Isolation and characterization of thermophilic mannanase-producing bacteria from soil

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Abstract

The aim of this research was to isolate the mannanolytic thermophilic bacterium useful for mannooligosaccharide (MOS) production. Thermophilic mannanase-producing bacteria were isolated from 5 soil samples collected from Roi Et Rajabhat University. The bacteria were cultured using Luria-Bertani (LB) medium containing 1% locust bean gum (LBG) and incubated at 60 ºC for 48 hours. The mannanase activity was screened on LB agar containing 1% LBG stained with iodine solution. The colonies that showed clear zone were selected. The isolates were cultured using LB broth containing LBG at 60 ºC for 72 hr. Two isolates were survived and exhibited the high amount of mannanase activities were selected and named KS2 and KS6. Mannanase activity at 50-100 ºC was measured using dinitrosalicylic acid method using LBG (pH 7.0) as mannan source and cultured broth as crude mannanase. The results indicated that at 60 ºC, KS2 and KS6 showed the mannanase activity at 4.25 and 4.02 U/ml, respectively. The thermostable of crude mannanase activity of both at 50 ºC were 3.72 and 3.96 U/ml. According to the morphological examination and 16S rRNA gene sequencing, both isolates KS2 and KS6 were identified as *Bacillus amyloliquefaciens* subsp. *plantarum.*

Keywords: Mannanase, *Bacillus*, Thermophilic

Introduction

The mannan endo-1,4-β-mannosidase or 1,4-β-D mannanase (EC 3.2.1.78), commonly named β-mannanase, is an enzyme responsible for mannooligosaccharides production [1, 2]. Mannanases are involved in catalyzing β-1, 4-mannosidic linkages in the main chain of β-1, 4-mannans, glucomannans and galactomannans [3]. The complete breakdown of mannan requires a variety of enzymes, including β -mannanase, β -mannosidase, β -glucosidase, α-galactosidase and esterase [4].

Mannans are a major group of hemicellulose found in softwoods and hardwoods [5, 6], seeds of leguminous plants and beans. Hemicelluloses are linear or branched polysaccharides, which most of them are in form of heteroglycans. Based on the primary sugar within the molecules hemicelluloses can be classified into mannan, xylan, arabinogalactan and arabinan [7]. Linear mannans consist of a backbone of β -1,4-mannose, glucomannans consist of randomly dispersed β -1,4-galactose and β -1,4-mannose, and the back-bones of galactomannans and galactoglucomannans are decorated with side chains of α-1,6-linked galactose residues [4].

Microorganisms are the main source of mannanase because of their rapid growth, easily controlled condition [8], low cost and high production rate including bacteria [9] and fungi [10, 11]. The thermophilic β-mannanases from thermophilic microorganism have great advantages during the high temperature processes [12], reducing the risk of contamination, increasing the substrate solubility, and improving the mass transfer rate [13] Many microorganisms that presented the thermophilic β-mannanases such as *Bacillus* sp., *Bacillus pumilus* GBSW19, *Talaromyces leycettanus* JCM12802, *Neosartorya fischeri* P1 [4, 7, 14, 15], *Aspergillus nidulans* XZ3 [16], Aanniz et al. [17] and *Rhizomucor miehei* [18] have been reported be the ideal microbial sources of excellent β-mannanases. The purpose of this research was to isolate of thermophilic mannanase-producing bacteria which useful for prebiotic production from copra meal.

Materials and methods

Soil samples and bacterial culture

Six soil samples were collected from Roi Et Rajabhat University, Thailand. One gram of soil sample was suspended in 5 ml sterilized 0.85% NaCl and was then well-mixed using vortex. One percent of inoculum was added into 50 ml of sterilized Luria-Bertani (LB) containing with 1% locust bean gum (LBG) (Sigma, USA). The culture flasks were incubated with shaking at 60 ^{o}C for 1 hr and continued culturing at 37 ^{o}C for 47 hrs.

Screening of mannanase-producing bacteria

One hundred microliters of appropriate dilution of each cultured broth were spread on LB agar and incubated at 37 ^{o}C for 24 hr. Single colony was selected and transferred onto new plate of LB agar and LB agar containing 1% LBG and incubated at 37 ^{o}C for 24 hrs. The mannanase-producing bacteria was screening by presenting clear zone after flooding iodine solution. The clear zone that presented around each colonies indicated that bacteria exhibited mannanase activity. The colony that showed the clear zone were collected.

Isolation of thermophilic mannanase-producing bacteria

Single colony of isolated bacteria were cultured using LB broth containing with 1% LBG and incubated at 60 ^{o}C, 150 rpm for 3 days. The survival bacteria were observed by streaking onto LB agar plate and incubated at 37 ^{o}C for 24 hrs. The survival thermophilic mannanase-producing bacteria were selected. An aliquot of 500 µl of crude mannanase was mixed with 500 µl of LBG (pH 7.0) and incubated at 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100 ^{o}C for 5, 15 and 30 min. The mannanase activity of thermophilic bacteria was determined using dinitrosalicylic acid (DNS) method. *Escherichia coli* K12 cultured supernatant was used as negative control. The mixture was boiled for 5 minutes and put on ice until cold. Two thousand and five hundred microliter of water was added. The amount of reducing sugar was determined by measuring absorbance at 540 nm. The mannanase activity was measured in terms of the amount of reducing D-mannose obtained from LBG during the mannanase activity. One unit

of enzyme is defined as the amount of enzyme liberates reducing sugars equivalent to 1 µmol D-mannose standard per minute under the experiment.

Thermostable mannanase activity

An aliquot of 500 µl of crude mannanase and incubated at 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100 ^{o}C for 1 hr prior mixed with 500 µl of LBG (pH 7.0). The mannanase activity was determined using dinitrosalicylic acid (DNS) method by incubated at 60 ^{o}C for 5 min. *Escherichia coli* K12 cultured supernatant was used as negative control.

Bacterial morphology and Gram staining

The selected bacteria were cultured on LB plate at 37°C for 24 hr. All of the selected bacteria were examined for their cell shape, Gram staining, colonial appearance, spore formation using standard method.

16S rRNA gene sequencing

Isolated bacteria were sent to Macrogen Company (Korea) for automated DNA sequencing. The amplicons were synthesized by using 27F and 1492R primers. DNA sequencing was done by using 785F and 907R primers. The resulting sequences were compared with the non-redundant nucleotide database from GenBank using the BLAST.

Results and discussion

Screening of mannanase-producing bacteria

Many sample sources have been used as mannanase-producing bacteria source such as desert [18], soil [20, 21] soil, fermented coconut, fertilizer [9, 15], palm oil shell [12], hot spring [22]. In this research, 6 soil samples from Roi Et Rajabhat Univeristy were used as mannanase-producing bacteria source. One gram of soil sample was transferred into 50 ml of sterilized LB containing with 1% LBG and incubated at 60 ^{o}C, 150 rpm for 1 hr and continued culturing at 37 ^{o}C, 150 rpm for 47 hrs. The primary screening was based on the clear zones formed on LB

agar containing LBG after flooding iodine solution (Fig. 1). Twenty eight isolates showed mannanase activity by showing the clear zone around colony.

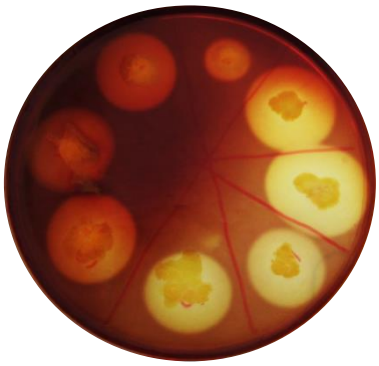

Figure 1. Mannanase activity assay on agar plate containing LBG stained with iodine solution.

Isolation of thermophilic mannanase-producing bacteria

Various temperatures were used to obtain thermophilic bacteria such as Harnentis and Maria [7] used at 60 ºC for 48 hrs but Kanjanavas et al. [22] used temperature at 45 ºC for 24 hrs. Screening step in this research was done using temperature at 60 ºC for 72 hrs. At this temperature condition, only thermophilic bacteria will survive [23]. Results from the isolation of thermophilic mannanase-producing bacteria step, only two isolates were survived and named KS2 and KS6. Mannanase activity assay was determined using an aliquot of 500 µl of cultured supernatant of each isolates were mixed with 500 µl of LBG (pH 7.0) and incubated at 50,55, 60, 65, 70, 75, 80, 85, 90 and 100 ºC for 5, 15 and 30 min. The mannanase reaction was measured by using dinitrosalicylic acid method. *E. coli* K12 cultured broth were used as negative control. The results indicated that KS2 and KS7 were survived and showed mannanase activity (Figure 2-4).

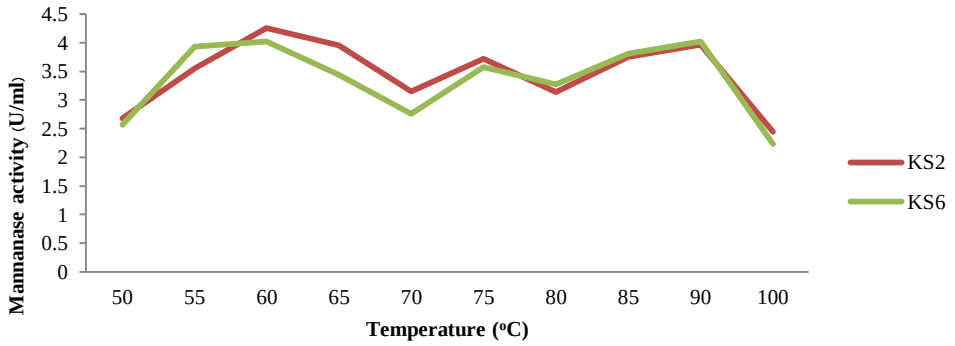

Figure 2. Mannanase activity of KS2 and KS6 at 50, 55, 60, 65, 70, 75, 80, 85, 90 and 100 °C and incubated for 5 min.

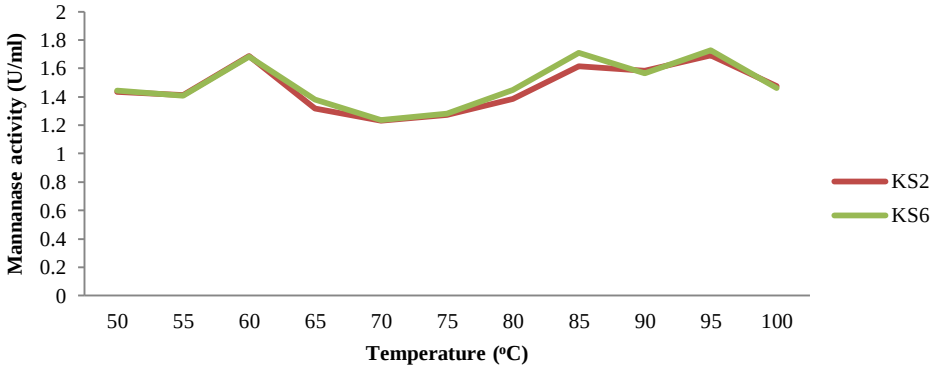

Figure 3. Mannanase activity of KS2 and KS6 at 50, 55, 60, 65, 70, 75, 80, 85, 90 and 100 °C and incubated for 15 min.

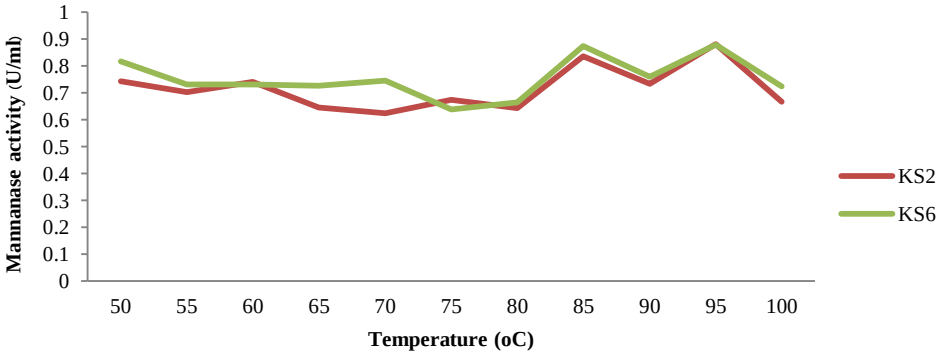

Figure 4. Mannanase activity of KS2 and KS6 at 50, 55, 60, 65, 70, 75, 80, 85, 90 and 100 °C and incubated for 30 min.

Thermostable mannanase activity

Cultured supernatant were used as crude mannanase [20, 22]. In this study, the cultured supernatant were incubated at 50 55, 60, 65, 70, 75, 80, 85, 90 and 100 ºC for 1 hr. The mannanase activity was determined using dinitrosalicylic acid (DNS) method by incubated at 60 ºC for 5 min. *Escherichia coli* K12 cultured supernatant was used as negative control. The thermostability of KS2 and KS6 was 3.72 and 3.96 U/ml at 50 ºC (Figure 5).

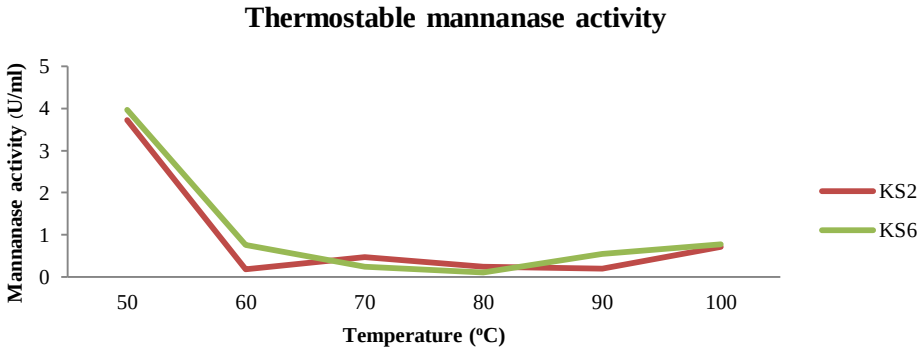

Figure 5. Thermostable of KS2 and KS6 crude mannanase incubated at 50, 55, 60, 65, 70, 75, 80, 85, 90 and 100 ºC for 1 hr.

Bacterial morphology and biochemistry

KS2 and KS6 were cultured on LB plate at 37°C for 24 hrs before examined for their cell shapes, colony appearance, spore formation, motility and Gram staining. The result found that KS2 and KS6 is Gram-positive, catalase positive, aerobic, rod-shaped and motile (data not shown).

Analysis of 16S rRNA sequence

Many thermophilic mannanase-producing bacteria have been reported including genera *Bacillus*, *Caldocellum*, *Caldibacillus*, *Rhodothermus* [1, 24, 25]. The 16s rDNA gene sequencing result of both KS2 and KS6 were compared with other bacterial sequences deposited in the GenBank database using the BLAST algorithm. The results indicated that both

16S rDNA sequences of KS2 and KS6 was 99% identical to *Bacillus amyloliquefaciens* subsp. *plantarum* strain FZB42 (Accession NR_075005).

Conclusion

Soil samples were collected from Roi Et Rajabhat University and was used as thermophilic mannanase-producing bacteria source. In this paper, twenty-eight bacterial isolates from soil were grown under high temperature at 60 ºC. Only two isolates KS2 and KS6 showed mannanase activity and these were cultured at 60 ºC for 72 hrs. Resutle showed that both KS2 and KS6 exhibited activity at 50 – 100 ºC, and KS2 and KS6 were both Gram-Positive, catalase positive, aerobic, rod-shaped and motile. 16S rRNA gene sequencing revealed that both isolated bacteria were *Bacillus amyloliquefaciens* sub sp. *plantarum*. This study determined that *Bacillus amyloliquefaciens* subsp. *plantarum* isolated from soil samples exhibited thermophilic mannanase activity at 50-100 ºC.

Acknowledgment

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