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Cellulolytic enzymes produced by a newly isolated soil fungus *Penicillium* sp. TG2 with potential for use in cellulosic ethanol production

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ABSTRACT

A newly isolated soil fungus, *Penicillium* sp. TG2, had cellulase activities that were comparable to those of *Trichoderma reesei* RUT-C30, a common commercial strain used for cellulase production. The maximal and specific activities were 1.27 U/mL and 2.28 U/mg for endoglucanase, 0.31 U/mL and 0.56 U/mg for exoglucanase, 0.54 U/mL and 1.03 U/mg for β-glucosidase, and 0.45 U/mL and 0.81 U/mg for filter paper cellulase (FPase), respectively. Optimal FPase activity was at pH 5.0 and 50 °C. We used a simultaneous saccharification and fermentation (SSF) process, which employed the yeast *Kluyveromyces marxianus* and *Penicillium* sp. TG2 cellulolytic enzymes, to produce ethanol from empty palm fruit bunches (EFBs), a waste product from the palm oil industry. The present findings indicate that *Penicillium* sp. TG2 has great potential as an alternative source of enzymes for saccharification of lignocellulosic biomass.

1. Introduction

There is increasing interest in the use of biomass as a renewable energy source because of increasing oil prices and the effect of fossil fuel burning on climate change [1]. However, use of sugarcane, plant starches, and plant oils as energy sources can inflate the prices of agricultural commodities. Thus, there are many advantages of using lignocellulosic materials, inedible biomass that is not a food source [2–5]. Empty palm fruit bunches (EFB) are a potential source of lignocellulosic materials because they are a readily available and low-cost residual by-product of the palm oil industry that consist of about 39.5% cellulose, 17.3% hemicellulose, 28.8% lignin, 10.3% extractives, and 3.8% ash [6].

However, in contrast to starch- and sugar-based biomass sources, more complex physical, chemical, or biological processes are needed to prepare lignocellulosic biomasses [7–9]. In particular, saccharification to fermentable sugars is usually carried out by enzyme catalysis because the process would minimize environmental pollution [10,11]. Endoglucanase, exoglucanase, and β-glucosidase perform this saccharification process [12]. Diverse bacteria and fungi have cellulolytic enzyme activities. Fungi of the *Trichoderma* genus are a well-known source of cellulolytic enzymes and some of these enzymes have been developed as commercial products [13]. In particular, Cellic® CTec2 (Novozyme) is a commercial cellulase enzyme mixture that consists of 15–20 enzymes, including *Trichoderma reesei* RUT-C30 enzymes.

Here we describe a newly isolated microbe that has excellent cellulolytic activity relative to a representative commercial strain, and characterize and evaluate the use of its enzymes for the saccharification of EFBs from the palm oil industry.

2. Materials and methods

2.1. Microorganisms and media

Penicillium sp. TG2 was newly isolated from soil sample (KCTC 12405BP) and *T. reesei* RUT-C30 was obtained from the Korean Collection for Type Culture. The fungal strains were maintained on potato dextrose agar (PDA) plates at 28 °C for 7 days, and stored at

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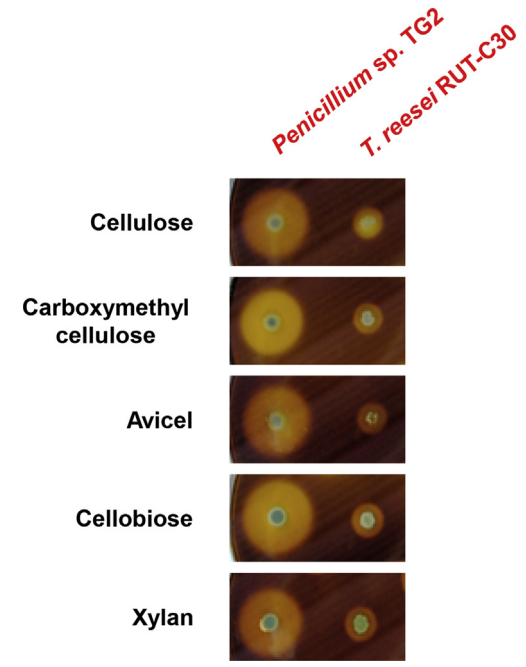

Fig. 1. Activity analysis of cellulolytic enzymes of *Penicillium* sp. TG2.

4 °C when spores were formed. *Kluyveromyces marxianus* CBS1555 (KCTC7001) was used for ethanol fermentation and maintained on YPD medium (1% yeast extract, 2% peptone, 2% glucose).

2.2. Cultivation of Penicillium sp. TG2 and T. reesei RUT-C30 for production of cellulases

Spores of *Penicillium* sp. TG2 and *T. reesei* RUT-C30 were suspended in 30 ml of sterile water containing 0.2% Tween 80 and inoculated into 250 ml Erlenmeyer flasks that contained 100 ml of inoculum medium with Mandel's mineral salts solution a concentration of 5×10^8 spores/mL. Each liter of medium contained 0.3 g of urea, 1.4 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 0.3 g of $MgSO_4{\cdot}7H_2O$, 0.4 g of $CaCl_2{\cdot}2H_2O$, 0.75 g of peptone, 0.25 g of yeast extract, 10.0 g of corn steep liquor (CSL), 5.0 mg of $FeSO_4{\cdot}7H_2O$, 1.6 mg of $MnSO_4{\cdot}7H_2O$, 1.4 mg of $ZnSO_4{\cdot}7H_2O$, 2.0 mg of $CoCl_2{\cdot}6H_2O$, and 2.0 g of microcrystalline cellulose as a carbon source. The initial pH was adjusted to 5.5 by addition of 10% (v/v) H_2SO_4. The cells were cultivated on a shaker at 30 °C and 150 rpm for 3 days, and then transferred to fresh medium for production of cellulase enzymes. Enzyme production continued for 5 days under the same culture conditions. Secreted extracellular enzymes were prepared by centrifugation (13,000 *g*, for 10 min at 4 °C) and then analyzed for activity [14].

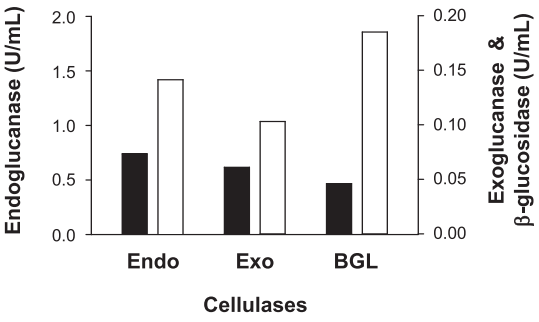

Fig. 2. Comparison of cellulolytic activities of culture broth of *Penicillium* sp. TG2 and *Trichoderma reesei* RUT-C30. Endo, endoglucanase; Exo, exoglucanase; BGL, β-glucosidase. Closed bars, *T. reesei* RUT-C30; open bars, *Penicillium* sp. TG2.

2.3. Cellulase activity of Penicillium sp. TG2

Cells were grown on solid medium containing Mandel's mineral salts solution with 0.2% cellulosic material as a substrate and 0.1% Triton X-100 as a colony restrictor. Cellulase activity was measured using Gram's iodine solution and measurement of the clearing zone ratio (clearing zone diameter/colony diameter) [15].

Cellulase activities were also measured for cells grown in culture broth and endoglucanase (CMCase) activity was determined using carboxymethyl cellulose sodium salt (CMC) as a substrate [16]. In these experiments, 0.1 mL of 2.0% (w/v) CMC solution was incubated with 0.1 ml of enzyme solution, diluted with 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. Then, 0.2 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added and the solution was boiled at 100 °C for 5 min. Finally, 0.6 ml of distilled water was added and the absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min under the conditions of the assay.

Exoglucanase activity in international units (IU) was assayed by a DNS method. In particular, 40 μL of enzyme solution was added to test tubes, and 160 μL of 1.25% avicel (cellulose substrate) was diluted with 0.1 M acetate buffer (pH 4.8). The mixture was incubated at 50 °C for 2 h, and 0.2 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added to stop the reaction. The tubes were transferred to boiling water at 100 °C for 5 min, 0.6 mL of distilled water was added, the contents of the tubes were mixed to yield a red color, and absorbance was measured at 540 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol glucose per min under the conditions of the assay.

β-glucosidase activity was assayed in a reaction mixture consisting of 25 μL of enzyme solution, 25 μL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 10 min. After the incubation, 0.1 mL of a 0.4 M Na_2CO_3 solution was added. After the color developed (indicating release of *p*-nitrophenol), absorbance was measured at 405 nm. For all these dosages, one unit of activity is the amount of enzyme required to release 1 μmol *p*-nitrophenol per min under the conditions of the assay. Total protein was measured by the Bradford method using bovine serum albumin (BSA) as a standard.

Filter paper cellulase (FPase) activity was assayed by incubating the 125 μL of enzyme solution with 250 μL of sodium citrate buffer (50 mM, pH 4.8) with filter paper (Whatman no. 1, 0.25 μm pore size, 1.5 cm diameter]). The mixture was incubated at 50 °C for 60 min. Then, 200 μL of enzyme solution (supernatant) was added to 200 μL of DNA reagent and boiled at 100 °C for 5 min. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method [16]. One international unit of FPase activity is the amount of enzyme that forms 1 μmol of glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. All presented data are average of three independent experiments.

2.4. Saccharification of empty palm fruit bunches by cellulases from Penicillium sp. TG2

Empty palm fruit bunches (EFBs) (5%, w/v) were pretreated with 1 M NaOH at 121 °C and stirred to homogeneity for 15 min [17]. The material was washed with flowing tap water to remove NaOH and then dried at 105 °C for 24 h. Pretreated EFB solids were hydrolyzed by cellulase enzymes in a 0.05 M sodium citrate buffer (pH 4.8) at 42 °C with agitation at 200 rpm. The amounts of sugars produced by enzymatic saccharification of EFB were measured by high performance liquid chromatography (HPLC) at different times. All presented data are the averages of three independent experiments.