Isolation of ß-mannanase-producing bacteria from Roi Et Rajabhat University

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ABSTRACT: Thirty eight mannanase-producing bacteria were isolated from 12 soil samples, which located inside and outside Roi Et Rajabhat University. The mannanase screening was carried out on Luria-Bertani agar containing locust bean gum (LBG) stained with iodine solution. After screening, two isolates that exhibited the highest halo ratio were selected and named KP1 and KP4. Mannanase activity was determined by DNS method using LBG as substrate at 50, 55 and 60 °C. The mannanase activity of KP1 and KP4 at 60°C for 5 mins. was 0.89 and 1.17 U/min, respectively, and at 60°C for 30 mins. was 0.35 and 0.26 U/min, respectively. KP1 and KP4 strains were identified by partial 16S rDNA gene sequence, biochemical test and morphology. The results of bacterial identification revealed that KP1 as *Bacillus subtilis* and KP4 as *Bacillus amyloliquefaciens*.

Keywords: mannanase; bacteria; isolation

Introduction

Mannanases are potential enzyme that can randomly hydrolyze the 1,4-β-D-mannosidic linkages (McCleary, 2012) in the main chain of mannans and heteromannans and also applied in bio-bleaching of pulp and paper, hydrolytic agent in detergent industry, hydrolysis of coffee extract, improvement of animal feed, fish feed additive slime control agent and pharmaceutical application (Chauhan et al., 2012). Microorganisms are preferred for source of enzyme because of their rapidly growth and production. Microbial mannanases has been found in many species such as *Acinetobacter* sp. (Titapoka et al., 2008), *Bacillus amyloliquefaciens* (Mabrouk and El Ahwany, 2008), *Bacillus* sp. (Lin et al., 2007; Zhang et al., 2009; Singh et al., 2010), *Cellulosimicrobium* sp. (Kim et al., 2011), *Chryseobacterium indologenes* (Rattanasuk and Ketudat-Cairns, 2009), *Klebsiella oxytoca* (Titapoka et al., 2008), *Aspergillus niger* (Kote et al., 2009; Norita et al., 2010), *Penicillium occitanis* (Blibech et al., 2011), *Scopulariopsis candida* (Mudau and Setati, 2008), *Trichoderma reesei* (Eneyskaya et al., 2009) and *Streptomyces* sp. (Bhoria et al., 2009).

Mannans are complex polysaccharides that are found in seed of leguminous plant and plant cell walls such as palm seeds, konjac, locust bean gum (LBG), coffee beans, and copra meal. Copra meal contains high amount of mannose in the form of galactomannan (Hossain et al., 1996). It is considered as an abundant agricultural waste in Thailand. Therefore, it seems to be a suitable source for the prebiotic mannooligosaccharides (MOS) production.

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In this study, mannanase-producing bacteria were isolated from soil residing in Roi Et Rajabhat University using the selective media containing LBG as carbon sources. The reducing sugars liberated from thermo-stability of mannanase activities at 50, 55 and 60 °C were determined using Dinitrosalicylic Colorimetric Method. The isolates bacteria exhibiting high mannanase activities were identified and characterized using 16S rDNA gene. The bacteria that produce the highest mannanase activity would be applied for production of MOS.

Materials and methods

Sample source

Twelve soil samples collected from various locations inside and outside Roi Et Rajabhat University were used for screening mannanase-producing bacteria.

Coconut meal preparation

Residual coconut meal after processing was bought from a Tawat Din Dang market, Roi Et. This copra meal was dried by incubating at 60 °C for 3 hrs. and then milled to obtain a particle size of 0.5 mm.

Mannanase-producing bacterial screening

One gram of soil sample was suspended in 5 ml sterilized 0.8% NaCl and was then well-mixed. Five hundred microliters of the mixed suspension were transferred into 50 ml of sterilized Luria-Bertani (LB) broth (Bertani, 1951) containing with 1% copra meal. The inoculated broth was incubated at 35 °C, 150 rpm for 48 hrs. The appropriate dilution of each cultured broth was spread on LB agar medium containing 1% LBG (Sigma, USA), and the plates were incubated at 35 °C for 18 hrs. Mannanase-producing bacteria were evaluated by flooding iodine solution. The twelve colonies with high halo ratio (ratio of clearing zone to colony diameter) after flooding an iodine solution were collected and kept in -20 °C.

Mannanase activity determination

Mannanase activity of each isolated was performed using LBG (Sigma) as substrate. Substrate was prepared by boiling 1 g LBG in 100 ml of 50 mM sodium phosphate buffer, pH 7.0 for 30 min. It was cooled and centrifuged at 4000 rpm to remove the insoluble and then autoclaved at 121 °C for 20 mins. The supernatant obtained after centrifugation of the culture broth with induced by using 1% LBG for 24 hrs., was used as a crude mannanase. An aliquot of 500 µl of crude mannanase was mixed with 500 µl of substrate and the solutions were incubated at 50, 55 and 60 °C for 5 and 30 mins. *Escherichia coli* K12 cultured supernatants were used as negative control. The mannanase activity was inhibited by adding DNS-reagent. The reducing sugar liberated in the mannanase reaction was measured as D-mannose reducing equivalents by the DNS method (Nelson, 1944; Somogyi, 1952). 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 experimental conditions described above.

Biochemical characterization

The selected bacteria were cultured on LB agar plate and determined by Gram's staining. Bacterial biochemical tests were evaluated by using an API test kit.

16S rDNA sequence analysis

Single colony of selected bacteria was resuspended with 10 µl sterilized distilled water and boiled for 5 mins. The supernatant of cell lysate was added to PCR with fD1 and rP2 primers (Rattanasuk and Ketudat-Cairns, 2009; Weisburg et al., 1991). The amplified 16S rDNA products were ligated into the pGem®-T Easy vector (Promega, USA) and sent to Macrogen Company (Korea) for automated DNA sequencing. The resulting sequences were compared with other DNA sequences deposited in GenBank database using the BLAST program.

Results and discussion

Mannanase-producing bacterial screening

Thirty eight mannanase-producing bacteria were isolated from 12 soil samples collected from various locations inside and outside Roi Et Rajabhat University. The screening based on the clear zones formed on LB agar containing 1% LBG and stained with iodine solution (Fig. 1).

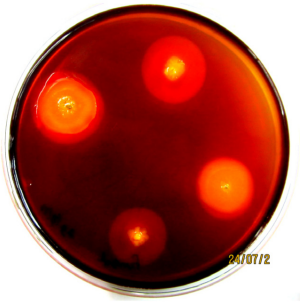

Figure 1 Clear zone formed on LB agar containing 1% LBG stained with iodine solution.

Mannanase activity determination

Mannanase activity of twelve isolates was performed using LBG in 50 mM sodium phosphate buffer, pH 7.0 as substrate. Twelve bacteria exhibiting high halo ratio were further selected for determining the mannanase activity. The results indicated that crude mannanase from the supernatants of the bacterial cultures of the strains named KP1 and KP4 had high mannanase activity at 50, 55 and 60 °C (**Table 1**). KP4 shows the highest mannanase activity with 1.17 Unit/ml at 60 °C for 5 mins.

Table 1 Mannanase activity at various temperatures (Unit/ml)

	50 °C		55 °C		60 °C	
Bacterial	5 min	30 min	5 min	30 min	5 min	30 min
KP 1	0.49	0.27	0.85	0.38	0.89	0.35
KP 6	0.17	0.02	0.58	0.22	0.74	0.18
KP 7	0.19	0.02	0.14	0.02	0.07	0.01
KP 8	0.17	0.02	0.09	0.01	0.06	0.02
KP 4	1.01	0.34	0.95	0.28	1.17	0.26
KP 29	0.16	0.12	0.45	0.08	0.16	0.03
KP 12	0.13	0.02	0.22	0.04	0.02	0.04
KP 9	0.14	0.01	0.12	0.07	0.19	0.04
KP 30	0.03	0.02	0.13	0.05	0.10	0.02
KP 38	0.89	0.07	0.18	0.02	0.08	0.01
KP 36	0.05	0.13	0.19	0.06	0.24	0.15
KP 35	0.15	0.03	0.05	0.12	0.15	0.08
E. coli	0.00	0.00	0.00	0.00	0.00	0.00

Biochemical characterization and 16S rDNA sequence analysis

For the Gram staining, KP1 and KP4 are Gram positive bacilli. From API analysis, the results indicated that KP1 was belonged to *Bacillus subtilis* and KP4 was belonged to *Bacillus amyloliquefaciens* (data not shown). The sequencing of 16S rDNA sequence of the both KP1 and KP4 were compared with other bacterial sequences deposited in the GenBank database using the BLAST algorithm. The results showed that both 16S rDNA sequences of KP1 and KP4 strain were identical to *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively, with the level of confidence of 99 and 99%.

Mannanase producing bacteria are important to a prebiotic MOS production. Many researchers attempt to find new source of mannanase including bacteria, fungi and plant seed (Soumya et al., 2010; Chauhan et al., 2012). The application of mannanase used for MOS production is more attention. Cuong et al. (2013) produced MOS from copra pulp by partial enzymatic hydrolysis using recombinant *Aspergillus niger* β-mannanase and used as dietary supplement in shrimp farms. The result demonstrated that MOS could increase in weight gain, specific growth rate, feed conversion, feed intake and probiotic bacteria (Cuong et al., 2013). Pourabedin et al. (2014) also studied the effects of MOS and virginiamycin on the cecal microbial community of chickens. The results indicated that MOS promoted the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. (Pourabedin et al., 2014). *Bacillus* species are an important source of enzymes which have long been used for the production of various industrial enzymes (Schallmey et al., 2004). From this research, we focused on the screening of mannanase-producing bacteria. The results showed KP1, *Bacillus subtilis* and KP4, *Bacillus amyloliquefaciens* exhibited high mannanase activity at 50, 55 and 60 ^{o}C. Both strains will be used as bacterial cultures for producing MOS in the near future.

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