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Isolation and Characterization of Mannanase Producing *Bacillus amyloliquefaciens* CS47 from Horse Feces

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The mannanase-producing bacteria, designated CS47, was isolated from the fresh feces of three horses (from a farm in Jinju National University). The isolate CS47 was facultatively anaerobic and grew at temperatures ranging from 20°C to 50°C with an optimal temperature of 38°C. The DNA G+C content of the isolate CS47 was 44 mlo%. The major fatty acids were anteiso-15:0 (39.6%), 17:0 (7.6%), and iso-15:0 (37.8%). The 16S rRNA gene sequence similarity between the isolate CS47 and other *Bacillus* strains varied from 93% to 98%. In the phylogenetic analysis based on these sequences, the isolate CS47 and *Bacillus amyloliquefaciens* clustered within a group and separated from other species of *Bacillus*. Based on the physiological and molecular properties, the isolate CS47 was classified within the genus *Bacillus* as *Bacillus amyloliquefaciens* CS47. The optimal pH and temperature for mannanase activity of *B. amyloliquefaciens* CS47 were pH 6.0 and 50°C, respectively. The thermal stability of mannanase from *B. amyloliquefaciens* CS47 is valuable when using this enzyme in industrial application.

Key words : Horse feces, mannanase activity, locust bean gum, *Bacillus amyloliquefaciens* CS47

Introduction

Mannanes are constructed from the simple sugar mannose as plant polysaccharides and are widely found in nature. Mannan materials include mannan, glucomannan, galactomannan, and galactoglucomannan, which consist of a β-1,4-linked linear backbone of mannose residues that carry other carbohydrates or acid substitutions. In general, there are three known enzymes; endo-1,4-β-mannanase, exo-1,4-β-mannanase, and β-mannosidase, that participate in the complete decomposition and conversion of mannan [28]. Mannanases are useful enzymes in several processes in the food, feed, pulp and laundry industries [1,14]. In the feed industry, mannanase was commonly used as feed enzyme with xylanase, β-glucanase and phytase to improve the digestion and absorption of dietary nutrients. Because lots of grains are recently used as carbon source for the microbial production of bioenergy, the prices of feed grains have been escalated. Hence, much attention has been concentrated on feed enzymes to increase the nutritional value of soybean meal, a major component of animal feed [20,23,31]. Despite this numerous applications, the use of mannanase is still limited due to low yields and high-production costs [30].

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A number of fungi, yeast, bacteria, and marine algae, as well as from germinating seeds of terrestrial plants, and various invertebrates can produce β-mannanase [2,4,26,27]. Among these organisms, production of β-mannanases by microorganisms is more promising due to its low cost, high production rate, and readily controlled conditions. Various microorganisms have been reported as mannanase producers. The mananases from *Streptomyces* sp. [25], *Bacillus subtilis* [17,29], *Sclerotium (Athelia) rolfsii* [21], *Bacillus stearothermophilus* [30], *Aspergillus awamori* [11] and *Trichoderma harzianum* [8] have been purified and characterized, as well as some genes from *B. subtilis* and *B. stearothermophilus* encoding mannanases were also cloned, sequenced and expressed [7,15]. Among those, *B. subtilis* was recommended because of its safety, fast growth and ability to secrete a high level of mannanase into medium [16,29].

The genus *Bacillus* consists of Gram-positive, rod-shaped, aerobic or facultatively anaerobic, spore-forming bacteria of diverse phenotypic characteristics, including differences with respect to nutritional requirements, growth conditions and DNA base composition [5]. The *Bacillus* activates a variety of defense processes, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production. The *Bacillus* spp. was easily cultivated in a low-cost culture medium. Their spore-forming characteristics confer better survival in adverse conditions and resistance to many physicochemical factors.

The non-ruminants such as horse, rabbits and guinea pigs have large intestine as the fermentative chamber which contains an abundant and highly complex community of microorganisms [6]. The microbiota in the intestine performs the same task as the microbes in the rumen. The microbial hydrolysis of dietary plant fibre within the large intestine leads to the release of soluble sugars which are fermented to short chain fatty acids, most notably, acetate, propionate and butyrate [3]. Despite its importance, the microbial community of the equine hindgut is poorly characterized. Stevens and Hume [24] reported that the most common microbiota in the equine gastrointestinal tract (EGT) were similar to those present in the rumen of cattle. Garret et al. [9] reported that each gram of feces of healthy horses contained 7.94 (log 10) cfu (mean count) of Gram-positive bacteria and 6.13 cfu/g of Gram-negative bacteria.

In this study, we isolated and characterized the mannanase-producing strain from interior of horse feces to use as a feed enzyme. In addition, the mannanase activity of isolate was investigated under different condition.

Materials and Methods

Isolation of mannanase-producing bacteria from horse feces

Fecal samples were collected from three horses (three geldings of 3, 8, and 9 years old from farm in Jinju National University) every 2 days for an 8-day period. At each time, a single large specimen (about 200 g) of freshly feces was collected from each animal. The interior part of freshly feces was suspended in the sterilized water and spread on LB agar (Dickinson Co., Sparks, MD. USA) containing 0.5% locust bean gum (Sigma-Aldrich Co., St. Louis, Mo. USA) and 1% trypan blue to select mannanase-producing strain. The plate was incubated at 38˚C for 24 hr. Mannanase activity was detected by the appearance of clearing zones around the bacterial colonies. The activity was calculated as the ratio of the diameter of the clearing zone to the diameter of the colony.

Morphological characteristics

The isolate was grown in Schaeffer's sporulation medium [22] at 38°C for 12 hr. Spores were visualized by staining with malachite green and light microscopy. Cell morphology was examined by light microscopy and transmission electron microscopy. The flagellum type was also examined by transmission electron microscopy (JEM 1010, Jeol Ltd., Tokyo, Japan) after negative stain cells from exponentially growing cultures with 1% phosphotungstic acid. The Gram staining was performed using the BBL™ Gram stain kit (Dickinson Co., Sparks, MD. USA) according to the manufacturer's instructions.

Fatty acid methyl ester analysis

The isolate was grown on TSA (Dickinson Co., Sparks, MD. USA) at 38°C for 24 hr and the cellular fatty acid composition was determined by gas chromatography (model 5890, Hewlett Packard Inc., Santa Clara, CA. USA) fitted with a phenylmethyl silicone fused silica capillary column (25 m, 0.2 mm, Hewlett Packard Inc., Santa Clara, CA. USA). The extraction and the analysis were performed according to the standard manual of the MIDI/Hewlett Packard Microbial Identification System. The profile was compared with the MIDI Microbial Identification database TSBA50, version 5.00 (MIDI Inc., Newark, DE. USA).

Utilization of carbon source

Tests for utilization of substrates as sole carbon source were performed with BIOLOG automatic identification system (Biolog Inc., Hayward, CA. USA). The isolate was grown on biolog universal growth medium (Biolog Inc., Hayward, CA. USA) at 38°C for 24 hr and suspended in a sterile solution containing 10% salts. The cell density was adjusted to an A_{590} of 0.34 to 0.39 using a BIOLOG model 21101 photometer. Immediately after the cells were suspended in the salt solution, the suspensions were transferred into the BIOLOG GP2 MicroPlates containing 95 different carbon sources and incubated at 38°C for 24 hr. The results were read with a MicroPlate Reader by using MicroLog 4.0 computer software to perform automated reading. The profile was compared with the BIOLOG identification database GN4.01.

DNA base composition

The DNA G+C content was determined by the thermal melting procedure described by Mandel & Marmur [13]. *Escherichia coli* DNA with a G+C content of 51 mol% was used for comparison.

Sequencing of 16S rRNA gene

Genomic DNA was isolated and purified using i-genomic BYF DNA extraction kit (iNtRON Biotechnology, Inc., Seongnam, Korea). PCR reaction was typically performed in

a final volume of 100 μl using 40 pmol of each primer, 50 ng of template, *Taq* polymerase (Takara), and 1 mM of each dNTP. Reactions comprised 30 cycles of 94°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min with a final extension at 72°C for 10 min using primers, which designed based on conserved region between nucleotide sequences of 16S rRNA gene of *Bacillus* species. The forward primer was 5′-TTCTACGGAGAGTTTGATCC-3′ and the reverse primer was 5′-CACCTTCCGGTACGGCTACC-3′. Amplified 16S rRNA gene fragments were analyzed with an automated DNA sequencer (Applied Biosystems, Foster city, CA. USA).

Sequence comparison and phylogenetic analysis

The 16S rDNA sequence was aligned with that of other *Bacillus* species obtained from GenBank database and analyzed using DNAMan analysis system (Lynnon Biosoft, Quebec, Canada). Phylogenetic comparison was inferred by using the neighbor-joining method. The resulting tree was drawn with the DNAMan software and treated as rooted, although the outgroup designation option was included to polarize character states.

Enzyme assays

Mannanase activity was determined by measuring the amount of reducing sugars liberated during the hydrolysis of locust bean gum by the dinitrosalicylic acid (DNS) method [18]. The standard assay reaction mixture consisted of 0.5% (w/v) of the polysaccharide substrates supplemented with 50 mM sodium citrate buffer (pH 6.0) and enzyme to make a final volume of 0.3 ml. The reaction mixture was incubated at 50°C for 15 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min. The optimal pH of mannanase activity was examined at pH 3.0-10.0 under standard assay conditions using 50 mM of various buffers. The effect of temperature on mannanase activity was determined by incubating the enzyme with the substrate at temperatures ranging from 10°C to 80°C in 50 mM sodium citrate buffer at pH 6.0.

Results and Discussion

Screening of mannan-degrading bacteria from horse feces

The nine bacterial strains were originally isolated from interior of horse feces. Among them, five isolates were able to hydrolyze mannanase during growth on LB agar containing 0.5% locust bean gum and 1% trypan blue, zones of different sizes were cleared around the isolated bacteria. Therefore, the strain with highest mannanase activity ratio, designated CS47, was selected for further studies (Fig. 1).

Morphological and physiological characteristics

The isolate CS47 was facultatively anaerobic and grew at temperature ranging from 20°C to 50°C with an optimal temperature of 38°C. After incubation for 24 hr on TSA, colonies of the isolate CS47 were smooth, convex and wrinkled with approximately 2.5±0.1 mm in diameter. Cells were Gram-positive, rod-shaped and the measured size by transmission electron microscopy was 1.4×0.7 μm in 36-hr culture grown at 38°C (Fig. 2).

The isolate CS47 was identified using BIOLOG automatic identification system. The BIOLOG data of the isolate CS47 allows *Bacillus* strain to be classified according to the ability

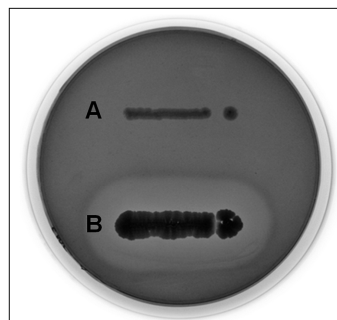

Fig. 1. Mannanase activity of the isolate CS47 by agar diffusion method. The isolate CS47 grown on LB medium containing 0.5% locust bean gum and 1% trypan blue. The plate was incubated at 38°C for 24 hr. A: *Escherichia coli* as a negative control, B: isolate CS47.

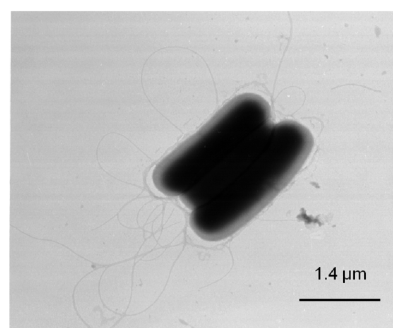

Fig. 2. Transmission electron micrograph of the isolate CS47. Exponentially growing culture was observed by negative stain with 1% phosphotungstic acid.

to ferment 95 different carbohydrates. The substrate utilization as carbon source of the isolate CS47 is presented in Table 1. Analysis based entirely on substrate utilization profiles showed that the isolate CS47 belonged to *B. amyloliquefaciens* and was different from *Bacillus* subgroup: *B. subtilis, B. lincheniformis, B. mojavensis,* and *B. atrophaeus*.

The DNA G+C content of the isolate CS47 was 44 mol%. This data also show that isolate CS47 belong to *B. amylolique-*

Table 1. Biochemical characteristics of isolate CS47

Carbon source	Reaction	Carbon source	Reaction
L-arabinose	+	Glycogen	+
α-D-lactose	-	D-fuctose	+
β-methyl-D-glucoside	+	D-mannitol	+
D-tagatose	-	D-rafinose	+
Lactomide	-	D-xylose	+
L-alaninamide	-	L-malic acid	+
Adenosine	+	L-asparagine	+
Mannan	-	Uridine	+
D-galactose	+	N-acetyl-β-D-mannosamine	+
D-melezitose	+	α-D-glucose	+
D-Ribose	+	3-methyl glucose	+
α-hydroxybutyric acid	-	Starchyose	+
Succinic acid mono-methyl ester	+	α-ketoglutaric acid	+
Glycyl-L-glutamic acid	-	Succinic acid	+
Thymidine-5′-monophosphate	+	2, 3-butanediol	+
Dextrin	+	β-cyclodextrin	+
D-cellobiose	+	Arbutin	±
Maltotriose	+	Maltose	+
D-psicose	+	Palatinose	+
Xylitol	-	Turanose	+
D-malic acid	-	L-lactic Acid	+
L-alanyl-glycine	-	L-alanine	±
Thymine	+	Inosine	+
N-acetyl-D-glucosamine	+	Tween 80	+
D-gluconic acid	+	Gentiobiose	-
β-methyl-D-galactoside	+	α-methyl-D-galactoside	-
D-sorbitol	+	Sedoheptulosan	-
p-hydroxy-phenyl acetic acid	-	γ-hydroxybutyl acid	+
Succine amic acid	+	Pyruvic acid	+
Putrescine	+	L-serine	-
α-D-glucose-1-phosphate	+	D-fructose-6-phosphate	-
α-cyclodextrin	+	Inulin	+
D-arabitol	-	L-fucose	-
Lactulose	-	D-mannose	+
α-methyl-D-mannoside	-	L-rhamnose	±
D-trehalose	+	Acetie acid	+
D-lactic acid methyl ester	-	Pyruvatic acid methyl ester	±
D-alanine	+	L-glutamic acid	+
2′-deoxy adenosine	+	Adenosine-5′-monophosphate	±
Tween40	-	Amygdalin	-
D-galacturonic acid	+	m-inositol	-
D-melibiose	+	α-methyl-D-glucoside	+
Salicin	-	Sucrose	+
β-hydroxybutyric acid	+	α-ketovaleric acid	+
Propionic acid	±	N-acetyl-L-glutamic acid	±
Pyroglutamic acid	+	D-L-α-glycerol phosphate	+

* positive reaction: +, negative reaction: −, weak reaction: ±.

faciens in the *B. subtilis* group and should not be classified as a strain or variety of *B. subtilis*. The mol% G+C of the DNA of *B. amyloliquefaciens* (43.5-44.9) is slightly higher than for *B. subtilis* (42-43) [12].

Chemotaxonomy

The whole-cell fatty acid composition was determined for the isolate CS47 and other members of *Bacillus* closely related to the *B. subtilis* subgroup. Fatty acid analysis showed that the isolate CS47 gave matches with *B. subtilis* with 0.36 similarity indices. Although comparison of bacterial fatty acid profiles revealed 'closest match' species, the similarity indices of these matches were very low. However, almost 'closest match' species belonged to the genus *Bacillus*. The predominant fatty acids in whole-cell methanolysates of the isolate CS47 were anteiso-15:0 (39.6%), 17:0 (7.6%), and iso-15:0 (37.8%).

16S rRNA sequence

An approximate 1.5-kb unique PCR product was generated from the isolate CS47. The partial 16S rRNA gene sequences of the isolate CS47 was determined and aligned to other known sequence of *Bacillus* strains obtained from GenBank. The sequence similarity between the isolate CS47 and other *Bacillus* strains varied from 93% to 98%. In the phylogenetic analysis based on these sequences, the isolate CS47 and *B. amyloliquefaciens* clustered within a group and separated from other species of *Bacillus* (Fig. 3).

Based on physiological and molecular properties, the isolate CS47 was classified within the genus *Bacillus* as *Bacillus amyloliquefaciens* CS47.

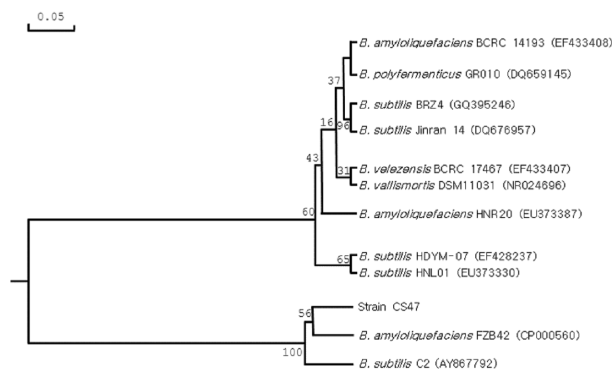

Fig. 3. Phylogenetic relationships of the isolate CS47 and other closely related bacteria based on the partial 16S rRNA sequence. The branching pattern was generated by the neighbor-joing method. Bootstrap values (expressed as percentages of 10,000 replications) are shown at major branching points. Bar, 0.05 substitution per nucleotide position.

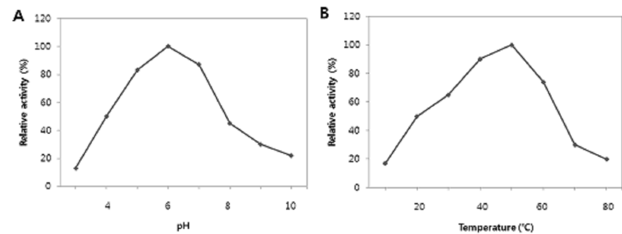

Fig. 4. Effects of pH (A) and temperature (B) on mannanase activities. (A) Enzyme activity was assayed at 38°C for 30 min in sodium citrate buffers with the indicated pH values. (B) Enzyme activity was assayed at pH 6 for 30 min at the indicated temperature.

Effect of temperature and pH on mannanase activity

The isolated *B. amyloliquefaciens* CS47 produced an extracellular mannanase during grown with locust bean gum. The optimal pH for the mannanase activity was pH 6.0 (Fig. 4A). The optimal pH for the mannanase of *B. amyloliquefaciens* CS47 was compared to pH 5.0 from *B. subtilis* NM-39 [17] and pH 7.0 from *B. subtilis* KU-1 [29] and *Bacillus* sp. [19]. The enzyme demonstrated broad pH stability within pH range of 5-9. The mannanase from *B. amyloliquefaciens* CS47 was optimally active at 50°C (Fig. 4B). The optimal temperature for the mannanase of *B. amyloliquefaciens* CS47 was compared to 50-55°C from *B. subtilis* NM-39 [17], KU-1 [29] and 5H [10], and 60°C from *Bacillus licheniformis* [30]. This thermal stability is important to use mannanase as an animal feed additive. For industrial application of mannanase from *B. amyloliquefaciens* CS47, it is necessary to carry out studies to determine the substrate specificity and stability of the purified mannanase under various conditions.

Acknowledgement

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초록 : 말 분변으로부터 mannanase를 분비하는 *Bacillus amyloliquefaciens* CS47의 분리 및 특성

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진주산업대학교 농장에서 사육되고 있는 말에서 채취한 분변으로부터 mannanase 생성능이 우수한 균주를 분리하였으며 이를 CS47이라 명명하였다. 통성혐기성인 분리균 CS47의 최적 생육온도는 38°C였으며 20°C에서부터 50°C까지 다양한 온도범위에서도 생육이 가능한 것으로 확인되었다. BIOLOG를 이용하여 분리균 CS47의 생화학적 특징을 분석한 결과, 분리균은 *Bacillus amyloliquefaciens* 균주와 유사한 특성을 나타내었으며 DNA G+C함량 (44 mol%)도 *B. amyloliquefaciens* 균주의 범위(43.5-44.9)에 속하였다. 16S rDNA 염기서열을 분석한 결과에서도 분리균은 *B. amyloliquefaciens* FZB42 균주와 가장 높은 상동성을 나타내었으며 *Bacillus*속의 다른 균주들과는 93-98%의 상동성을 나타내었다. 그러나 분리균의 세포벽을 이루고 있는 주요 지방산[anteiso-15:0 (39.6%), 17:0 (7.6%), iso-15:0 (37.8%)]은 *Bacillus subtilis* 균주와 유사한 특성을 나타내었다. 최종적으로 생화학적 특성과 16S rDNA 염기서열 분석 결과를 근거로 하여 분리균 CS47은 *Bacillus amyloliquefaciens* CS47로 동정되었다. *B. amyloliquefaciens* CS47 균주가 분비하는 mannanase는 50°C, pH 6.0에서 가장 높은 활성을 보였다.