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Screening and Identification of a Fungal β-Glucosidase and the Enzymatic Synthesis of Gentiooligosaccharide

Yongling Qin · Yunkai Zhang · Haiyan He · Jing Zhu · Guiguang Chen · Wei Li · Zhiqun Liang

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Abstract After screening with 0.1% esculoside and 0.03% $FeCl_3$, we identified from rotten wood a fungal isolate HML0366 that produces high amount of β-glucosidase. Phenotypic and rDNA internal transcribed spacer sequence analyses indicated that the isolate belongs to *Aspergillus oryzae*. The β-glucosidase produced by HML0366 had an activity of 128 U/g. high performance liquid chromatography analysis also demonstrated a high transglycosylation activity of the crude enzyme. The β-glucosidase was stable between pH 4–10 at 60 °C. A gentiobiose yield of 30.86 g/L was achieved within 72 h of the enzymatic reaction at pH 5 and 55 °C using 50% glucose as the substrate. For the first time, we report here the isolation of an *A. oryzae* strain producing β-glucosidase with high hydrolytic activities. The crude enzyme has a high transglycosylation activity, which enables the enzymatic synthesis of gentiooligosaccharides.

Keywords β-Glucosidase · Transglycosylation · *Aspergillus oryzae* · Gentiooligosaccharide

Introduction

Oligosaccharides are important carbohydrates consisted of two to10 monosaccharides linked by glycosidic bonds. They are presented in free format in many natural food including fruits, vegetables, milk, and honey [1, 2]. Functional oligosaccharides are useful and functional food additives that selectively stimulate the growth of endogenous bifidobacteria but inhibit harmful bacteria in the gut to improve the health of the host [3]. Moreover, functional oligosaccharides are involved in multiple aspects of health promotion,

Yongling Qin, Yunkai Zhang, and Haiyan He contributed equally to this work.

Y. Qin · Y. Zhang · J. Zhu · G. Chen · W. Li · Z. Liang (✉)
College of Life Science and Technology, Guangxi University, Nanning, Guangxi 531005, China
e-mail: zqliang@gxu.edu.cn

Y. Qin · H. He
Department of Chemistry and Life Science, Hechi University, Yizhou, Guangxi 546300, China

including the absorbance of minerals, inhibition of dental caries, lowering cholesterol and blood sugar levels, and improving the physical and chemical properties of food. As a functional food substrate, oligosaccharides draw broad attention, and the research and development of new oligosaccharides are in progress [2, 4].

Gentiooligosaccharide is a new functional oligosaccharide consisted of more than two D-glucose linked with β-1,6-glycosidic bonds. The main components of gentiooligosaccharide are gentiobiose and small amount of trisaccharide and tetrasaccharide [4, 5]. Gentiooligosaccharide is low in calorie and has low risk of causing dental caries. Moreover, gentiooligosaccharide promotes the proliferation of probiotics such as bifidobacteria and lactobacillus, whose functions involve vitamin syntheses, promoting immune response, inhibiting the growth of harmful bacteria, tumor inhibition, as well as promoting nutrition uptake and metabolism [3, 6].

Since 1970, glycosylsucrose, fructooligosaccharides, maltooligosaccharides, isomaltooligosaccharides, and galactooligosaccharides have been enzymatically synthesized at industrial scales. However, there is only limited report on the industrial scale production of gentiooligosaccharide using starch as the feedstock in Japan [2].

β-Glucosidase (EC3.2.1.21) is an important component of the cellulase complex and plays a key role in cellulose degradation by catalyzing the breakdown of cellulosic oligosaccharides into glucose [7, 8]. Some β-glucosidases also have transglycosylation activities, which is responsible for the synthesis of gentiooligosaccharide. Using high concentrations of glucose as the substrate, β-glucosidase transfers free glucose to other sugar substrates through β-1,6-glycosidic bonds and synthesizes gentiooligosaccharide [9–11]. This process is mild, safe, low cost, and only causes low levels of pollution. In addition, the end product is easy to be isolated from the reaction mixture [12, 13].

In this report, we show the screening and isolation of an *Aspergillus oryzae* strain HML0366 with high β-glucosidase activity. The β-glucosidase produced by this strain has high hydrolytic activities and is tolerant to wide range of pH. The crude enzyme has a high transglycosylation activity and is therefore suitable for synthesizing gentiooligosaccharide.

Materials and Methods

Screening of β-Glucosidase-Producing Microbes

For primary screening, samples were collected from the soil underneath rotten wood in the national reserve forest of Mulun, Huanjiang County, Guangxi Province, China. Soil samples were 10-fold serial diluted to 10^{-5} and plated on β-glucosidase primary screening agar containing 0.1% esculoside, 0.03% $FeCl_3$, Mandels salts, and 1.5% agar [14, 15]. The cultures were incubated at 30 °C for 4 days. Single colonies with black halos were picked and streaked onto potato dextrose agar (PDA) slant.

For secondary screening, conidia of the β-glucosidase-producing isolate obtained from the primary screening were washed off the PDA agar slant using 10 mL of normal saline solution. A total of 10^7 conidia were inoculated onto β-glucosidase secondary screening agar containing 6 g sugarcane bagasse, 4 g bran, and 30 mL Mandels salts and incubated at 30 °C for 4 days. Two hundred milliliters of sterile distilled water were then added to the culture, followed by incubating the flask in a 40 °C water bath for 1 h with shaking. Crude enzyme extract was obtained by centrifuging the liquid mixture at 8,000 rpm for 10 min and collecting the supernatant. The β-glucosidase activity in the crude extract was confirmed.

β-Glucosidase Enzyme Activity Assay

One unit (U) of β-glucosidase enzyme activity was determined as the amount of enzyme needed to produce 1 μmol glucose per minute using salicin (Fluka) as the substrate. The reaction mixture containing 1% salicin, citric-citrate buffer (0.05 M, pH 4.8), and appropriately diluted enzyme solution was incubated at 50 °C for 30 min in a water bath. The reaction was stopped by the addition of 3 mL dinitrosalicylic acid reagent to the mixture, boiling the mixture for 5 min and cooled in ice bath. The enzymatic activity was quantified through the release of glucose reflected by changes in the absorbance at 510 nm [16]. The activity was expressed as unit per gram of dry carbon source weight.

Strain Identification

Fungal microscopic structure was observed using an Olympus CX41 microscope and a Hitachi S-3400N scanning electronic microscope. Molecular identification was performed by rDNA internal transcribed spacer (ITS) region sequencing. ITS degenerate primes 1 and 4 are described previously [17]. The fungal ITS was amplified from genomic DNA, purified, sequenced, and analyzed using the BLAST program from NCBI. DNA was analyzed using Vector NTI. Phylogenetic tree was generated using the neighbor-joining algorithm in the MEGA4 software.

Characteristics of the β-Glucosidase Produced by HML0366

To determine the optimal temperature of the enzyme, the reaction was performed at different temperatures between 30 and 80 °C in 50 mM sodium acetate buffer (pH 4.8). To investigate the heat stability of the enzyme, the residue activity was measured under optimal conditions after incubating the enzyme for 60 min at various temperatures between 40 and 90 °C.

At optimal temperature, the enzyme activity was measured when the reaction was performed in solution buffered between pH 3 and 9, and the optimal pH was determined. To evaluate pH stability of β-glucosidase, relative enzyme activity was determined at optimal pH and temperature condition after the enzyme was kept in buffer with pH ranging from 3 to 11 at 4 °C for 24 h, and further incubated at 30 °C for 3 h.

The relative enzyme activity was defined as the ratio of enzyme activity under a specific experimental condition to that under the experimental condition when maximum enzyme activity was achieved.

Hydrolysis Activity of the HML0366 β-Glucosidase and the Synthesis of Gentiooligosaccharide Using the Transglycosylation Activities of the Crude Enzyme

The hydrolysis activity of β-glucosidase was assayed. Ten milliliters of 1% (*w*/*v*) cellobiose substrate was dissolved in citrate buffer (50 mM, pH 4.8), and the reaction was performed at 30 °C for 30 min with 2 mL crude enzyme. The carbohydrate compositions were determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC; Shimadzu LC-10AD pump, refractometric detector RID-6A, Kyoto, Japan). NH2 columns (250×4.6 mm, 5 μm) were obtained from Hanbon Sci. & Tech. Lichrospher, China. The injection volume was 5 μL, the liquid phase was acetonitrile–water (4:1, *v*/*v*), and the speed was 1 mL/min.

The effects of glucose concentration and reaction time on enzymatic synthesis of gentiooligosaccharide were investigated.

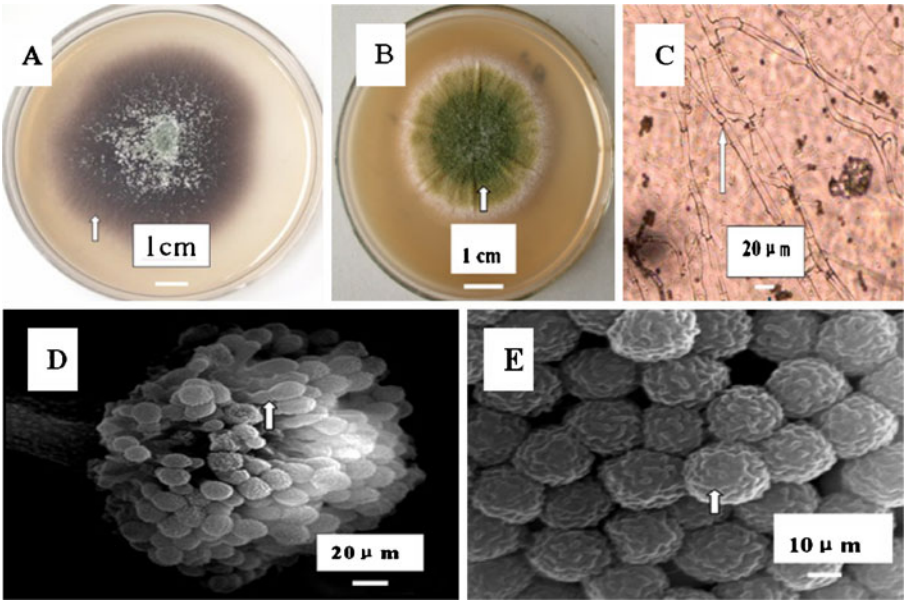

Fig. 1 Colony and fungal morphology of HML 0366. **a** HML0366 grown on primary screening agar plate. **b** Colony of HML0366 on PDA plate. **c** Hyphae of HML0366 (800×). **d** Scanning electron microscope image of the conidiophore of HML0366 (2,000×). **e** Scanning electron microscope image of the conidia of HML0366 (8,000×)

Results and Discussion

Screening of β-Glucosidase-Producing Isolate

β-Glucosidase activity catalyzes the reaction that leads to the formation of black yellow precipitation using 0.1% aesculin and 0.03% $FeCl_3$ substrates. One isolate HML0366 produced the largest black halo on the primary screening agar (Fig. 1a). HML0366 is capable of using bagasse to produce β-glucosidase by solid fermentation. When using

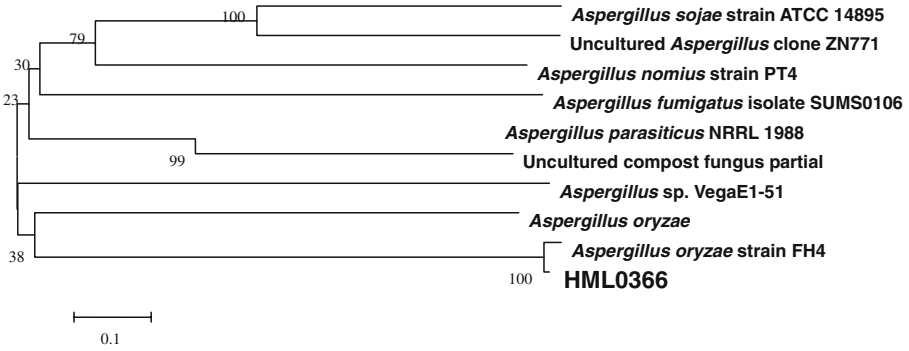

Fig. 2 Phylogenetic tree based on ITS sequences of isolate HML0366 and other related species. The phylogenetic tree was constructed by the neighbor-joining method. Levels of bootstrap support (percent, n= 1,000) were indicated at nodes. The *scale bar* represents 0.1 nucleotide substitution per position

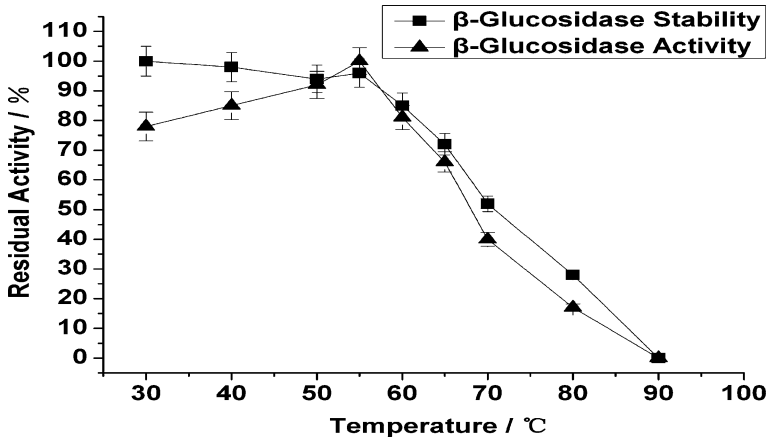

Fig. 3 Optimal temperature and thermal stability of the HML0366 β-glucosidase. *Error bars* are ±SD

salicin as the substrate, the β-glucosidase activity was 128 U/g. Sugarcane bagasse consists of approximately 24% hemicelluloses and 38% cellulose and is a good solid fermentation feedstock [18].

Identification of HML0366

HML0366 colony was yellow green (Fig. 1b). The fungus has branched and septated hyphae (Fig. 1c). The conidiophore formed at the tip, on which one or two layers of primary and secondary phialides supported chains of spherical rough conidia (Fig. 1d, e). We determined that this isolate HML0366 is *A. oryzae* according to its morphology [19].

The ITS of HML0366 was successfully amplified by PCR. Phylogenetic analysis indicated that HML0366 and *A. oryzae* strain FH4 belong to the same clade (Fig. 2). Combined with morphological characteristics, we confirmed that HML0366 is *A. oryzae*.

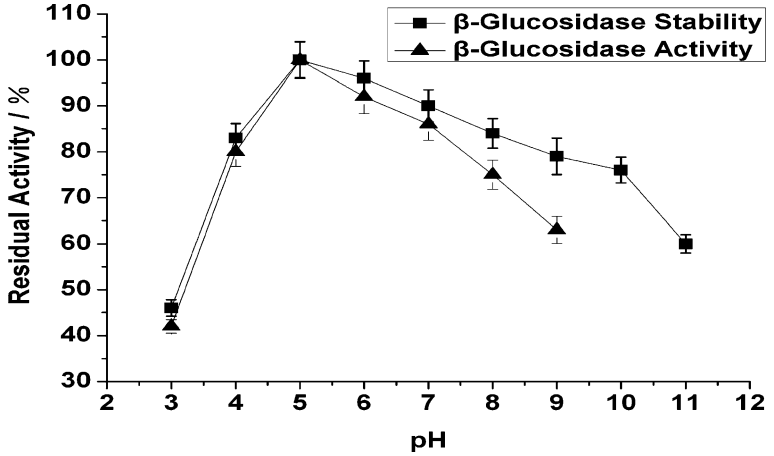

Fig. 4 Optimal pH and pH tolerance of the HML0366 β-glucosidase. *Error bars* are ±SD

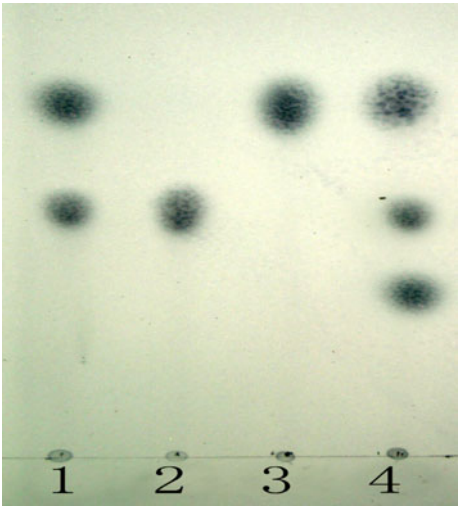

Fig. 5 Hydrolytic activity of the HML0366 β-glycosidase shown by TLC. *Lane 1* glucose and cellobiose standards, *lane 2* cellobiose standard, *lane 3* glucose standard, *lane 4* cellobiose+enzyme

Characteristics of the β-Glucosidase Produced by HML0366

The optimal temperature of the enzymatic reaction was 55 °C. At temperatures below 60 °C, the enzyme was quite stable. Eighty-five percent of the enzyme activity was retained after the enzyme was incubated at 60 °C for 1 h (Fig. 3). The enzyme activity was the highest at pH 5 and remained stable between pH 4 and 9. At pH 4, 83% of the enzyme activity was retained, and at pH 9, 80% of the enzyme activity was maintained (Fig. 4).

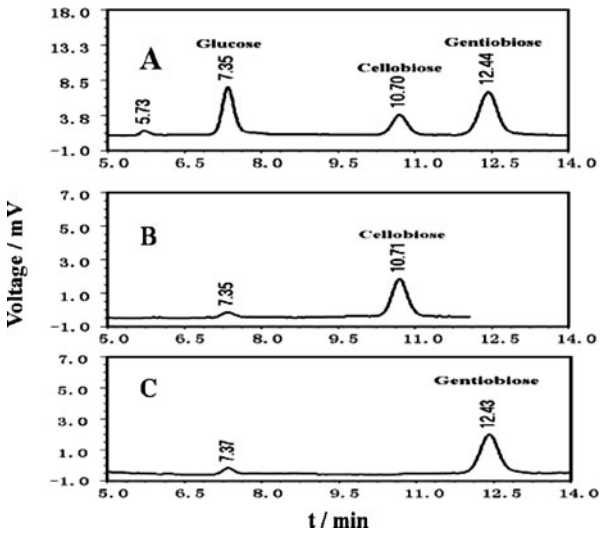

Fig. 6 Synthesis of gentiooligosaccharide by the HML0366 crude enzyme. **a** Glucose+enzyme. **b** Cellobiose standard. **c** Gentiooligosaccharide standard

Hydrolysis and Transglycosylation Activities of the HML0366 Crude Enzyme

Hydrolysis of cellobiose was performed at 55 °C for 30 min, and the product was analyzed using TLC [20]. The HML0366 β-glucosidase hydrolysed the β-1,4-glycosidic bonds in cellobiose and resulted in the production of glucose. Meanwhile, transglycosylation activity restuled in the formation of polysaccharides with a higher molecular weight than cellobiose (Fig. 5, lane 4).

The transglycosylation of 8 mL 20% glucose was performed at 55 °C for 12 h in phosphate buffer (50 mmol/L, pH 4.8) with the presence of 2 mL crude enzyme. The reaction was terminated by heating at 100 °C for 5 min and filtration through a 0.45-μm filter. HPLC showed that both cellobiose (retention time, 10.7 min) and gentiooligosaccharide (retention time, 12.43 min) were synthesized by HML0366 crude enzyme using glucose (retention time, 7.35 min) as the substrate (Fig. 6).

Enzymatic Synthesis of Gentiooligosaccharide by the HML0366 Crude Enzyme

The reaction volume was 10 mL (8 mL substrate and 2 mL crude enzyme). The amount of synthesized gentiooligosaccharide was calculated based on the standard curve generated with gentiooligosaccharide standard samples.

Reaction time had an effect on the enzymatic synthesis. Aliquots of samples were taken every 12 h after the onset of the reaction, which was performed using 20% glucose as the substrate at pH 5 and 55 °C. During the first 48 h, the amount of gentiooligosaccharide increased dramatically and then slowed down and reached a peak level of 26.28 mg/mL at 72 h. At 84 h, the concentration of the end product decreased to 76% of that at 72 h.

The substrate concentration also played a role in enzymatic synthesis. The reaction was performed at pH 5 and 55 °C. At 72 h, with an increase in the substrate concentration, the concentration of gentiooligosaccharide increased as well. The peak level of the product was 30.86 mg/mL when the substrate concentration was 50%. The transglycosylation activity was higher than that of other *Aspergillus* spp. [5, 10].

In this study, we obtained a β-glucosidase-producing isolate HML0366 and determined that the isolate belongs to *A. oryzae* based on colony morphology, microscopic morphology, and ITS phylogenetic analysis. The β-glucosidase produced by HML0366 had an activity of 128 U/g; the hydrolysis activity was higher than that of other *Aspergillus* spp. [21, 22].

We report here the high hydrolysis activities of the HML0366 β-glucosidase; the enzyme has a wide pH tolerant range. The crude enzyme of HML0366 has high transglycosylation, enables the enzymatic synthesis of gentiooligosaccharide, and has potential applications in bioenergy, food industry, and chemical engineering.

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