for one hour at 70°C.

FIG. 6. Effect of Magnesium Concentration with Different D-Glucose Concentrations.

The composition of the reaction mixture: 2.5 ml of 0.2M phosphate buffer solution (pH 7.2), 1.0 ml of the enzyme solution (27 unit/ml) and various amounts of D-glucose and magnesium, as shown in figure. The total volume was made up to 10 ml with distilled water and incubated at 70°C. Concentration of D-glucose.

○—○ 0.01% ▲—▲ 1.8% □—□ 10%
 ●—● 50% △—△ 20%

FIG. 7. Effect of Cobalt.

The composition of the reaction mixture: 25 ml of 0.2M phosphate buffer solution (pH 7.2), 50 g of D-glucose, 5 ml of 0.1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution, (or none), 1 ml of 0.1M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ solution (or none) and the enzyme solution (291 units). The total volume was made up to 100 ml with distilled water and incubated at 70°C. pH was adjusted to 6.8 to 7.2 with NaOH at every twelve hours interval.

○—○ In the absence of Mg^{++} and Co^{++}
 ▲—▲ In the presence of Co^{++}
 △—△ In the presence of Mg^{++}
 ●—● In the presence of Mg^{++} and Co^{++}

Effect of Metal Ions. Effect of several metal ions on the enzyme activity was investigated. Table IX shows the effect of various added metal ions to the dialyzed enzyme. Magnesium ion was effective on the enzyme activity and cobaltous ion was also effective to some extent. The effect of magnesium depended on the substrate concentration and was less the higher the latter became. Fig. 6 shows the result of activation effect to the enzyme by magnesium with different amounts of D-glucose added. It will be seen that the effect of addition of magnesium is very great at low D-glucose concentration, whereas, at high D-glucose concentration, the enzyme shows a considerable activity even in the absence of magnesium.

The presence of cobaltous ion in the reaction mixture enhanced the heat-stability of the enzyme. Especially, the effect of addition of cobaltous ion was great when used together with magnesium ion. These are illustrated in Fig. 7. It was found that magnesium was also effective to enhance the heat-stability of the enzyme.

Practical Conversion of D-Glucose to D-Fructose with Living Cells and with Starch Hydrolyzate. Under the conditions determined above, the practical conversion of D-glucose to D-fructose was carried out with 10l of reaction mixture. As a substrate, a starch hydrolyzate (by glucoamylase), of 50% in concentration and of DE 97.0 (indicating sugar composition of 95% of D-glucose plus 5% of oligosaccharide in which disaccharide is contained), was used. The enzyme used in this experiment was washed living cells of the strain which was grown at 30°C for twenty-five hours in 10l of medium consisting of wheat bran 3%, corn steep liquor 2% and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024% in a fermenter, under the same conditions as presented in Fig. 2.

The wheat bran debris of broth (1.5l, 20.5

FIG. 8. Time Course of Formation of D-Fructose from D-Glucose and pH Control.

The composition of the reaction mixture: enzyme hydrolyzate of starch (5 Kg as D-glucose), 10.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and the enzyme (living cells 500 g (wet), enzyme activity, 59 unit/g of the cells). The total volume was made up to 10l with water and incubated at 70°C. pH was adjusted to around 7.0 with NaOH at every five hours interval, as shown in figure.

unit/ml broth) were removed by a sieve (40 mesh), and the cells were harvested by filtration and washed with water. The amount of living cells thus obtained was about 500 g (wet). The living cells were incubated with the enzyme hydrolyzates of starch (5 kg as D-glucose), 10.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2.4 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in a total volume of 10l. In the course of the reaction, the temperature was maintained at 70°C, and pH was adjusted to around 7.0, by adding sodium hydroxide. The result obtained is presented in Fig. 8.

The ratio of conversion of D-glucose to D-fructose was 45% after 80 hours incubation. The rate of reaction with living cells did not differ significantly from that with cell-free extracts.

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