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Continuous Production of Xylooligosaccharides from Xylan by Crude Enzyme Derived from *Eupenicillium javanicum*

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The immobilized enzyme packed column was used to produce oligosaccharides by the continuous hydrolysis of soluble xylan. The immobilized enzyme was significantly stable and did not inactivate up to at least 1,200 h of reaction. Oligosaccharides only were produced effectively by crude enzyme derived from *Eupenicillium javanicum*. The ratio of hydrolysis of soluble xylan was ca. 20 % and the molar ratio of xylotriose to xylobiose was almost 1 to 1. The productivity of xylooligosaccharides seems to increase under higher concentration of soluble xylan and higher reaction temperature than 28℃.

Keywords: *Eupenicillium javanicum*, xylan, endo-xylanase, β-xylosidase, xylooligosaccharides, packed column reactor.

Introduction

Xylan is one of main components in agro wastes and wood wastes, which are wasted large amount. The effective use of xylan is hoped.

The xylooligosaccharides which are enzymatic hydrolysates of xylan, are utilized as indigestible sweeteners and are useful as a functional substance which has a stimulatory effect on the selective growth of human intestinal *Bifidobacteria*, important for the maintenance of a healthy intestinal microflora (1-3). The enzymatic hydrolysis of xylan is beneficial because it occurs under mild conditions with no side-reaction. We found that *Eupenicillium javanicum* isolated from humus produced a highly active xylanase in spite of a weak β-xylosidase activity (4). This xylanase system was considered to have a merit to obtain xylooligosaccharides from xylan.

Since β-xylosidase activity in the culture liquid was weak, the culture liquid recovered was used as a crude enzyme solution without purification, and the continuous production of xyloologosaccharides from xylan by the enzyme solution was investigated.

1. Experimental

1.1. Preparation of crude enzymes

The basal medium for growth of *Eupenicillium javanicum* was 1.0 g Bacto peptone, 0.3 g Urea, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 5.0 mg $FeSO_4 \cdot 7H_2O$, 1.6 mg $ZnSO_4 \cdot 7H_2O$, 1.4 mg $MnSO_4 \cdot H_2O$, 4.0 mg $CoCl_2 \cdot 6H_2O$ per liter. The initial pH and temperature of main culture were 5.5 and 28℃, respectively (4). The cells obtained by pre-culture (100 ml basal medium solution containing 10 g/l glucose) were recovered by centrifugation, and main culture on 2 liter-shaking flask was carried out by adding the above cells in the culture liquid (1,000 ml basal medium solution containing 40 g soybean bran). The culture liquid was recovered at 11 days of cultivation time, the supernatant obtained by centrifugation was used as a crude enzyme solution, and also freeze-dried for storage.

1.2. Preparation of immobilized enzyme

The freeze-dried sample (25.5 g) was obtained from 3,500 ml of the supernatant solution. The immobilization of xylanase was performed with carbodiimide coupling method. The concentration of enzyme solution under the following experiments was 0.1 g/ml. TOYOPEARL AF-Amide-650M (12 ml gel) was used as the immobilization carrier. The suitable amount of the enzyme solution was added in the gel, that is, gel suspension. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chloride) was added in the gel suspension as a condensing agent, then the gel suspension was shaken

for 2 days at 4℃. After immobilization, that was washed with distilled water, 1 M NaCl solution, distilled water, 50 mM acetate buffer (pH5.0) in turn on glass-filter (G2). Consequently, the immobilized enzyme was obtained.

1.3. Experimental apparatus and operation

The enzyme reaction was carried out continuously in the column (working volume: 12ml) packed with the above immobilized enzyme gel. The column size was 14.7 mm in inner diameter and 120 mm in length. Substrate solution (1 % soluble xylan) was supplied at the rate of 0.01 ml/min at 28℃ through a micro-tube pump. At the column exit, reaction solution was recovered with a fraction collector every 3 hours. The measurement of reaction products was performed by HPLC (TSK gel Amide 80 x 2, Elution; 65 % acetonitrile, Elution rate; 0.8 ml/min, Column temperature: 60℃, RI detector) for xylose, xylobiose and xylotriose, and Somogyi-Nelson method for reducing sugar (R.S.) by using xylose as a standard.

1.4. Stability of immobilized enzyme

The above apparatus was used for a test of stability of the immobilized enzyme. Working volume and feeding rate of reaction solution were 5.5 ml and 0.25 ml/min, respectively. Soluble xylan (1.0 %) of 10 ml was circulated between the packed column and substrate storage tank for a suitable period. After a suitable period, substrate storage tank was exchanged to new soluble xylan solution of 10 ml. This operation was repeated up to 1,100 h.

2. Theoretical consideration

The hydrolysis of xylan by endoxylanase assumes as the followings.

<Endoxylanase>

Xylan → Olygosaccharides (1)
(xylotriose and xylobiose)

We assume that xylose is not produced by hydrolysis of xylan with endoxylanase, since it is produced by hydrolysis of xylobiose and xylotriose with β -xylosidase. For simplification, if this reaction is Michaelis-Menten type and inhibition as substrate and product inhibitions does not occur, the enzyme reaction rate is obtained from the following equation.

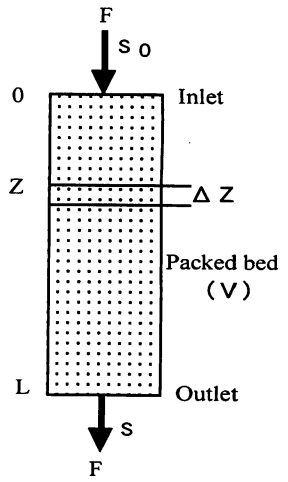

Fig. 1. Fixed bed axial-flow column.

$$v = k_2E_0S/(K_m + S) \quad (2)$$

Where, E_0 (g/l) is initial enzyme concentration, k_2 (1/min) is first order reaction rate constant, k_2E_0 (g/l/min) is maximal reaction rate, Km (g/l) is Michaelis constant, and S (g/l) is substrate (soluble xylan) concentration.

From **Figure 1**, mass balance of substrate (soluble xylan) at small region in axial direction on the immobilized endoxylanase packed column is obtained as the following equation.

$$-F(dS/dZ) = A(1-\varepsilon)k_2E_0'S/(K_m+S) \quad (3)$$

Where, boundary condition is Z=0, and S=S_0. Z is distance from column inlet, and S_0 is initial substrate concentration at column inlet. F (ml/min) is flow rate (feeding rate) of reaction solution, A (cm^2) is area of cross section of column, ε (-) is ratio of void volume of bed in column, Z (cm) is distance from column inlet, and k_2E_0' (g/l/min) is maximal rate of immobilized enzyme per unit bed volume.

When the equation (3) is integrated under the conditions of range of S_0 to S for S and 0 to L for Z, the following equation is obtained.

$$-K_m\ln(S/S_0) + (S_0 - S) = AL(1-\varepsilon)k_2E_0'/F \quad (4)$$

When the result obtained is made dimensionless, and rearranged, the following equation is obtained.

$$-K_m\ln(1-x) + S_0x = \tau k_2E_0' \quad (5)$$

Because S/S_0 is the ratio of remaining substrate after hydrolytic reaction, $\ln S/S_0$ is expressed as $\ln(1-x)$, when $x(-)=(S_0-S)/S_0$ is the ratio of hydrolysis of soluble xylan and τ (min)$=V(1-\varepsilon)/F$ is the residence time per volume of immobilized enzyme. Where, V (ml) is bed volume of column.

In the case of $S_0 \ll K_m$,

$$-\ln(1-x) = \tau k_2E_0'/K_m \quad (6)$$

In the case of $K_m \ll S_0$,

$$x = \tau k_2E_0'/S_0 \quad (7)$$

From the equation (5), the value of k_2E_0' is obtained by changing the flow rate of F under a high concentration of S_0, and from the equation (6), K_m value is obtained by changing the flow rate of F under a low concentration of S_0 using the above value of k_2E_0'.

3. Results and discussion

3.1. Activity of immobilized xylanase

Before the immobilization of crude enzyme to carrier, the enzyme solution was desalted. In this step, xylanase activity was almost not decreased (data not shown). High activity of xylanase was not attained, because the effect of immobilization to carrier was significantly low (data not shown). The improvement of immobilization method was necessary. However, the carrier immobilized as the above was used, since xylanase activity was not decreased in the step of immobilization.

3.2. Stability of immobilized enzyme

The experiment was carried out as described in Section 1.4. The results are shown in **Figure 2.** The reaction solution circulated was exchanged to new substrate solution of 10 ml every 100 h up to 1,100 h. The circulation time of reaction solution was 40 min. About 2.0 ~ 2.5 mg R.S./ml was produced. The ratio of hydrolysis of xylan was ca. 20 ~25 %.

By the way, **Figure 3a** shows the amount of reducing sugar produced by free crude enyme. In this case, enzyme concentration and soluble zylan concentration were 1.0 mg/ml and 1.0 %, respectively. The reaction solution was prepared with 0.05 M acetate buffer (pH5.0) and the reaction temperature was 30 ℃. Apparently the ratio of hydrolysis attained plateau at ca. 20 %.

This result shows that in the case of immobilized enzyme as shown in Fig. 2, the enzyme reaction time in the bed was sufficient and the activity of immobilized enzyme was not decreased up to 1,100 h. On the other hand, the equation (2) is expressed as the following equation.

$$v = -dS/dt = k_2E_0S/(K_m + S) \quad (8)$$

When this equation is integrated and rearranged, the following equation is obtained.

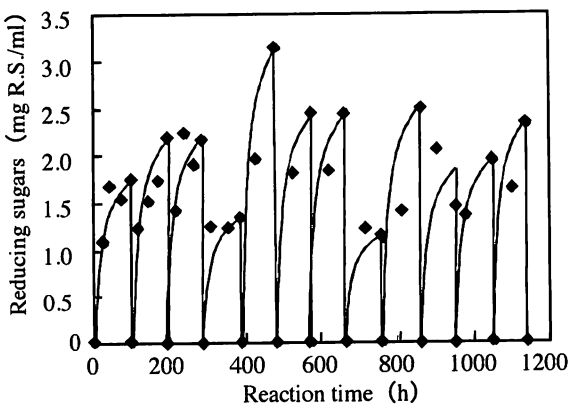

Fig. 2. Stability of immobilized enzyme.

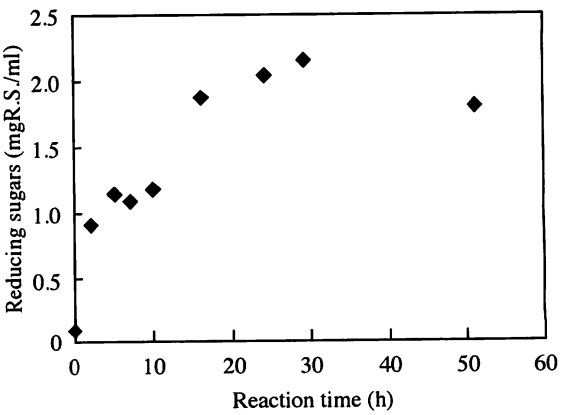

Fig. 3a. Hydrolysis of soluble xylan by free crude enzyme.

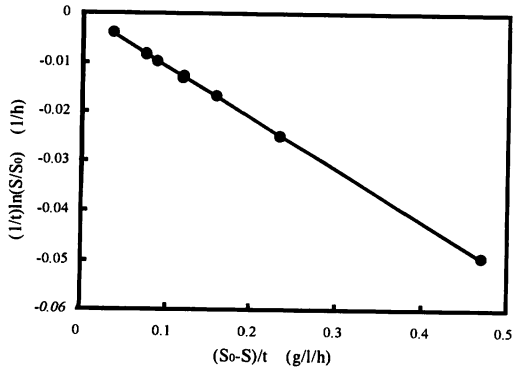

Fig. 3b. Determination of K_m and V_m of free crude enzyme.

$$-(1/t)\ln(S/S_0) = -(1/K_m)(S_0 - S)/t + V_m/K_m \qquad (9)$$

Where, $V_m = k_2E_0$ (g/l/h), that is maximum velocity of hydrolysis.

From the data of Fig. 3a and the equation (9), **Figure 3b** is obtained.

From the slope and the intercept at y-axis of straight line, the K_m value and the V_m value are 10 g/l and 5.0 x 10^{-3} g/l/h, respectively. That is, the finding shows that the free crude enzyme reaction was carried out at the concentration of soluble xylan in the vicinity of K_m value.

3.3. Continuous hydrolysis of soluble xylan by immobilized enzyme using the packed column

using the packed column. The initial reaction period did not produce reducing sugars, since the elution of initial solution in the column bed was needed. After the initial period, stable hydrolysis was attained. The ratio of hydrolysis of xylan was ca. 20 %. This value was similar to that of the above circulation experiment. Moreover, the sugars

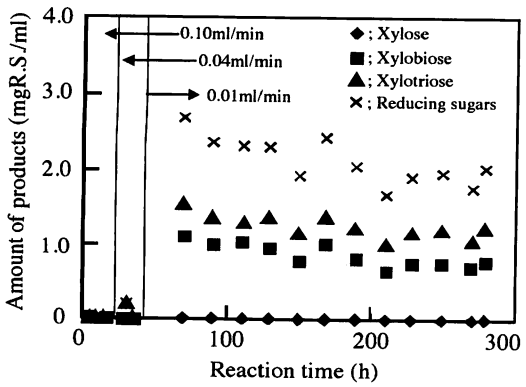

Fig. 4a. Continuous hydrolysis of soluble xylan by immobilized enzyme using the packed column.

Figure 4a shows the result of continuous hydrolysis of soluble xylan by immobilized enzyme

produced were xylotriose and xylobiose only, and xylose was not. Xylotriose was more than xylobiose under the weight ratio. However, the molar ratio of xylotriose to xylobiose was almost 1 to 1. This finding shows that the assumption of equation (1) was valid.

Figure 4b shows the total amount of products under the continuous hydrolysis of soluble xylan. In the range of reaction period, the amount of reducing sugars, xylotriose and xylobiose increased monotonically. The amount of production of reducing sugars attained to ca. 300 mg after the reaction of 250 h. The amount of production was increased with the increase in the reaction temperature from 20℃ to 45℃ (data not shown).

Apparently the continuous hydrolysis of soluble xylan by immobilized endoxylanase is very effective.

3.4. Determination of K_m and k_2E_0'

From the experiment of continuous hydrolysis, V = 12 ml, S_0 =10 g/l and F = 0.01 ml/min. If ε is 0.32 as the closed packing and S_0 is extremely higher than K_m value, since τ is 816 min and x is 0.2, from the equation (7), k_2E_0' = 2.5 x 10^{-3} g/l/min. If K_m value is extremely higher than S_0, from the equation (6), k_2E_0'/K_m is 2.7 x 10^{-4} 1/min. To get the accurate values of these kinetic constants (k_2E_0' and K_m), the experiments under conditions of $K_m \ll S_0$ and $K_m \gg S_0$ were necessary. Since our goal was not to obtain such kinetic constants, additional experiment was not performed. However, in the step of immobilization of enzyme, if K_m value was no change, the reaction of hydrolysis by the immobilized enzyme was

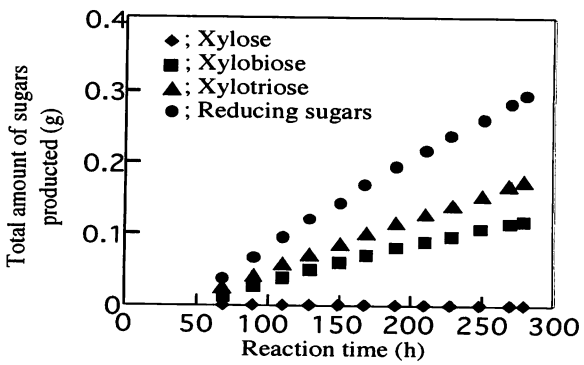

Fig. 4b. Total amount of sugars produced under the continuous hydrolysis of soluble xylan.

apparently performed under the concentration of soluble xylan at K_m value.

Conclusions

The immobilized enzyme packed column was used to produce oligosaccharides by the continuous hydrolysis of soluble xylan. The immobilized enzyme was significantly stable and did not inactivate up to at least 1,200 h of reaction.

The immobilized enzyme packed column was used to produce oligosaccharides by the continuous 1,200 h of reaction. Oligosaccharides only were produced effectively by crude enzyme derived from *Eupenicillium javanicum*, since this mold did almost not produce β -xylosidase in the culture liquid. The ratio of hydrolysis of soluble xylan was ca. 20 % and the molar ratio of xylotriose to xylobiose was almost 1 to 1. From the theoretical consideration, to get the kinetic constants of immobilized enzyme reaction in the packed column reactor, the experiments under conditions of $K_m \ll S_0$ and $K_m \gg S_0$ must be carried out. The productivity of oligosaccharides seems to increase under higher concentration of soluble xylan and higher temperature of reaction, since the hydrolysis by immobilized enzyme was carried out under the K_m value and 28℃.

References

1. Okazaki, M. : Production of xylooligosaccharides using a substrate-packed column reactor (in Japanese), *Bioscience & Industry*, **49** (9), 956-960 (1991).
2. Okazaki, M., Fujikawa, S., Matsumoto, N. : Effect of xylooligosaccharide on growth of *Bifidobacteria* (in Japanese), *J. Jpn. Soc. Nutr. Food Sci.*, **43** (6), 395- 401 (1990).
3. Petschow, B. W., Talbott, R. D. : Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk, *J. Clin. Microbiol.*, **28**, 287-292 (1990).
4. Tanaka, M., Sumida, T., Mitsui, R. : Effect of culture conditions on production of cellulases and xylanases by *Eupenicillium javanicum* (in Japanese), *Seibutu-Kogaku Kaishi*, **78** (2), 43-47 (2000).