Isolation of endo-1,4-β-D-glucanase producing Bacillus subtilis sp. from fermented foods and enhanced enzyme production by developing the mutant strain

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ABSTRACT

Cellulolytic bacteria living in food can be applied to microbial feed additives to improve fiber digestion in animal feeds. In this study, a cellulase-producing bacteria was isolated from salted clam and treated with physical or chemical agents to enhance their enzyme production. The bacteria was identified as a strain of Bacillus subtilis on the basis of 16S rRNA analysis. Endo-1,4-β-D-glucanase (endoglucanase) was produced by the wild type using 0.4% carboxy-methyl-cellulose as a carbon source with maximal activity (0.04 U/mL) after 24 h incubation. Insoluble cellulose and oat spelt xylan were also used as carbon sources for investigation of exoglucanase and xylanase, however, these enzymes were not found in the culture supernatant. Maximum endoglucanase activity of Bacillus subtilis sp. was measured at 50°C and pH 5, respectively. Then, the strain was subjected to classical mutagenesis (UV-irradiation and chemical treatment) to improve endoglucanase production. A mutant strain, P11 treated with ethyl methyl sulfonate was finally selected. Mutant P11 was sub-cultured and tested for endoglucanase production, which was 0.05 U/mL after 24 h growth. The significant difference of endoglucanase production between wild type and mutant P11 was prolonged to 10th generation. Thus, the mutant strain was found to have enhanced endoglucanase production.

Key words: Bacillus subtilis sp., Endoglucanase, Ethyl methyl sulfonate, Mutagenesis, Salted clams.

INTRODUCTION

Cellulose is abundant sources of carbohydrate, continually replenished by photosynthetic reduction of carbon dioxide with sunlight energy (Fan et al., 1987). Cellulose is good sources of the ruminant feed, but they can be degraded only by the action of microorganisms that are capable of hydrolyzing cellulososes into hexoses. There is increasing interest in these organisms since they and the enzymes they produce may play a significant role in the conversion of plant materials into valuable products (Doi, 2008).

Cellulolytic microorganisms including both anaerobic and aerobic have been studied on various purposes, but the most important interest in these studies is how to reduce enzyme production cost. For that purpose, mutagenesis has been used to improve enzyme activities. However, those efforts were mostly focused on fungi rather than bacteria. Members of the fungal genus Trichoderma and Aspergillus have been extensively studied, particularly due to their ability to secrete cellulose-degrading enzymes (Chand et al., 2005).

Unlike past attempts, this study aimed to develop bacterial cellulases. Bacterial cellulases have some advantages over fungal cellulases: 1) usually more thermostable, 2) having a short generation time, 3) being able to easily grow to very high cell density using inexpensive carbon and nitrogen sources; more convenient expression system and manipulation resulting in the high level expression of endogenous cellulase (Li et al., 2008).

Cellulolytic bacteria isolated from fermented foods were investigated to avoid safety issue since the fundamental goal of this study was to develop microbial feed additives. In addition, mutagenesis of cellulolytic bacteria by chemical treatment was performed to obtain bacterial strains having high level of extracellular cellulase activities. Optimization
the conditions for enzyme assay was also conducted for the selected bacteria.

MATERIALS AND METHODS
Microorganisms and growth media: Bacterial strains investigated in this study were isolated from Korean traditional fermented foods, salted clams and were grown in Luria-Bertani (LB) agar (Difco laboratories, Sparks, USA) (tryptone, 10g/L; yeast extract, 5g/L; sodium chloride, 5g/L; agar, 15g/L) containing 0.4% (w/v) of carboxymethylcellulose (CMC, Sigma, St. Louis, MO, USA) as a carbon source at 37°C. Strains were routinely cultured in broth and stored with 30% of glycerol at -80°C.

Screening and identification of endoglucanase-producing bacteria: Food samples, salted clams were screened on Luria-Bertani (LB) agar (Sigma, St. Louis, MO, USA) (tryptone, 10g/L; yeast extract, 5g/L; sodium chloride, 5g/L; agar, 15g/L) containing 0.007% (w/v) of trypan blue (Sigma, St. Louis, MO, USA) and 0.4% (w/v) of CMC. The colonies forming clear zones were carefully picked and re-streaked onto same agar plate to check their enzyme activities, and then they were isolated. The strains which showed consistent endoglucanase activity were regarded as strains finally screened.

For identification of the selected bacterial strain, 16S rRNA sequence was amplified by polymerase chain reaction (PCR). A total of 1.5 mL of culture media was centrifuged (10,000 g x 1 min) to obtain a cell pellet for DNA extraction, which was performed using a DNeasy Blood & Tissue Kit (Qiagen, Seoul, South Korea). Polymerase chain reaction amplification of the 16s rRNA gene fragments was performed using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACGCTACCTTGTTACGACTT-3') (Stackebrandt and Goodfellow, 1991). The amplification was performed using HotStar Taq Master Mix Kit (Qiagen, Seoul, South Korea) with the following cycling parameter: 95°C for 15min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C with final extension for 7 min. The amplified PCR product was visualized by gel electrophoresis. The 16s rRNA band was cut and purified using a Gel DNA extraction kit (Qiagen, Seoul, South Korea). A sequence similarity search of the purified PCR product was carried out using BLAST with the NCBI database (http://www.ncbi.nlm.nih.gov) and alignment was performed using V-NTI (Life Science Technology, Co. Ltd., USA).

Growth curve: The culture medium used in this study was liquid LB media containing 1% of CMC. The seed culture was developed prior to measurement of growth phase using the same media. The culture media (100 mL) in 500 mL shake flasks was inoculated with 1% of seed culture showing 0.5 of optical density at 600 nm (OD_600). Aliquots of the bacterial cultures were taken from the growth media at 2 h intervals, and their absorbance was measured at 600 nm in a UV-VIS spectrophotometer (UV-1601 PC, Shimadzu, Japan). Growth curves were plotted as absorbance vs time. Enzyme activity was also calculated at same time intervals.

Enzyme assay: Endoglucanase activity was determined using 3, 5-dinitrosalicylic acid (DNS) reagent (Ghose, 1987; Miller, 1959). Culture media were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was used as extracellular enzyme preparation. The enzyme with 1% (w/v) of CMC prepared in 0.05 M potassium phosphate buffer (pH 6.6) was incubated in a water bath at 37°C for 1hr. The reaction was stopped by addition of DNS reagent. The reaction mixture was boiled for 5 min in a boiling water bath for color development and cooled rapidly. The reducing sugar (liberated) was measured at 540 nm in a UV-VIS spectrophotometer (UV-1601 PC, Shimadzu, Japan).

Exoglucanase and xylanase were measured under same conditions as described above using insoluble cellulose (Avicel PH101, Sigma-Aldrich, St. Louis, MO, USA) and oat spelt xylan (Sigma, St. Louis, MO, USA) as carbon sources, respectively.

Glucose (Sigma, St. Louis, MO, USA) was used for the standard curve of endoglucanase and exoglucanase, and xylose (Sigma, St. Louis, MO, USA) was used for the standard curve of xylanase. One unit of enzyme activity (U/mL) was defined as the amount of enzyme releasing 1µM of reducing sugar per minute.

Optimal pH and temperature for endoglucanase assay:
The optimal pH on the reaction was studied in the presence of different buffers such as 0.05 M sodium citrate buffer (pH 3 and 4), 0.05 M sodium acetate buffer (pH 5), 0.05 M potassium phosphate buffer (pH 6 and 7) and 0.05 M Tris-HCl buffer (pH 8 and 9) at 37°C. To determine the optimum temperature, the selected endoglucanase activity was assayed at a wide range of temperatures (30-80°C) in 0.05 M potassium phosphate buffer at an interval of 10°C.

Mutagenesis
UV-irradiation: Selected parent strain, Bacillus subtilis sp. was spread on LB agar containing 0.4% (w/v) of CMC and then UV-irradiated by 20W-UV lamp (distance from plates: 30 cm) for 10 sec. The plates wrapped by aluminum foil were incubated at 37°C overnight. Colonies showing 2% of survival rate were sub-cultured on trypan blue screening plates and incubated at 37°C overnight. Mutants having higher endoglucanase activity than the parent strain were selected, sub-cultured and stored at -80°C.

Chemical treatment: An aliquot of 20 µL of Ethyl methyl sulfonate (EMS, Sigma, St. Louis, MO, USA) was added to 1 mL of culture broth of Bacillus subtilis sp. and incubated in a shaking chamber at 37°C for 1h, and then 4 mL of 5% (w/v) sodium thiosulfate (Sigma, St. Louis, MO, USA) was added to stop the reaction. Bacterial media treated with EMS were spread on nutrient agar plates and cultured at 37°C.
overnight. Colonies showing 2% of survival rate were sub-cultured on trypan blue screening plates and incubated at 37°C overnight. Mutants having higher endoglucanase activity than the parent strain were selected, sub-cultured and stored at -80°C.

Statistical analysis: A completely randomized experiment was conducted in triplicates for each tested condition. Data were analyzed with PROC GLM using the statistical package SAS 9.3 (SAS Institute, Cary, NC). Tukey’s Studentized Range (HSD) test was used for comparisons and statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION
Isolation and identification of CMCase-producing bacteria: Seven out of 15 bacterial strains isolated from fermented foods were selected by screening for endoglucanase-producing bacteria using trypan blue screening plates (Figure 1). Among them, only 1 strain was finally selected because other candidates showed lower growth rate or endoglucanase activity compared to the strain finally selected. It was found to be a gram-positive, facultative, spore forming bacteria. Then it was identified as Bacillus subtilis strain (maximum identity: 99%) based on 16S rRNA sequence aligned with NCBI data.

Enzyme characteristics of Bacillus subtilis sp. isolated from salted clams: Carboxymethylcellulose, insoluble cellulose, oat spelt xylan, glucose and xylose were used as substrates and the activities of endoglucanase, exoglucanase and xylanase were measured. For control group, no carbon substrate was added to the media. Significantly high level of endoglucanase ($P < 0.05$) was obtained when CMC and xylan were used as substrates (Figure 2). Bacillus subtilis sp. did not grow well without substrate, insoluble cellulose or glucose (data not shown). Exoglucanase and xylanase were hardly observed regardless of carbon sources. Therefore, the optimal pH and temperature were investigated only for endoglucanase activity. The optimum pH and incubation temperature for endoglucanase were pH 5 and 50°C, respectively (Figure 3). The endoglucanase activity at optimum pH and temperature was 0.07 U/mL. Endoglucanase from Bacillus subtilis sp. was very weak at strong acidic condition like under pH 3.

Mutagenesis: A total of 211 UV-irradiated mutants and 188 EMS-treated mutants were screened and tested for endoglucanase production. Three mutants were selected and sub-cultured. Mutant 6EH13-2 and 6EH22-4 exhibited

![Fig 1: Endoglucanase producing bacteria forming a clear zone, was isolated from salted clams.](image1)

![Fig 2: Substrates specific enzyme production of Bacillus subtilis sp. isolated from salted clams. All assays were performed in triplicate. The data point and error bar indicated the average values and standard error.](image2)
highly enhanced endoglucanase production at 1st generation. However, their endoglucanase activities were decreased and finally, the effect of mutagenesis disappeared after a while. Mutant P11 was found to be stable for endoglucanase production for the studied period (10 generations) and the activity of mutant P11 was approximately 25% higher than that of the parent strain (Figure 4). Therefore, mutant P11 was selected and investigated for the further studies.

Growth and enzyme production of wild type and mutant strain: The growth and endoglucanase activity of two strains were shown in Figure 5. The growth of *Bacillus subtilis* sp. started at 0 h and has grown to 12 h later and the mutant strain also showed the similar trend of growth rate compared to the wild type. However, the optical density of the mutant at 12 h was significantly higher than that of the wild type suggesting that maximum growth of the mutant was higher than the growth of the parent strain. After 12 h, *Bacillus subtilis* sp. was in the stationary phase, but the highest endoglucanase activity (0.04 U/mL) was obtained at 16, and 24 h incubation as shown in Figure 5. The endoglucanase activity of the mutant P11 were significantly higher than that of the parent strain after 16 h incubation.

To date, studies on microbial cellulases have focused on fungal strains rather than bacterial strains because fungi are known to produce cellulases in large amounts, which include all the components of a multi-enzyme system with different specificities and mode of action (Mawadza et al., 2000). In addition, most fungi are capable of degrading both amorphous and crystalline celluloses (Afzal et al., 2010). However, they have a relatively long generation time resulting in time-consuming production process, and also the expression system and manipulation are less convenient.
compared to bacteria (Li et al., 2008). Therefore, bacteria were targeted in this study.

Bacillus subtilis sp. selected in this study was isolated from salted clams. Some cellulolytic strains of Bacillus strains have been isolated from other niche such as soil (Beukes and Pletschke, 2006), dead plant (Kotchoni et al., 2003), hot spring (Li et al., 2008), etc. However, unlike those strains, cellulolytic bacteria from fermented foods were investigated in this study so that they can be used as microbial feed additives without safety problems. In 16S rRNA analysis, the selected bacteria producing endoglucanase showed 99% of homology with Bacillus subtilis. Strains of Bacillus subtilis have been studied for their cellulases (Li et al., 2008; Li et al., 2009; Yang et al., 2009) and xylanases (Ayyachamy and Vatsala, 2007; Manimaran and Vatsala, 2007) producing ability.

Endoglucanase, one of three major cellulases, has a capability of cleaving the internal β-glycosidic linkage of cellulose chains randomly (Hong et al., 2001) and the CMC has been known as a carbon source widely used to check the activity of endoglucanase. Thus, CMC was added as a carbon substrate to both growth and selection media because endoglucanase was the target enzyme in this study. Substrate specificity was tested with CMC and other carbon sources to verify enzyme induction by substrates. When insoluble cellulose, glucose or xylene was added as a substrate, Bacillus subtilis sp. produced little amount of endoglucanase. However, CMC and xylan induced high level of endoglucanase. Interestingly, xylan did not induce xylanase as much as it induced endoglucanase.

Bacillus subtilis sp. exhibited the highest level of endoglucanase activity at pH 5 and 50°C. The optimal temperature and pH of enzymes from microorganism varied by the habitats where the host lives. A thermophilic Geobacillus sp. produced thermostable endoglucanase which had maximum activity at 70°C and this bacteria was isolated from core area of compost facility where the temperature was 70°C (Rastogi et al., 2010). The reason why the optimal temperature of the endoglucanase was 50°C was not uncertain because the temperature around the location where Bacillus subtilis sp. was collected, was under 50°C. It was speculated that high salted condition might affect the characteristics of endoglucanase produced from Bacillus subtilis sp. The optimal pH discovered in this study (pH 5) was consistent with previous study which reported that the recombinant bacteria having endoglucanase gene (from Bacillus licheniformis B-41361) showed maximum activity at pH 5.5 (Bischoff et al., 2007).

Strain improvement by mutation is a traditional method used with great success to obtain mutants having enhanced level of enzyme activities. Bacillus subtilis sp. was subjected to UV-irradiation and 211 mutants were found to exhibit enhanced clearance zone on trypan blue screening plates. A total of 188 mutants were also selected after chemical treatment using EMS. Among them, mutant P11 treated with EMS showed stable level of CMCase activity through subculture. Mutant P11 showed 122.5% of endoglucanase activity at first generation, and it constantly maintained the improved endoglucanase activity by 25% (Figure 4). UV-irradiation and chemical toxic agents were traditional ways to give damage to the bacterial DNA thereby resulting in the change of protein structure. Since these physical or chemical agents prevent the self DNA repair mechanism of host cells, the effect of mutation may be permanent (Gopinath et al., 2009). Gopinath et al. (2009) developed the mutant Bacillus sp. which had the improved biodegradation of Congo red, using UV-irradiation and Ethidium bromide.
Both Bacillus subtilis sp. and mutant P11 reached stationary phase at 12 h in time course study, but their enzyme activities were exhibited at the highest level after 16 h of growth. The highest enzyme production after the decline of growth rate (16 h) suggested that both strains actively utilized the carbon source, CMC during growth phase (Seo et al., 2013). The similar pattern of enzyme production was also reported in several studies. Rastogi et al. (2010) reported that the maximum endoglucanase activity of Bacillus sp. DUSELR13 was observed when the culture had faced the dying phase. Bacillus licheniformis JK7 cultured in LB media containing 1% of CMC exhibited the maximum endoglucanase activity (0.68 U/mL) at 24 h incubation which was the dying phase (Seo et al., 2013).

CONCLUSION
In the present study, endoglucanase-producing bacteria, Bacillus subtilis sp. was isolated from salted clams and the improvement of endoglucanase activity in the parent strain by EMS treatment resulted in 25% increase. The mutant P11 did not lose the ability to produce endoglucanase until 10th generation indicating that the change of enzyme production by mutation might be permanent. Thus, Bacillus subtilis sp. and its mutant P11 could be used as potential sources of microbial feed additives with high level of cellulolytic activities.

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REFERENCES