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Production of Cellulase by *Bacillus subtilis* subsp. *subtilis* in Batch Fermentation and Its Enzymatic Properties

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Abstract

High-throughput screening was performed for the isolation of soil microorganisms secreting cellulase. A motile rod shaped bacterium, *Bacillus subtilis* subsp. *subtilis* designated strain DW02 (EF538682), was isolated from forest soil samples collected from Mt. Baekwoon near Gwangyang, Korea. The neighbour-joining tree based on *gyrase* A gene sequences showed that strain DW02 formed a monophyletic clade with *Bacillus subtilis* subsp. *subtilis* KCTC 3135^T (AF272021) with 100% bootstrap support. The highest cellulase activity was found in the bacterial cell pellet lysis extracts. The pH and temperature of the highest cellulase activity were measured by a reducing sugar assay, where their optimal pH and temperature were 7.0 and 50°C, respectively. After cellulose hydrolysis reaction using 1% carboxymethyl cellulose (CMC) at 50°C for 2 hr, the bacterial cell pellet lysis extracts (2.29 mg/mL) produced cellobiose, cellotriose and cellotetraose in a thin layer chromatography.

Key words: *Bacillus subtilis* subsp. *subtilis*, cellulase, high-throughput screening, cellulose

Introduction

Bacillus subtilis is an aerobic, Gram-positive, endospore-forming, rod-shaped bacterium commonly found in soil, water and many plant-derived molecules. A large proportion of translated genes are related to the metabolism of carbohydrates and related molecules. During nutritional starvation, these strains can utilize a variety of carbon sources using industrially important hydrolases such as proteases and carbohydrases (Kunst et al., 1997). Compost microorganisms such as *Bacillus* spp., *Actinomycetales*, *Thermus* spp., *Streptomycetes* spp., *Aspergillus* spp. are able to break down a large variety of carbohydrates, lipids and proteins into smaller units (Strom, 1985; Beefa et al., 1996; McCaig et al., 2001; Song et al., 2001). The microorganisms convert organic material into carbon dioxide, biomass and thermal energy (Tuomela et al., 2000). Research or development interest in these microorganisms has increased due to potential source for the industrial production of antibiotics and hydrolases (Malherbe & Cloete, 2002). Leemhuis et al. (2003) developed sensitive screening assay to detect cellulolytic strains producing carbohydrate hydrolase. The aims of this study were to screen the isolates for cellulase activity, to perform temperature optimum and temperature stability studies on the enzymes and to identify the microorganisms with enzyme activity using *gyrA* sequence analysis.

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Materials and Methods

Bacterial strains and culture media

Using standard dilution plating techniques, *Bacillus subtilis* subsp. *subtilis* designated strain DW02 (EF538682) was isolated from forest soil samples collected from Mt. Baekwoon near Gwangyang, Korea (35°06' N, 127°37' E), in June 2007. Isolation was achieved using plate count agar (PCA, Difco) at 35°C for 7 days. The isolates were transferred to 200 µl LB (Luria Bertani) medium in 96-well microtiter plates by picking colonies grown on PCA and the microtiter plates were incubated overnight and maintained as a glycerol suspension (20%, w/v) at -80°C.

High-throughput screening

After incubation at 35°C overnight in microtiter plates, 25 µl of each culture was transferred to a second microtiter plate (Polystyrene, Corning Inc., NY, USA) and was mixed with 10 µl bacterial protein extraction reagent (Pierce,

Rockford, IL, USA) per well in order to release enzymes located within cells. After addition of 200 μL of 1% (w/v) carboxymethyl cellulose (CMC) in 10 mM sodium citrate buffer (pH 7.0), the microtiter plates were incubated in an oven at 50°C for 2 hr. Nelson-Somogyi assay was used for the screening of strains producing cellulase by measuring reducing sugars with a spectrophotometer (Mecasys Co., Ltd., Daejeon, South Korea) at an absorbance of 525 nm (Green et al., 1989).

Bacterial identification

To identify a bacterium based on *gyrA* fragments, corresponding to *B. subtilis gyrA* numbering positions 43-1065 (Kunst et al., 1997), genomic DNA was extracted using a bacterium grown overnight on LB by an Accuprep Genomic DNA extraction kit following the manufacturer's protocol. DNA amplification was carried out in the following manner: 5 min of pre-soaking followed by 30 cycles at 94°C, 30 sec at 55°C for denaturing, 30 sec at 72°C for annealing, 40 sec for extension, and finishing with an incubation at 72°C for 5 min using two oligonucleotide primers, gyrA-f (50-CAG TCAGGA AAT GCG TAC GTC CTT -30) and gyrA-r (50-CAA GGT AAT GCT CCA GGC ATT GCT -30), as described previously (Chun & Bae, 2000). The resultant amplicons were purified using Wizard PCR Prep Kits (Promega, Madison, WI, USA) and sequenced in both directions using an ABI 310 automated DNA sequencer and BIG-dye cyclic sequencing kits (PE Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The same primers were used for the sequencing reactions. The closest known relatives of the new isolates were determined by performing sequence database searches and the sequences of closely related strains were retrieved from GenBank or the Ribosomal Database Project (RDP) libraries. Nucleotide (NT) sequence similarities were calculated using the PHYDIT program (Weisburg et al., 1991; Chun, 1995).

Production of bacterial cellulase in batch fermentation

Bacillus subtilis subsp. *subtilis* was grown at 35°C for 24 hr in a 7-liter fermenter (KoBioTech Co., Incheon, Korea) containing 5 L of LB medium (per liter): 10 g of tryptone, 5 g of yeast extract, and 5 g NaCl. After centrifugation at 7,000 ×g for 30 min, the bacterial cell pellet was mixed with 500 mL of bacterial protein extraction reagent and then the bacterial lysis extracts were concentrated using an Amicon concentrator (10 kDa. cut-off filter) against 50mM Tris buffer (pH 7) at 4°C. The enzyme optimal pH and temperature were estimated by the reducing sugar assay over the range pH 4.0 to pH 8.0 and 40°C to 60°C.

Thin layer chromatography

The product formed by hydrolysis of CMC was assessed by thin-layer chromatography (TLC). Hydrolysis products were separated by TLC on silica gel plates (Analtech, Inc., Neward, DE, USA) by using a mixture of chloroform, glacial acetic acid, and water (6:7:1, vol/vol/vol) as the solvent (Li et al., 1997).

Results and Discussion

As the result of high-throughput screening, bacterium capable of degrading CMC was isolated from forest soil samples collected from Mt. Baekwoon near Gwangyang, Korea (35°06' N, 127°37' E). The strain isolated is a motile rod shaped bacterium; *Bacillus subtilis* subsp. *subtilis* designated strain DW02 (EF538682). The neighbour-joining tree based on *gyrase* A gene sequences showed that strain DW02 formed a monophyletic clade with *Bacillus subtilis subsp. subtilis* KCTC 3135^T (AF272021) with 100% bootstrap support (Fig. 1). Partial *gyrA* sequences, coding for DNA gyrase subunit A of *B. subtilis* and allied taxa was used to determine the bacterial identification of closely related aerobic, endospore-forming bacilli instead of bacterial classification using 16S rRNA gene, which shows limited variation for closely related species (Fox et al., 1992; Chun & Bae, 2000). The pH range (pH 3-8) and NaCl tolerance

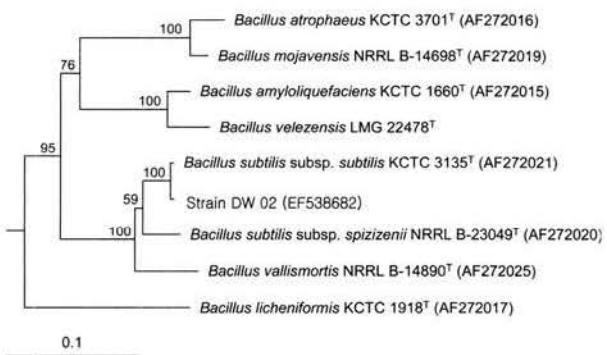

Fig. 1. Neighbour-joining tree (Saitou and Nei, 1987) based on *gyrase* A gene sequences of *Bacillus subtilis* complex and *Bacillus velezensis*. The *gyrase* A gene sequence *of Salmonella typhimurium* SLPS1-3 (AY612609) were included as outgroups. Numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled dataset. Scale bar indicates 0.1 nucleotide substitution per nucleotide position. T, type strain.

[0.5, 1, 2, 3, 4, 10% (w/v)] were checked using LB, for 49 hr. Growth occurs in pH 4-7 (optimum pH 5) and 0.5-10% (w/v) NaCl (optimum 0.5%) (data not shown).

As enzymes hydrolyze the glycosidic bonds of carbohydrates, the number of reducing sugar ends increases the color intensity in a reducing sugar assay (Leemhuis et al., 2003). A reducing sugar assay showed the highest cellulase activity in the bacterial cell pellet lysis extracts rather than in the cell culture supernatant obtained from 5 liters of LB medium (data not shown). The optimal pH and temperature of *Bacillus subtilis* subsp. *subtilis* designated strain DW02 were 7.0 and 50°C, respectively (Fig. 2). Hakamada et al. (1997) isolated *Bacillus* sp. KSM-237 from a soil sample and the strain had a pH optimum of 8.6-9.0 and showed the highest carboxymethylcellulase (CMCase) activity at 45°C. Fukumori et al. (1985) and Horikoshi et al. (1984) isolated *Bacillus* sp. strains N4 and 1139 secreting

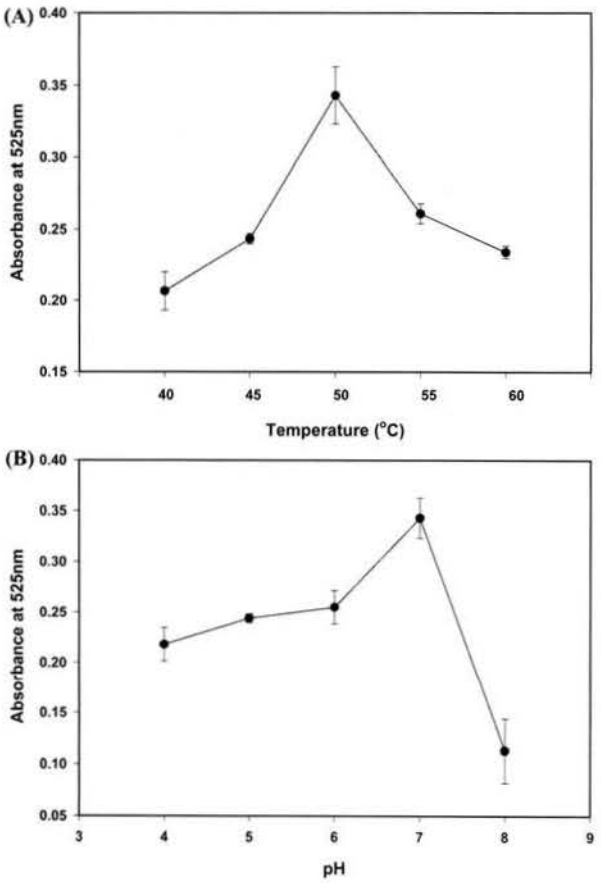

Fig. 2. The effect of pH (A) and temperature (B) on the cellulase activity of bacterial cell pellet lysis extracts (above 10k dalton) of *Bacillus subtilis* subsp. *subtilis* in a reducing sugar assay.

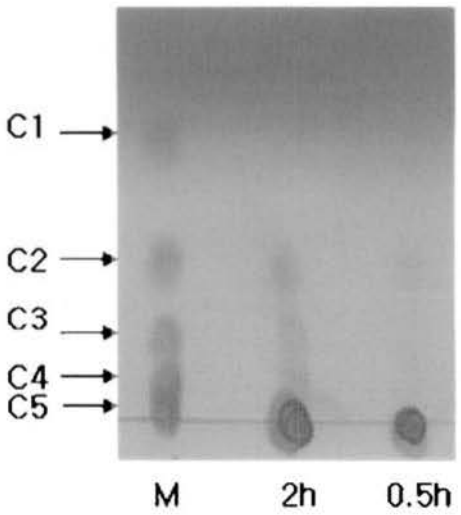

Fig. 3. Carboxymethyl cellulose hydrolysis reaction using bacterial cell pellet lysis extracts in a thin layer chromatography. 200 μl of 1% carboxymethyl cellulose (CMC) and 25 μl of bacterial cell pellet lysis extracts (2.29 mg/mL) were incubated at 50°C for 0.5hr and 2hr prior to a thin layer chromatography. M, size marker: C1 (glucose), C2 (cellobiose), C3 (cellotriose), C4 (cellotetraose), C5 (cellopentaose).

alkaline CMCases. *Bacillus* sp. strain N-4 showed its optimum CMCases activity over a pH range from 5 to 10. *Bacillus* sp. strain 1139 displayed optimum CMCase activity at pH 9.0 and the enzyme activity was unwavering over the pH range from 6 to 11 for 24 hr at 4°C and 10 min at up to 40°C (Fukumori et al., 1987). The *Sinorhizobium fredii* strain CCRC 15769 required pH 7.0 and 35°C for the optimum CMCase activity (Chen et al., 2004).

After cellulose hydrolysis reaction using 200 μL of 1% carboxymethyl cellulose (CMC) and 25 μL of bacterial cell pellet lysis extracts (2.29 mg/mL) at 50C for 2 hr, a thin layer chromatography showed cellobiose, cellotriose and cellotetraose, indicating the presence of cellulase activity (Fig. 3). Apiraksakorn et al. (2008) characterized the crude enzyme with β-1,3-1,4-Glucanase activity obtained from *B. subtilis* GN156. The crude enzyme had optimum pH values of 6–6.5 and 60°C. Incubation with the crude enzyme preparation for 6 hr produced cellotriose as the major product. After 12 hr incubation, the crude enzyme produced three different oligosaccharides with 1,3 linkages triose, tetrose and pentose.

The high-throughput screening assay used in this study has the advantage that cellulose degrading activity can be directly measured using liquid cultures grown in microtiter plates without the need of separation or purification steps

and is fast and easy to perform more adaptable for screening of a large number of samples. With this high level of cellulose degrading activity, it would be expected that this cellulase has the potential to be used in industrial applications.

Acknowledgements

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