Locust Bean Gum Hydrolysis for Mannooligosaccharide (MOS) Production Using *Bacillus methylotrophicus* KS1

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Abstract:

Locust bean gum is a source of galactomannan that can be used as substrate for mannooligosaccharide production with prebiotic potential through mannanase-catalyzed hydrolysis. The aim of this research was to hydrolyze the locust bean gum using *Bacillus methylotrophicus* KS1 isolated from soil collected from Roi Et, Thailand. Locust bean gum hydrolysis conditions were performed using 1% *Bacillus methylotrophicus* KS1 inoculum (10^8 CFU/ml) in nutrient broth containing 1% locust bean gum in 500 ml Erlenmeyer flask and incubated at 37 °C and 150 rpm for 24 h. Five milliliters of cultures were collected at the specific time intervals for mannanase activity determination. The result indicated that the highest mannanase activity was found at 18 h with 14.10 U/ml. Mannooligosaccharide composition of locust bean gum hydrolysis was identified using High Performance Liquid Chromatography method. The result showed that the produced mannooligosaccharide consisted of mannotriose, mannotetraose and mannohexose. These may have prebiotic attributes that will be investigated in the future.

Keywords: *Bacillus methylotrophicus* KS1, Locust bean gum, Mannanase, Mannooligosaccharide, HPLC

Introduction

Locust bean gum (LBG) is a source of galactomannan consists obtained from seed endosperm of fruit pod of *Ceratonia siliqua* L. Chemical structure of LBG of a β-1,4-linkage mannose backbone with galactose monomers linked to it randomly by α-1,6 bonds [1, 2]. It has been used for food, pharmaceuticals, paper, textile, oil well drilling, cosmetics and also beneficial for human health [3]. LBG can be used as substrate for mannooligosaccharides (MOS) production as prebiotics by mannanase-catalyzed hydrolysis.

MOS are an oligosaccharides comprised of mannose residues obtained from mannan hydrolysis including mannan, glucomannan, galactomannan and galactoglucomannan by β-mannanase [4]. MOS are prebiotics that can be used as a feed additive to reduce pathogenic bacteria such as *Vibrio*, *Coliforms*, *Clostridia* and *Salmonella* and modulate the immune system of host animals and also stimulate the growth of probiotics such as *Bifidobacterium* sp. and *Lactobacillus* sp. [5-7].

Mannanase is an enzyme that randomly hydrolyzes of mannan and heteromannan at the 1,4-β-D-mannosidic linkage [1, 8]. Many microorganisms are sources of mannanase such as *Chryseobacterium indologenes*, *Geobacillus stearothermophilus*, *Neosartorya fischeri* P1, *Bacillus pumilus* GBSW19, *Bacillus* sp. and *Kitasatospora* sp. [1, 9-12]. The aim of this research was to produce MOS from LBG by isolated bacteria, *Bacillus methylotrophicus* KS1.

Materials and methods

Chemical and reagents

LBG was purchased from Sigma (USA). Nutrient broth (NB) was purchased from Himedia Laboratories (India). 3,5-Dinitrosalicylic acid and sodium potassium tartrate were purchased from Sigma (USA). Standard sugars including mannose, mannobiose, mannotriose, mannotetraose, mannopentose and mannohexose were purchased from Megazyme (Wicklow, Ireland).

Bacterial strain

Bacillus methylotrophicus KS1 was isolated from soil in Roi-Et and stored at microbiology laboratory, Department of Science and Technology, Roi Et Rajabhat University, Roi Et, Thailand.

Bacterial growth and staining

Single colony of *B. methylotrophicus* KS1was cultured using nutrient broth (NB) medium and incubated at 37 ºC with 150 rpm for 18 h. One percentage (v/v) of *B. methylotrophicus* KS1 was mixed with 200 ml NB containing 1% LBG and incubated at 37 ºC with 150 rpm for 24 h. Five milliliters of cultured medium broth were collected at 0 - 72 h and centrifuged. Supernatant was discarded and cell pellet was resuspended using sterile normal saline. Bacterial cell count was determined by ten-fold dilution method. One hundred microliters of each dilution were spread on to nutrient agar (NA) incubated at 37 ºC for 18 h. Bacterial population was calculated from plate containing 30-300 colonies. *B. methylotrophicus* was stained by Gram-staining and endospore staining method.

Mannanase activity assay

B. methylotrophicus KS1was cultured using nutrient broth (NB) medium at 37 ºC with 150 rpm for 18 h. The turbidity of the bacterial cell suspension was adjusted at OD600 to the required concentration of 10^8 CFU/ml. One percentage (v/v) of *B. methylotrophicus* KS1 was mixed with 200 ml NB containing 1% LBG and incubated at 37 ºC with 150 rpm for 24 h. Five milliliters of cultured medium broth were collected at 0, 3, 6, 12, 18 and 24 h and centrifuged. Mannanase activities assay was described previously [9]. Briefly, 500 µl of supernatant was mixed with 500 µl of 1% LBG, pH 7 (phosphate buffer) and incubated at 60 ºC for 5 min. The reaction was inhibited by adding DNS solution. The mixture was boiled for 5 minutes and cooled on ice. Two thousand and five hundred microliters of water were added. The amount of reducing sugar was determined at OD540 nm. One unit of mannanase activity is defined as the amount of mannanase that hydrolyzes LBG and liberates 1 µmol D-mannose within 1 min of reaction at 60 ºC.

Mannooligosaccharide composition analysis

MOS composition was analyzed by high pressure liquid chromatography (HPLC) condition described as previously [13]. Mannose, mannobiose, mannotriose, mannotetraose, mannopentose and mannohexose were used as standard sugars.

Results and discussion

Bacterial growth and staining

B. methylotrophicus KS1 was cultured using NB and cultured broth samples were collected for bacterial count. The result indicated that at lag phase was at 0 - 12 h, exponential phase was at 12 - 18 h, stationary phase was at 18 - 48 h and decline phase was after 48 h (Fig. 1). Result of Gram staining and endospore staining showed that *B. methylotrophicus* KS1 was Gram-positive bacilli, rod shape and produced oval subterminal spores after 18 h. (Fig. 2). This growth curve presented that late exponential phase that had the highest growth rate. This incubation time of 18 h will be used for future experiment and it was similar to the reported value in ref [14].

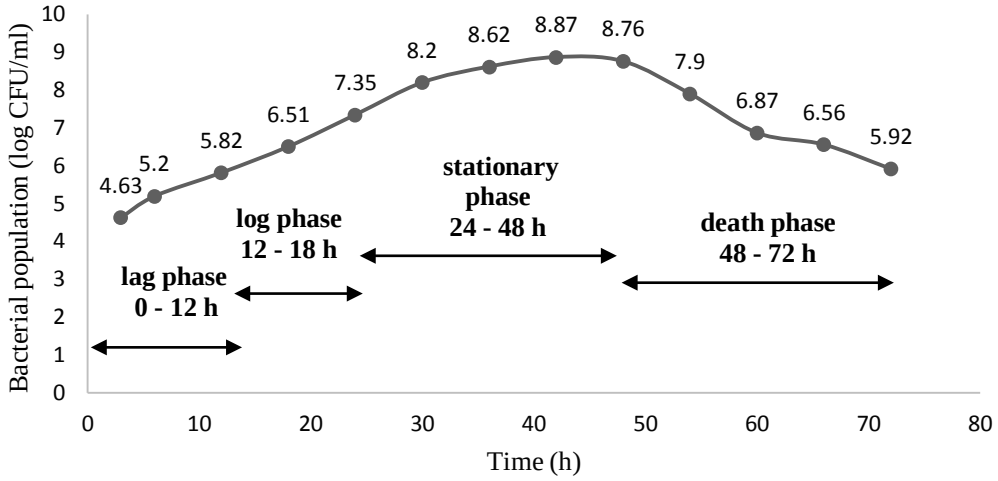

Figure 1 Growth curve of *Bacillus methylotrophicus* KS1 over 72 h.

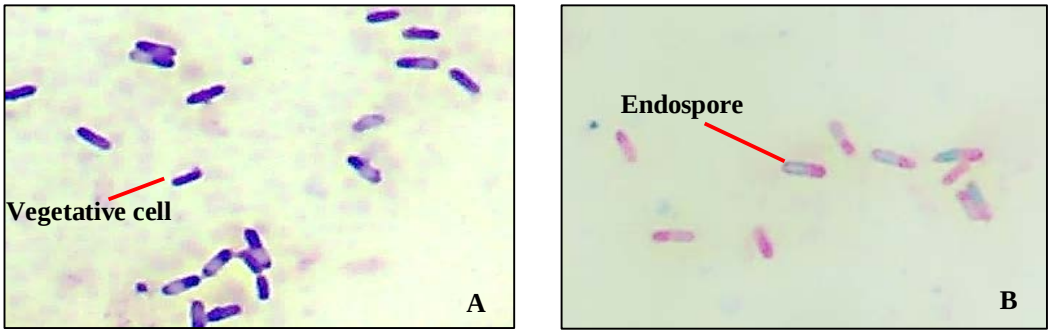

Figure 2 *Bacillus methylotrophicus* KS1 Gram staining and endospore staining under light microscope 1000x.
A. Gram positive bacilli; B. Oval subterminal endospore

Mannanase activity assay

Mannanase activity of *B. methylotrophicus* KS1 was gradually increased until presented the high activity at 18 h (14.10 U/ml) (Fig. 3). Form the above result that showed at 18 h after incubation was late log phase that indicated that high cell growth.

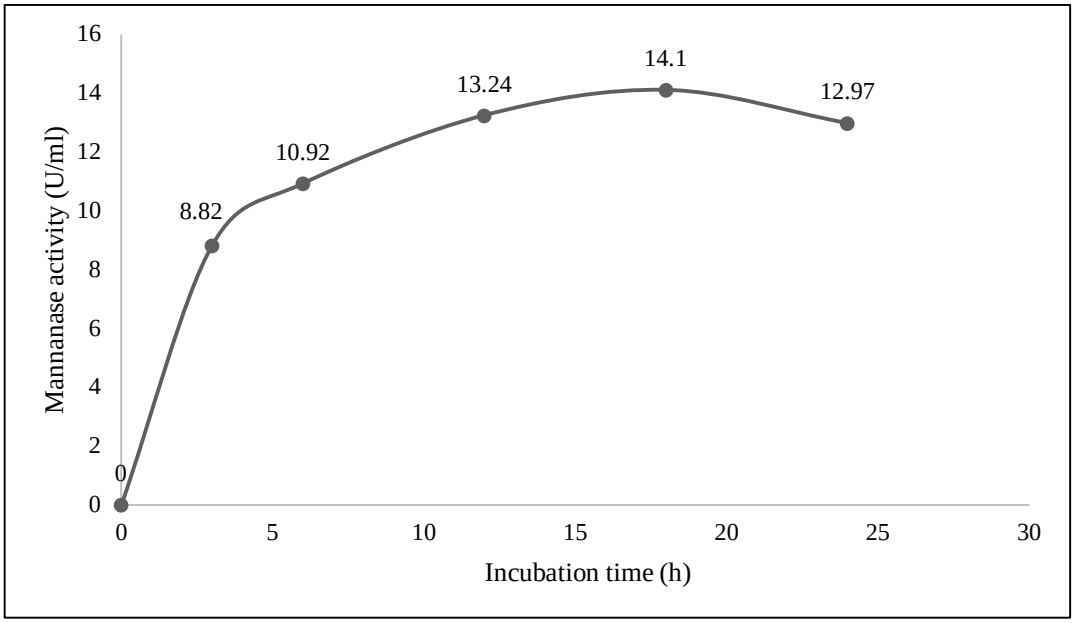

Figure 3 Mannanase activity of *Bacillus methylotrophicus* KS1

Mannooligosaccharide composition analysis

The result from this research found the MOS composition from LBG hydrolysis composed of mannotrios M3 (2.42±0.04 mg/ml), mannotetraose M4 (36.74±0.45 mg/ml) and mannohexose M6 (1.77±0.08 mg/ml) (Table. 1.) obtained from locust bean gum hydrolysis. This result was similar to previous reports [15-17]. However, the composition of MOS is also found from the copra meal by mannase enzyme from *Bacillus circulans* NT6.7 composed of mannotrios M3, mannotetraose M4 and mannohexose M6 [18]. MOS composition from copra meal hydrolysis by mannanase enzyme from *Streptomyces* sp. BF3.1 composed of mannotrios M3, mannotetraose M4, mannopentose M5 and mannohexose M6 [19].

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Table 1 Mannooligosaccharide composition from LBG hydrolysis by *Bacillus methylotrophicus* KS1

Replicate	Mannooligosaccharide (mg/ml)				
	M6	**M4**	**M3**	**M1**	**Total (M6-M3)**
1	1.71	36.42	2.44	0.61	40.57
2	1.83	37.05	2.39	0.64	41.27
$\bar{X}$ ± SD	1.77±0.08	36.74±0.45	2.42±0.04	0.63±0.02	40.92±0.49

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