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RESEARCH ARTICLES

PRODUCTION OF SORBITOL AND ETHANOL FROM SUCROSE BY *ZYMOMONAS MOBILIS* : SEPARATION AND PROPERTIES OF GLUCOSE-FRUCTOSE OXIDOREDUCTASE

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ABSTRACT

Sorbitol was found as a product from reduction of fructose by NADH catalyzing with intracellular enzyme extracted from Zymomonas mobilis *IFO 13756. Grinding with sand provided the maximum activity of glucose-fructose oxidoreductase comparing to the others four methods of extraction; treatment of lysozyme, cell disruption with ultrasonic wave, extraction with cold acetone and extraction of cell debris by Triton X-100. Partial purification of enzyme by Blue-Sepharose CL-4B column resulted in 7.22 folds of purification with 23.39% yield which was higher than the purification by DEAE Sephadex A-50. The partial purified enzyme from the higher yield method gave the optimum pH, optimum temperature,* V_{max} *and* K_m *at 7.0, 25°C, 0.006 μmole. min*$^{-1}$ *and 4 mM respectively. The catalytic reduction property of the partial purified enzyme was confirmed by the activity assay of the protein band on polyacrylamide gel electrophoresis.*

INTRODUCTION

The production of sorbitol and ethanol by the anerobic *Zymomonas mobilis* grown on sucrose or mixtures of glucose and fructose has been reported over the last ten years.[1-4] In our previous report it was demonstrated that *Z. mobilis* IFO 13756 produced the highest yield in sorbitol and ethanol among a few microorganisms tested.[4] The studies with cell-free extracts indicated that the reduction of fructose to sorbitol was coupled with the dehydrogenation of glucose to gluconolactone.[3] Sorbitol was proved to be derived only from fructose by NMR and HPLC.[2] The enzyme catalyzing this reaction was identified as a glucose-fructose oxidoreductase containing tightly bound NADP as hydrogen carrier.[6]

This paper describes the partial purification of glucose-fructose oxidoreductase extracted from *Z. mobilis* IFO 13756 and its properties for the production of sorbitol.

MATERIALS AND METHODS

Growth of microorganism

The organism used was *Z. mobilis* IFO 13756. The grown-cells were obtained by 10% inoculum in batch culture containing 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.2% $MgSO_4$, 1% yeast extract, 1% peptone (Difco, Michigan, USA) and 25% sucrose at pH 7.5. Incubation was carried out at 30° C for 36 hours without shaking the culture. The cells were harvested and separated by centrifugation at 4°C 5000 rpm 20 min using a refrigerated centrifuge RC-5B (Sorvall, Dupont Instruments, USA).

Extraction of enzyme

Glucose-fructose oxidoreductase is an intracellular enzyme of *Z. mobilis*[7], Therefore, crude enzyme has to be extracted from wet cells. Five methods of cell disruption were used to prepare crude glucose-fructose oxidoreductase in order to selected the suitable method.

a. Grinding with sand The wet cells of *Z. mobilis* 10 g were ground with 20 g of sea sand and 25 cm^3 of 0.01 M phosphate buffer, pH 5.8 at 0-4°C for 30 min. Crude enzyme solution was obtained by centrifugation at 4 °C 12,000 rpm for 30 min to separate the cell debris.

b. Treatment of lysozyme Lysozyme solution composed of 30 mM K_2HPO_4 (0.2613 g), 0.5 mM $(NH_4)FeSO_4$(0.0098 g), 10 mM Sodium asorbate (0.0991 g), 0.1% Titron X-100 (0.05 cm^3), 2 $\mu g/cm^3$ DNase I (0.0001 g) and 0.2 mg/cm^3 lysozyme (0.0100 g) in distilled water with total volume of 50 cm^3. Six millilitres of lysozyme solution were mixed with 1 g wet cells and kept at 0-4°C. The mixture was stirred for 2 hours and the cell debris was separated by centrifugation at 4°C 12,000 rpm 60 min. Crude enzyme was obtained in the supernatant.

c. Cell disruption with ultrasonic wave The mixture of 10 g of wet cells and 25 cm^3 0.01 M acetate buffer, pH 5.8 was sonicated at 4°C for 15 seconds 20 times. Crude enzyme in cell-free supernatant was obtained by centrifugation at 4°C, 12,000 rpm, 60 min.

d. Extraction with cold acetone Ten grams of wet cells were stirred with 100 cm^3 cold acetone for 30 min and passed through 0.2 µm membrane filter. The cells were allowed to dry at room temperature and then mixed with 25 cm^3 0.01 M acetate buffer, pH 5.8. The cells were separated by centrifugation at 4°C. The supernatant was tested for glucose-fructose oxidoreductase activity.

e. Extraction of cell debris by Triton X-100 The cell debris(from a) was washed with 50 cm^3 0.01 M acetate buffer pH 5.0. Ten grams of the cell debris was mixed with 5 cm^3 of solution containing 1% Triton X-100, 0.1 M KCl and 0.1 M sorbitol in 0.01 M acetate buffer, pH 5.0. The mixture was stirred at 0-4 °C for 2 hours. The cell debris was separated by centrifugation at 4°C. The proteins were precipitated from the supernate with PEG 6000 (20%w/v) at 0-4°C for 12 hours. The precipitate was dissolved in 0.01 M acetate buffer, pH 5.0 containing 0.1% Triton X-100.

Enzyme assay

Enzyme activity was measured by the modification method of Gerlach and Hiby.[8] The reaction mixture contained 0.2 M Triethanolamine buffer pH 7.4 (2.1 cm^3), 2.0 M fructose (0.3 cm^3), 12 mM NADH (0.1 cm^3), and enzyme solution (0.5 cm^3). The increase of absorbance at 340 nm of reaction mixture was monitored by a Spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that reduced 1 µmole of fructose per min at 25° C.

Determination of protein

Protein concentration was determined according to the method of Bradford[9] using bovine serum albumin (Sigma, Messachusette, USA) as a protein standard.

Purification of enzyme

Two types of columns; DEAE-Sephadex A-50 and Blue-Sepharose CL-4B were used. The crude enzyme solution (20 cm^3) was obtained by grinding wet cells with sand, and passed through a column (2.6x20 cm) of DEAE-Sephadex A-50 or a column (1.6x10 cm) of Blue-Sepharose CL-4B. The enzyme was separated and eluted with linear gradient of NaCl (0.1 M) in 0.01 M acetate buffer, pH 5.8 for DEAE-Sephadex A-50 column, and with 1 mM NAD^+ in 0.01 M acetate buffer, pH 5.0 for Blue-Sepharose CL-4B. The eluated fractions (3.5 cm^3/fraction) were assayed for protein content and the activity of the enzyme.

Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Gordon[10] with 6.0% polyacrylamide gel and 0.05M Tris-glycine buffer, pH 9.6. Proteins were stained with Coomassie brillant blue. For activity assay the gel was cut 2 mm pieces, eluted with the assay buffer and assayed for glucose-fructose oxidoreductase activity.

HPLC Analysis

Sugars were determined by HPLC (Water Associates) using carbohydrate column (3.9x300 mm, Water Associates no.84034) with a mixture of acetonitrile : ethanol : water (90: 5: 5) as eluent (flow rate 1.5 $cm^3\ min^{-1}$) at 25°C, using refractive index (LBK Pharmacia) as detector.

RESULTS AND DISCUSSION

The assay of glucose-fructose oxidoreductase for sorbitol production

The determination of glucose-fructose oxidoreductase was based on the methods of Gerlach[8] with some modifications as indicated in our method. Due to the oxidation of NADH to NAD^+ on the reduction of fructose to sorbitol, the decrease of absorbance at 340 nm is the measurement of the enzyme activity. Sorbitol formation in the assay was proved by HPLC (Table 1).

Extraction of glucose-fructose oxidoreductase

Various methods of extraction were investigated for preparation of crude glucose-fructose oxido-reductase from *Z. mobilis* IFO13756 (Table 2). Crude enzyme obtained by grinding the cells with sand contained the maximum activity and specific activity. Failure in the extraction of the cell debris with Triton X-100 indicated that the enzyme was not the membrane bound protein. At 25°C, the maximum rate of the enzyme catalyze reduction of fructose in phosphate buffer, pH 6.0 in the present of NADH was 0.045 μmol/min and the Michaelis's constant was 8 mM. The stability of crude enzyme at 28°, 4° and -21°C during 1-3 days were shown in Table 3. The enzyme totally lost its activity in two days at 28°C whereas 42% and 16% of the activity decreased at 4° and -21°C respectively in the same period. Therefore, the purification method for the enzyme should be done in a short time at low temperature in order to maintain the enzyme activity.

Partial purification of enzyme

Two methods of chromatography; anion-exchange chromatography and affinity chromatography, were applied for purification of crude enzyme. The chromatogram of purification by DEAE-Sephadex A-50 and Blue-Sepharose CL-4B were shown in figure 1 and 2 respectively. Table 4 shows that Blue Sepharose CL-4B provided higher efficiency in both percentage yield and purification fold of purification of glucose-fructose oxidoreductase than DEAE-Sephadex A-50. The separation of protein by polyacrylamide gel electrophoresis of partial purified enzyme from DEAE-Sephadex A-50 showed more than 2 bands of protein (Figure 3) whereas one strong band and one weak band were obtained in sample of enzyme purified from Blue-Sepharose CL-4B. The strong band gave the activity of glucose-fructose oxidoreductase but the weak band was not. Therefore, affinity chromatography using Blue Sepharose CL-4B is more efficient and convenient in purification of the enzyme than anion exchange on DEAE Sephadex A-50 column. Furthermore, the better method needed two days for the purification whereas the other used eight days.

The optimum pH and temperature of the partial purified enzyme from both chromatographic method were shown in Table 4 and Figure 4 a and b respectively. The studies of the rateof reaction with different concentrations of fructose provided the maximum rate of reaction (V_{max}) and Michaelis constant (K_m) as shown in Figure 5 a and b.

Table 1 Identification of sorbitol in the reaction mixture of crude enzyme catalyzed reduction of fructose by NADH.

Substrate	Product concentration (g dm^{-3})		
	Fructose	Glucose	Sorbitol
absent of enzyme	1.74	0.00	0.00
present of enzyme	0.72	0.00	0.64

Table 2 Specific activity of glucose-fructose oxidoreduction extracted by various methods.

Methods of separation	Volume (cm^3)	activity of enzyme units/cm^3	Total activity (units)	Protein mg/cm^3	Total (mg)	specific activity (units/mg)
Acetone extract	25	0.005	0.125	2.32	58.00	0.002
Lysis with lysozyme	14	0.224	3.136	4.00	56.00	0.050
Grinding with sand	25	0.349	8.725	4.65	116.20	0.075
Lysis with ultrasonic wave	25	0.283	7.075	6.90	172.50	0.041
Triton X-100 extract	3	0.000	0.000	0.52	1.56	0.000

Table 3 Stability of crude glucose-fructose oxidoreductase at different temperatures.

Temperature (°C)	Activity of crude enzyme (units/cm^3) 0 h	24 h	48 h	72 h	Loss of activity (% of initial value) 0 h	24 h	48 h	72 h
- 21	0.873	0.811	0.729	0.532	0.00	7.10	16.49	39.06
4	0.880	0.633	0.507	0.417	0.00	28.07	42.39	52.61
28	0.869	0.234	0.008	0.001	0.00	73.07	99.08	99.98

Table 4 Purification of glucose-fructose oxidoreductase and properties of partial purified enzyme.

Enzyme	Volume	Activity of enzyme		Protein		Specific	%	purification	Optimum		Kinetic value	
	(cm^3)	mg/cm^3	Total (units)	$units/cm^3$	Total (mg)	activity (units/mg)	yield	fold	pH	Temp. (°C)	K_m (M)	V_{max} (μmole/min)
Crude enzyme SDH	25.0	0.32	8.08	1.20	30.00	0.27	100	1.00	6.0	25	0.008	0.045
Purified enzyme by DEAE-Sephadex A-50	5.0	0.18	0.90	0.23	1.13	0.80	11.14	2.96	6.5	25	0.007	0.004
Purified enzyme by Blue-Sepharose CL-4B	4.4	0.43	1.91	0.22	0.97	1.97	23.39	7.22	7.0	25	0.004	0.006

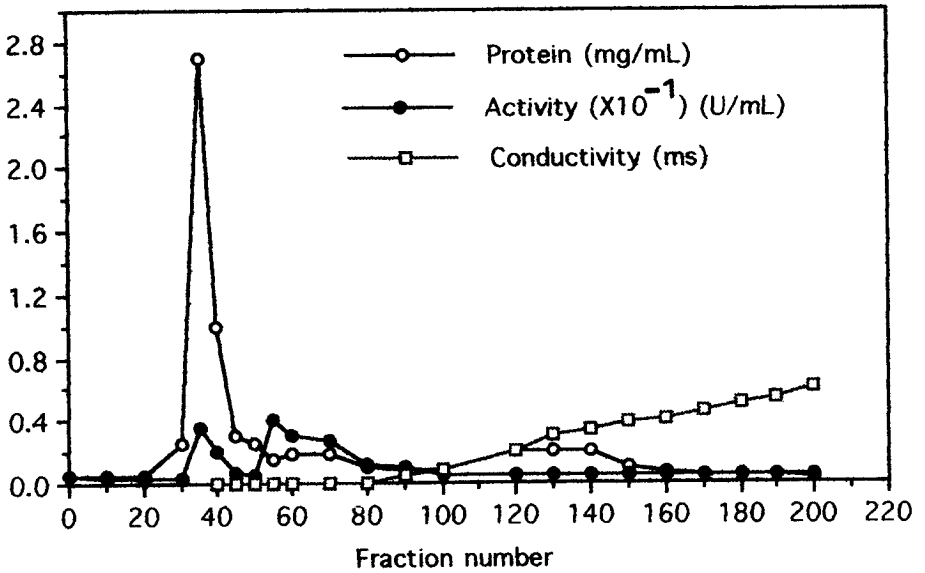

Fig. 1 Chromatography of crude enzyme on DEAE-Sephadex A-50 column (2.6 x 20 cm) equilibrated with 0.01 M acetate buffer, pH 5.0.The column was washed with the same buffer which contained 0.1 M NaCl.

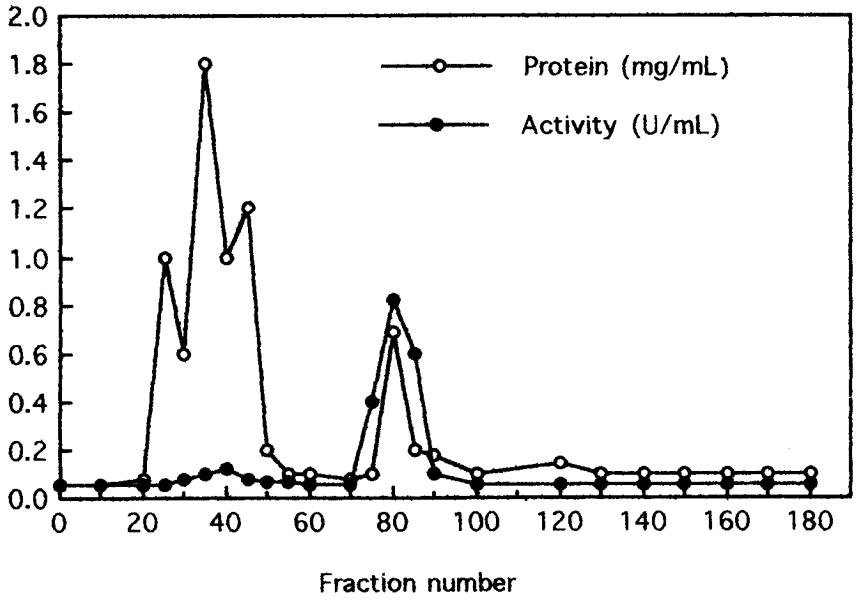

Fig. 2 Purification of glucose-fructose oxidoreductase elute from Blue-Sepharose CL-4B column (1.8x10 cm) by 0.01 M acetate buffer, pH 5.0 in present of 1 mM NAD^+

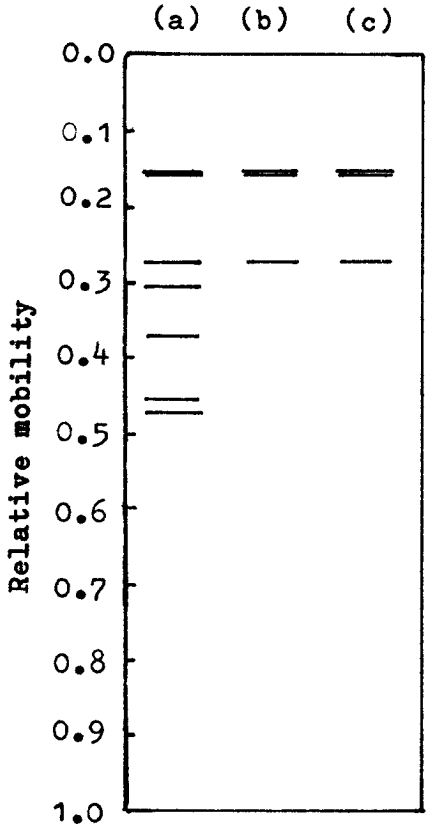

Fig. 3 Polyacrylamide gel electrophoresis of crude enzyme (a), purified enzyme solution from DEAE-Sephadex A-50 (b) and purified enzyme solution from Blue Sepharose CL-4B (c).

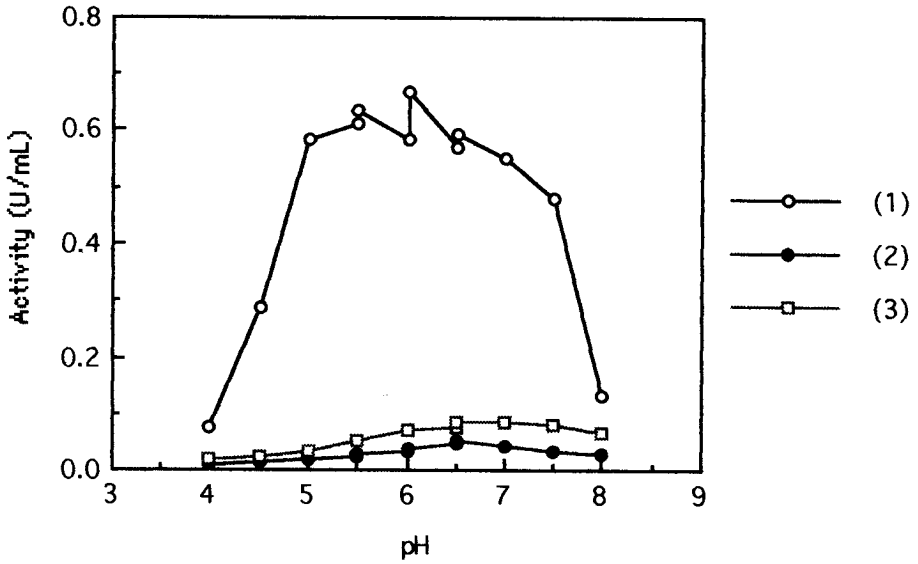

Fig. 4a Optimum temperature of crude enzyme (1), purified enzyme from DEAE-Sephadex A-50 column (2) and purified enzyme from Blue Sepharose CL-4B (3).

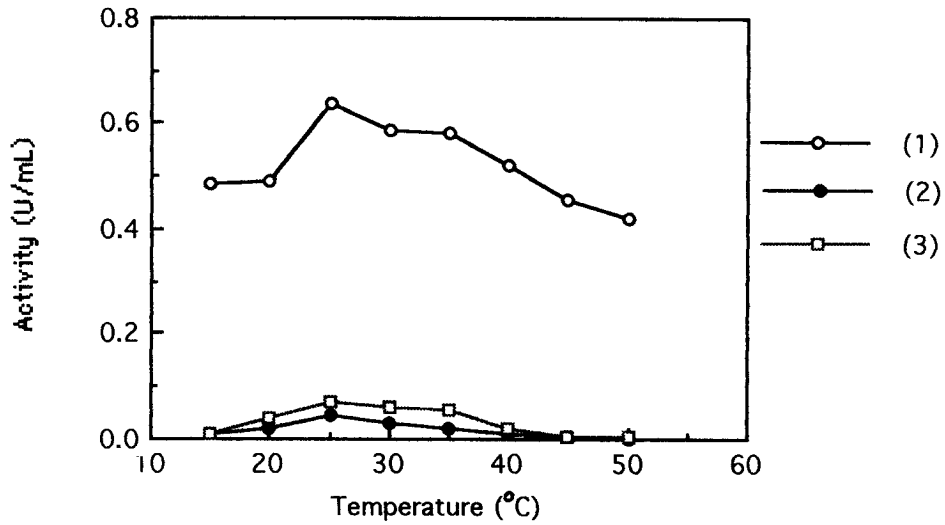

Fig. 4b Optimum pH of crude enzyme (1), purified enzyme from DEAE-Sephadex A-50 column (2) and purified enzyme from Blue Sepharose CL-4B (3).

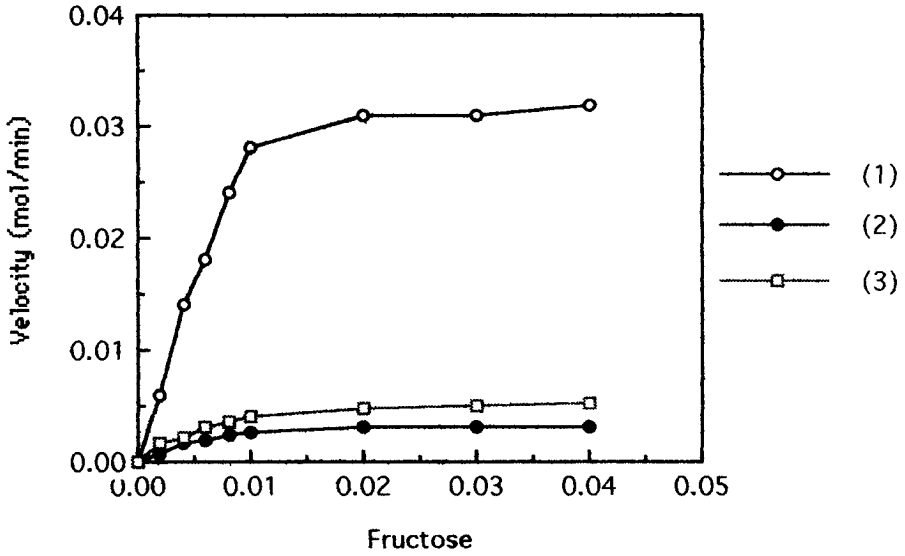

Fig. 5a Glucose-fructose oxidoreductase activity, measured as the rate of oxidation of NADH on fructose in 0.2 M phosphate buffer, pH 7.0 at 25°C using crude enzyme (1), purified enzyme from DEAE-Sephadex A-50 column (2) and purified enzyme from Blue Sepharose CL-4B column (3).

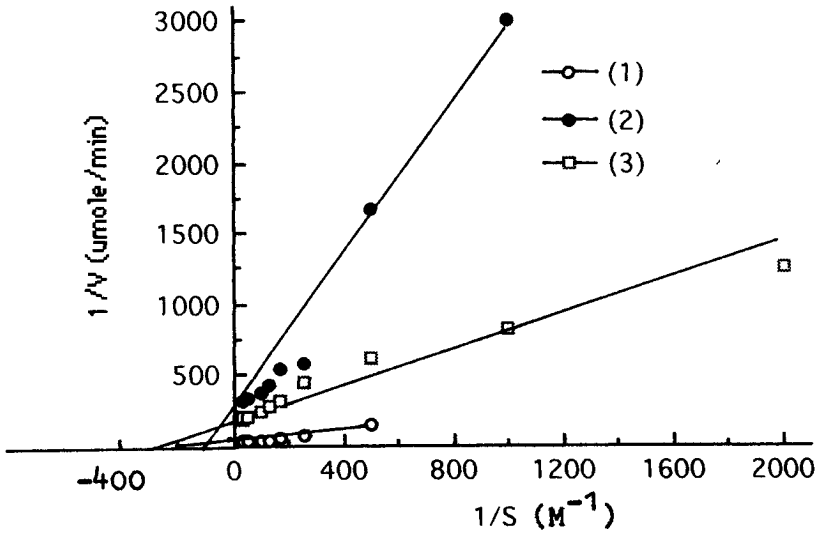

Fig. 5b Lineweaver-Burk plot from reaction of crude enzyme (1), purified enzyme from DEAE-Sephadex A-50 column (2) and purified enzyme from Blue-Sepharose CL-4B (3).

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บทคัดย่อ

ซอร์บิทอลเป็นผลิตภัณฑ์จากปฏิกิริยาการรีดิวส์ฟรุคโทสโดยเอนไซม์ภายในเซลล์ของ *Zymomonas mobilis* IFO 13756 ซึ่งมี NADH ร่วมด้วย การบดเซลล์ด้วยทรายทำให้ได้กลูโคส-ฟรุคโทสออกซิโดรีดักเทสที่มีแอคติวิตีสูงสุดเมื่อเปรียบเทียบกับการสกัดเอนไซม์ด้วยวิธีอื่นอีก 4 วิธี ซึ่งได้แก่ การใช้ไลโซไซม์ การแตกเซลล์ด้วยคลื่นอัลตราโซนิค การสกัดด้วยอะซิโตนเย็น และการสกัดกากเซลล์ด้วยไตรทอน X-100 เอนไซม์กึ่งบริสุทธิ์ซึ่งได้จากคอลัมน์ของ บลู-เซฟาโรส CL-4B มีความบริสุทธิ์ 7.22 เท่าของเอนไซม์เริ่มต้น และได้ผลผลิต 23.39% ซึ่งมีค่าสูงกว่ากรณีการใช้คอลัมน์ของ DEAE-เซฟาเดกซ์ A-50 เอนไซม์กึ่งบริสุทธิ์จากวิธีที่ทำให้ได้ผลผลิตสูงกว่านั้น มีพีเอชที่เหมาะสม อุณหภูมิที่เหมาะสม V_{max} และ K_m ที่ 7.0, 25°ซ, 0.006 ไมโครโมลต่อนาที และ 4 มิลลิโมลาร์ ตามลำดับ สมบัติการเร่งปฏิกิริยาการรีดิวส์ของเอนไซม์กึ่งบริสุทธิ์ ได้รับการทดสอบและยืนยันโดยการวิเคราะห์แอคติวิตีของแถบโปรตีน ที่แยกโดย polyacrylamide gel electrophoresis