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Production of Fructose Corn Syrup by Glucose Isomerase

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Glucose Isomerase 효소를 이용한 이성화당(과당) 생산에 관한 연구

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ABSTRACT

Two strains S-P-1 and S-P-2, both *Streptomyces sp.*, have been isolated and were found to have relatively high specific enzyme activity compared to other organisms reported. The specific activity of the enzyme produced from these two strains were 0.25 and 0.2 international units respectively. The productivity of the enzyme achieved was about 50 IU/*l*/hr.

Glucose isomerase from these strains was found to be stable under the temperature of heat treatment(at 65°C) for fixation of enzyme inside the cell. This organism has an advantage in that it did not require toxic metalic ion for enzyme activity and could utilize xylan in leu of xylose as an inducer. The optimal temperature and pH of enzymatic reaction kinetics of whole-cell-enzyme was studied and kinetic parameters were determined for the purpose of using these data for the optimal operation and designing of enzyme reactor system. The reaction mechanism was found to follow the single substrate reversible reaction kinetics.

The kinetic constants determined experimentally are:

K_{mf}=0.33M, K_{mb}=1.0M, V_{mf}=0.88μmole per min., V_{mb}=2.96μmole per min. and K_{eq}=0.74.

INTRODUCTION

Since glucose syrup derived from starch is only 70~75% as sweet as sucrose and fructose is 1.7 times sweeter than sucrose, glucose converted to fructose syrup (containing 40~45% fructose on dry basis) has sweetness that is equal to sucrose. This fructose syrup could substitute sugar in practically all food applications except those requiring a dry sweetner and those in which the total invert sugar gives unsatisfactory results. Because of this advantage, fructose syrup derived from starch could, to a large extent, replace imported sugar in a country where sugar is totally imported.

Many different microorganisms have been

reported to be capable of producing glucose isomerase. Marshall and Kooi in 1957 discovered glucose isomerase activity in *Pseudomonas hydrophilia*. This is believed to be the inital finding of glucose isomerase from microbial source. Since then, glucose isomerase have been detected by others in *Aerobactor Lactobacillus*, *Bacillus*, *Streptomyces* and etc.

For the purpose of isolating an microorganism that has glucose isomerase activity and is heat-stable for preperation of heat treated-whole cell enzyme for industrial use, we have isolated two strains of microorganisms and found activity in *Streptomyces sp.* (S-P-1). The immobilized enzyme in the form of whole-cell enzyme was chosen because in our work soluble form of enzyme is not practical for industrial use and the purification and immobilization of the enzyme is costly. Among three microorganisms that we have isolated *Streptomyces sp.* (S-P-1) was found to be the best for industrial application. With the glucose isomerase from *Streptomyces sp.* (S-P-1), the optimum fermentation time for maximum production of this enzyme have been determined. The whole-cell enzyme was prepared, and the kinetic constants and optimal conditions for reaction have been studied.

METERIAL AND METHOD

1. Isolation and Cultivation

The soil samples locally collected was inoculated into a 50m*l* synthetic medium containing xylose as a sole carbon source. After 3 days of shake-culture at 30°C, the organisms were streaked on a plate for further purification of the strain. After transfering the isolated single colony to an agar slant, it was incubated at 30°C for 48~50 hours. The agar slant cultures so prepared were stored at 5°C as a stock culture. Seed cultures of test strain were developed in a synthetic liquid medium containing xylose and was shake-cultured at 30°C for 72hours. 100m*l* of same liquid medium contained in 500m*l* flask was inoculated with 5m*l* of this seed culture and was cultured at 30°C for 48 hours. After cultivation the cells were harvested by centrifugation. The harvested cells were washed with 0.75% saline solution and with deionized water. The cells thus prepared were tested for glucose isomerase activity.

Unless otherwise stated, the medium composition used was D-Glucose 0.1%, Xylose 0.9%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CoCl_2 \cdot 6H_2O$ 0.024 %, K_2HPO_4 0.3%, and Caseine(enzyme digest) 2.0%. The medium was adjusted to pH 7.0 with H_3PO_4.

Seed culture was developed by inoculating 50m*l* of liquid medium and shake-cultured for 48hours at 30°C. 5m*l* of this seed culture was inoculated into a 100m*l* of liquid medium and was cultured for 46~48 hours at 30°C.

The cell concentration was determined by the measurement of packed cell volume after centrifugation of the broth 3000 rpm for 6 min and converting it to the dry cell weight using a calibration curve of packed cell volume versus the dry cell weight.

2. Enzyme Assay

The reaction mixture prepared for glucose isomerase activity measurement of whole-cell enzyme from S-P-1 strain consisted of 0.8M D-glucose, 0.01M $MgSO_4 \cdot 7H_2O$, 0.001M $CoCl_2 \cdot 6H_2O$, 0.05M pH8.0 phosphate buffer and heat-treated whole-cell enzyme suspension and total volume was 4m*l*. The reaction was carried out at 70°C and pH 8.0. The reaction was stopped by adding 8m*l* of 0.5N perchloric acid to the reaction mixture. One unit of enzyme was defined as μmole of fructose formed per one minute under the assay conditions used. The specific activity of whole cell enzyme was defined as unit per mg of dried

Table 1. Relative enzyme activity of strains isolated

Strain Isolated & Screened	Relative Enzyme Activity***	Metal Ion Requirement*	Inducer Requirement**
S-P-1	1.00	Mg	xylan or xylose
S-P-2	0.85	Mg	xylose
B-P-1	0.07	Mg	xylose
B-P-2	0.09	As	xylose
B-P-3	0.09	Mg	xylose

* Concentration of metal ion; 0.04M for As and 0.01M for Mg

** Inducer in the medium; 0.9%

*** 1.0 Relative enzyme activity is equivalent to 0.232 I.U.

cell. Dry weight of cell was measured after drying for 10~12 hours at 85°C. The fructose formed was determined by the modified cysteine-carbazole method developed by Dische and Borenfreund. Glucose was determined by glucose-oxidase method using Worthington Glucostat Reagent Kit.

The cells washed with deionized water were heat-treated at 65°C for 15 minutes and was stored at −5°C as the whole-cell enzyme stock.

To test the possibility of prolonged and repeated use of the enzyme the reaction was carried out for 10 hr period each time and the whole-cell enzyme was washed prior to reuse. This procedure was repeated several times and the enzyme activity was measured each time.

RESULTS

1. Activity of Organisms Isolated

From the soil samples collected from the farming area in Inchon and Pyungtaek, 37 strains of microorganisms which grew on xylose synthetic medium have been isolated. Among the isolated microorganisms, five strains having relatively good specific activity have been screened. Table 1 shows the comparison of specific activity, inducer requirement for enzyme production and metalic ion requirements for activity. Table 2 shows that the specific activities of strains S-P-1 and S-P-2 are relatively high compared to microorganisms reported by others. Among the five organisms screened, S-P-1 was chosen for detailed study because this strain had the highiest specific activity and xylose could be

Table 2. Comparison of specific activity of microorganisms

Strain	Specific activity (I.U./mg D.C.W.)
Streptomyces albus(12)	0.236
S-P-1	0.232
S-P-2	0.198
Streptomyces phaeochromogenous(13)	0.199
Pseudomonas hydrophilia(1)	0.027
Bacillus coagulans(4)	0.025

Tbble 3. Effect of Inducer on production of glucose isomerase

Inducer	Relative enzyme activity
Glucose	Not detected
Xylan	1
Xylose	15

* Fermentation conditions: 48 hours at pH 7.0 and temperature of 30°C.

replaced by less expensive inducer and required no toxic metal ion for activity.

2. The Effect of Inducer on Production of Glucose Isomerase

Similar to most microorganisms previously reported by others, this strain did not produce glucose isomerase when D-glucose was used as the only carbon source in the medium, but produced glucose isomerase when xylose or xylan was present in the medium as an inducer. As shown in Table 3 the cells that have been cultured in the xylose containing medium gave about 15 times the specific acti vity of the cells that were cultured in xylan containing medium. The percentage of inducer used in this test was 0.9%.

3. Time Course of Enzyme Production by Fermentation

The production of glucose isomerase showed a maximum at about 46.5 hours of cultivation. Based on volume of culture broth the maximum cell yield was 10g per liter of broth. The decrease in specific activity of the cell after reaching the maximum value at 46.5 hours seems to be due to autolysis of cell and deactivation of glucose isomerase by proteolytic enzyme during the death phase of cell growth(Fig. 1).

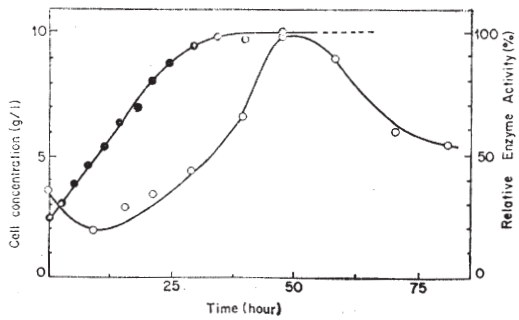

Fig. 1. Profile of fermentation for production of glucose isomerase. Enzyme activity (○) and cell concentration (●).

4. Heat Treatment

To find the loss of activity caused by heat, untreated whole-cell enzymes were treated at various temperature for 15 minutes then the residual activity of whole-cell enzyme was measured. Fig. 2 shows that at 50°C there is no apparent loss of activity but beyond this temperature the activity loss is greater as the temperature increases. At 85°C, which is the optimum temperature of reaction, about half of the initial activity is lost. Because of the significant loss of activity at optimum temperature range, reaction should be carried out at a lower temperature range for practical application considering the cost of enzyme and the operational expenses. At the temperature of heat-treatment we selected, which was at 65°C, the cells retained about 90% of original activity.

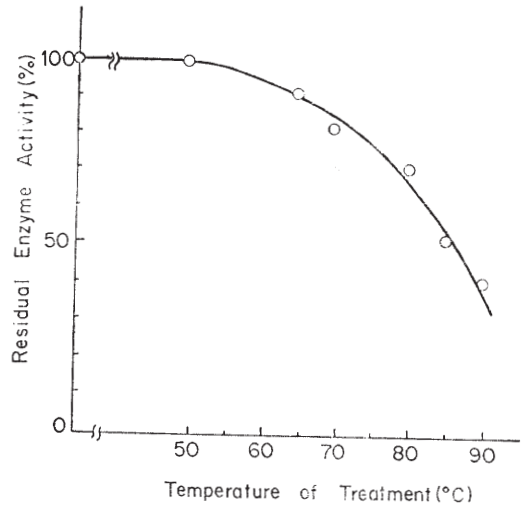

Fig. 2. The effect of heat-treatment temperature on the enzyme activity of the whole-cell enzyme. Cells were heat-treated at a given temperature for 15 min. control (●) and treated (○).

5. Optimal Temperature and pH for Isomerization

The pH optimum of glucose isomerase of the whole-cell enzyme was 7.5 under the assay conditions (Fig. 3). The optimum temperature of the reaction was observed at 85°C, under the assay conditions with reaction time of 15 minutes(Fig. 4). But at this temperature enzyme is not stable and loss of activity is

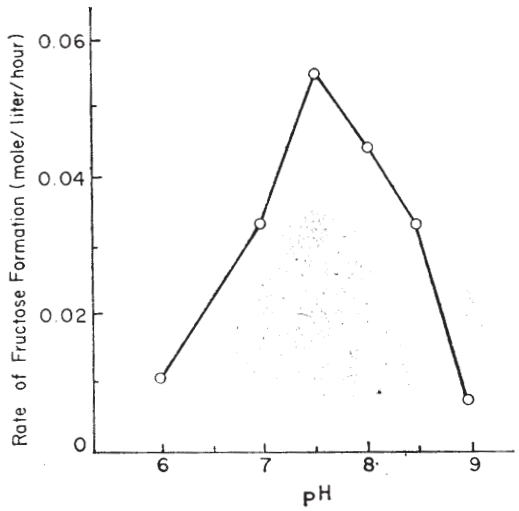

Fig. 3. Effect of pH on the reaction rate of the whole-cell glucose isomerase. D-glucose 0.8 M, 70°C, 30 min. reaction time.

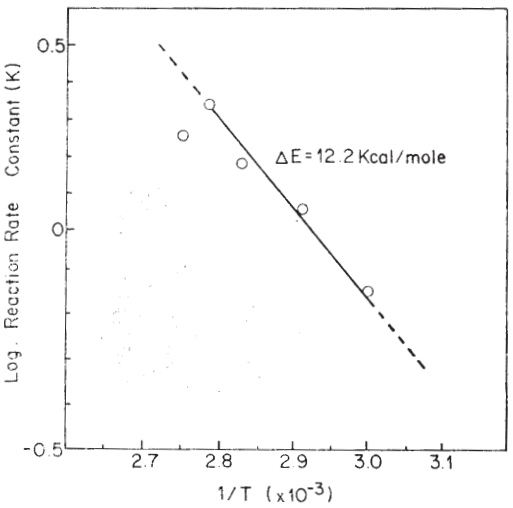

Fig. 4. Effect of temperature on reaction rate of the whole-cell glucose isomerase. D-glucose 0.8M, pH 8.0, 15min.

significant. The activation energy for the reaction was determined as 12.2kcal/mole (Fig. 5).

6. Determination of Kinetic Parameters

The kinetic parameters of the reaction were determined at pH 7.5 and 70°C. As shown in Fig. 6 and Fig. 7, a line of best fit was drawn for the Lineweaver-Burk plot of the

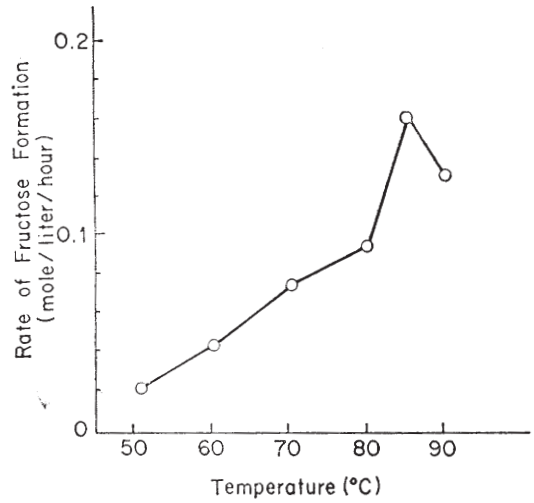

Fig. 5. Activation energy of the glucose isomerase reaction.

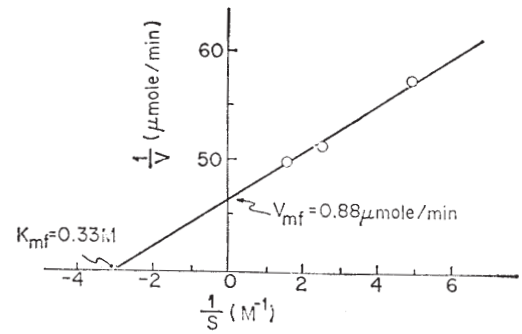

Fig. 6. Lineweaver-Burk plot of the whole-cell glucose isomerase forward reaction. 0.05M phosphate buffer 0.01M $MgSO_4$, pH7.5, 70°C.

data. The initial rate was determined by measuring the rate after 15, 30 and 60 minutes of reaction time. From the slope and intercept, following values were obtained for the parameters. K_{mf}=0.33(M), K_{mb}=1.0(M), V_{mf}=0.88(μM/min.), V_{mb}=2.96(μM/min.).

Equilibrium constant was determined by chemical analysis method. In Fig. 8 the lower curve represents the time course of formation of D-fructose from D-glucose and the upper curve shows the time course of the decrease

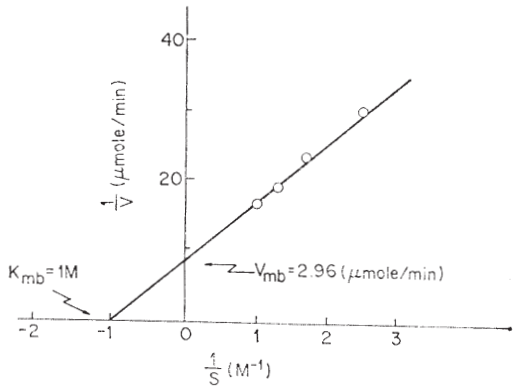

Fig. 7. Lineweaver-Burk plot of the whole-cell glucose isomerase reverse reaction. 0.05M phosphate buffer, 0.01 $MgSO_4$, pH7.5, 70°C.

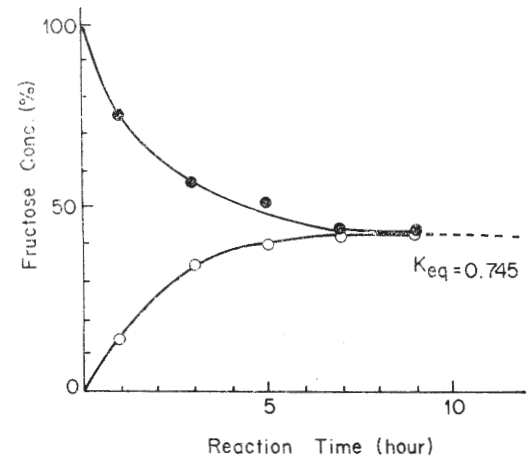

Fig. 8. Time course showing the reversible reaction and the equilibrium for the glucose isomerase. The reaction conditions are the same as those in Fig. 6 and 7. Forward reaction (○) and reverse reaction (●).

in D-fructose concentration. Equilibrium is reached about 9 hours after the start of the reaction, and the equilibrium concentration of D-fructose is obtained directly from its concentration at this time. Equilibrium constant is expressed as:

$$K_{eq}=\frac{(\text{D-fructose})_{eq}}{(\text{D-glucose})_{eq}}$$

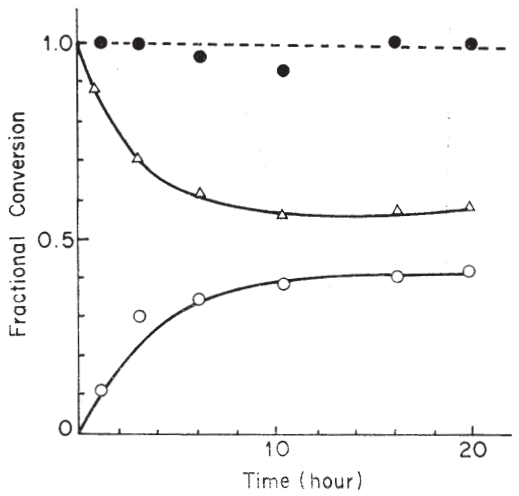

Fig 9. Time course of glucose isomerization reaction by both the glucose determination and fructose determination. Fructose (○), glucose (△), total sugar (●).

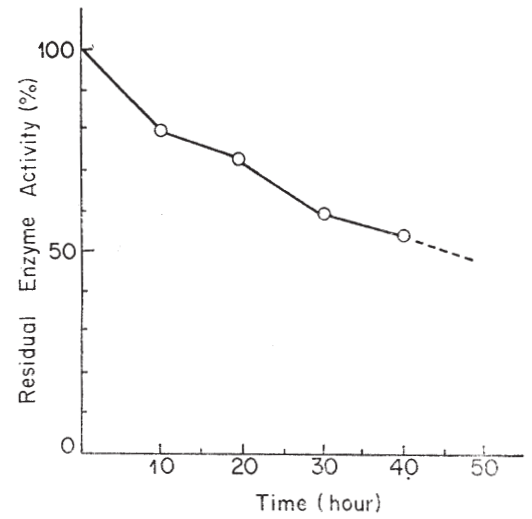

Fig. 10. Repeated and prolonged use of whole-cell glucose isomerase.

The value obtained was 0.745, corresponding to 42.7% conversion of glucose to fructose.

7. Fructose Determination for the Reverse Reaction

Because cysteine-carbazole, H_2SO_4-cysteine and cysteine-H_2SO_4 methods were inaccurate in determining the low concentration

of fructose in presense of high concentration of glucose due to glucose interference, possibility of fructose determination in this enzyme system by determining glucose concentration using glucose oxidase method was tested. During heat-treatment to fix the glucose isomerase inside the cell, other enzymes unstable to heat using glucose as substrate would be deactivated, Thus, glucose is not consumed as a substrate by any enzyme other than glucose isomerase. Since the total amount of sugar in the reaction mixture should remain constant throughout the reaction, it would be possible to determine the amount of fructose produced by measuring the decreased amount of glucose. Time course of enzyme reaction in Fig. 9 shows that total amount of glucose and fructose remain constant throughout 48 hour period of reaction. From this result, determination of fructose by determination of glucose in this enzymatic reaction system seems reasonable especially for the reverse reaction.

8. Prolonged use of Whole-Cell Glucose Isomerase

The glucose isomerase is fixed inside the cell by the heat treatment and could be used repeately as an insoluble form of glucose isomerase. Fig. 10 shows that about half of the activity was lost after four 10-hour batch reactions. This is probably due to leakage of glucose isomerase from the cell and deactivation by heat during the reaction. In a batch process, to fix the time required to achieve certain conversion the enzyme must be supplemented in order to compensate for the loss of activity.

DISCUSSION

The maximum enzyme activity of glucose isomerase from the strain S-P-1 was observed at 46.5 hours of cultivation as shown in Fig. 1. At this time the strain S-P-1 gave the maximum specific activity of 0.232(I.U./mg D.C.W.) and had a maximum cell yield of 10 (g/liter). This represents a productivity of about 50 IU/*l*/hr by a batch process. When the process conditions are optimized, glucose isomerase productivity of this strain could be increased to make it more attractive for industrial application.

For induction of glucose isomerase, this strain requires either xylose or xylan and the cells that have been cultured in the medium containing xylose gave about 15 times the specific activity of the cells that were cultured in xylan containing medium. This enzyme did not require toxic metal ion for the reaction and was stable at the temperature of heat-treatment for preperation of the whole-cell enzyme. Only 10% of initial activity was lost during 15 min. heat-treatment at 65°C. Optimal pH and temperature of reaction was found to be at 7.5 and 85°C. At the optimal reaction temperature about 50% of initial activity was lost after heat-treatment for 15 miniutes. Because of the significant loss of enzyme activity at the optimal reaction temperature, the reaction should be carried out at a lower temperature where the activity loss is not so significant.

The reaction mechanism was found to follow the single substrate reversible reaction kinetics. The kinetic constants determined experimentally are: K_{mf}=0.33(M), K_{mb}=1.0(M), V_{mf}=0.88(μM/min.), and V_{mb}=2.96(μM/min.) K_{mb}, which may indicate that the affinity of D-glucose to enzyme is greater than that of D-fructose, whereas V_{mf} is smaller than V_{mb}. Thus when K_{eq} is calculated using Haldane Relationship; $K_{eq}=\frac{V_{mf}}{K_{mf}}\cdot\frac{V_{mb}}{K_{mb}}$ value of near unity 0.9 was obtained. The K_{eq} value calculated by using Haldane Relationship was close to experimental value of K_{eq}, which was 0.745.

The enzymatic reaction of glucose isomerization was found to be an endothermic reaction and the value of activation energy(Ea) calculated from the experimental data was 12.2 kcal/mole.

The whole-cell form of glucose isomerase from the strain S-P-1 had the half-life of about 40 hours under batch reaction conditions. The reason for this short half life seems to be due to the leakage of glucose isomerase from the cell and also due to deactivation by heat. Further work in the area of enzyme stabilization and increased productivity should be pursued.

摘 要

*Streptomyces sp.*에 속하는 Strain S-P-1, S-P-2가 분리되었으며 보고되었던 다른 균주들 보다 높은 효소 역가를 나타내었다. 이들 두 균주에 의해 생산된 효소의 역가는 각각 0.25, 0.2IU이었으며 효소 생산율은 약 50IU/*l*/hr였다. 이들 균주로부터 생성된 포도당 이성화효소는 세포내 효소 고정화를 위해서 65°C 열처리를 했을때 역가의 손상이 나타나지 않았다. 사용된 균주는 효소 작용에 유해한 금속이온을 요구하지 않을 뿐 아니라, inducer로서 xylose 대신에 xylan을 사용할 수 있다는 장점을 갖고 있었다. Whole-cell-enzyme의 효소반응에 있어서 최적온도와 최적수소이온 농도가 연구되었으며, 반응 최적화와 효소반응기의 설계를 위해 반응 속도 상수들이 결정되었다. 반응 기작은 단일기질 가역 효소 반응으로 밝혀졌으며 반응 속도 상수들은 다음과 같았다.

K_{mf}=0.33M, K_{mb}=1.0M, V_{mf}=0.88 μmole/min., V_{mb}=2.96 μmole/min. K_{eq}=0.74

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