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The Preparation of Crystalline Mannobiose from Brown Copra Meal Using the Enzyme System and Yeast Fermentation

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β-1,4-Mannobiose was prepared by the enzymatic hydrolysis of brown copra meal and the subsequent elimination of mono-saccharides from the resultant hydrolysate with a yeast. The enzyme system hydrolyzed brown copra meal and produced monosaccharides and β-1,4-mannobiose without other oligomers at the final stage of the reaction. Brown copra meal (30 g) was hydrolyzed at 50°C and pH 5 for 48 hr with the crude enzyme solution (300 m*l*) from *Penicillium purpurogenum*. By the elimination of monosaccharides from the hydrolysis products with a yeast (*Candida parapsilosis* var. *komabaensis* k-75), 5.2 g of crystalline mannobiose was obtained without the use of chromatographic techniques. After 50 hours of yeast cultivation, the total sugar content fell from 3.5% to 2.4%, and the average degree of polymerization rose from 1.8 to 2.2.

The Southeast Asian countries, especially Indonesia, India and the Philippines, are coconut-growing areas, and coconut is one of their important crops. Thus, the total utilization of coconuts is essential in these countries. However, as the main objective of the coconut industry is to extract coconut oil and to use it as a raw material, a large amount of residual cake (brown copra meal and white copra meal) is discharged as a by-product in the process of extracting oil from copra.

The copra meal contains 40～50% of galactomannan (Gal : Man=1 : 10～1 :15). As sources of mannan of a high concentration and of a high level of purity are very limited in nature, we carried out our studies on the preparation of β-1,4-mannooligosaccharides (4) by using the *Streptomyces* β-mannanase system. In above paper we considered that direct hydrolysis of the copra meal by mannanase is easier and more economical for the preparation of mannooligosaccharides.

The objective of this paper is to apply the specific character of the enzyme and carry out the preparation of mannobiose from brown copra meal with a combined process. The combined process consists of namely hydrolyzing the copra meal by the crude enzyme and eliminating monosaccharides from the resulting hydrolysate with a yeast.

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MATERIALS AND METHODS

Brown Copra Meal(BCM) and β-1,4-Mannooligosaccharides

The preparation method and sugar compositions of BCM have been described in previous paper (7). And the substrate was also prepared by the method described in the previous paper (4).

Preparation of β-Mannanase Solution

The mannanase from *Penicillium purpurogenum* was prepared by a submerged-culturing, as described in a previous paper (3). The resulting culture filtrate was dialyzed at 8℃ overnight against a 4-fold volume of distilled water, and then was used as the mannanase solution in the hydrolysis of the copra meal.

Determination of β-Mannanase Activity

The assay mixture, containing 129.4 mg of copra mannan (9) (equivalent to 100 mg of polymannose), 4.0 m*l* of McIlvaine buffer solution (pH 5) and 5 m*l* of water, was put into an L-shaped tube. Then, the tube was preincubated at 55℃ for 10 min on a monod shaker with agitation at the speed rate of 60 oscillation per min. One m*l* of the enzyme solution was added to the mixture, then the mixture was incubated for 30 min at the same temperature. The reducing power produced by the enzyme reaction was determined as mannose by the method of Somogyi (8). One unit of the enzyme activity was defined as the amount which liberated redu-

cing sugar equivalent to 1 μmol of mannose per min under the above conditions.

Determination of Reduction Sugar

Reducing sugar was determined by the method of Somogyi (8). Total sugar content in the enzymatic hydrolsate was determined by the same method, after hydrolyzing oligosaccharides by 4% H_2SO_4 at 100℃ for 2 hr.

Nutrients Compositions and Conditions of Yeast Cultivation

Nutrients (consising of 0.2 g peptone, 0.3 g yeast extract, 0.1 g potassium phosphate (monobasic) and magnesium sulfate each), and 0.2 g calcium carbonate, were added to 100 m*l* of the supernatant liquid of the enzymatic hydrolysate of brown copra meal. The resulting medium (pH 5) was placed a 500 m*l* shake flask, and sterilized at 120℃ for 5 min in an autoclave. The seed culture of the yeast was inoculated into the medium, and cultivated at 30℃ on a reciprocal shaker. At certain time intervals, a small amount of culture broth was removed from the flask, followed by the removal of yeast cells from the broth by centrifugation. The supernatant solution thus obtained was subjected to the determination of sugar content and the TLC.

Preparation of β-1,4-Mannobiose

The brown copra meal (30 g), which contained 14.9 g of total sugar (with 9.5 g of mannan), was hydrolyzed with 300 m*l* of the enzyme solution at pH 5 and 50℃ for 48 hr. After the removal of insoluble materials from the hydrolysate by centrifugation, a solution containing 9.93 g 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 100 m*l* of each of the solution supplement with the nutrients were placed into five 500 m*l* shake flasks each and sterilized under the above conditions. After cooling, 5 m*l* of the seed culture of *Candida parapsilosis* var. komabaensis k-75 was inoculated into the medium in the flask. Cultivation was carried out at 30℃ for about 50 hr. After cultivation, the yeast cells were removed by centrifugation, and the supernatant liquid containing in total 7.1 g of sugar was obtained. The solution was decolorized with active carbon, and was then desalted on 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. Hot absolute ethanol was added to the syrup to reach the concentration of about 80% ethanol. After the seeding of cyrstalline β-1,4-mannobiose and the cooling, the mannobiose was crystallized. The crystals formed were isolated by centrifugal filtration, and crystalline mannobiose was obtained.

Thin-layer Chromatography (TLC)

TLC was carried out according to the method of McCleary (6). The sugar sample was dotted on a plate of Merck DC-Alufolien Kiesel gel 60 (0.2 mm), and developed with a solvent system of 1-propanol : nitromethane : water (5 : 2 : 3, v/v) for about 4 hr at room temperature. The sugar on the plate was revealed by heating the plate at 120℃ for about 10 min after spraying it with 30% H_2SO_4-ethanol.

RESULTS

Production of β-Mannanase System

Fig. 1 shows the course of cultivation. There was a rapid increase in the production of the enzyme at about 80 hr after the begining of cultivation, and the mannanase activity in the culture filtrate reached maximum (9 units/m*l*) between the 120th~124th hr. After the 124th hr, mycelium was filtered off through a Buchner funnel with a Toyo-roshi No. 2 filter paper. The resulting filtrate was then dialyzed against a 4-fold volume of distilled water, and the dialzyed solution was used as the mannanase solution for the following experiments.

Formation of β-1,4-Mannobiose from Brown Copra Meal with Mannanase Solution

The brown copra meal (3 g), which contained 1.49 g of total sugar (with 0.95 g of mannan), was hydrolzed with 30 m*l* of the enzyme solution at pH 5 and 50℃ for 48 hr. After the removal of insoluble materials from the resultant hydrolysates at cretain time intervals (1, 3, 5, 8 and 24 hr) by centrifugation, each 4 μ*l* of the supernatant liquid was subjected to TLC for the characterization of the hydrolysis products.

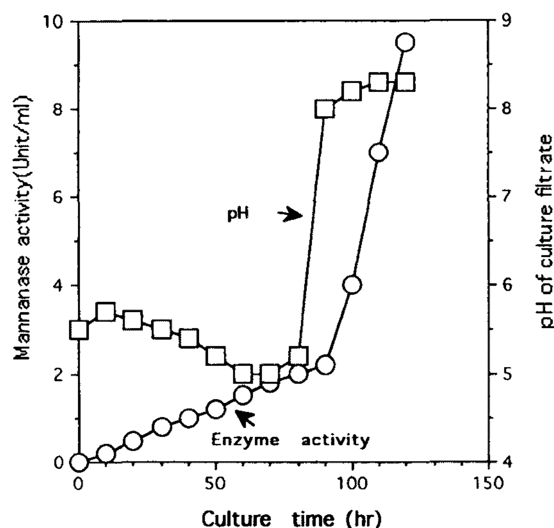

Fig. 1. Time course of the production of mannanase from *Penicillium purpurogenum*.

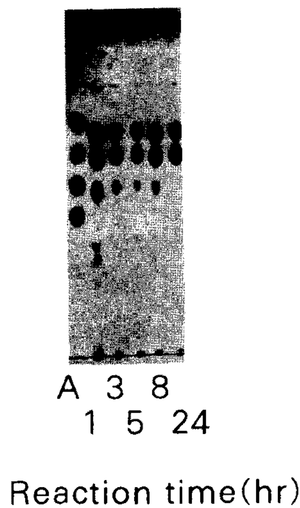

Fig. 2. Time-course of hydrolysis of brown copra meal with enzyme solution.
A: authentic mannose, mannobiose, mannotriose and mannotetraose from top to bottom.

Fig. 2 shows TLC of the time-course of hydrolysis of brown copra meal with enzyme solution. At the reaction time between the first and the 8th hr, monosaccharides, mannobiose and mannotriose were the products of the hydrolysis. But the main products of 24th were monosaccharides and mannobiose. From this result, we studied to prepare mannobiose from the enzymatic hydrolysate of brown copra meal without using chromatographic separation techniques. And we came up with the combination of the hydrolysis of the copra meal by crude enzyme and the elimination of monosaccharides from the resultant hydrolysate by the selective fermentation with a yeast.

Elimination of Monosaccharides from Enzymatic Hydrolysate of Brown Copra Meal by Yeast

We tried to find a yeast strain which is capable of metabolizing monosaccharides in enzymatic hydrolysate, and of leaving mannobiose in the hydrolysate, and *Candida parapsilosis* var komabaensis k-75 was selected.

The time-course of the decrease of sugar in the yeast cultivation was followed by the method of Somogyi (8). Fig. 3 shows TLC of the time-course of yeast cultures. After 48 hours of cultivation, the yeast strain deigested monosaccharides, but left mannobiose in the medium.

Fig. 4 shows the course of yeast cultivation. As shown in Fig. 4, the sugar content decreased as the time passed till about the 35th hr, but no remarkable decrease was observed thereafter. After 50 hours of cultivation, the total sugar content fell from 3.5% to 2.4%, and the average degree of polymerization (DP), on the other hand, rose from 1.8 to 2.2.

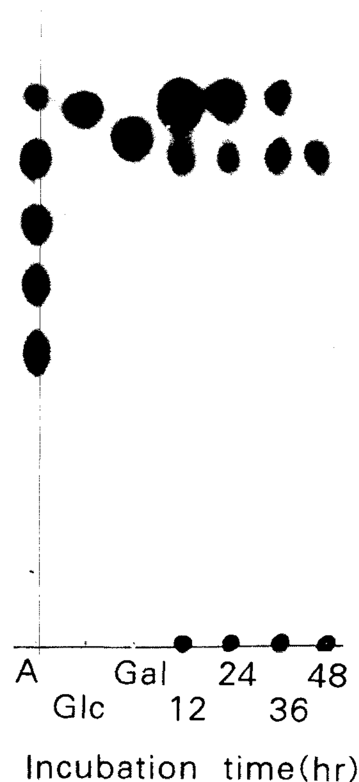

Fig. 3. Elimination of monosaccharides from enzymatic hydrolysate of brown copra meal by yeast.
A: authentic mannose, mannobiose, manntriose, mannotetraose and mannopentose from top to bottom, Glc: Glucose, Gal: Galactose.

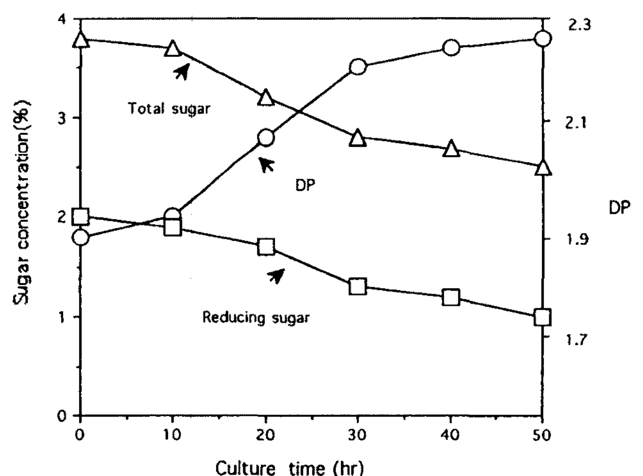

Fig. 4. Time course of cultivation of enzymatic hydrolysate of brown copra meal with yeast.

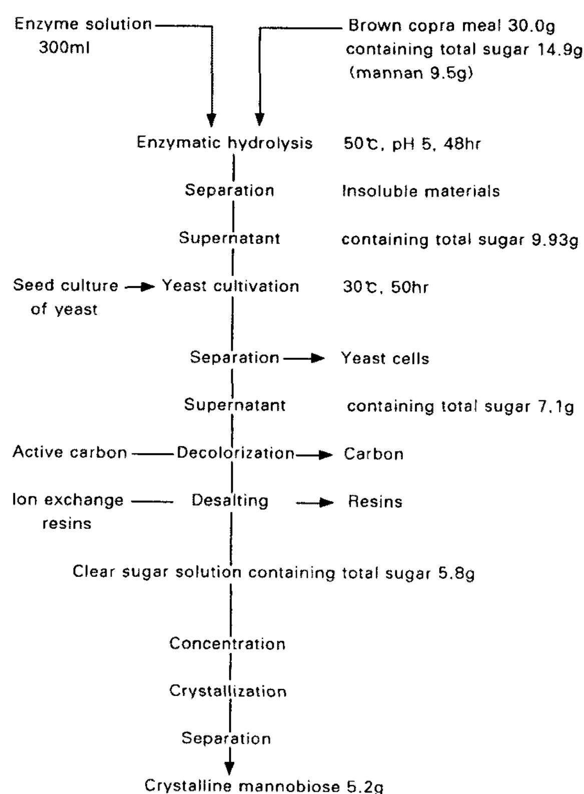

Fig. 5. Flow chart for the process of enzymatic preparation of crystalline mannobiose from brown copra meal.

Some Properties of β-1,4-mannobiose

Fig. 5 shows the flow sheet on the preparation of crystalline mannobiose. Crude crystals of the mannobiose from above were recrystallized twice from about 80% aqueous ethanol. 5.2 g of crystalline mannobiose was obtained and showed the following properties; $[\alpha]_D^{20}$ value$=-6.9°$ (c=2, H_2O), melting point=187~191℃. Hydrolysis of the methylated mannobiose, on the other hand, afforded 2,3,4,6-tetra-O-methyl-D-mannopyranose (1 mol) and 2,3,6-tri-O-methyl-D-mannopyranose (1 mol).

DISCUSSION

The partial chemical structure (2) of the copra galactomannan is as follows.

The galactomannan consists of galactose and mannose in the ratio of 1 : 10~1 : 15. α-Galactosyl branches is irregularly, and in some parts of the galactomannan chain, the distance between two branches is short. Therefore, in this experiment, the final products (2, 5), from the degradation of the galactomannan by *Streptomyces* mannanase, were mannose and mannooligo-saccharides, in addition to several kinds of hetero-oligomers consisting of galactose and mannose residues. On the other hand, the saccharides produced from the degradation of several galactomannans by mannanases originating from various kinds of fungi, were almost the same with the saccharides described above (1). Accordingly, an enzyme system with the coexistence of β-mannanase and α-galactosidase is essential to avoid the formation of the galactomanno-oligosaccharides from copra galactomannan. Morevoer, it is desirable to simplify the process of mannobiose production so that an enzyme system can hydrolyze directly the galactomannan in brown copra meal and produce only monosaccharides and mannobiose from the copra meal. In this connection, we tried to isolate a microorganism which secretes an enzyme system suitable for the above purpose.

The strain produced β-mannanase extracellularly and the enzyme system attacked directly the galactomannan in the brown copra meal. Moreover, the optimum pH and temperature for the mannanase activity were 5 and 60℃, respectively. On the other hand, the optimum pH and temperature for the α-galactosidase activity, produced by the same strain, were 4.5 and 55℃, respectively. Therefore, the enzyme system seems to be most suitable for the hydrolysis of galactomannan in the brown copra meal, because the properties of the two enzymes are very similar to each other. The final products of the digestion of galactomannan by the enzyme system included monosaccharides (galactose, glucose and mannose) and mannobiose without a considerable amount of other oligomers. We were able to eliminate only the monosaccharides with selective fermentation by yeast, and to prepare mannobiose without using any chromatographic technique. In addition, it was assumed that the enzyme system may also contains other kinds of enzymes, probably cellulase and β-glucoisidase, as glucose was detected in the enzymatic hydrolysate.

In conclusion, our preparation method, the combination of the hydrolysis of brown copra meal by the enzyme system of *Penicillium purpurogenum* and the elimination of monosaccharides from the resultant hydrolysate with *Candida parapsilosis* var. komabaensis k-75, was suitable for the preparation of mannobiose from the copra meal.

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