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Original Article

Chryseobacterium indologenes, novel mannanase-producing bacteria

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Abstract

Mannanase is a mannan degrading enzyme which is produced by microorganisms, including bacteria. This enzyme can be used in many industrial processes as well as for improving the quality of animal feeds. The aim of the present study was to screen and characterize the mannanase-producing bacteria. Two genera of bacteria were isolated from Thai soil samples, fermented coconut, and fertilizer. Screening was carried out on agar plates containing mannan stained with iodine solution. The bacteria were identified by partial 16S rRNA gene sequence, biochemical test and morphology, respectively. The mannanase activity was determined by zymogram and DNS method. Two strains of bacteria with mannanase activity were identified as *Bacillus* and *Chryseobacterium*. This is the first report of mannanase-producing *Chryseobacterium*.

Keywords: *Chryseobacterium*, *Bacillus*, zymogram, mannanase-producing bacteria

1. Introduction

Mannanase, β-D-mannanase or 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78) (Techapun *et al*., 2003) can perform random hydrolysis of 1,4-β-D-mannosidic linkages (McCleary, 1988) in the main chain of β-1,4-mannans, galactomannans, glucomannans, and galactogluco-mannans. This structure consists of a backbone of β-1,4-linked mannose and glucose residues substituted with α-1,6-linked galactosyl side groups. Mannanase was reported to act on various heteromannans to produce mannooligosaccharides (MOS) and a small amount of mannose, glucose and galactose.

Mannanases are of special interest because of their function and applicability in many industries, including food technology. They are used for hydrolyzing high molecular weight mannans as well as in the feed industry for increasing the digestibility of animal feed (Viikari *et al.*, 1993; Wong and Saddler, 1993). The application of mannanases has also been suggested in the processing of coffee beans, marine algae and other plant materials. Other applications include print processing for textiles, and pulp biobleaching (Clark *et al*., 1990).

Microbial species known to actively produce mannanases include *Bacillus subtilis* (Emi *et al*., 1972), *Aeromonas hydrophila* (Ratto and Poutanen, 1988), *Enterococcus casseliflavus* (Oda *et al*., 1993), *Pseudomonas* sp. (Yamaura *et al*., 1990), *Vibrio* sp. (Tamaru *et al.*, 1995), *Streptomyces* sp. (Takahashi *et al*., 1984), fungi (Rees and Shibata, 1964) and yeast (Oda and Tonomura, 1996). Mannanases are also distributed in higher plants (Shimhara *et al*., 1975) and animals (Yamaura and Matsumoto, 1993).

Mannan is one of the most abundant polysaccharides found in unused resources in Thailand. It can be found in seeds of leguminous plant and plant cell walls, such as locust bean gum (LBG), copra meal, coffee beans and palm seeds. Among the various types of mannan, the one from copra has a linear β-1,4-mannan backbone with only a few β-galactosyl stubs. It has been reported that between 60-70% of copra meal consists of β-mannan or a complex derivative of β-mannan. In addition, it has been shown that 20-30% of the dry weight of copra appears to be pure β-mannan,

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(www.chemgencom/coprameal.html). Thus, copra meal, which is a cheap agricultural by-product, seems to be a suitable source for the production of excellent prebiotic mannooligosaccharides (MOS).

In this study, bacteria exhibiting high mannanase activity were isolated from various sources with selective media. These sources included soils, fertilizer and fermented coconut. The bacteria were identified and characterized using 16S rRNA gene sequence. Mannanase activities were determined by an in-gel activity assay, a zymogram. The bacteria that can produce the highest β-mannanase activity from the screen will be used for production of β-mannanase for improvement of prebiotics production from copra.

2. Materials and Methods

2.1 Sample source

Ten samples of soils, fertilizer and fermented coconut collected from various locations in Suranaree University of Technology, Nakhon Ratchasima, Thailand, were used as sources for the isolation of mannanase-producing microorganisms.

2.2 Preparation of coconut meal

The residual coconut cake that remains after pressing, usually referred to as copra meal, was bought from a local market (Nakhon Ratchasima, Thailand) and used as a substrate for the enzyme assay and as a carbon source for medium formulation. This copra meal was dried at 60°C for 4 hr, and then blended and milled with a hammer mill (IKA Labortechnik; Janke & Kunkel, Germany) to obtain a product with an average particle size of 0.5 mm.

2.3 Screening and characterization of mannanase-producing bacteria

For the primary screening step, 1 gram or 1 ml of soil, fertilizer or fermented coconut was suspended in 10 ml of sterilized 0.8% normal saline and was then mixed by vortex. One percent (v/v) of the solution was transferred into 20 ml of sterilized bacterial isolated medium (BIM) (modified from Abe *et al*., 1994) and cultured under aerobic conditions and shaking at 150 rpm for 18-24 hr at 30°C. The BIM was composed of (w/v) 0.1% yeast extract, 0.1% polypeptone, 0.1% NH_4NO_3, 0.14% KH_2PO_4, 0.02%; $MgCl_2$ with 1% copra meal as the main carbon and energy source. Then, an appropriate dilution of the culture broth was spread on BIM agar medium containing 1% LBG (Sigma Chemical; St. Louis, USA). After incubation at 30°C for 18-24 h, the mannanase activity of each isolate was evaluated based on the ratio of the diameter of the clear zone formed to the diameter of the colony. The colonies with a clear zone showing mannanase activity against LBG were collected and maintained as frozen stocks in the presence of 20% glycerol at -70°C for further studies.

To confirm that the isolated bacteria have mannanase activity, the samples that had been kept at -70°C were streaked onto LB agar and incubated at 30°C for 24 hr. A single colony was picked and touched on LB agar containing 1% LBG. *Bacillus subtilis* 168 was used as positive control (El-Helow *et al*., 1997). *Escherichia coli* K12 was used as negative control (Blattner *et al*., 1997). After being incubated at 30°C for 24 hr, plates were stained with 0.1% (w/v) Congo red solution or iodine solution. The halo ratios were then compared between the isolated bacteria and the positive and negative control for the bacterial selection.

2.4 Morphology and biochemical determination

The selected isolates were cultured on NA plate at 30°C for overnight. All of the selected isolates were examined for their cell shapes, Gram staining, colony appearance, spore formation, motility and pigmentation. Bacterial biochemical tests were evaluated by using an API test kit, which included the fermentative production of acid from various sugars as carbon sources and the production of hydrolytic enzymes such as amylase, xylanase, etc. (bioMérieux; Marcy l'Etoile, France).

2.5 Analysis of 16S rRNA sequence

Bacterial strains were cultured overnight at 30°C on LB agar. A single colony was suspended in 10 µl of distilled water. Bacterial cells were lysed by heating at 100°C for 5 min. The lysate was centrifuged at 4,000 rpm for 2 min and 5 µl of the supernatant was directly used for PCR with the forward primer fD1 (5' -AGAGTTTGA TCCTGGCTCAG-3') and reverse primer rP2 (5'-AAGGAGGTGATCCAGCC -3') (William *et al*., 1991), which amplify a fragment of about 1,500 bp from the 16S rRNA gene. An initial denaturation at 94°C for 2 min was followed by 35 cycles with denaturation at of 94°C (45 s), annealing at 55°C (45 s) and extension at 72°C (2 min), and a final extension at 72°C for 10 min. The amplied 16S rDNA products were ligated into the pGem®-T Easy vector, as described by the manufacturer (Promega; Madison, WI, USA). Competent cells of *E. coli* DH5α were prepared by the calcium chloride method (Rodriguez and Tait, 1983). Transformation of recombinant DNA into competent cells was performed according to the manufacturer (Promega). White colonies were picked and checked for DNA size. The sequence of the amplied 16S rDNA product was determined by dideoxy sequencing at Macrogen Company (Korea). The resulting sequences were compared with the non-redundant nucleotide database from GenBank using the BLAST program.

2.6 Zymogram analysis

For the analysis of mannanase, a zymogram of mannanase activities was generated by an in-gel activity assay

on 0.25% LBG as a substrate copolymerized with polyacrylamide. After centrifugation at 4,000 rpm 15 min at 4°C of the culture broth that was induced by using 1% LBG after 24 hr, the supernatant was used as a crude enzyme and was applied on a 12% (w/v) polyacrylamide gel in 50 mM sodium phosphate buffer, pH 7.0. The positive control was *B. subtilis* 168 and the negative control was *E. coli* K12, respectively. After electrophoresis, the gel was briefly rinsed with de-ionized water, and then soaked in 2.5% Triton X-100 for 1 hr at 4°C. The gel was then incubated for 1 hr at 50°C in 100 mM sodium phosphate buffer (pH 7.0). After incubation, the gel was stained with 0.1% Congo red solution then gently shaken prior to being destained in 1 M NaCl overnight. Next, it was placed in 5% acetic acid (Penttila *et al*., 1987). Mannanase activity was detected as clear zones against red (after staining with Congo red) or blue background (after soaking in 5% acetic acid).

2.7 Determination of mannanase activity

Mannanase activity was assayed using 0.5% LBG (Sigma, product number G0753) as substrate. The substrate was dissolved in 50 mM sodium phosphate buffer (pH 7.0) by homogenization at 100°C. It was cooled and stored at room temperature overnight, then continually stirred and autoclaved at 121°C for 20 min. The insoluble (less than 5% of substrate) was removed by centrifugation at 4,000 rpm, 4°C for 30 min. The supernatant obtained after centrifugation of the culture broth with induced by using 1% LBG for 24 hr, was used as a crude enzyme. An aliquot of 0.2 ml of crude enzyme was then incubated at 50°C for 5 min with 1.8 ml of the substrate solution. Solutions that contained *B. subtilis* 168 and *E. coli* K12 cultured supernatant were used as positive and negative controls, respectively. The reaction was stopped by adding DNS-reagent. The reducing sugar liberated in the enzyme reaction was measured as D-mannose reducing equivalents by the Somogyi and Nelson method (Nelson, 1994; Somogyi, 1952). Substrate and enzyme blanks were made with the addition of distilled water instead of the crude enzyme or substrate. One unit of enzyme is defined as the amount of enzyme which liberates reducing sugar equivalent to 1 μmol D-mannose standard per minute under the experimental conditions described above.

3. Results

3.1 Screening and characterization of mannanase-producing bacteria

Ten samples of soil, fertilizer and fermented coconut from Suranaree University of Technology, Nakhon Ratchasima, Thailand were used as sources for the isolation of mannanase-producing bacteria. The primary screening was based on the clear zones formed on mannan containing-LB agar plate. Primarily, the copra mannan was our target substrate for the bacterial isolation, but it did not dissolve well in BIM medium. Consequently, LBG was used as the substrate for this primary screening. More than ten strains of bacteria that could produce mannanase were identified using halo ratio (ratio of clearing zone to colony diameter). Two strains (SUT1 and SUT2) were isolated because they each had a large hydrolysis halo on LB+0.5% LBG agar plate compared with the positive and negative control (Figure 1). Since we planned to look for specific mannanases which were active to copra mannan, both isolates were further determined for their activities against copra meal. The results showed that the crude enzyme from the supernatant of the bacterial strain SUT1 had higher mannanase activity than *B. subtilis* 168 which, used as positive control, whereas the bacterial strain SUT2 had lower activity and no activity was detected in *E. coli* K12 (Table 1). The bacterial strain SUT1 was aerobic, rod-shape, endosporeforming, Gram-positive and the bacterial strain SUT2 was non-fermentative, Gram-negative, yellow in colour. Based on its morphology and biochemical properties (from API test kit) referred to above,

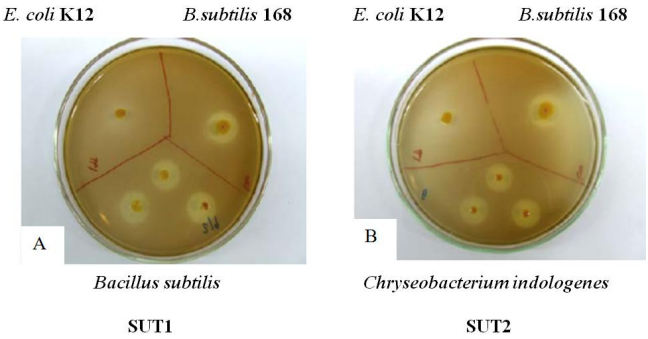

Figure 1. Enzyme activity assay on agar plates. Hydrolysis halos produced on LB+0.5% LBG agar plate by touching and incubated at 30°C for 24 hr before stained with iodine solution. A; Bacterial strain SUT1 (*B. subtilis*) and B; Bacterial strain SUT2 (*C. indologenes*). The isolated bacteria were compared with *E. coli* K12 (negative control), *B. subtilis* 168 (positive control) in each plate, respectively.

Table 1. Mannanase production from the isolated strains. Mannanase activity was calculated using crude enzyme was obtained from cultured medium after cultivation in LB + 1% LBG after 24 hr. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1 μmol of mannose per minute under the assay conditions.

Bacterial strain	Mannanase activity (U/ml)
Bacillus subtilis 168 (positive control)	1.5×10^{-3}
Escherichia coli K12 (negative control)	0.0
Strain SUT1 (*Bacillus subtilis*)	1.7×10^{-3}
Strain SUT2 (*Chryseobacterium indologenes*)	0.9×10^{-3}

* Unit of enzyme from 50 ml of culture medium

bacterial strain SUT1 could belong to family *Bacillaceae* genus *Bacillus* species *Bacillus subtilis* whereas bacterial strain SUT2 was belong to family *Flavobacteriaceae* genus *Chryseobacterium* species *Chryseobacterium indologenes* (data not shown).

3.2 Sequence analysis of 16S rRNA

Amplification of 16S rDNA from the isolated bacteria with the universal primers, fD1 and rP2 produced a product of ~1,500 bp. The sequences of the isolated bacteria were edited and aligned using ClustalW program from the NCBI data base. Based on the 16S rRNA sequences, strain SUT1 was identified as *B. subtilis* being 99% identical with *B. subtilis* gene for 16S rRNA accession number AB065370 and the sequence from isolated strain SUT2 was identified as *Chryseobacterium* sp. being 99% identical with *Chryseobacterium* sp. LDVH 42/00 16S ribosomal RNA gene accession number AY468475. The sequencing confirmed the results that were obtained from morphological and physiological characteristics as described above.

3.3 Zymogram analysis

The active enzymes could be highly expressed and could be efficiently secreted out of the cells as shown by clearing zones on a zymogram of media proteins. The zymogram analysis showed a large hydrolytic zone by the isolated bacterial strain SUT1 than in the positive control while SUT2 had a smaller hydrolytic zone. The negative control *E. coli* K12 showed no hydrolytic zone (Figure 2).

3.4 Determination of mannanase activity

As shown in Table 1, the activity of the mannanase enzyme that was secreted out of the cell was quantified at the same culture period using *B. subtilis* 168 and *E. coli* K12 as the positive and negative controls, respectively. Routinely, 1.7 x 10^{-3} U/ml of *B. subtilis*, 0.9 x 10^{-3} U/ml of *C. indologenes*, 1.5 x 10^{-3} U/ml of *B. subtilis* 168 were obtained after overnight cultivation in shake flasks under the conditions described above. As expected, no activity was measurable in culture broth from *E. coli* K12.

4. Discussion

Bacillus species are an important source of enzymes. They have long been used for the production of various industrial enzymes, mainly alpha-amylases and proteases (Schallmey *et al.*, 2004). Many *Bacillus* species are non-pathogenic and have designated as GRAS (generally recognize as safe) status (Olempska-Beer *et al.*, 2006). This study found that the most active mannanase-producing bacteria, strain SUT1 was identified as *B. subtilis* and strain SUT2 was identified as *C. indologenes*. This is the first report that shows that *C. indologenes* can produce manna-nase. However, in the in-gel activity assay (Figure 2), the mannanase activity of *C. indologenes* was low when incubated at 50°C, but the activity of secreted mannanase from *C. indologenes* was highest when incubated at 30°C (Figure 1).

The active mannanase could be expressed and secreted out of the cell efficiently. This was shown by the clearing zones on agar plates containing LBG and also by in-gel activity staining (zymogram). The sequence of 16S rRNA PCR product confirmed the results of API test kit identification so the isolated bacteria are *B. subtilis* and *C. indologenes*.

Further work is in progress to analyze the oligosaccharide using the crude β-mannanase with copra meal by thin layer chromatography (TLC). For enzyme purification, pH and temperature will be evaluated. This enzyme will be improved using directed evolution for the production of mannooligosacharides (MOS) from copra meal, a cheap agricultural by-product. Copra meal seems to be a suitable source for the production of prebiotic mannooligosacharides (MOS).

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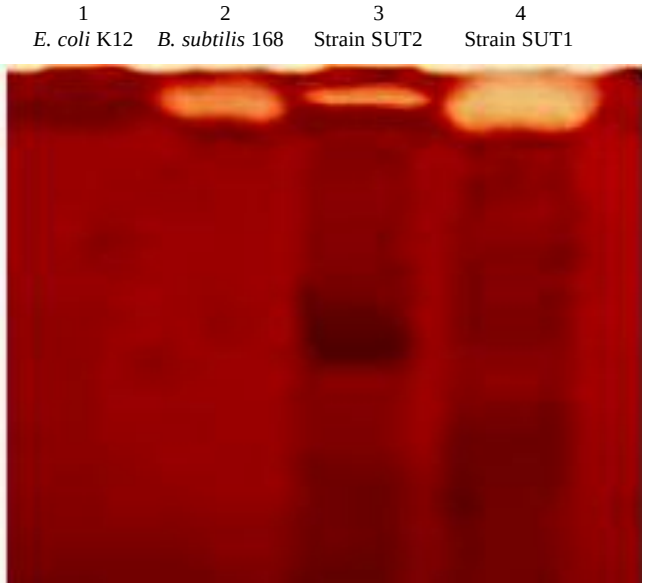

Figure 2. Zymogram analysis. Zymogram analysis illustrates in-gel analysis of mannanase activity. Lanes 1 and 2 are hydrolytic bands of mannanase of control which used *E. coli* K12 (negative control) and *B. subtilis* 168 (positive control) respectively; lanes 3 and 4 are hydrolytic bands of isolated bacterial strain SUT2 (*C. indologenes*) in lane 3 and strain SUT1 (*B. subtilis*) in lane 4, respectively. Crude enzyme was obtained from cultured medium after cultivation in LB + 1% LBG for 24 hr.

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