ISOLATION AND SCREENING OF MANNANASE PRODUCING BACTERIA FROM MANNAN WASTE

Phanwipa Pangsri[1*]

Abstract

Mannanase is the most important enzyme for the hydrolysis of mannan, which is produced by microorganisms, including bacteria and fungi. Mannanase could be used as valuable food, its application for mannooligosaccharides preparation as prebiotic. This study focus on the isolation and screening an effective strain of mannanase producing bacteria. The result found that 2 isolates showing mannanase production were isolated from soil samples, which compose of waste from mannan. Isolate P2-5 and P3-1 showed high ratio of clear zone diameter to colony diameter 1.75 and 1.50, respectively. Isolate P2-5 and P3-1 were identified by 16S rDNA gene sequencing, biochemical test and morphology. The results of bacterial identification revealed P2-5 and P3-1 as *Bacillus subtilis*, which were 99% identity. The both isolates were able to ferment some of carbohydrates such as D-glucose D-mannitol D-mannose Myo-inositol D-ribose and D-trehalose. *Bacillus* species are an important source of mannanase enzymes production. Therefore, the both isolates will be used for mannanase enzyme production and its application for various industrials and mannooligosaccharides preparation.

Keywords : Isolation, Mannanase, Mannan Waste, *Bacillus subtilis*

[1] *Biotechnology Program, Faculty of Science and Technology, Valaya Alongkorn Rajabhat University under the Royal Patronage, Pathumthani, Thailand*

**Corresponding Author E-mail : Phanwipa@vru.ac.th*

Introduction

Mannan is the most abundant polysaccharides found in leguminous plant and plant cell walls. They are the major constituent of many plants such as konjac, ivory nut, guar gum, locust bean gum, palm kernel, coffee seed and copra meal. Mannan consists of repeating beta-1,4 mannose units and a few alpha-1,6-galactose units attached to a beta -1,4 mannose backbone "called galactomannan" (Mohammad *et al*.,1996). Coffee waste are a by-product from the coffee extract processing and coconut waste are a by-product from coconut milk processing, which contains a large amount of mannose in the form of mannan. Mannanase is the most important enzyme for the hydrolysis of mannan. The enzymes which could be hydrolyzed mannan structures were classified to two types ; endo- beta-1, 4-mannanase EC 3.2.1.78) and beta- mannosidase (EC 3.2.1.25). Endo- beta-1, 4-mannanase can randomly cleave bonds within mannan chain while beta-mannosidase enzyme is capable of removing one or more mannose units from the ends of chains (McCleary, 1988). Bacterial species known to produce beta-mannanases include *Aeromonas hydrophila* (Ratto and Poutanen, 1988), *Enterococcus casseliflavus* (Oda *et al*. 1996), and *Streptomyces* sp. (Takahashi *et al*., 1984). *Bacillus subtilis* (Emi *et al*., 1972). Mannanase can hydrolyze mannan into annooligosaccharides. Therefore, mannanases could be used as valuable food, its application for mannooligosaccharide preparation to be used as prebiotic. Mannooligosaccharides (MOS) are non-oligosaccharides digestibles (NODs), which are beneficial to bacteria in the intestine. Sachslehner *et al*. (2000) hydrolysed mannan in coffee waste by *Sclerotium rolfsii* mannanase into mannotetraose, mannotriose, and mannobiose. Titapoka *et al*. (2008), who reported that the products hydrolyzed from copra meal by the S1 enzyme were mannotriose and mannotetraose. Moreover, The defatted copra meal hydrolysate from crude enzyme and purification (purified M1) enzyme of *Bacillus circulans* NT 6.7 can be stimulate growth of beneficial bacteria and can inhibit the growth of pathogens (Pangsri *et al*, 2015a,b).

Objectives

This study focus on the isolation and screening an effective strain of mannanase producing bacteria from mannan waste.

Materials and Methods

Sample source

The eight samples of soils, which compose of coffee and coconut waste from various locations in Pathumthani, Thailand. It was used as sources for the isolation and screening of mannanase producing bacteria.

Isolation and screening of mannanase producing bacteria

The sample, 1 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). One percent (v/v) of the solution was transferred into 20 ml of sterilized isolation medium ; BIM containing locust bean gum (Abe *et al*.,1994). The microbial cells were grown under aerobic condition by shaking at 150 rpm for 24 hours at 37 °C. The culture broth from enumeration step were serial diluted and spread to isolation medium (BIM) containing locust bean gum. The cells were allowed to grow at 37°C for 24 hr. The colonies with a clear zone of mannanase activity were observed and the ratio of diameter of clear zone to colony was calculated (Phothichitto *et al*., 2006). The positive isolates were selected and kept in 20% glycerol at -20° C for further study.

Morphological and biochemical determination

The selected isolates were cultured on NA plate at 37°C for overnight. The selected isolates were examined for their cell shapes, Gram staining, colony appearance and biochemical tests.

16S rDNA sequence analysis

The selected isolates were cultured on NA slant and send to Macrogen Company (Korea) for automated DNA sequencing. The DNA were analyzed by using forward primer 785F (5'-GGA TTA GAT ACC CTG GTA-3') and reverse primer 907R (5'-CCG TCA ATT CCT TTR AGT TT-3'). The resulting sequences were compared with other DNA sequences deposited in GenBank database using the BLAST program.

Result and Discussion

Isolation and screening of mannanase producing bacteria

The samples of soil, which compose of coconut and coffee waste from Pathumthani Province, Thailand were used as sources for the isolation of mannanase producing bacteria. The primary screening was based on the clear zones on mannan containing locust bean gum agar plate. Twenty-nine strains of bacteria that could produce mannanase as shown in Table 1. This result related to Phothichitto *et al.* (2006) were isolated 19 bacteria and 4 fungi from twenty-three soil samples, coconut waste and fermented coconut. Isolate NT 6.7 showed high mannanase activity of 0.306 U/mL. Based on morphological, biochemical and molecular methods, this bacterium was identified as *Bacillus circulans*. The optimal conditions for mannanase production from *Bacillus circulans* NT 6.7 were at pH 6.0, temperature 45°C with locust bean gum (LBG) as a carbon source. Isolate CW2-3 and ST1-1 were isolated from soil and coconut waste, which high mannanase activity (Titapoka *et al*.,2008). Two isolate of bacteria

were isolated from soil samples, fermented coconut, and fertilizer in Nakhon Ratchasima Province, Thailand can produce mannanase enzyme (Rattanasuk and Ketudat-Cairns., 2009).

Table 1 The twenty-nine strains of bacteria for mannanase enzyme

Source	Isolates	Code Strains
1	5	P1-1,P1-2.P1-3,P1-4,P1-5
2	9	P2-1,P2-2,P2-3,P2-4,P2-5,P2-6, P2-7,P2-8,P2-9
3	4	P3-1,P3-2,P3-3,P3-4
4	5	P4-1,P4-2,P4-3,P4-4,P4-5
5	6	P5-1,P5-2,P5-3,P5-4,P5-5,P5-6
6	ND	ND
7	ND	ND
8	ND	ND

ND = Not detected

Source 1-5 : soil compose of coffee waste, Source 6-8 : soil compose of coconut waste

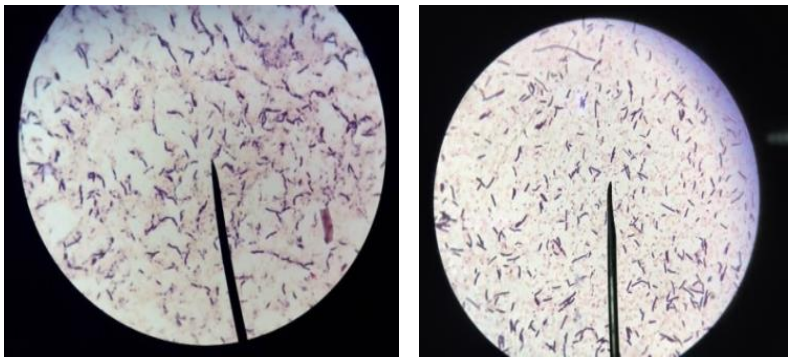
A total of twenty-nine isolates bacterial showed clear zone of mannanase activity in isolation medium (BIM) using locust bean gum as substrate. The isolates were identified using ratio (ratio of clear zone diameter to colony diameter). The isolates P2-5 P3-1 P4-4 P5-2 and P1-1 showed the highest ratio of clear zone diameter to colony diameter in locust bean gum to 1.75 1.50 1.47 1.42 and 1.24, respectively as shown in Table 2. The result similar to Phothichitto *et al*. (2006) was showed that the isolated bacteria could produce enzymes mannanase from soil samples, and coconut waste. The result found that NT 6.3 NT 6.4 isolates showed that ratio of clear zone diameter /colony diameter to 6 and NT 6.5, NT 6.6, NT 6.7 isolate showed ratio of clear zone/colony to 4. The primary screening showed more than ten strains of bacteria that could produce mannanase were identified using halo ratio (ratio of clearing zone to colony diameter) (Rattanasuk and Ketudat-Cairns., 2009). The isolates P2-5 and P3-1 were high ratio of clear zone diameter to colony diameter, which were selected for further study.

Table 2 Mannanase activity of 29 isolates

Isolates	Ratio of clear zone diameter/ colony diameter
P1-1	1.24
P1-2	1.08
P1-3	0.96
P1-4	0.88
P1-5	0.82
P2-1	1.14
P2-2	1.03
P2-3	1.10
P2-4	1.03
P2-5	1.75
P2-6	0.91
P2-7	1.06
P2-8	1.19
P2-9	1.05
P3-1	1.50
P3-2	0.98
P3-3	0.94
P3-4	1.03
P4-1	0.95
P4-2	1.15
P4-3	1.18
P4-4	1.47
P4-5	0.97
P5-1	0.95
P5-2	1.42
P5-3	0.90
P5-4	1.19
P5-5	1.08
P5-6	0.88

Morphology and biochemical determination

The study of morphological characteristics of isolate P2-5 and P3-1 were evaluated by the method in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). The result found that P2-5 was white colony, rod-shape and gram-positive. P3-1 was white colony, rod-shaped and gram-positive. Cell shape and Gram staining of P2-5 and P3-1 under microscope as shown Fig 1. The isolate P2-5 and P3-1 were tested for carbohydrate fermentation and ability to various carbon sources. It was observed that both isolates were able to ferment some of carbohydrates such as D-glucose D-mannitol D-mannose Myo-inositol D-ribose and D-trehalose as shown in Table 3.



(A) (B)

Fig. 1 Cell shape and Gram staining of P2-5 (A) and P3-1 (B)

Table 3 Characteristics of isolates P2-5 and P3-1

Characteristics	Isolates	
	P2-5	P3-1
Gram	positive	positive
shape	rod	rod
Carbohydrate		
D-glucose	+	+
D-galactose	-	-
D-mannitol	+	+
D-mannose	+	+
Myo-inositol	+	+
L-Rhamnose	-	-
D-ribose	+	+
D-trehalose	+	+
D-melezitose	-	-

+ Positive reaction , - Negative reaction

16S rDNA sequence analysis

The sequencing of 16S rDNA sequence of the P2-5 and P3-1 isolates were compared with other bacterial sequences deposited in the GenBank database using the BLAST algorithm. The results showed that both 16S rDNA sequences of P2-5 and P3-1 strain were identified to *Bacillus subtilis*, with the level of identity of 99%. *Bacillus* species are an important source of mannanase enzymes production which have been used for application in industrial such as coffee extraction, animal feed, food and mannooligosaccharides preparation.

Many researchers attempt to find new source of mannanase including bacteria, fungi and plant seed (Soumya *et al*., 2010; Chauhan *et al*., 2012). Nowadays, the mannanase enzyme are important to a prebiotic preparation ; mannooligosaccharides (MOS). Titapoka *et al*. (2008) isolated CW2-3 and ST 1-1 from soil, which high mannanase activity. These two enzymes can be used for the production of prebiotic mannooligosaccharides from copra or LBG. Moreover, Pangsri *et al* (2015a) prepared mannooligosaccharides from defatted copra meal by crude mannanase enzyme from *Bacillus circulans* NT 6.7 and testing the prebiotic properties on beneficial bacteria and pathogenic bacteria. The result defatted copra meal hydrolysate could effectively promote the growth of probiotic bacteria. Pangsri *et al* (2015b) studied to the effect of mannooligosaccharides from purified M1. The result found that purified M1 can be hydrolyzed defatted copra meal into mannooligosaccharides (mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaose (M2–M6)). Defatted copra meal hydrolysate could effectively promote the growth of beneficial bacteria and inhibit pathogenic bacteria more than commercial mannooligosaccharides, which prepared from yeast cell wall.

Conclusion

The soil samples from mannan waste can be isolate the mannanase producing bacteria 29 isolates. Isolate P2-5 and P3-1 showed high ratio of clear zone diameter to colony diameter. Two isolates of mannanase enzyme were identification by 16S rDNA gene sequencing and classification by biochemical test and morphology. The results of bacterial identification revealed P2-5 and P3-1 as *Bacillus subtilis*, which was 99% identity. The both isolates were able to ferment carbohydrates such as D-glucose D-mannitol D-mannose Myo-inositol D-ribose and D-trehalose. *Bacillus* species are an important source of mannanase production. Therefore, isolate P2-5 and P3-1 will be used for mannanase enzyme production, which used for various industrials and mannooligosaccharides preparation in the future.

Acknowledgement

This research grant was supported by the Research Development Institute, Valaya Alongkorn Rajabhat University under the Royal Patronage, Pathumthani, Thailand.

Reference

Abe J, Hossain ZM, Hizukuri S. (1994). Isolation of beta-mannanase producing microorganisms. Journal of Fermentation Bioengineering. 3:259–261.

Chauhan, P. S., N. Puri, P. Sharma, and N. Gupta. (2012). Mannanases: microbial sources, production, properties and potential biotechnological applications. Applied Microbiology biotechnology. 93(5): 1817-1830.

Emi, S., Fukumoto, J. and Yamamoto, T. (1972). Crystallization and some properties of mannanase. Agricultural and Biological Chemistry. 36 : 991-1001.

Holt, J. G., Krieg, N. R., Sneath, P.H. A., Staley, J. T., and Williams, S.T. (1994). Bergey's manual of determinative bacteriology. 9thed. Williams & Wilkins USA.

McCleary, B.V. (1988). Beta -D-Mannanase. Methods in Enzymology. 160, 596-610.

Mohammad Z.H, J. Abe, and S. Hizukuri. (1996). Multiple forms of beta-mannanase from *Bacillus* sp. KK01. Enzyme Microbial Technology. 18: 95-98.

Oda, Y. and Tonomura, K. (1996). Characterization of beta-mannanase and beta-mannosidase secreted from yeast *Trichosporon cutaneum* JCM 2947. Letters in Applied Microbiology. 22: 173-178.

Pangsri, P., Nitisinprasert,S., Ingkakul,A., and S. Keawsompong. (2015a). Defatted Copra meal Hydrolysate as Novel Candidate for Prebiotic. VRU Research and Development Journal. Vol 10 No. 3 :1-10.

Pangsri, P., Piwpankaew, Y., Ingkakul, A., Nitisinprasert, S., & Keawsompong, S. (2015b). Characterization of mannanase from *Bacillus circulans* NT 6.7 and its application in mannooligosaccharides preparation as prebiotic. SpringerPlus, 4, 771. http://doi.org/10.1186/s40064-015-1565-7.

Phothichitto,K., S. Nithisinprasert and S. Keawsompong. (2006). Isolation, Screening and Identification of Mannanase Producing Microorganisms. Kasetsart Journal (Nat. Sci.) 40 (Suppl.) : 26-38.

Rattanasuk,S. and Ketudat-Cairns,M. (2009). *Chryseobacterium indologenes*, novel mannanase-producing bacteria. Songklanakarin Journal Science Technology. 31 (4) : 395-399.

Ratto, M. and Poutanen, K. (1988). Production of mannan-degrading enzymes. Biotechnology Letters. 10, 661-664.

Sachslehner, A., Foidl,G. , Foidl,N. , bitz,G.G., and Haltrich D. (2000). Hydrolysis of isolated coffee mannan and coffee extract by mannanases of *Sclerotium rolfsii*. Journal of Biotechnology. 80: 127-134.

Soumya, R. S., and E. T. Abraham. (2010). Isolation of beta-mannanase from *Cocos nucifera* Linn haustorium and its application in the depolymerization of beta-(1,4)-linked D-mannans. International Journal Food Science Nutrition. 61(3): 272-281.

Takahashi, R., Kusakabe, I. and Kobayashi, H. (1984). Purification and some properties of mannanase from *Streptomyces* sp. Agricultural and Biological Chemistry.48: 2189-2195.

Titapoka. S., S. Keawsompong, D. Haltrich and S. Nitisinprasert. (2008). Selection and characterization of mannanase-producing bacteria useful for the formation of prebiotic manno-oligosaccharides from copra meal. 24 (8) : 1425–1433 World Journal Microbiology Biotechnology. DOI 10.1007/s11274-007-9627-9.