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A New Method for the Preparation of Mannotriose from White Copra Meal Using the Enzyme System and Yeast Fermentation

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Abstract

A new method was developed to prepare β-1,4-mannotriose by the enzymatic hydrolysis of white copra meal and the subsequent elimination of monosaccharides and mannobiose from the resultant hydrolysate with a yeast. The optimum pH and temperature for the mannanase were 6 and 50° C, respectively. The mannanase was stable between pH 5.5 and 7 after 2hr treatment at 30° C. White copra meal (70g) was hydrolyzed with the mannanase (3,450units / 500ml) at pH 6 and 50° C for 24hr. The hydolysis products were monosaccharides, mannobiose and mannotriose. By the elimination of monosaccharides and mannobiose from the hydrolysis products with *Candida guilliermondii* IFO 0566, 12.1g of mannotriose was obtained without the use of chromatographic techniques.

Key words : β-1,4-mannotriose, β-mannanase, *Candida guilliermondii* IFO 0566

INTRODUCTION

The objective of this research is to apply β-mannanase from *Penicillium* sp. to the preparation of mannotriose from white copra meal. A large amount of coconut residual cake is discharged as a by-product in the process of oil extraction from copra, and the full utilization of the cake has not been accomplished in the coconut industry in the Southeast Asian countries (1). Only a small amount of the cake has been utilized as livestock feed (1), even though the cake contains about 20% protein, 65% carbohydrate and others (2) partly because the above countries are technologically underdeveloped to utilize the cake effectively. In this connection, Kusakabe *et al.* have been studied β-mannanase, especially the enzymatic preparation of β-1,4-mannooligosaccharides (3,4) from coconut residual cake, and also some properties (5-7) of the purified enzyme. However, their process showed two drawbacks : (1) The enzyme, in addition to mannooligosaccharides, also produced considerable amounts of galactomannooligosaccharides, which interfered with the crystallization of mannooligosaccharides. (2) The copra galactomannan, extracted by a heavy alkali solution (20~24%), was used in their study for the preparation of mannooligosaccharides. However, it is difficult to extract the mannan from the cake by the alkali solution. Threrfore, it is necessary to find out other enzyme which is able to directly hydrolyze the galactomannan in the cake, and does not produce galactomanno-oligosaccharides in the hydrolysate. In order to overcome the drawbacks, author attempted to isolate microorganisms producing β-mannanase, and succeeded in isolating a strain from soil, *Penicillium* sp. which produced extracellulary a galactomannan-degrading enzyme.

Thus, author, in this paper, attempted to apply the specific characteristics of the enzyme, and carried out the preparation of the mannotriose from white copra meal by a combined process ; that is, hydrolyzing the copra meal by the crude enzyme and eliminating both monosaccharides and mannobiose from the resultant hydrolysate with yeast.

MATERIALS AND METHODS

White copra meal

The copra meal, which is a by-product of oil extraction from copra, was kindly supplied by Blue Bar Inc.(the Phillippines). The meal contained 49.9% of as total sugar which was composed of 63.4% mannose, 24.9% glucose, 6.6% galactose and 4.4% arabinose. White copra meal was hydrolyzed with 72% sulfuric acid at 30° C for 30min,

followed by 4% sulfuric acid at 100° C for 2hr. The total sugar content and sugar composition were determined by Somogyi's method (8) and gas liquid chromatography (9), respectively.

Preparation of β-mannanase solution

The medium for the enzyme production was composed of 4% white copra meal, 0.9% peptone, 0.1% yeast extract, 1% potassium phosphate (monobasic), 0.05% magnesium sulfate, 0.5% corn steep liquor and a small amount of Toshiba Silicone as a antifoaming agent. The mediun was placed in a jar fermentor (model ; MB-C, Iwashiya Bio-science Co. Ltd, Japan), and sterilized at 120° C for 10min. The seed culture of *Penicillium* sp., which had been grown in the same medium in shake flasks at 35° C for about 2 days on a reciprocal shaker, was inoculated into the fermentor. The fermentation was carried out at 35° C with an air supply at a flow rate of 500ml per min and with an agitation at the speed of 700rpm. A few ml of the broth during the cultivation were withdrawn from the fermentor, and filtered through a Toyo-roshi No. 2 filter paper. Then the filtrate was analyzed for the activity of the enzyme.

Thin layer chromatography (TLC)

TLC was carried out according to the method of McCleary (11). The sugar sample was dotted on a plate of Merck DC-Alufolien Kiesel gel 60 (0.2mm), and developed with a solvent system of 1-propanol : nitromethane : water (5 : 2 : 3, v/v/v) for about 4hr at room temperature. The sugars on the plate were visualized by spraying with 30% sulfuric acid-ethanol and heating to 120° C for 10min.

Determination of β-mannanase activity

β-Mannanase activity was determined by the method described in the previous paper (10).

Preparation of β-1,4-mannotriose

The white copra meal (70g), which contained 34g of total sugar (with 20g of mannan), was hydrolyzed with 500 ml of the enzyme solution (3,450units) at pH 6 and at 50° C for 48hr. After the removal of insoluble materials from the hydrolysate by centrifugation, a solution containing 21.2g of total sugar was obtained. The final concentration of the nutrients added to the solution was 0.2% for peptone, 0.3 % for yeast extract, 0.1% for potassium phosphate (monobasic), 0.05% for magnesium sulfate and 0.2% for calcium carbonate. About 100ml each of the solution supplemented with the nutrients was placed into five 500ml shaking flasks each and sterilized under the above conditions. After cooling, 5ml of the seed culture of *Candida guilliermondii* IFO 0566 was inoculated into the medium. The cultivation was carried out at 30° C for about 48hr.

Determination of sugar

Reducing sugar was determined by Somogyi's method (8). Total sugar content in the enzymatic hydrolysate was determined by the same method after hydrolysis by 4% sulfuric acid at 100° C for 2hr. Average degree of polymerization (D.P) of the sugar was calculated from the ratio of the total sugar to the reducing sugar.

RESULTS

Production of β-mannanase system

Fig. 1 shows the progress of fermentation. There was a rapid increase in the production of the enzyme at about 110hr after the beginning of fermentation, and the mannanase activity in the culture filtrate reached maximum values at 120hr (8.2 units/ml). After 120hr, the mycelium was filtered off through a Buchner funnel with a Toyo-roshi No. 2 filter paper. The resultant filtrate was then dialyzed against a 4-fold volume of distilled water, and the dialyzed solution (6.9 units of mannanase activity/ml) was

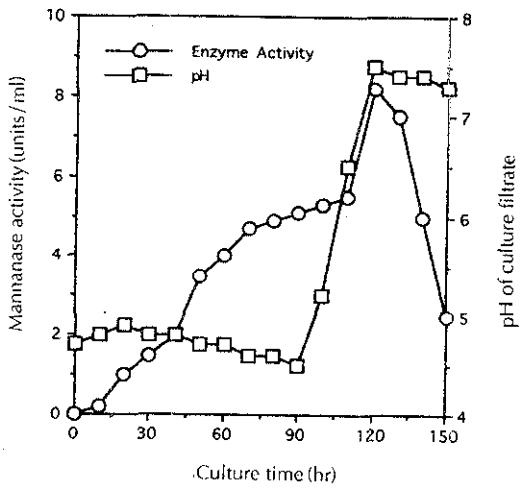

Fig. 1. Time course of the production of mannanase.

used as the mannanase solution for the following experiments.

Enzymatic properties of the mannanase

Effect of pH on mannanase activity : The enzyme reaction was carried out at various pHs with a McIlvaine buffer solution at 50° C for 30min. As shown in Fig. 2, the enzyme showed the maximum activity at pH 6.

Effect of temperature on mannanase activity : The enzyme reaction was carried out at various temperatures at pH 6.0 for 30min. As shown in Fig. 3, the optimum temperature for the enzyme reaction was 50° C.

Effect of pH on stability : The enzyme solution was maintained at 30° C for 2hr at various pHs, and the remaining activity was assayed. The pH stability of the enzyme was presented in Fig. 4. The enzyme was stable in between pH 5.5 and 7.

Effect of white copra meal concentration on enzymatic hydrolysis

Each reaction mixture contained 1.5g (5% based on enzyme solution used), 2.1g (7%), 3.0g (10%), 4.5g (15%) or 6g (20%) of white copra meal, and 30ml of the enzyme solution. The enzyme reaction was performed at pH 6 and 50° C in a T-form tube on a Monod shaker. A small portion of each mixture was withdrawn from the tube and heated to about 100° C for 5 min to inactivate the enzyme. After removal of insoluble materials from the mixture by centrifugation, the reducing sugar content in the supernatant was determined by Somogyi's method (8) to estimate the degree of hydrolysis.

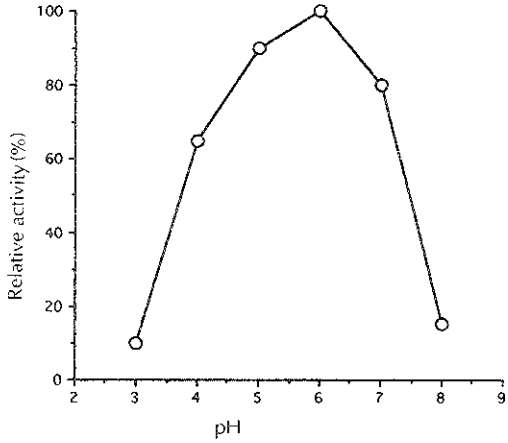

Fig. 2. Effect of pH on mannanase activity at 50° C.

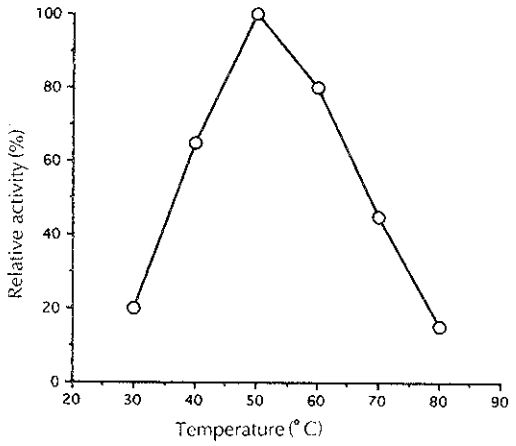

Fig. 3. Effect of temperature on mannanase activity at pH 6.0.

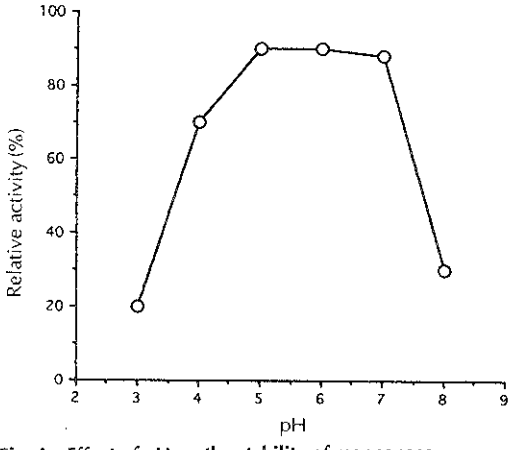

Fig. 4. Effect of pH on the stability of mannanase.
The enzyme solution was maintained at various pHs, ranging from 3 to 8, using the McIlvaine buffer solution.

Fig. 5 shows the time course of enzymatic hydrolysis. The hydrolysis of the white copra meal proceeded rapidly at an early stage of the reaction, but gradually slowed thereafter especially at a higher concentration of copra meal such as 20%. As shown in Fig. 6, the major products at the final stage were monosaccharides, mannobiose and mannotriose without detectable amounts of other saccharides. In addition, the enzyme scarcely hydrolyzed β-1,4-mannotriose under the condition of low enzyme concentration.

Elimination of monosaccharides and mannobiose from enzymatic hydrolysate of white copra meal by *Candida guilliermondii*

The mannanase from *Penicillium* sp. produced mainly monosaccharides, mannobiose and mannotriose from the white copra meal. Therefore, it might be possible to prepare mannotriose from the enzymatic hydrolysate of the meal without using chromatographic techniques, if a certain yeast could eliminate monosaccharides and mannobiose from the hydrolysate by selective fermentation. To achieve this objective, *Candida guilliermondii* IFO 0566 was selected in a preliminary experiment. The yeast metabolized arabinose, galactose, glucose, mannose, xylose and β-1,4-mannobiose but not β-1,4-mannotriose (Fig. 7).

As shown in Fig. 8, the sugar content decreased with the time of fermentation up to about 24hr, but any significant decrease was not observed thereafter. After 48hr cultivation, the total sugar content fell from 4.5% to 3.7%, and the average degree of polymerization, on the other hand, rose from 2.6 to 3.2.

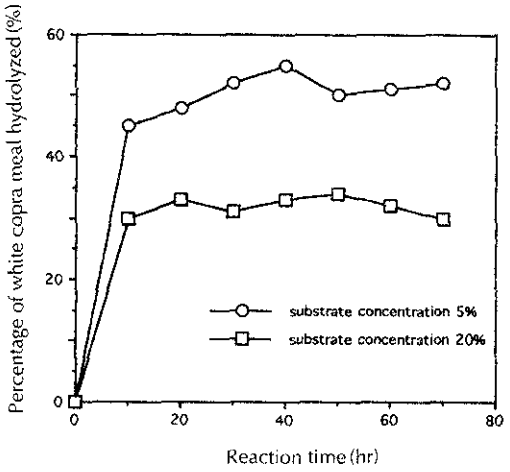

Fig. 5. Effect of white copra meal concentration on enzymatic hydrolysis.

Some properties of β-1,4-mannotriose

At the end of fermentation, the yeast cells were removed by centrifugation, and the sugar solution containing 17.8g as total sugar was obtained. The solution was decolorized with active carbon, followed by desalting on the columns of cation (IR-200c) and anion (IRA-68) exchange resins. The resulting sugar solution was concentrated to a syrup by a vacuum rotary evaporator. Ethanol was added to the syrup to reach the concentration of about 80% ethanol. After seeding of crystalline β-1,4-mannotriose and cooling, mannotriose was crystallized. Crystals formed were

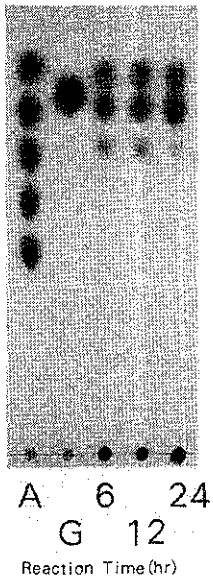

Fig. 6. Time course of hydrolysis pattern of white copra meal with enzyme solution.
A : Standard mannose, mannobiose mannotriose, mannotetraose and mannopentose from top to bottom
G : Standard galactose

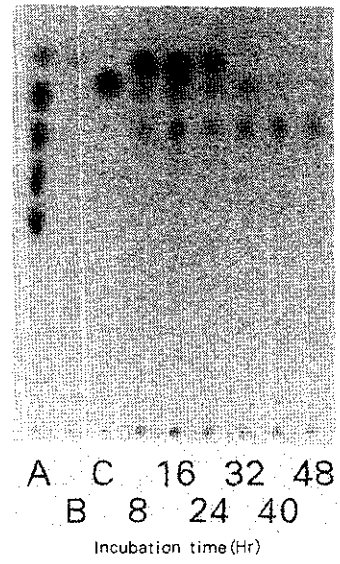

Fig. 7. Elimination of monosaccharides and mannobiose from enzymatic hydrolysate of white copra meal by yeast.
A : Standard mannose, mannobiose, mannotriose, mannotetraose and mannopentaose from top to bottom.
B : Standard glucose, C : Standard galactose

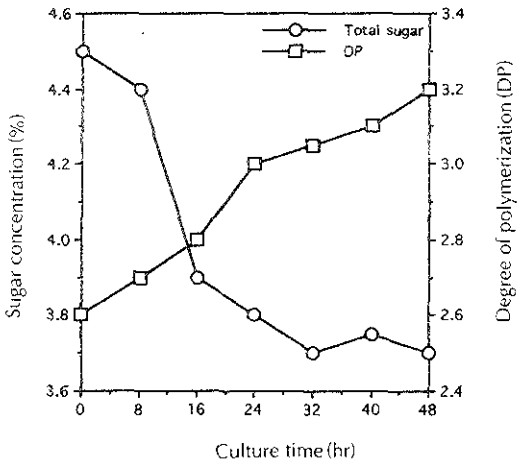

Fig. 8. Time course of cultivation of enzymatic hydrolysate of white copra meal with *Candida guilliermondii*.

isolated by centrifugal filtration, and 12.1g of crystalline mannotriose was obtained.

Fig. 9 shows the flow sheet for the preparation of crystalline mannotriose. Crude crystals of the mannotriose obtained above were recrystallized twice from about 80% aqueous ethanol. The recrystallized mannotriose was subjected to thin layer chromatography and was homogeneous on the thin layer chromatogram. Hydrolysis of the methylated saccharide, on the other hand, afforded *2,3,4,6-tetra-O-Me-D-mannopyranose (1mol) and 2,3,6-tri-O-Me-D-mannopyranose* (2mol) using the gas liquid chromatography. These results indicate that the saccharide had the structure of β-1,4-mannotriose.

DISCUSSION

The galactomannan gives galactose and mannose in the ratio of 1 : 10~1 : 15. The distribution of the α-galactosyl branches is irregular and the distance between two branches, in some parts of the chain of galactomannan, is short. Therefore, the final products (3,7), arising from the degradation of the galactomannan by *Streptomyces* mannanase, were mannose and mannooligosaccharides, in addition to several kinds of hetero-oligomers (galactomanno-oligosaccharides) consisting of galactose and mannose residues.

In this paper, *Penicillium* sp. was isolated from soil samples. The strain produced β-mannanase extracellularly

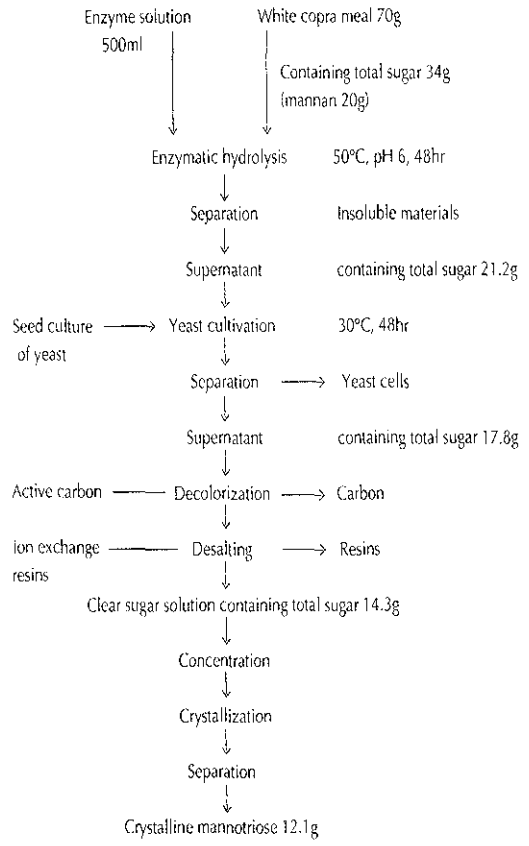

Fig. 9. Flow chart for the process of enzymatic preparation of crystalline mannotriose from white copra meal.

and the enzyme system attacked directly the galactomannan in the white copra meal. Thus, a process extracting galactomannan with the concentrated solution of sodium hydroxide was not necessary for the preparation of mannotriose by using the enzyme system. The final products of the galactomannan digest with the enzyme system included monosaccharides (galactose, glucose and mannose), mannobiose and mannotriose without the detection of a considerable amount of other oligomers. I was able to eliminate the monosaccharides and mannobiose with selective fermentation by yeast, and to prepare mannotriose without using any chromatographic technique. In addition, it is assumed that the enzyme system also contains other kinds of enzymes, probably cellulase and β-glucosidase, as glucose was detected in the enzymatic hydrolysate.

In conclusion, my preparation methods, namely the process combining the hydrolysis of white copra meal

by the enzyme system of *Penicillium* sp. and the elimination of monosaccharides and mannobiose from the resultant hydrolysate with *Candida guilliermondii* IFO 0566, is suitable for the preparation of mannotriose from the copra meal, because there is no free mannotriose in nature nor any other efficient method for the preparation of the sugar.

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효소법과 효모발효법을 이용한 White Copra Meal로 부터의 Mannotriose의 새로운 조제법

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요 약

본 연구는 white copra meal에 대한 효소적 가수분해법과 효모발효법을 이용한 β-1, 4-mannotriose의 조제법이다. *Penicillium* sp.로 부터 생성된 mannanase의 최적 pH와 온도는 각각 6과 50°C였다. pH 안정성에서는 30°C, 2시간 처리 후 pH 5.5~7의 범위에서 90%의 잔존활성을 유지하였다. pH 6, 50°C, 24시간 효소액 500ml (3,450units)로 white copra meal 70g를 가수분해한 결과 반응말기에 단당류, mannobiose, mannotriose가 생성되었다. *Candida guilliermondii* IFO 0566의 단당류와 이당류에 대한 자화성에 의해 최종 가수분해산물로 부터 12.1g의 mannotriose가 조제되었다.