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Production of Mannooligosaccharides by the *Penicillium purpurogenum* Mannanase

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Abstract

***Penicillium purpurogenum*, which produces a copra galactomannan degrading enzyme extracellularly, was isolated from soil, and its properties and formation condition of mannooligosaccharides were investigated. The optimum pH and temperature for the activity of the mannanase were 5.5 and 55° C, respectively. The mannanase was stable in between pH 3.5 and 7.0 after 2hr incubation at 30° C and lost 90% of the original activity after incubation at 55° C and pH 5.5 for 2hr. With two different substrate concentrations, hydrolysis of white copra meal proceeded rapidly at the early stage of the reaction, but gradually slowed thereafter especially at a higher concentration of copra meal (20%). The enzyme hydrolyzed white copra meal to monosaccharides, mannobiose and mannotriose at the final stage of the reaction.**

Key words : mannooligosaccharides, mannanase

INTRODUCTION

β-D-Mannanase[(1→4)-β-D-mannan mannohydrolase, endo-β-D-mannanase, EC 3.2.1.78][1-5] is an enzyme that hydrolyzes the (1→4)-β-D-mannopyranosyl linkages of mannans, galactomannans, glucomannans and galactoglucomannans. There have been a few reports about mannanase produced from various microorganism[5] ; especially, some species among the *Genus Penicillium* (*P. chrysogenum*[6], *P. paxillus*[7], *P. wortmanni*[8], etc) have also been reported to produce a mannanase.

In order to overcome the disadventageous point of extraction of mannan from a cake by the alkali solution, the author attempted to isolate microorganisms producing β-mannanase, and succeeded in isolating a strain from soil, *Penicillium purpurogenum* which produced a galactomannan degrading enzyme extracellulary.

The enzyme hydrolyzed copra galactomannan to monosaccharides, mannobiose and mannotriose without a significant amount of other oligosaccharides. For application of the enzyme system, author have an schedule of mannooligosaccharides preparation from white copra meal by means of a process involving hydrolysis of the meal with the enzyme in the next paper. This paper described the properties and formation conditions of mannooligosaccharides by a crude β-mannanase.

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[9].

Isolation of microorganisms producing a copra galactomannan degrading enzyme

Microorganisms, which formed a clear zone around the colony when grown on the agar plate containing 1% of copra galactomannan, were isolated from soil collected in various districts of Japan. The detailed procedure for the isolation of the microorg-

anisms (first screening method) was described in the previous report[10]. One loop of the isolates obtained by the first screening was inoculated into 100ml of the second screening medium in a 500ml shaking flask with a shoulder (Sakaguchi flask), and incubated at 35° C for 4~5 days on a reciprocal shaker with 125 oscillations per minute (Model ; RLR. Iwashiya Bioscience Co. Ltd, Japan). The second screening medium was composed of 3% white copra meal, 0.6% peptone, 1% potassium phosphate (monobasic) and 0.05% magnesium sulfate. The culture broth thus obtained was filtered through a Toyo-roshi No. 2 filter paper (Toyo-roshi Co. Ltd, Japan), and the filtrate was used as an enzyme solution.

A quantity of 10ml of the enzyme solution adjusted to pH 5 was added to an L-form tube containing 0.5g of white copra meal, and the enzyme reaction was performed at 50° C for about 24hr on a Monod shaker. After removal of insoluble materials from the hydrolysate by centrifugation, a small portion of the supernatant was subjected to thin layer chromatography to characterize the hydrolysis products. On the other hand, the reducing sugar content in the supernatant was determined by the Somogyi's method[9] to estimate the degree of hydrolysis. Through the above method, fungus was selected from seven species of fungi isolated by the second screening and the enzyme system from the fungus directly hydrolyzed galactomannan in the white copra meal without any treatment to produce monosaccharides, β-1,4-mannobiose and β-1,4-mannotriose as final products. In addition, fungus showed high productivity and stability for the enzyme production. This strain was identified to be *Penicillium purpurogenum* according to the method of Pitt[11].

Determination of β-mannanase activity

The activity was determined as follows : A reaction mixture containing 129.4mg of copra mannan (equivalent to 100mg of polymannose), 4.0ml of McIlvaine buffer solution (pH 5) and 5ml of water, was put into an L-form tube. Then, the tube was preincubated at 55° C for 10min on a Monod shaker with agitation at the speed for 60 oscillations per minute. One ml of the enzyme solution was added to the mixture which was incubated for 30min at the same temperature. One ml of the reaction mixture was placed into 5ml of Somogyi's reagent[9] in a test tube. The test tube was heated in a boiling water bath for 20min, and the reducing power produced by the enzyme reaction was determined as mannose by Somogyi's method[9]. The mannanase activity was expressed as "mg of mannose/10ml of reaction mixture/1ml of enzyme solution/30min". One unit was defined as the activity producing 5.7mg of reducing sugar under the conditions described above. The relation between the mannanase activity (unit) and the increase of the reducing power is shown in Fig. 1.

Thin-layer chromatography (TLC)

TLC was carried out according to the method of McCleary[12]. 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 : nitroethane : water (5 : 2 : 3, v/v) for about 4hr at room temperature. The sugars on the plate were visualized by heating to 120° C for about 10min after spraying with 30% sulfuric acid-ethanol.

RESULTS

Production of β-mannanase system

The medium for the enzyme production was composed of 4% white copra meal, 0.9% peptone, 0.1%

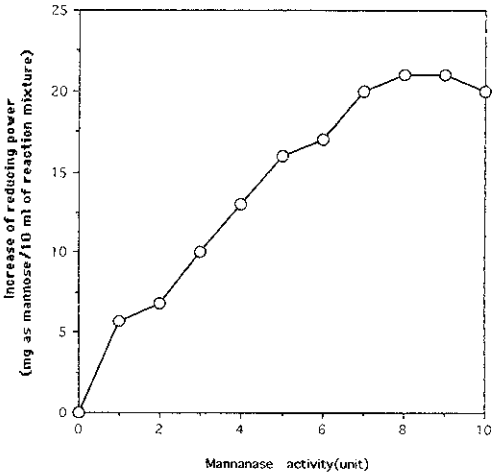

Fig. 1. Relation between mannanase activity and increase of reducing power.

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 medium (3 liters) was placed in a 5 liter Jar Fermentor (Model ; MB-C, Iwashiya Bioscience Co. Ltd, Japan), and sterilized at 120° C for 10min. The seed culture (200ml) of *Penicillium purpurogenum*, 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 cultivation was carried out at 35° C with aeration (500ml per min) and with agitation (700rpm). A few ml of the broth during the cultivation were occasionally 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 system.

Fig. 2 shows the time course of cultivation. There was a rapid increase in the production of the enzyme at about 100hr after the begining of cultivation, and the mannanase activity in the culture filtrate reached maximum values at 140hr with the activity of about 9units/ml. After 140hr, 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 voume of distilled water, and the dialyzed solution (7.9units of mannanase activity/ml) was used as the mannanase solution for the further experiments.

Enzymatic properties of the mannanase system

Effect of pH on mannanase activity. The enzyme reaction was done at various pHs with a McIlvaine buffer solution at 55° C for 30min. As shown in Fig. 3, the enzyme system showed the maximum activity at around pH 5.5.

Effect of temperature on mannanase activity. The enzyme reaction was carried out at various temperatures for 30min and at pH 5.5 (McIlvanine buffer solution). As shown in Fig. 4, the optimum temperature for the enzyme reaction was 55° C.

Effect of pH on stability. The enzyme solution was incubated at 30° C for 2hr at various pHs, and the remaining activity was assayed. The pH stability curve of the enzyme is presented in Fig. 5. The enzyme was stable in between pH 5 and pH 7.

Effect of white copra meal concentration on enzymatic hydrolysis

Each reaction mixture contained 2.6g (10%) or 5.2g (20% based on enzyme solution used) of white copra meal, and 30ml of the enzyme solution. The enzyme reaction was performed at pH 5.5 and 55° C in a T-form tube on a Monod shaker. A small portion of each mixture was occasionally with drawn from the tube and heated to about 100° C for 5min to inac-

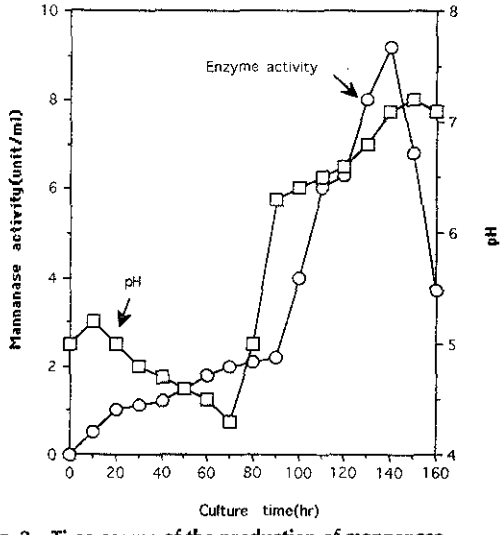

Fig. 2. Time course of the production of mannanase.

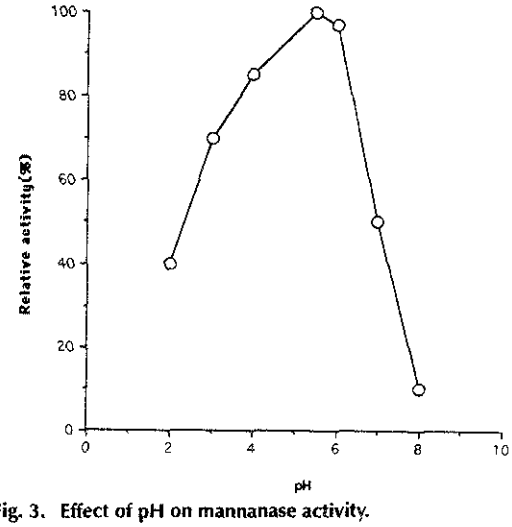

Fig. 3. Effect of pH on mannanase activity.

tive 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[9] to estimate the degree of hydrolysis.

Fig. 6 shows the time course of enzymatic hydrolyisis. 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 (20%).

The formation of mannooligosaccharides

White copra meal (100g) was hydrolyzed with the mannanase (500ml) at pH 5.5 and 55°C for 24hr. As shown in Fig. 7, the major product of the final stage were monosaccharides, mannobiose and mannotriose without detectable amounts of other saccharides. In addition, the enzyme scarcely hydrolyzed β-1,4-mannobiose and β-1,4-mannotriose.

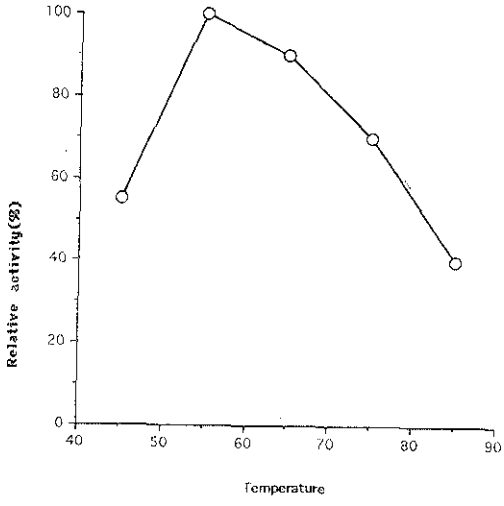

Fig. 4. Effect of temperature on mannanase activity.

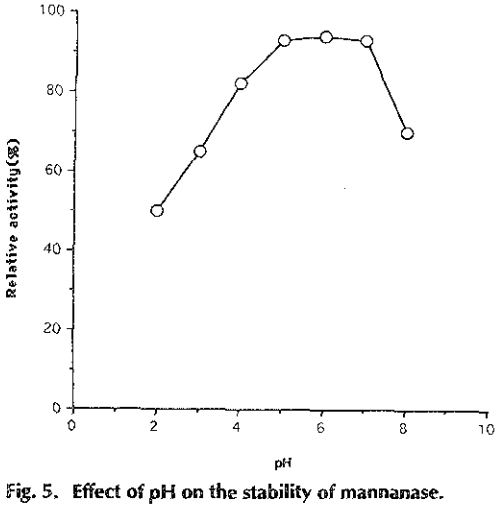

Fig. 5. Effect of pH on the stability of mannanase.

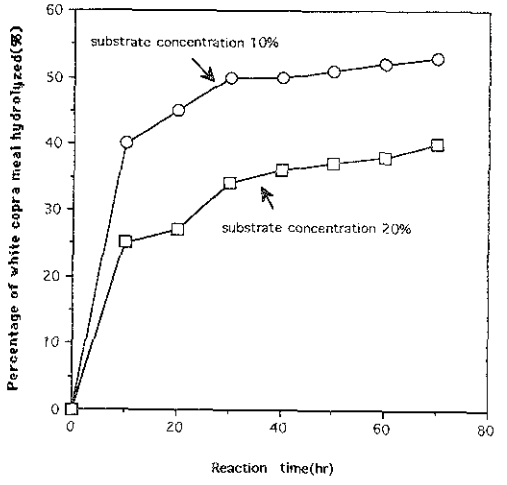

Fig. 6. Effect of white copra meal concentration on enzymatic hydrolysis.

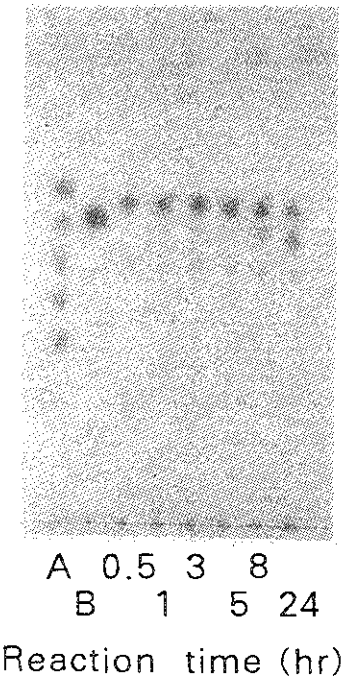

Fig. 7. Time-course of hydrolysis of white copra meal with enzyme solution.
A : mannose, mannobiose, mannotriose, mannotetrose and mannopentose from top to bottom
B : galactose

DISCUSSIONS

Kusakabe *et al.* have been studied β-mannanase, especially the enzymatic preparation of β-1,4-mannooligosaccharides[13,14] from coconut residual cake, and also some properties[15~17] of the enzyme. However, it was pointed out that the enzyme has drawbacks as follows :

1. The copra galactomannan, extracted by a heavy alkali solution (20~24%), was used in their study to prepare mannooligosaccharides. However, it is difficult to extract the mannan from the cake by the alkali solution.

2. The enzyme, in addition to mannooligosaccharides, also produced considerable amounts of galactomannooligosaccharides which interfered with the crystallization of mannooligosaccharides. Therfore, it is necessary to find other enzymes which are able to directly hydrolyze the galactomannan in the cake, and do 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 purpurogenum* which produced a galactomannan-degrading enzyme extracellulary. The enzyme was hydrolytic enzyme capable of directly hydrolyzing galactomannan in the cake to produce monosaccharides, mannobiose and mannotriose.

Moreover, the optimum pH and temperature for the mannanase activity were 5.5 and 55° C, 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° C, respectively. Therefore, the enzyme seems to be the most appropriate for the hydrolysis of galactomannan in the white copra meal, because the properties of the two enzymes are very similar to each other. 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. But, the hydrolysis pattern of the purified mannanase included 3 kinds of galactomannooligosaccharides containing of mannobiose and mannotriose (Fig. 8). This result suggests that crude enzyme system containing of β-mannanase and α-galactosidase are able to hydrolyze galactomannooligosaccharides to galactose, mannose, mannobiose and mannotriose. Recently, author tried to prepare the mannooligosaccharides obtained from this paper.

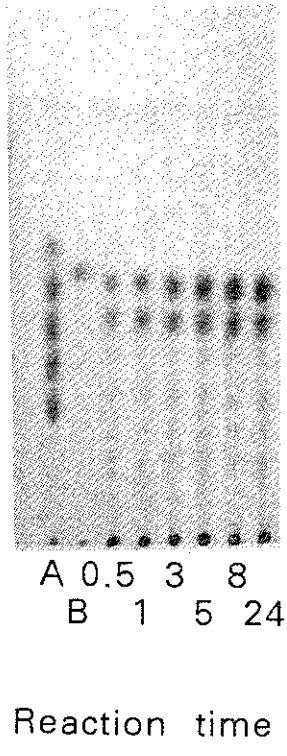

Fig. 8. Time-course of hydrolysis of white copra meal with purified β-mannanase.
A : mannose, mannobiose, mannotriose, mannotetrose and mannopentose from top to bottom
B : galactose

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Penicillium purpurogenum 유래의 Mannanase에 의한 Mannooligosaccharide의 생산

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요 약

Copra galactomannan분해효소 mannanase를 생산하는 *Penicillium purpurogenum*이 토양으로 부터 분리되었다. Mannanase의 효소학적 성질과 mannooligosaccharides의 형성조건이 연구되었다. 최적 pH와 최적 온도는 각각 5.5와 55°C이며, 30°C · 2시간 배양에서 pH 3.5~7.0 사이에 pH 안정성이 있었으며, 55°C · pH 5.5 · 2시간의 배양에서는 초기활성의 90%가 실활되었다. 10%, 20%의 기질농도에서 white copra meal은 반응초기에 가수분해 속도가 증가하였으나, 20%의 고농도에서는 반응시간의 증가와 더불어 가수분해속도가 억제되었다. Mannanase는 white copra meal을 가수분해하여 반응 말기에 monosaccharides, mannobiose, mannotriose를 생산하였다.