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Production of Gentiobiose from Hydrothermally Treated *Aureobasidium pullulans* β-1,3-1,6-Glucan

Katsuki Hirabayashi,[1,2,‡] Yoshiya Tashiro,[2,‡] Nobuhiro Kondo,[1] and Sachio Hayashi[2,†]

[1] *Itochu Sugar Co., Ltd.*

(*3 Tamatsuura, Hekinan-shi, Aichi 447−8506, Japan*)

[2] *The Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki*

(*1−1 Gakuen Kibanadai Nishi, Miyazaki-shi, Miyazaki 889–2192, Japan*)

Abstract: We report production of the functional disaccharide gentiobiose β-D-Glc*p*-(1→6)-D-Glc by a hydrolysis reaction of hydrothermally treated *Aureobasidium pullulans* β-1,3-1,6-glucan as the substrate and Kitalase as the enzyme. Gentiobiose was produced over the pH range 4-6 and the concentration of gentiobiose produced decreased above pH 7. The maximum value of gentiobiose production was unaffected by the enzyme concentration. The maximum concentration of gentiobiose produced was dependent on the substrate concentration whereas the maximum ratio of gentiobiose to glucose was not. The production of gentiobiose from yeast β-1,3-1,6-glucan was lower than that from *A. pullulans* β-1,3-1,6-glucan.

Key words: gentiobiose, hydrolysis, β-1,3-1,6-glucan, *Aureobasidium*

INTRODUCTION

The disaccharide gentiobiose β-D-Glc*p*-(1→6)-D-Glc is found in birch sap[1] and tomato fruit.[2] Gentio-oligosacharides containing gentiobiose are produced industrially by transglucosylation and condensation reactions catalyzed by fungal β-glucosidase.[3] Gentio-oligosacharides taste bitter and exhibit high moisture-retaining activity due to their high hygroscopcity, and dissolved gentio-oligosacharides reduce the freezing point of water;[3] consequently, these compounds are useful for modifying the taste and quality of some foods. Gentio-oligosacharides support the growth of probiotics such as *Bifidobacterium infantis* and *Lactobacillus acidophilus*, have potential as anti-cancer agents[4] and for improving the absorption of calcium,[3] and so are useful as a component of some health foods. Novel roles for gentiobiose in regulating overwintering buds dormancy[5] and in initiating the ripening of tomato[2] have also been reported.

Gentiobiose can be synthesized via a transglucosylation reaction of β-glucosidase from *Aspergillus oryzae*,[6] *Penicillium multicolor*,[7] and *Rhizomucor miehei*[8] using glucose or oligosaccharides as substrate. However, the production of gentiobiose from the *A. pullulans* polysaccharide β-1,3-1,6-glucan via an enzymatic hydrolysis reaction has not been reported to date. The enzymatic hydrolysis products of functional saccharides containing gentiobiose can be used as health-promoting food ingredients. We previously confirmed the chemical structure of hydrothermally treated *A. pullulans* β-1,3-1,6-glucan and noted its water solu-bility and sensitivity towards enzymatic hydrolysis.[9]

In the present study, we generated gentiobiose from *A. pullulans* β-1,3-1,6-glucan using Kitalase and investigated the yield of gentiobiose and the ratio of gentiobiose to glucose in an effort to enhance the industrial production of gentiobiose.

MATERIALS AND METHODS

Preparation of hydrothermally treated A. pullulans β-1,3-1,6-glucan. *A. pullulans* ATCC 20524 was cultured in liquid medium consisting of 6 g/L sucrose, 2 g/L rice bran and 2 g/L ascorbic acid at 23 ˚C for 72 h. β-1,3-1,6-Glucan was harvested by the addition of burnt alum, then subjected to hydrothermal treatment using continuous flow tubular type reactor (300 mL/min, 180 ˚C for 15 min at pH 5.5), concentrated by ultrafiltration, autoclaved for 15 min at 121 ˚C, and lyophilized for 48 h.

Enzymatic hydrolysis. The enzymatic reaction was conducted as follows, except when stated otherwise in the Results. Kitalase (primarily the endo-β-1,3-glucanase EC 3.2.1.6 from *Rhizoctonia solani*; Wako Pure Chemical Industries Ltd., Osaka, Japan) was used. MacIlvain buffer (pH 5.0, 50 mmol/L) containing substrate (100 g/L and Kitalase (0.1 U/mg substrate) was incubated for 18 h at 40 ˚C. One unit is the amount of enzyme which liberate soluble sugar equivalent to 1 μmol of glucose per min.

Carbohydrate measurement. The obtained glucose and gentiobiose were quantified using a high-performance liquid chromatography (HPLC) instrument (Shimadzu LC-10; Shimadzu Corporation, Kyoto, Japan) fitted with a Shodex KS-801 column (8 × 300 mm; Showa Denko K.K., Tokyo, Japan) and the following conditions: temperature, 40 ˚C;

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[†]Corresponding authors (Tel. & Fax. +81−985−58−7317,
E-mail: shayashi@cc.miyazaki-u.ac.jp).
[‡]These authors contributed equally to this work.
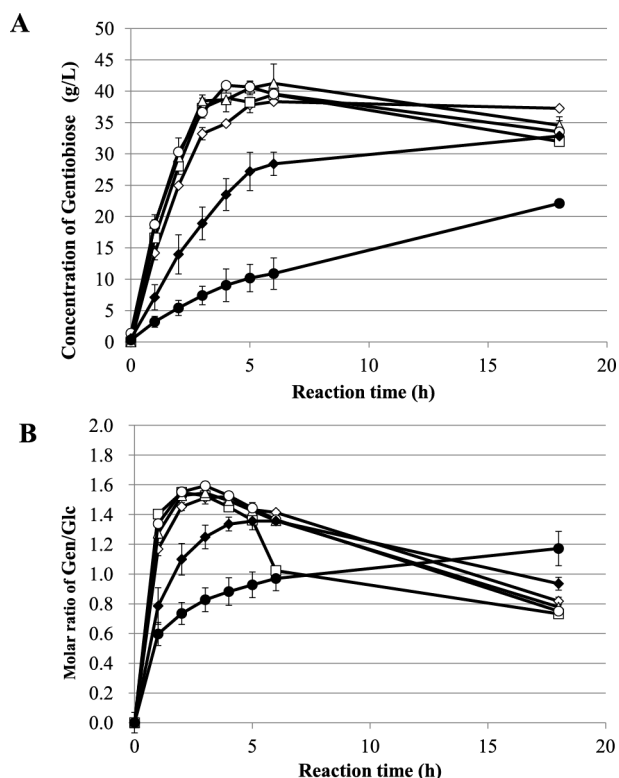
A

B

Fig. 1. Time course of gentiobiose production (A) and the molar ratio of gentiobiose to glucose (B) using Kitalase at various pH values.

The values at pH 4.0, 5.0, 5.5, 6.0, 7.0, and 8.0 are shown by open rhombuses, open squares, open triangles, open circles, closed rhombuses, and closed circles, respectively.

mobile phase, H_2O; flow rate, 0.5 mL/min; detector, RI detector. The total yields of gentiobiose are shown as percentages of the initial substrate concentration in the reaction mixture.

RESULTS

Gentiobiose production at various reaction pH values.

The time course of gentiobiose production and the ratio of gentiobiose to glucose (Gen/Glc) from 100 g/L *A. pullulans* β-1,3-1,6-glucan by Kitalase at various reaction pH values is shown in Fig. 1. The maximum concentration of gentiobiose was obtained at pH 5.5: 41.2 g/L after 6 h reaction, providing a yield of 41.2 % (w/w) based on the initial substrate concentration. The maximum value of gentiobiose obtained at various pH values and the Gen/Glc value at each pH are shown in Fig. 2. Gentiobiose was produced at between 38.3−41.2 g/L in the pH range 4−6 and the Gen/Glc value ranged from 1.52−1.59. Both the concentration of gentiobiose and the Gen/Glc values decreased above pH 7.

Gentiobiose production at various enzyme concentrations.

The time course of gentiobiose production and the value of Gen/Glc from 50 g/L β-1,3-1,6-glucan by various concentrations of Kitalase is shown in Fig. 3. The maximum concentration of gentiobiose (21.4 g/L) was obtained at 0.5 U/mL after 1 h reaction, providing a yield of 42.8 % (w/w)
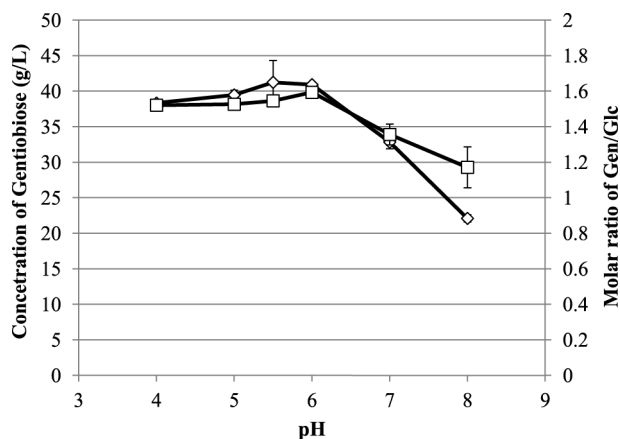

Fig. 2. Effect of pH on the maximum concentration of gentiobiose produced (rhombuses) and the maximum molar ratio of gentiobiose to glucose (squares).
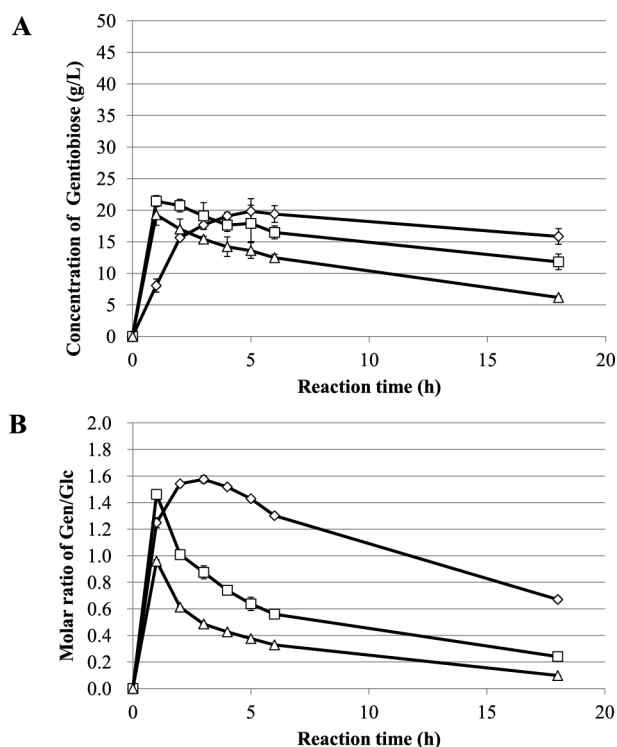
A

B

Fig. 3. Time course of gentiobiose production (A) and the molar ratio of gentiobiose to glucose (B) obtained using various enzyme (Kitalase) concentrations.

The values at enzyme concentration of 0.1, 0.5, and 1.0 U/mL are shown by rhombuses, squares, triangles, respectively.

based on the initial substrate concentration. The maximum gentiobiose concentration and the Gen/Glc obtained at various enzyme concentrations is shown in Fig. 4. The maximum value of Gen/Glc decreased from 1.57 to 0.96 as the enzyme concentration increased, whereas the maximum concentration of gentiobiose (19.3−21.4 g/L) remained essentially unchanged.

Gentiobiose production at various substrate concentrations.

The time course of gentiobiose production and the Gen/Glc values obtained using Kitalase at various *A. pullulans* β-1,3-1,6-glucan concentrations is shown in Fig. 5.
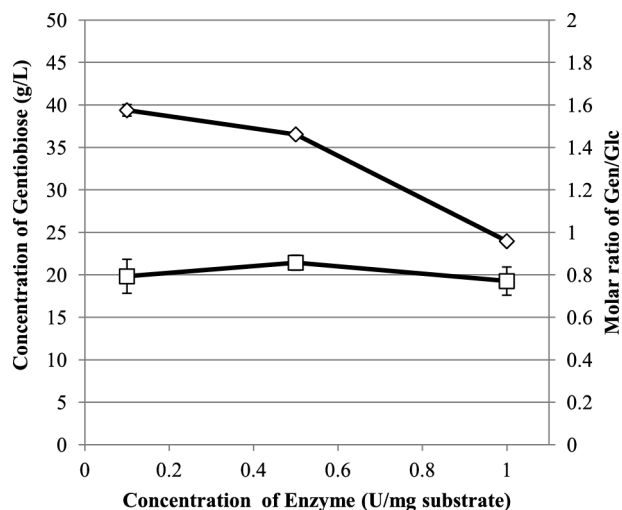
Fig. 4.   Effect of enzyme concentration on the maximum concentration of gentiobiose produced (squares) and the maximum molar ratio of gentiobiose to glucose (rhombuses).
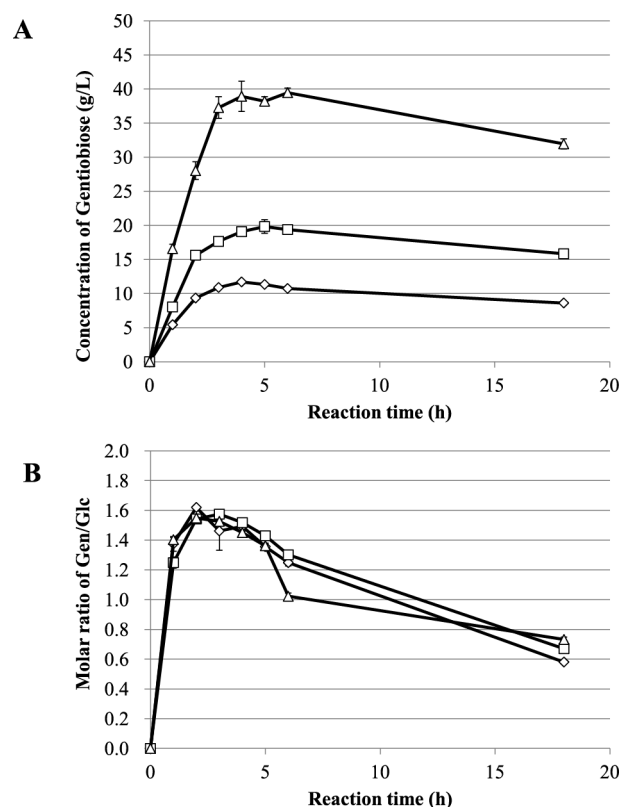

Fig. 5.   Time course of gentiobiose production (A) and the molar ratio of gentiobiose to glucose (B) by Kitalase at various substrate concentrations.
The values at substrate concentration of 30, 50, and 100 g/L are shown by rhombuses, squares, triangles, respectively.

The maximum concentration of gentiobiose was obtained at 100 g/L β-1,3-1,6-glucan. The maximum gentiobiose concentration and Gen/Glc values obtained at various enzyme concentrations is shown in Fig. 6. The maximum concentration of gentiobiose was dependent on the initial substrate concentration and increased as the substrate concentration increased, whereas the maximum Gen/Glc value (1.55–1.62) at each substrate concentration remained essentially unchanged.
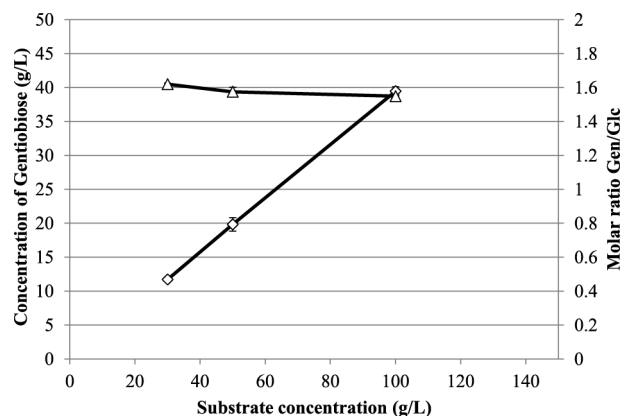

Fig. 6.   Effect of substrate concentration on the maximum concentration of gentiobiose produced (rhombuses) and the maximum molar ratio of gentiobiose to glucose (triangles).

Enzymatic hydrolysis of β-1,3-1,6-glucan from different sources.

The time course of gentiobiose production by Kitalase and the values of Gen/Glc obtained using 30 g/L β-1,3-1,6-glucan from *Saccharomyces cerevisiae* (Oriental Yeast Co., Tokyo, Japan) or *A. pullulans* are shown in Fig. 7. The maximum concentration of gentiobiose produced from *S. cerevisiae* β-1,3-1,6-glucan was 5.6 g/L after 2 h reaction, providing a yield of 18.7 % (w/w) based on the initial substrate concentration. This concentration is lower than that obtained using *A. pullulans* β-1,3-1,6-glucan (37.8 % w/w). The maximum value of Gen/Glc obtained using *S. cerevisiae* β-1,3-1,6-glucan was 0.48 after 1−2 h, which is much lower than the value observed using *A. pullulans* β-1,3-1,6-glucan (1.62).

HPLC analysis of the products generated by the enzymatic reaction.

Figure 8 shows the HPLC profile of the reaction mixture obtained by enzymatic reaction of *A. pullulans* β-1,3-1,6-glucan, together with gentiobiose and glucose standards. The retention time of the produced gentiobiose (12.0 min) was identical to that of the gentiobiose standard (12.0 min), and the gentiobiose peak was sufficiently separated from the other product peaks to allow its isolation and collection.

DISCUSSION

Gentiobiose is a bitter-tasting disaccharide[7] used in the food industry and has useful characteristics, as described in the Introduction. The saccharide detected at 12.0 min by HPLC analysis shown in Fig. 8 was identified as gentiobiose using NMR in the previous research.[9] An efficient industrial production method would therefore be useful. Gentio-oligosaccharides, including gentiobiose, are usually produced by the transglucosylation reaction described above, but we attempted to produce gentiobiose from polysaccharide using enzymatic hydrolysis.

We investigated the hydrolysis of hydrothermally treated *A. pullulans* β-1,3-1,6-glucan and the optimal conditions for producing gentiobiose. Gentiobiose was successfully

produced from β-1,3-1,6-glucan, given that the maximum yield obtained was approx. 40 % (w/w), which is higher than that produced using β-glucosidase from recombinant *T. caldophilus*,[10] *P. multicolor*,[7] and *R. miehei*.[8] The high Gen/Glc value (1.59) from *A. pullulans* β-1,3-1,6-glucan is

due to its high branching characteristic, as reported previously.[9]

It is suggested that the ratio of β-1,6-glucosidase activity to β-1,3-glucanase activity of Kitalase was increased in the pH range 7−8 because the value of Gen/Glc decreased at pH values above pH 7 as shown in Fig. 2. It is also considered that the amount of enzyme did not affect the maximum gentiobiose concentration obtained or the maximum value of Gen/Glc as shown in Fig. 3 because the activity ratio of β-1,6-glucosidase to β-1,3-glucanase was not changed by the amount of added enzyme. As shown in Fig. 6, the maximum concentration of gentiobiose increased as the substrate concentration increased whereas the value of Gen/Glc was independent of substrate concentration, suggesting that the enzyme activities of β-1,6-glucosidase and β-1,3-glucanase in Kitalase were unaffected by substrate concentration.

As shown in Fig. 8, the present hydrolysis method did not produce the variety of oligosaccharides (*n* > 2) produced by the transglucosylation reaction[7)10)] near gentiobiose peak, indicating that the present methodology could be convenient for the generation and isolation of gentiobiose as a reagent for basic research. Scale up of this methodology for the industrial production of gentiobiose and optimization of the isolation and purification conditions will be carried out in the near future.

We evaluated the present hydrolyzing method by comparing the production of gentiobiose from *A. pullulans* β-1,3-1,6-glucan to that obtained using *S. cerevisiae* β-1,3-1,6-glucan, which has a different branching frequency. An average relative linkage percentage of 22 % for β-1,6-branch from *S. cerevisiae* was previously reported.[11] It is considered that the production of gentiobiose and the observed value of Gen/Glc were low as predicted because *S. cerevisiae* β-1,3-1,6-glucan is a low branching polysaccharide.

In this report, we produced gentiobiose from hydrothermally treated *A. pullulans* β-1,3-1,6-glucan using a hydrol-
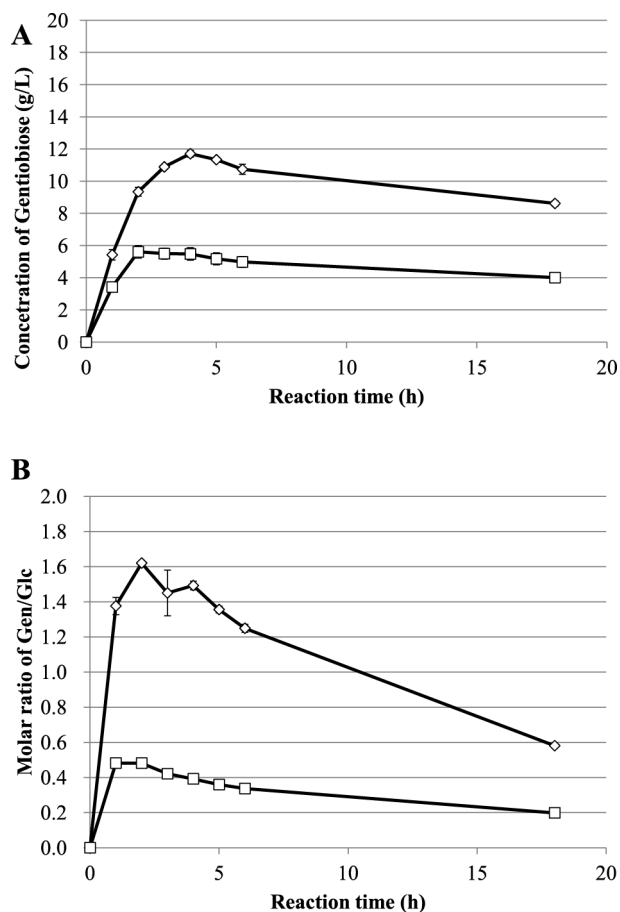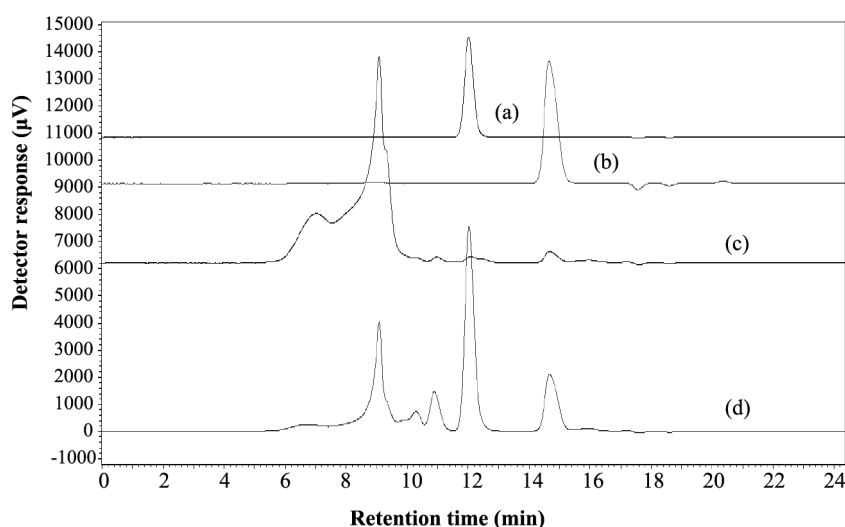

Fig. 7.   Time course of gentiobiose production (A) and the molecular ratio of gentiobiose to glucose produced (B) by Kitalase from *A. pullulans* or *S. cerevisiae* β-1,3-1,6-glucan.
Open rhombuses, *A. pullulans*; open squares, *S. cerevisiae*.

Fig. 8.   HPLC analysis of the products generated by enzymatic reaction using Kitalase.
(a) Gentiobiose standard, (b) Glucose standard, (c) Reaction mixture (30 g/L of substrate enzyme concentration of 0.1 U/mg substrate, pH 5, 40 ˚C, 0 h reaction.), (d) Reaction mixture (30 g/L of substrate, enzyme concentration of 0.1 U/mg substrate, pH 5, 40 ˚C, 1 h reaction).

ysis method. We propose the present novel method, which is different from the current glucose-conjugation reaction using β-glucosidase, for the industrial production of gentiobiose.

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