2011 International Conference on Chemistry and Chemical Process
IPCBEE vol.10 (2011) © (2011) IACSIT Press, Singapore

Identification of a Novel *Phoma* sp. with Tangerine Peel Degrading Activity and Cellulolytic Enzymes from the Fungus

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Abstract. Tangerine is a popular fruit in Jeju island, Korea. But tangerine peel is one of the major agricultural wastes in Jeju island and their annual yields is more than 38,000 tons. The aims of this study were to screen, isolate, identify and characterize a tangerine peel degrading fungus with cellulolytic activity. In this study, a fungal strain with tangerine peel degrading activity was isolated from rotten tangerine peel and was examined for the cellulases from the fungus. ITS region analysis showed that the fungus belong to Ascomycota. When the fungus was incubated using the cellulose powder, the major cellulases such as endoglucanase, cellobiohydrolase and β-glucosidase were produced. Especially, the β-glucosidase showed the highest activity. The *β*-glucosidase from *Phoma* sp. isolated from rotten tangerine peel was purified 8.5-fold with a specific activity of 84.5 U mg^{-1} protein. The purified enzyme had a molecular mass of 440 kDa with a subunit of 110 kDa.

Keywords: *Phoma* sp., Cellulolytic enzymes, Identification, Tangerine peel

1. Introduction

Tangerine is a popular fruit in Jeju island, Korea. In 2008, about 500,000 tons of tangerine were produced and widely used as a fresh food, raw material for juice and other processed food. However, tangerine peels is one of the major agricultural wastes in Jeju island and their annual yields is more than 38,000 tons. Although about 70% of these residues accumulate is recycled as useful resources such as animal feed and oriental medicine materials, about 30% of them are dumped on the ocean. Therefore, research into microbial degradation and utilization of agricultural wastes is important and urgently required in order to accomplish waste reduction.

Cellulose and Pectin are known to be the major components of tangerine peel. Especially, cellulose, which is a linear polymer of D-glucose units linked by *β*-1,4-glucosidic bonds, is renewable carbon source for long-term solutions to energy, chemical, and food resource problems [1]. The most useful technology for utilizing cellulose is biological conversion to alcohol via cellooligosaccharides; however, depolymerization of cellulose into cellooligosaccharides with commercial enzymes remains expensive [2]. The enzymatic hydrolysis for the conversion of cellulose to fermentable monomeric sugar, glucose, involves synergistic action of three types of cellulase such as endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), and *β*-glucosidase (BGL, EC 3.2.1.21). Especially, BGL catalyzes the hydrolysis of cellobiose. It also plays an important role in the process of saccharification of cellulose by removing cellobiose, which is known to be a strong inhibitor of CBH and EG [3].

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In this presentation, we describe for the first time that the identification of *Phoma* sp. having a significant tangerine peel degrading activity isolated from the rotten tangerine peel and characterization of an extracellular cellulolytic enzymes, such as EG, CBH and BGL, from the fungus.

2. Methods and Materials

2.1. Isolation of microorganism and growth conditions

The fungal strains on rotten tangerine peel were plated on potato dextrose agar (PDA) plate, and then incubated for 7 days at 28ºC. After two subsequent transfers on the same plate, the isolates were maintained pure at 28ºC. For cellulase production, the isolate was cultivated in the cellulolytic medium containing (w/v): 0.05% $(NH_4)_2SO_4$, 0.05% L-asparagine, 0.05% KCl, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.05% yeast extract and 0.5% dextrose with 5% cellulose powder. The culture was then grown at 28ºC and pH 4.5 under aerobic conditions for 14 days. The culture supernatant was used to purify cellulolytic enzyme.

2.2. Identification of isolated strain

For genomic DNA preparations, the strain was grown on 2% PDA plate overlaid with sterile cellophane sheets and was incubated at room temperature for 7 days. DNA was extracted from hyphae of the isolate with AccuPrep® Genomic DNA extraction kit (Bioneer Inc., Korea). The ITS region (ITS) of the nuclear ribosomal DNA operon was amplified with the primer pair ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTATATGATATGC-3') (White et al. 1990). PCR reaction mixtures containing AccuPrep™ PCR premix (Bioneer Inc., Korea), 5-50 ng DNA, 5-10 pmol each primer in a total volume of 20 µl were subject to the following protocol: 5 min initial denaturation at 95ºC, followed by 40 cycles of denaturation (95ºC for 30 min), annealing (48ºC for 30s) and extension (72ºC for 80s). The final extension was conducted at 72ºC for 7 min. PCR products were subjected to electrophoresis in a 1% agarose gel containing EtBr and visualized by UV illumination. The PCR products were purified by using AccuPrep® PCR Purification kit (Bioneer Inc., Korea). Sequencing was performed at Macrogen (Seoul, Korea). For the phylogenetic analysis, sequences from the isolate were aligned with sequences obtained from BLAST searches in GenBank. Sequences were aligned with ClustalW version 1.83. The resulting multiple alignments were corrected manually using PHYDIT version 3.2. The phylogenetic tree was constructed by Neighbor-joining method using MEGA version 4.0 [4].

2.3. Enzyme assay and protein determination

The cellulolytic enzyme activities from this fungus were measured by using methods as described previously [5]. The protein concentration in the culture solution was measured by using a Bradford's method.

2.4. Enzyme purification and kinetic studies

The supernatant solution (2 liters) was filtered through a filter paper and concentrated using a stirred ultrafiltration cell equipped with a 10 kDa cutoff polyethersulfone membrane and dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.0). The concentrated solution was loaded on a DEAE Sepharose™ Fast Flow column (GE healthcare, Sweden) equilibrated with same buffer. The bound BGL eluted with a step gradient of 0 to 500 mM NaCl prepared in 20 mM Tris-HCl buffer (pH 7.0) at a flow rate of 5.0 ml min^{-1}. Fractions containing BGL activity were collected, concentrated and dialyzed against 50 mM sodium acetate buffer (pH 5.0) containing 0.15 M NaCl. The dialyzed enzyme was further purified by Fast Protein Liquid Chromatography (FPLC) on a HiPrep 16/60 Sephacryl S-300 HR column (GE healthcare, Sweden). Elution was performed with the same buffer at a flow rate of 2.0 ml min^{-1} and activity fractions were concentrated and dialyzed in 20 mM sodium acetate buffer (pH 4.5). The dialyzate was applied to a MonoQ ion exchange column 5/50 GL (GE healthcare, Sweden) equilibrated with the same buffer, and then eluted with 20 mM sodium acetate buffer (pH 4.5) containing 0.5 M NaCl. The active fractions were pooled, concentrated and used as a purified enzyme for subsequent studies.

Various concentrations of *p*NPG (from 0.05 mM to 1 mM) and cellobiose (from 0.05 mM to 1 mM) were used to determine kinetic parameters (K_m and k_{cat}) of the purified enzyme. The reactions were performed in 100 mM sodium acetate buffer (pH 4.5) at 60ºC. The inhibition constant (K_i) for glucose and

glucono-δ-lactone was investigated in the presence of 0-10 mM glucose and 0-1 mM glucono-δ-lactone at pH 4.5 and 60ºC with *p*NPG as substrate. The enzyme kinetic parameters, K_m and k_{cat} values were defined by fitting to the Michaelis-Menten equation.

3. Results and Discussion

3.1. Identification of isolation strain

The cellulolytic enzyme producing fungal strain was isolated from a rotten tangerine peel. The phylogenetic tree of isolated fungus and related fungal species based on ITS regions was shown in Fig. 1. It was clearly seen that isolated fungus was included in the genus *Phoma,* and was closely related to the species *Phoma pomorum*, showing the highest sequence similarities with *P. pomorum* var. *circinata* CBS 286.76 99.4% and *P. pomorum* var. *cyanea* CBS 388.80 99.6% and *P. pomorum* var. *pomorum* CBS 539.66 99.6%. *P. pomorum* is one of the common fungal species representing a biological risk for plants. It causes mildew and brown lesions on the leaves of various fruit trees such as apples, cherries, pears and strawberries [6]. *P. pomorum* is also well-known to be a fungus that is being developed as a biocontrol agent of hounds tongue disease. Because isolated strain is phylogenetically so similar to *P. pomorum* group, it was regarded as a variety of this species but additional experiments are required to know an accurate varietal name.

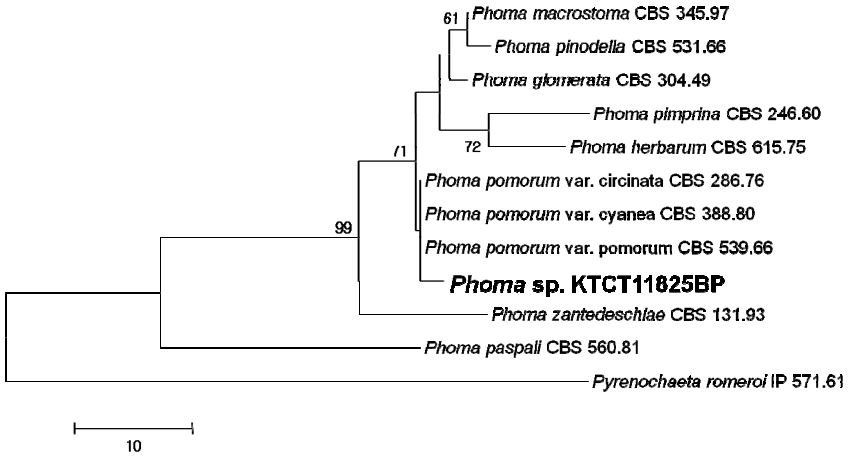

Fig. 1. The phylogenetic tree of KCTC11825BP and related fungal species based on ITS regions.

3.2. Production of cellulolytic enzymes

The ability of the *Phoma* sp. to produce extracellular cellulolytic enzymes (EG, CBH and BGL) was carried out using the liquid culture containing 5.0 % (w/v) cellulose powder. The data presented in Figure 2 show that the BGL and EG activities increased sharply after 7 days of cultivation. Especially, BGL activity showed the highest.

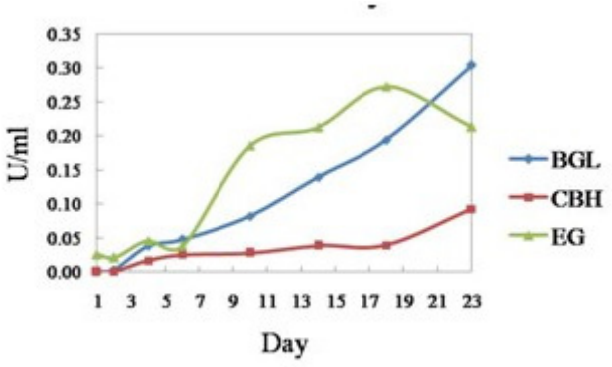

Fig. 2. Changes in the activity of extracellular EG, CBH, and BGL of *Phoma* sp. grown on 5.0% cellulose culture.

3.3. Purification of BGL and kinetic parameters

An extracellular BGL was purified to homogeneity from the culture filtrates of *Phoma* sp. grown on 5.0% cellulose powder as a carbon source. A summary of purification steps of the BGL from *Phoma* sp. is presented in Table 1. The BGL from *Phoma* sp. was purified 8.5-fold with 34.3% yield and a final specific activity of 84.5 U mg^{-1} of $protein^{-1}$ with *p*NPG as substrate. The native molecular mass of the purified enzyme was 440 kDa by gel filtration chromatography on a Sephacryl S-300 HR 16/60 preparative-grade column, and that of subunits was 110 kDa by the method of SDS-PAGE (Fig. 3). These results suggest that the purified enzyme appeared to be a tetramer. These results suggest that the purified enzyme appeared to be a tetramer. This BGL had a higher molecular mass and multimeric than well-known BGL in nature [7, 8].

Table 1. Purification of the *β*-glucosidase from *Phoma* sp. KCTC11825BP

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	77.1	767.9	10.0	100.0	1.0
Concentration (10 kDa cut-off)	41.5	701.8	16.9	91.4	1.7
DEAE Sepharose^{TM} Fast Flow	18.4	522.4	28.4	68.0	2.8
Sephacryl S-300 HR	6.1	329.1	54.1	42.9	5.4
Mono Q 5/50 GL	3.1	263.5	84.5	34.3	8.5

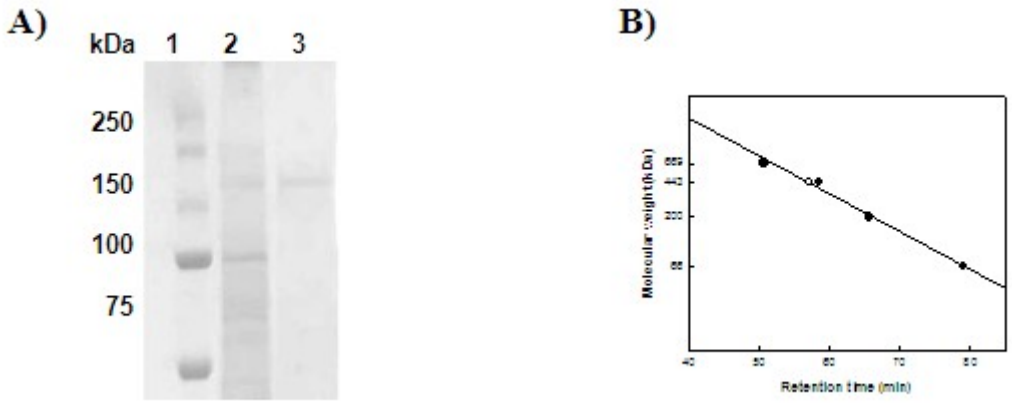

Fig. 3. SDS-PAGE analysis (A) and molecular mass (B) of purified enzyme

Kinetic parameters of the purified enzyme for *p*NPG and cellobiose are presented in Table 2. The Lineweaver-Burk plots indicated that the K_m for *p*NPG and cellobiose were 0.3 mM and 3.2 mM, and the k_{cat} values were 0.5 sec^{-1} and 699.4 sec^{-1}, respectively. The kinetic efficiency (k_{cat}/K_m) values for hydrolysis of *p*NPG and cellobiose were calculated as 1.6 and 215.8 mM sec^{-1}, respectively. The purified BGL has 10-fold higher affinity for *p*NPG than cellobiose. The effect of glucose and glucono-δ-lactone was performed with *p*NPG as the substrates. Glucose and glucono-δ-lactone inhibited BGL competitively, with inhibition constants (K_i) of 1.7 mM and 0.1 mM, respectively, indicating that glucose is a stronger inhibitor of BGL from *Phoma* sp. KCTC11825BP than glucono-δ-lactone like other cellulolytic fungi [9]. Thus, these result showed that the BGL from *Phoma* sp. KCTC11825BP classified as an aryl-BGL with cellobiase activity.

Table 2. Kinetic characteristic of the *β*-glucosidase from *Phoma* sp. KCTC11825BP

Substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m(mM sec^{-1})	Compound	Inhibition pattern	K_i (mM)
*p*NPG	0.3	0.5	1.6	Glucose	Competitive	1.7

Cellobiose	3.2	699.4	215.8	Glucono-δ-lactone	Competitive	0.1

4. Acknowledgments

This work was supported by the Korea Institute of Energy Technology Evaluation and Planning (KETEP) grant funded from the Ministry of Knowledge Economy (MKE), Republic of Korea.

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