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Isolation and Characterization of Mannanase-Producing *Bacillus amyloliquefaciens* YJ17 from Spent Mushroom (*Flammulina velutipes*) Substrates

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ABSTRACT: The mannanase-producing bacteria, designated YJ17, was isolated from spent mushroom (*Flammulina velutipes*) substrates. The isolate YJ17 was a facultative anaerobic and was grown at temperatures ranging from 20°C to 50°C with an optimal temperature of 40°C. The DNA G+C content of the YJ17 was 44 mol%. The major fatty acids were anteiso-15:0 (38.9%), 17:0 (7.6%), and iso-15:0 (36.5%). The 16S rRNA gene sequence similarity between the isolate YJ17 and other *Bacillus* strains was from 98% to 99%. In the phylogenetic analysis based on these sequences, the isolate YJ17 and *Bacillus amyloliquefaciens* clustered within a group together and separated from other species of *Bacillus*. Based on the physiological and molecular properties, the isolate YJ17 was classified within the genus *Bacillus* as *B. amyloliquefaciens* YJ17. The optimal pH and temperature for mannanase activity of *B. amyloliquefaciens* YJ17 were pH 7.0 and 50°C, respectively.

KEYWORDS: *Bacillus amyloliquefaciens* YJ17, Mannanase-producing bacteria, Spent mushroom substrates

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 (Yoon and Lim, 2007). Mannanases are useful enzymes in several processes in the food, feed, pulp and laundry industries (Admark *et al*, 1998; McCleary, 1988). 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 (Petty *et al,* 2002; Zou *et al,* 2006; Slominski *et al,* 2006). Despite this numerous applications, the use of mannanase is still limited due to low yields and high-production costs (Zhang *et al*, 2000).

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 (Akino *et al,* 1987; Arisan-Atac *et al,* 1993; Wozniewski *et al,* 1992; Yamaura and Matsmoto, 1993). 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. (Takahashi *et al,* 1984), *Bacillus subtilis* (Mendoza

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et al, 1994; Zakaria *et al,* 1998), *Sclerotium (Athelia) rolfsii* (Sachslenhner and Haltrich, 1999), *Bacillus stearothermophilus* (Zhang *et al,* 2000), *Aspergillus awamori* (Kurakake and Komaki, 2001) and *Trichoderma harzianum* (Ferreria and Filho, 2004) have been purified and characterized, as well as some genes from *B. subtilis* and *B. stearothermophilus* encoding mannanases were also cloned, sequenced and expressed (Ethier *et al,* 1998; Mendoza *et al,* 1994). Among those, *B. subtilis* was recommended because of its safety, fast growth and ability to secrete a high level of mannanase into medium (Zakaria *et al,* 1998; Mendoza *et al,* 1994b).

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 (Claus and Berkeley, 1986). The *Bacillus* activates a variety of defense processes, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production. The *Bacillus* sp. 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.

In this study, we isolated and characterized the mannanase-producing strain from freshly spent mushroom substrates (*Flammulina velutipes*). In addition, the mannanase activity of isolate was investigated under different condition.

Materials and Methods

Isolation of mannanase-producing bacteria from spent mushroom substrates

The mannanase-producing bacteria YJ17 was isolated from freshly spent mushroom (*Flammulina velutipes*) substrates obtained from the Doremifarm located in Chungnam, Korea. The spent mushroom (*Flammulina velutipes*) substrates was suspended in the sterilized water and spread on tryptic soy agar (TSA, Difco). The plate was incubated at 40°C for 24 h. Screening of mannanase-producing bacteria was performed onto TSA supplemented with 0.5% locust bean gum (Sigma-Aldrich Co., St. Louis, Mo. USA) and 1% trypan blue. The plate was incubated at 40°C for 24 h. 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 (Schaeffer *et al,* 1965) at 40°C for 12 h. Spores were visualized by staining with malachite green and light microscopy (JEM 1010, Jeol Ltd., Tokyo, Japan). Cell morphology was examined by light microscopy and transmission electron microscopy. The flagellum type was also examined by transmission electron microscopy 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 40°C for 24 h 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 40℃ for 24 h 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 92 different carbon sources and incubated at 40°C for 24 h. 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 (Mandel and Marmur, 1968). *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 ul 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* sp. 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 rRNA sequence was aligned with that of other *Bacillus* sp. 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 (Miller, 1959). The standard assay reaction mixture consisted of 0.5% (w/v) of the locust bean gum 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 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 from spent mushroom substrates

The eighteen bacterial strains were originally isolated from freshly spent mushroom (*Flammulina velutipes*) substrates. Among them, three isolates were able to hydrolyze mannanase during growth on TSA 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 YJ17, was selected for further studies (Table 1). The mannanase activity has been reported in a wide variety of *Bacillus* sp., but few data are available on mannanase of *B. amyloliquefaciens*.

Morphological and physiological characteristics

The isolate YJ17 was facultative anaerobic and was grown at temperature ranging from 20°C to 50°C with

Table 1. Mannanase activity of the isolated strains from spent mushroom substrate. The activity ratio was calculated from clear zones formed on TSA plate containing 0.5% locust bean gum as substrate and incubated at 40°C for 24 h

Isolated strain	YJ03	YJ12	YJ17
Activity ratio (mm)	6	13	25

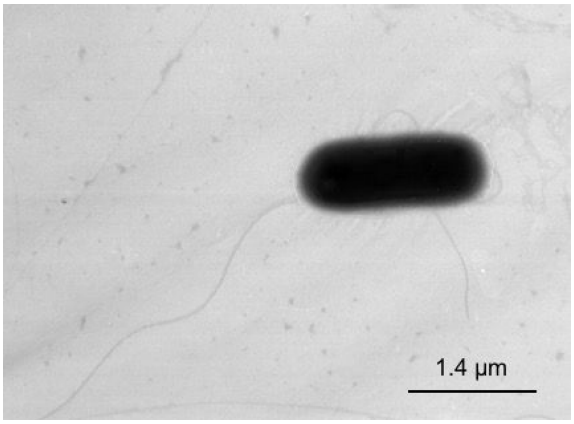

Fig. 1. Transmission electron micrograph of the isolate YJ17. Exponentially growing culture was observed by negative stain with 1% phosphotungstic acid. Scale bar, 1.4 um.

Table 2. Biochemical characteristics of isolate YJ17

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 monomethyl 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-*a*-glycerol phosphate	+

+, positive reaction; -, negative reaction; ±, weak reaction

an optimal temperature of 40°C. After incubation for 24 h on TSA, colonies of the isolate YJ17 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 h culture grown at 40°C (Fig. 1).

The isolate YJ17 was identified using BIOLOG automatic identification system. The BIOLOG data of the isolate YJ17 allows *Bacillus* strain to be classified according to the ability to ferment 92 different carbohydrates. The substrate utilization as carbon source of the isolate YJ17 is presented in Table 2. Analysis based entirely on substrate utilization profiles showed that the isolate YJ17 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 YJ17 was 44 mol%. This data also show that isolate YJ17 belong to *B. amyloliquefaciens* 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) (Logan and Berkeley, 1984).

Chemotaxonomy

The whole-cell fatty acid composition was determined for the isolate YJ17 and other members of *Bacillus* closely related to the *B. subtilis* subgroup. Fatty acid analysis showed that the isolate YJ17 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

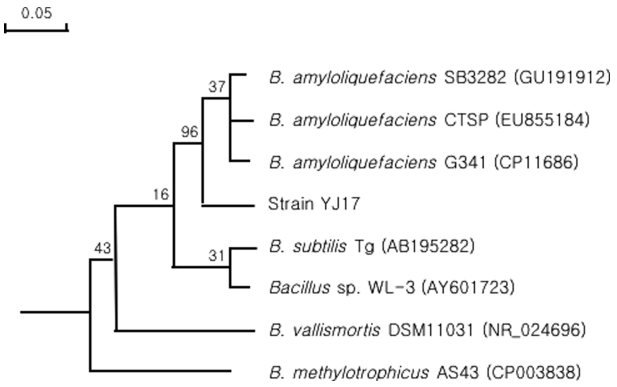

Fig. 2. Phylogenetic relationships of the isolate YJ17 and other closely related bacteria based on the partial 16S rRNA sequence. The branching pattern was generated by the neighbor-joing method. Bootstrap values (expressed as percentages of 10,000 replications) are shown at major branching points. Bar, 0.05 substitution per nucleotide position.

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 YJ17 were anteiso-15:0 (38.9%), 17:0 (7.6%), and iso-15:0 (36.5%).

16S rRNA sequence analysis

An approximate 1.5-kb unique PCR product was generated from the isolate YJ17. The partial 16S rRNA gene sequences of the isolate YJ17 was determined and aligned to other known sequence of *Bacillus* strains obtained from GenBank. The sequence similarity between the isolate YJ17 and other *Bacillus* strains was from 98% to 99%. In the phylogenetic analysis based on these sequences, the isolate YJ17 and *B. amyloliquefaciens* clustered within a group and separated from other species of *Bacillus* (Fig. 2).

Based on physiological and molecular properties, the isolate YJ17 was classified within the genus *Bacillus* as *Bacillus amyloliquefaciens* YJ17.

Effect of temperature and pH on mannanase activity

The isolated *B. amyloliquefaciens* YJ17 produced an extracellular mannanase during grown with locust bean gum. The optimal pH for the mannanase activity was pH 7.0 (Fig. 3A). The optimal pH for the mannanase of *B. amyloliquefaciens* YJ17 was compared to pH 5.0 from *B. subtilis* NM-39 (Mendoza *et al*, 1994a), pH 6.0 from *B. amyloliquefaciens* CS47 (Cho, 2009) and pH 7.0 from *B. subtilis* KU-1 (Zakaria *et al*, 1998) and *Bacillus* sp. (Ooi and Kikuchi, 1995). The enzyme demonstrated broad pH stability within pH range of 5-8. The mannanase from *B. amyloliquefaciens* YJ17 was optimally active at 50°C (Fig. 3B). The optimal temperature for the mannanase of *B. amyloliquefaciens* YJ17 was compared to 50-55°C from *B. subtilis* NM-39 (Mendoza *et al*, 1994a), KU-1 (Zakaria *et al*, 1998) and 5H (Khanongnuch *et al*, 1998), *B. amyloliquefaciens* CS47 (Cho, 2009) and 60°C from *Bacillus licheniformis* (Zhang *et al,* 2000). This thermal stability is important to use mannanase as an animal feed additive. For industrial application of mannanase from *B. amyloliquefaciens* YJ17, it is necessary to carry out studies to determine the substrate specificity and stability of the purified mannanase under various conditions.

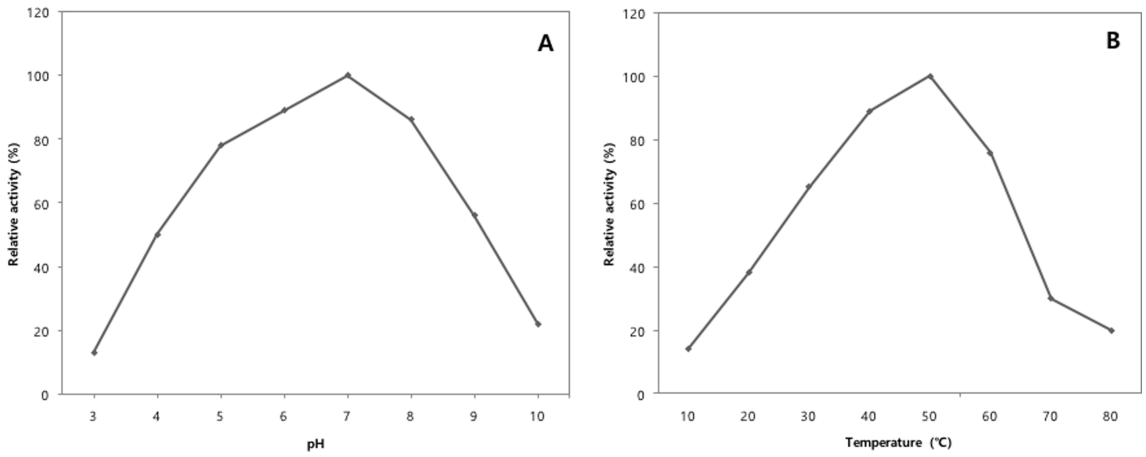

Fig. 3. Effects of pH (A) and temperature (B) on mannanase activities. (A) Enzyme activity was assayed at 50°C for 15 min in sodium citrate buffers with the indicated pH values. (B) Enzyme activity was assayed at pH 6.0 for 30 min at the indicated temperature.

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