Optimization of β-Glucosidase Production by a Strain of *Stereum hirsutum* and Its Application in Enzymatic Saccharification

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A high β-glucosidase (BGL)-producing strain, *Stereum hirsutum*, was identified and isolated and showed a maximum BGL activity (10.4 U/ml) when cultured with Avicel and tryptone as the carbon and nitrogen sources, respectively. In comparison with other BGLs, BGL obtained from *S. hirsutum* showed a higher level of activity to cellobiose (Vₘₐₓ = 172 U/mg, and kₙₑₓ = 281/s). Under the optimum conditions (600 rpm, 30°C, and pH 6.0), the maximum BGL activity of 10.4 U/ml with the overall productivity of 74.5 U/l/h was observed. BGL production was scaled up from a laboratory scale (7-L fermenter) to a pilot scale (70-L fermenter). When *S. hirsutum* was cultured in fed-batch culture with rice straw as the carbon source in a 70-L fermenter, a comparable productivity of 78.6 U/l/h was obtained. Furthermore, *S. hirsutum* showed high levels of activity of other lignocellulases (cellobiohydrolase, endoglucanase, xylanase, and laccase) that are involved in the saccharification of biomasses. Application of *S. hirsutum* lignocellulases in the hydrolysis of *Pinus densiflora* and *Catalpa ovata* showed saccharification yields of 49.7% and 43.0%, respectively, which were higher than the yield obtained using commercial enzymes.

**Key words:** Biomass, β-glucosidase, production, pilot scale, saccharification

Cellulose is the main constituent of wood tissue and is the most abundant renewable biomass on Earth [12]. Microbial cellulases that hydrolyze cellulose are industrially important and are used in industries such as food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry [10]. At least 3 types of enzymes, endoglucanase (EG, E.C.3.2.1.4), cellobiohydrolase (CBH, E.C.3.2.1.91), and β-glucosidase (BGL, 3.2.1.21), are involved in the conversion of cellulose to glucose. EGs act randomly along the cellulose chains to produce cellulose fragments. CBHs act as exoglucanase to release cellobiose, and BGLs hydrolyze cellobiose to yield glucose. Cellobiose exerts product inhibition on both CBH and EG. BGL not only produces glucose but also reduces the product inhibition exerted by cellobiose, allowing efficient functioning of the other cellulolytic enzymes. Therefore, high BGL activity is essential for efficient enzymatic saccharification of a lignocellulosic biomass [5,8,16].

Many bacteria, fungi, and yeast produce BGL. The enzyme system of *Trichoderma* spp. has been the most studied and reviewed among those of the cellulose-producing fungi. The amount of BGL produced by this fungal species is insufficient for the effective conversion of cellulose to glucose. High levels of BGL are important for the complete conversion of cellulose because of the product inhibition by cellobiose on CBH and EG. In the flavor industry, BGLs are the key enzymes in the production of aromatic compounds from the glucosidic precursors present in fruit and fermentation products. BGL is also useful in a de-inking process for removal of printing ink from wastepaper.

In the present study, we isolated and identified a potent BGL-producing fungus, *Stereum hirsutum* SKU512. In a previous study, a woody biomass was biologically pretreated using *S. hirsutum*. This species of white rot fungus could effectively degrade lignin and other lignin-like compounds, such as chlorine-containing aromatic compounds [11].
However, the cellulolytic activity of *S. hirsutum* has not been reported. Here, we evaluated the optimal culture parameters for maximum BGL production by *S. hirsutum*. We also studied the enzymatic saccharification of lignocellulosic substrates using lignocellulases from *S. hirsutum*.

**Materials and Methods**

**Screening, Isolation, and Identification of the Microorganism**

The soil samples were collected from Sorak Mountain, Korea by using the capillary tube method and were diluted in 0.9% saline. The aliquots were spread on potato dextrose agar (PDA) plates, which were then incubated for 3 days. Morphologically distinct colonies were inoculated into 3 ml of growth medium (composition in g/l: peptone, 8; yeast extract, 2; KH\(_2\)PO\(_4\), 5; K\(_2\)HPO\(_4\), 5; MgSO\(_4\)·7H\(_2\)O, 3; and cellulose, 20; Sigma, St. Louis, MO, USA). The colonies were cultured at 28°C with agitation at 200 rpm for 5 days. The BGL activity of the culture broth was analyzed using a previously described method using \(\beta\)-nitrophenyl-\(\beta\)-d-glucopyranoside (pNPG, Sigma). On the basis of the results of the analyses, the strain with the highest BGL activity was selected. The isolated strain was identified by using internal transcribed spacer (ITS) rDNA sequence analysis. The sequence was then submitted to GenBank with the accession number HM004553. The identified strain *S. hirsutum* SKU512 was added to the list of organisms at the Korean Culture Center of Microorganisms (KCCM) and was given the KCCM accession number 10982P.

**Culture Conditions and Shake Flask Cultures**

The fungal strain was subcultured every 3 weeks and stored at 4°C on PDA plates. A 500-ml flask containing 50 ml of PDB was used to seed the culture. After 4–5 days of incubation, 5 ml of this seed culture was inoculated in 50 ml of standard production medium (composition in g/l: peptone, 8; yeast extract, 2; KH\(_2\)PO\(_4\), 5; K\(_2\)HPO\(_4\), 5; MgSO\(_4\)·7H\(_2\)O, 3; thiamin-HCl, 0.02; and cellulose, 20) with the pH adjusted to 5.0. The flasks were incubated at 25°C with agitation at 150 rpm for 5 days.

The optimal concentrations of the best carbon and nitrogen sources for BGL production were evaluated by varying the concentration from 1% to 5% and from 0.5% to 2.5%, respectively. The effects of vitamins in BGL production were studied using various vitamins (inositol, riboflavin, pyridoxine hydrochloride, biotin, calcium pantothenate, folic acid, and thiamin hydrochloride) at different concentrations ranging from 1 to 50 mg/l. All fermentation trials were performed in triplicate, and the enzyme activity was assayed after 5 days of culturing. The activities of other lignocellulases such as cellobiohydrolase, endoglucanase, and endoxylanase were determined according to standard procedures.

**Laboratory-Scale and Pilot-Scale Cultures**

The fermentation trials were performed in a 7-L fermenter (working volume, 3.5 L) and the agitation rate, pH, and temperature were examined. For these experiments, 1% (v/v) of 5-day-old preculture was used as the inoculum. The standard medium was used, and the aeration rate was fixed at 0.5vvm. Antifoam agent was added when required. Samples were drawn from the fermentative broth at regular intervals and analyzed to determine the enzyme activity. For the 70-L stirred fermenter, the precultures were used at 1.3% (v/v) with a 35-L working volume. The optimal conditions including medium compositions determined in shake flask scale and fermentation in 7-L jar fermenter scale were applied for the scale-up production.

**Pretreatment and Enzymatic Saccharification of Lignocellulosic Biomass**

Two lignocellulosic biomasses (*Catalpa ovata* and *Pinus densiflora*) were used as the substrates for hydrolysis. The lignocellulosic content of the biomasses was analyzed according to the method described by Sluiter et al. [17]. Glucose and xylose contents were analyzed using a high performance liquid chromatography (HPLC, UltiMate 3000, Dionex) instrument equipped with an evaporative light scattering detector (ELSD; ESA Inc., USA). The sugars were separated using a Shodex Sugar SP0810 column at 30°C with 70% acetonitrile as the eluent at a flow rate of 0.5 ml/min.

The lignocellulosic materials were pretreated with NaOH prior to the hydrolysis. Therefore, 20 g of the biomass was suspended in 80 ml of a 2% aqueous solution of NaOH at 85°C for 1 h, with a solid-to-liquid ratio of 1:4. The solid residue was collected through filtration and extensively washed with distilled water until the pH was neutral. The pretreated biomass was dried in an oven at 70°C to maintain a constant weight and used as the substrate for the saccharification experiments. A typical hydrolysis mixture consisted of 0.2 g of the substrate, 20 FPU (filter paper units) of the enzyme [Celluclast 1.5L (Novozyme) or *S. hirsutum* BGL], and 10 ml of sodium acetate buffer (pH 5.0). This mixture was incubated at 37°C in a rotary shaker at 150 rpm and was sampled at different time intervals. The samples were immediately heated to 100°C to denature the enzymes, cooled, and centrifuged for 10 min at 8,000 rpm. The supernatant obtained was used for determining the reducing sugar content, which was measured using the 3,5-dinitrosalicylic acid (DNS) method. The conversion efficiency was calculated by using the following equation: % Saccharification = reducing sugars × 0.9 × 100/amount of carbohydrates in the substrate. The results obtained for enzymatic conversion using *S. hirsutum* cellulase were compared with those obtained during enzymatic conversion using Celluclast 1.5L.

**Results and Discussion**

**Identification of the Isolated Strain**

A high-BGL-producing fungus was isolated from the soil. The ITS rDNA of the isolate was sequenced, and the results showed that the isolated strain exhibited maximum identity with *S. hirsutum* HM004553. Morphological analysis showed that the fruiting bodies were often fused together and were semicircular or irregular, hairy, and laterally attached, without a stem. In addition, the isolated strain showed a similar cellular fatty acid composition as that of *S. hirsutum* (data not shown). On the basis of the morphology, fatty acid composition, and rDNA gene sequence, the isolated strain was identified as a variant of *S. hirsutum* and was named *S. hirsutum* SKU512.