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Isolation, Screening and Identification of Mannanase Producing Microorganisms

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ABSTRACT

Mannan is used in many industries and known to be the major polysaccharides of legume seeds, coconut kernel and tubers of konjac. Several bacteria and fungi have been reported to produce mannanase. In this paper, 19 bacteria and 4 fungi showing mannanase production were isolated from twenty-three soil samples. Isolate NT 6.7 showed high mannanase activity (0.306 unit/ml), displayed broad inhibition to pathogens; *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010, and could promote growth of probiotic *Lactobacillus reuteri* AC5. The strain was identified by morphological, physiological, biochemical (API 50 CHB, 99.5%identity) and 16 S rDNA sequence (99%identity) tests as *Bacillus circulans*. The mannanase activity from this strain had optimum pH of 7.0-9.0, optimum temperature at 50 ∂C and high stability at 50 ∂C. The results suggested mannanase produced from *B. circulans* NT 6.7 had interesting properties and could be applied in prebiotic preparation.

Key words: prebiotic, mannan, copra meal, probiotic, locust bean gum

INTRODUCTION

Copra meal is agricultural waste containing mannan. Mannans are hemicellulose, accounting for 15-20 % (dry basis) in softwoods but only 5% in hard woods (Timell and Syracuse, 1967). The main chain of softwood mannans is composed of b-1,4-linked D-mannopyranose and D-glucopyranose units. The residues in the main chain are partially substituted by a-1,6-linked D-galactosyl side groups. Mannans can be obtained from legume seeds (Marcos *et al.*, 1995), coconut kernel (Balasubramanian, 1976) and tubers of konjac (Wootton *et al.*,1993). The mannan of legume and coconut is galactomannan (Marcos *et al.*, 1995; Balasubramanian, 1976) and of konjac is glucomannan (Wootton *et al.*,1993).

Mannanase from a variety of different organisms have been studied, including bacteria, fungi, higher plants and animals (Dekker and Richard, 1976). Mannanases are useful in many fields including biobleaching of pulp and detergent industry (Gubitz *et al.*, 1997), bioconversion of biomass wastes to fermentable sugars (Chandrakant and Bisaria, 1998), and upgrading of animal feed stuff (Ray *et al.*, 1982). It can be used to reduce the viscosity of coffee extracts (Hashimoto and Fukumoto, 1969). The coffee preparation using b-mannanases showed better volatile aroma, taste properties and visual appearance of the final drink (Nunes and Coimbra, 1998). Mannanases could be used as valuable food

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sweetener or additives (Tomotari, 1990) and also have potential application for manno-oligosaccharide preparation to be used as prebiotic, which is expected to improve the growth performance of animal. Manno-oligosaccharides are generated when mannan is hydrolyzed by endo-1,4-b–D-mannanase (EC 3.2.1.78) which catalyzes the random cleavage of b-D-1, 4-mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galacto-glucomannan and mannan (McCeary and Matheson, 1974). It has been reported that manno-oligosaccharides is a special nutrient or growth promoter for probiotics, such as *Bifidobacterium* sp. and *Lactobacillus* sp. In this study, we isolated, screened and identified an effective strain of mannanase producing bacteria. Characteristics of crude mannanase, optimal pH, optimal temperature and thermal stability, were also investigated.

MATERIALS AND METHODS

Materials and chemicals

The coconut residual cakes, usually called copra meal, were collected from Pakkret Market, Thailand and used as a substrate for enzyme assay and a carbon source for medium formulation. The residual were dried at 60∂C for 2-4 hr. After that, the residual were blended and milled. The copra meal with the particle size of 0.5 mm was obtained by sieving. Locust bean gum was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Sample sources

Twenty-three samples of soils, coconut waste and fermented coconut (18 samples from Nakhon Pathom province, 2 samples from Nakhon Sawan province, 1 sample from Kamphaeng Phet province and 2 samples from Saraburi province) were collected and used as sources for isolation of mannanase producing microorganism.

Isolation and enumeration

The sample, 1 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). The solution was mixed by Stomacher" 80 Lab System with normal mode for 60 second. One percent (v/v) of the solution was transferred into 20 ml of sterilized isolation medium (IM); BIM, FIM and YIM (Abe *et al*., 1994) with 1% copra meal for bacteria, fungi and yeast, respectively. The microbial cells were grown under aerobic condition by shaking at 150 rpm for 24 hr at 45∂C.

Primary screening

The culture broth from enumeration step were serial diluted and spreaded to isolation medium (BIM, FIM and YIM) containing locust bean gum instead of copra meal. The cells were allowed to grow at 45∂C for 18-24 hr for bacteria and 3-7 days for fungi and yeast,. The colonies with a clear zone of mannanase activity were observed and the ratio of diameter of clear zone to colony was calculated. The positive isolates were selected and kept in 20% glycerol at -87∂C for further study.

Secondary screening

The effective isolates were evaluated on their properties on mannanase production, growth promotion of probiotic (*Lactobacillus reuteri* AC-5), and inhibition of pathogens (*Salmonella* serovar Enteritidis. S003 and *Esherichia coli* E010). The selected strain was used in the next step.

Mannanase activity on copra meal The positive colonies showing clear zone were confirmed for mannanase activity. Enzyme production was performed in 500 ml Erlenmeyer flask containing 300 ml of enzyme producing medium (PM) (Abe *et al.*, 1994). The composition was as followed: 1% Copra meal, 3% Poly peptone, 1.5% KH_2PO_4, 0.06 % $MgSO_4.7H_2O$, 2.5 % (v/v), Corn steep liquor, and 1% inoculums,

pH 7.0. The flasks were shaken in incubator shaker at 150 rpm, 45∂C for 24 hr. Then, the culture broth was centrifuged at 7,000 rpm, 4∂C for 15 min. The supernatant was collected and kept at -20∂C for further study. Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50 mM potassium phosphate buffer pH 7.0 and 1% copra meal with 0.5 ml of supernatant at 45?C for 60 min (Abe *et al.,* 1994). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) (Miller, 1959). One unit of mannanase activity was defined as amount of enzyme producing 1 micromole mannose per minute under the experimental condition.

Effect of culture filtrate on lactic acid bacteria growth *Lactobacillus reuteri* AC-5 was grown in 50 ml of MRS broth for 12-15 h at 37∂C. Later, 1% of the inoculum, which was adjusted to the absorbance of 0.5 at 600 nm was transferred into 5 ml of MRS broth with or without 1 % of the supernatant for 4 hr at 37∂C. After 4 hr of incubation, the number of cells were determined by plating on MRS agar and grown overnight at 37∂C. The isolates yielding culture filtrate which showed promotion of *Lactobacillus reuteri* AC-5 were selected.

Enhanced activity (%) was determined as

$$\frac{[SB - CB]}{CB} \times 100$$

Where SB is amount of cells in MRS with culture filtrate (cfu/ml) and CB is amount of cells in MRS without culture filtrate (cfu/ml).

Effect of culture filtrate on pathogen *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010 growth *Salmonella* serovar Enteritidis S003 or *Escherichia coli* E010 were cultivated in 50 ml of NB medium under aerobic condition by shaking at 150 rpm for 12-15 h at 37∂C. After that, 1% of the inoculum, which was adjusted to the absorbance of 0.5 at 600 nm were transferred into 5 ml NB medium and 5 ml of NB plus 1% of the supernatant and grown at 37∂C by shaking at 150 rpm for 4 h. The cell numbers were determined by plating on NB agar and incubated overnight at 37∂C. The isolates yielding culture filtrate showing inhibition of *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010 were selected.

Inhibition activity (%) was determined as

$$\frac{[CB - SB]}{CB} \times 100$$

Where SB is number of cells in NB with culture filtrate (cfu/ml) and CB is number of cells in NB without culture filtrate (cfu/ml).

Bacterial identification

Cell shape and gram stain of 24 hours culture were examined by phase contrast microscope to determine the cell morphology. The tests for motility, catalase, growth, colony and spore-forming were carried out by the method described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986; Atlas, 1997; Forbes *et al*., 1998).

The effective isolate was grown on NA plate by inoculating one colony into 5 ml of NB medium under aerobic condition by shaking at 150 rpm at temperatures of 5∂C, 10∂C, 30∂C, 45∂C, 55∂C and 65∂C, at pH 5.7 and 6.8 with NaCl concentration of 2%, 5%, 7% and 10% for 18-24 h, as described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986). Then, the growth of each strain was observed by the turbidity of the broth culture measured by spectrophotometer at 600 nm, as previously described (Nitisinprasert *et al*., 2000).

Carbohydrate fermentation patterns of the effective strain were determined, simultaneously at temperature 45∂C with API 50CH Rapid fermentation strips (API, BioMérieux, France) in CHB medium as specified

by the manufacturer. The results of biochemical test and carbohydrate fermentation were determined for 24-48 h. The data were analyzed by the APILAB II Plus program.

Part of 16S rRNA gene of the effective isolate was investigated as described by Nitisinprasert *et al.* (2000). Chromosomal DNA was isolated by a modified method of Anderson and Mckay (1983), in which the alkaline denaturation step was omitted. This DNA was used as a template for the polymerase chain reaction (PCR) that was conducted according to Newton and Graham (1997). The oligonucleotide primers 8 UA (forward primer: 5'-AGAGTTTGATC CTGGCTCAG-3') and 1407B (reverse primer: 5'-GACGGGCGGTGTGTAC-3'), were used to amplify about 1.4 kb fragment from the 16S rRNA gene. All amplification reactions were carried out in a DNA thermal cycler (PCR machine, Touchdown Hybaid) and the program was performed as follows: 1 cycle of 5 min denaturation at 94∂C and further 35 cycles consisting of (i) 1 min denaturation at 94∂C, (ii) 2 min primer annealing at 55∂C and (iii) 2 min primer extension at 72∂C. After the 35th cycle, the extension reaction was continued for another 15 min at 72∂C to ensure the completion of the final extension step. The PCR product was purified by using QIAEX II Gel Extraction Kit (QIAGEN Inc, USA) and then ligated into TA-cloning pGEM-T easy vector (Promega, USA), using the manufacturer's protocol. Recombinant plasmid DNA were prepared from positive clones and sequenced on both strands using T7 and SP6 sequencing primers on an ABI Prism DNA Sequencer model 3100, at the Bio Service Unit (BSU), Thailand. Analysis of the sequence was performed with the ABI Prism Sequencing Analysis version 3.7. The resulting sequences were compared with the non-redundant nucleotide database at GenBank by using the BLAST program.

Enzyme production

Enzyme production was performed in 500 ml Erlenmeyer flask containing 300 ml of enzyme producing medium (PM) (Abe *et al.*, 1994). The medium composition was as followed: 1% locust bean gum, 3% poly peptone, 1.5% KH_2PO_4, 0.06% $MgSO_4$. $7H_2O$ and 2.5% (v/v) corn steep liquor, pH 7.0 and the medium was sterilized at 121∂C for 15 min. The culture was incubated at 150 rpm, 45∂C for 24 hr.

Enzyme characterization

Optimum pH To determine the pH effects, the mannanase activity was assayed at various pHs as described previously by using locust bean gum (LG) as a substrate. The reaction mixture containing 0.01 g of locust bean gum, 0.5 ml pH buffer and 0.5 ml of enzyme extract was incubated in water bath at 45 ∂C for 30 min. The buffer systems used for pH optima study were: 0.1 M citric acid buffer (pH 3, 4, 5, 6), 50 mM potassium phosphate buffer (pH 7, 8) and 0.2 M glycine buffer (pH 9, 10).

Optimum temperature The effect of temperature was determined in a reaction mixture containing 0.01 g of locust bean gum, 0.5 ml of enzyme extract and 0.5 ml 50 mM potassium phosphate buffer (pH 7.0) at 30, 35, 40, 45, 50, 55 and 60 ∂C for 30 min.

Temperature stability Effect of temperature on enzyme stability was investigated. Enzyme was incubated in water bath at 40, 50, 60 and 70 ∂C and sample collected at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 hr, respectively. After sample collection, the reaction mixture containing 0.01 g of locust bean gum, 0.5 ml of enzyme extract and 0.5 ml potassium phosphate buffer (pH 7.0) was assayed at 50 ∂C for 30 min, according to the above method.

RESULTS AND DISCUSSION

Isolation and primary screening

Twenty-three soil samples were primary screened for effective microbes. A total of 23 isolates (19 bacterial isolates and 4 fungal isolates) showed clear zone of mannanase activity in isolation medium (BIM) or (FIM) using locust bean gum as substrate at 45∂C (Table 1). Isolates NT 6.3 and NT 6.4 showed the highest ratio of F clear zone/F colony of 6 in locust bean gum medium and also isolates NT 6.5, NT 6.6 and NT 6.7 showed a ratio of F clear zone/F colony of 4 which was higher than other isolates.

Secondary screening

The secondary screening was conducted with twenty-three isolates from primary screening. They were grown in enzyme producing medium (PM) and then the culture filtrate were assayed

Table 1 Mannanase activity of 23 isolates, expressed as ratio of F Clear zone/F Colony.

Source	Isolate	F Colony (mm)	F Clear zone (mm)	Ratio of F clear zone/F colony
	Bacteria			
Soil from	NT 1.1	1.5	3	2
Nakhon	NT 1.2	2.5	4	1.6
Pathom	NT 6.1	1	3	3
	NT 6.2	2	4	2
	NT 6.3	0.5	3	6
	NT 6.4	0.5	3	6
	NT 6.5	0.5	2	4
	NT 6.6	0.5	2	4
	NT 6.7	0.5	2	4
	NT 6.8	1	2	2
	NT 6.9	0.5	1	2
	NT 6.10	0.5	1	2
	NT 6.11	0.5	1	2
	NT 6.12	0.5	1	2
	NT 16.1	2	4	2
	NT 17.1	2	4	2
	NT 17.2	2	4	2
	NT 17.3	2	4	2
	Fungi			
	NT 2.1	10	11	1.1
	NT 2.2	11	12	1.09
	NT 2.3	9	10	1.11
	NT 2.4	9	9.5	1.05
	Bacteria			
Sludge from Nakhon Sawan	NV 1.1	4	6	1.5

for mannanase activity. The highest mannanase activity was 0.306 units/ml for bacterial isolate NT 6.7 (Figure 1). All isolates were observed for their effective pathogen inhibition and promotion of lactic acid bacteria growth. The results of *Salmonella* serovar Enteritidis S003 inhibition and promotion were shown in Figures 2 and 3 and the inhibition and promotion *Escherichia coli* E010 were shown in Figures 4 and 5, respectively. The promotion and inhibition of *Lactobacillus reuteri*

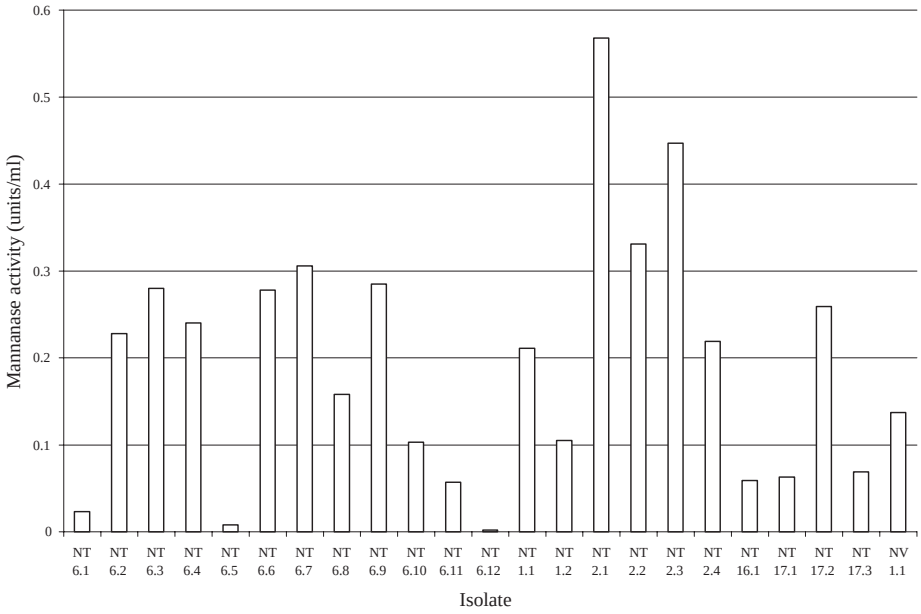

Figure 1 Mannanase activity of selected isolates using copra meal as substrate

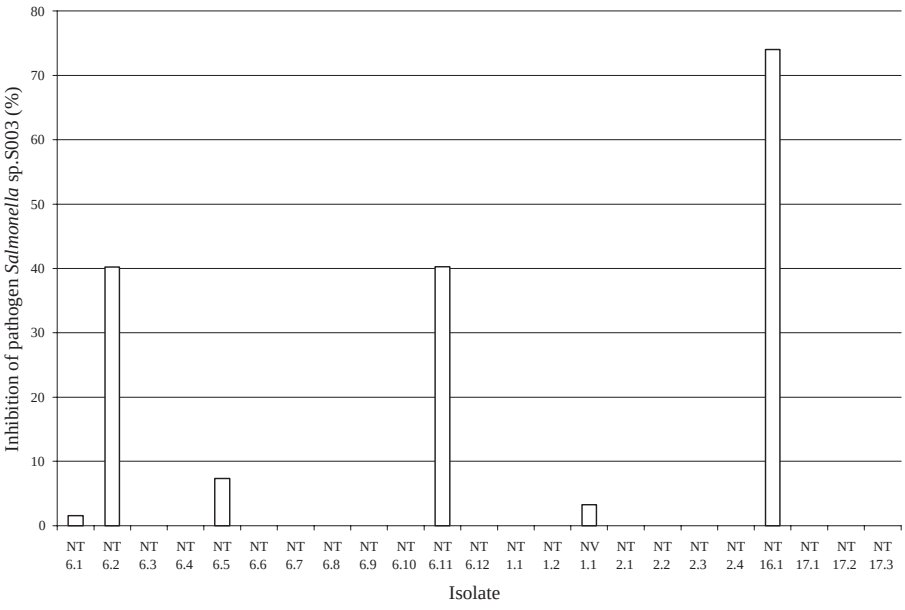

Figure 2 Inhibition of pathogen *Salmonella* serovar Enteritidis S003

AC-5 were shown in Figures 6 and 7. Therefore, the best isolate was isolate NT 6.7 because the isolate NT 6.7 showed the highest mannanase activity (0.306 unit/ml). The mannanase activity of isolate NT 6.3 and NT 6.4 were 0.280 unit/ml and 0.240 units/ml respectively. Moreover, the isolate NT 6.7 could promote *Lactobacillus reuteri* AC-5 up to 49.593% and strongly inhibit *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010. However, the isolate NT

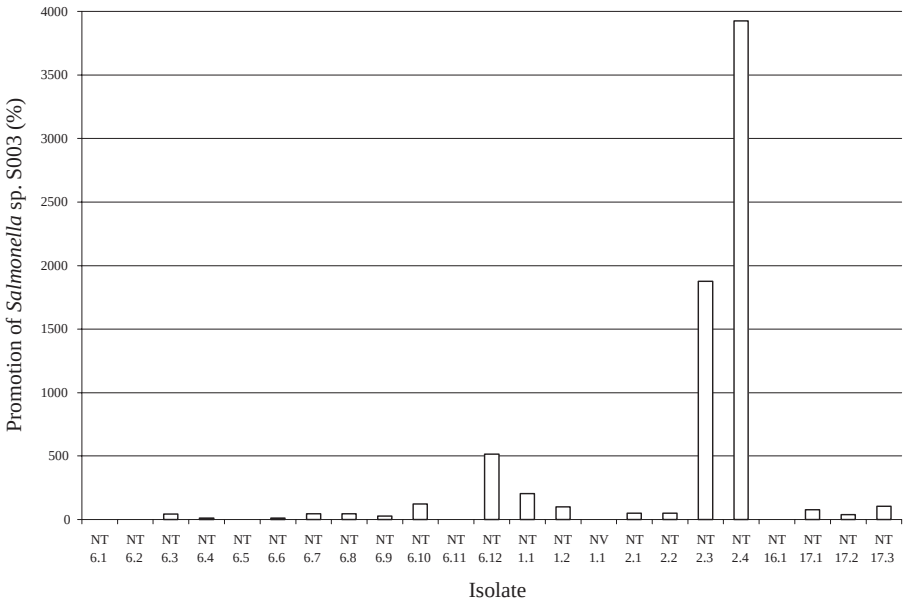

Figure 3 Promotion of pathogen *Salmonella* serovar Enteritidis S003

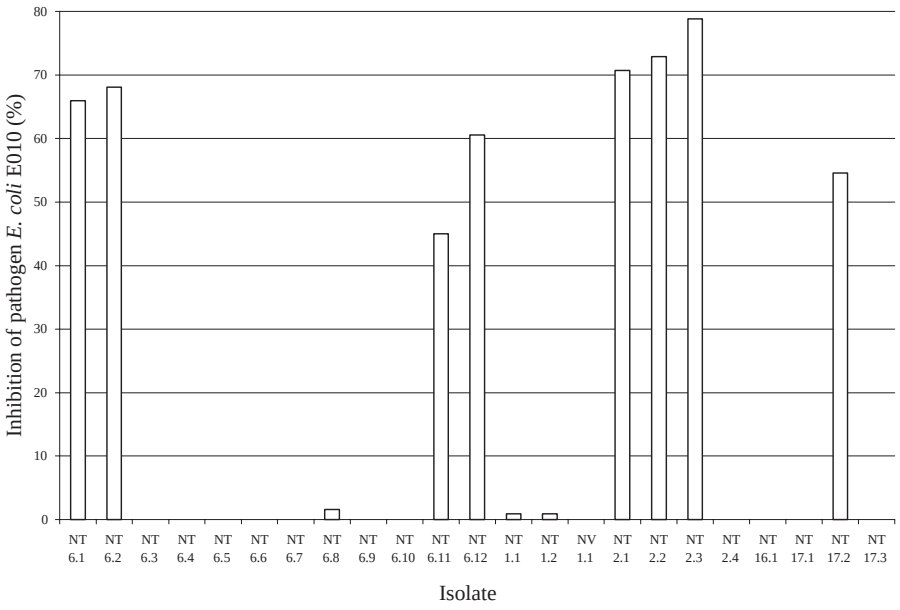

Figure 4 Inhibition of pathogen *Escherichia coli* E010

6.3 and NT 6.4 could promote *Lactobacillus reuteri* AC-5 up to 536.111% and 527.778%, but they could not inhibit *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010.

Identification of isolate NT 6.7

Isolate NT 6.7 was observed for its morphology and motility. The colony of isolate NT 6.7 on agar plate was puncti form, raised, entire and white. Its cell was gram-positive, short rod, catalase-positive, growth-aerobe, non-motile and spore-center forming when grown at 45∂C for 24 hrs. It could grow in NB broth supplemented with 2%, 5%, 7% and 10% NaCl and NB broth adjusted

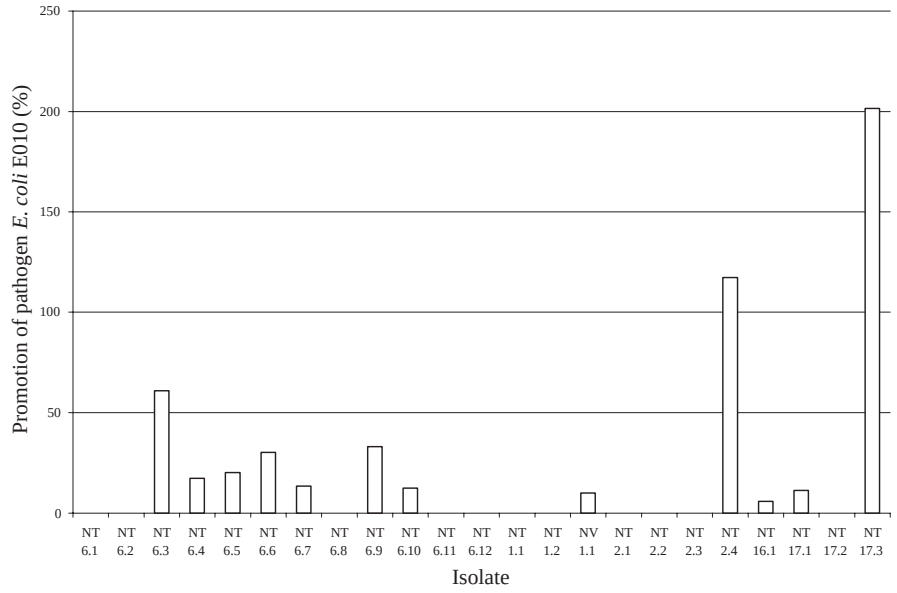

Figure 5 Promotion of pathogen *Escherichia coli* E010

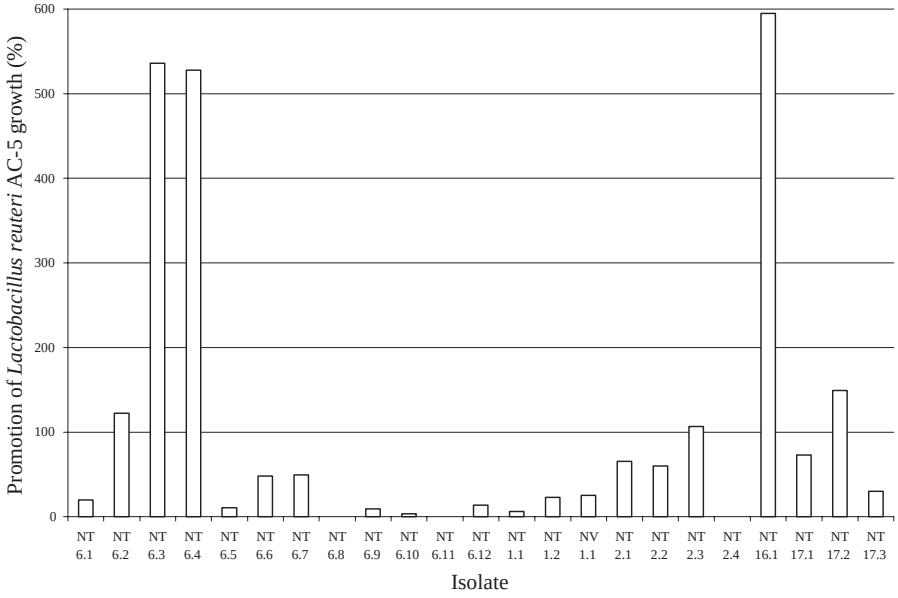

Figure 6 Promotion of *Lactobacillus reuteri* AC-5

to pH 5.7 and pH 6.8. Moreover, the isolate NT 6.7 was able to grow at 30-55∂C. All the results directly matched with database of *Bacillus circulans* as described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986).

Isolate NT 6.7 was tested for carbohydrate fermentation and ability to utilize various carbon sources by using API 50 CBH kit, at 45∂C for 24-48 hrs. It was able to metabolize carbon sources API 50 CBH kit, but was unable to ferment some of them such as glycerol, inositol, sorbitol, esculin and D-raffinose. With API kit database, the closest match for isolate NT 6.7 was proposed to be *Bacillus circulans* with 99.5 % identity. The carbohydrate fermentation pattern was also similar to the mannanase producing *Bacillus circulans* from previous studies (Heck *et al.,* 2005).

The PCR amplification of the 16S rRNA gene gave a product of about 1.4 kb for isolate NT 6.7. The 16S rDNA was sequenced on both strands at Bio Service Unit (BSU), Thailand. Analysis of the DNA sequences by BLAST program comparing to the database of the GenBank (http://www.ncbi.nlm.nih.gov) revealed that isolate NT 6.7 showed highest level of similarity (99 % identity) to *Bacillus circulans*. Isolate NT 6.7 could be assigned to *Bacillus circulans* because the results of their morphological, physical, and biochemical tests were consistent with the 16S rDNA alignment.

Enzyme characterization

Optimal pH The effects of buffer pH on mannanase activities were determined by using the culture supernatant of *Bacillus circulans* NT 6.7. The activities at pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, were determined at 45∂C for 30 minutes using locust bean gum as substrate. The *Bacillus circulans* NT 6.7 mannanase exhibited broad pH optimum from 6.0 to 9.0 (Figure 8). The relative enzyme activities were not different in pH range 6.0-9.0; higher than 95 % of highest enzyme activity (2.799 units/ml, pH 9.0). The optimal pH of *Bacillus circulans* NT 6.7 mannanase was similar to that reported in *Bacillus subtilis* KU-1 (Zakaria et al., 1998) and *Aspergillus niger* NCH-189 (Lin and Chen, 2004) but different from that

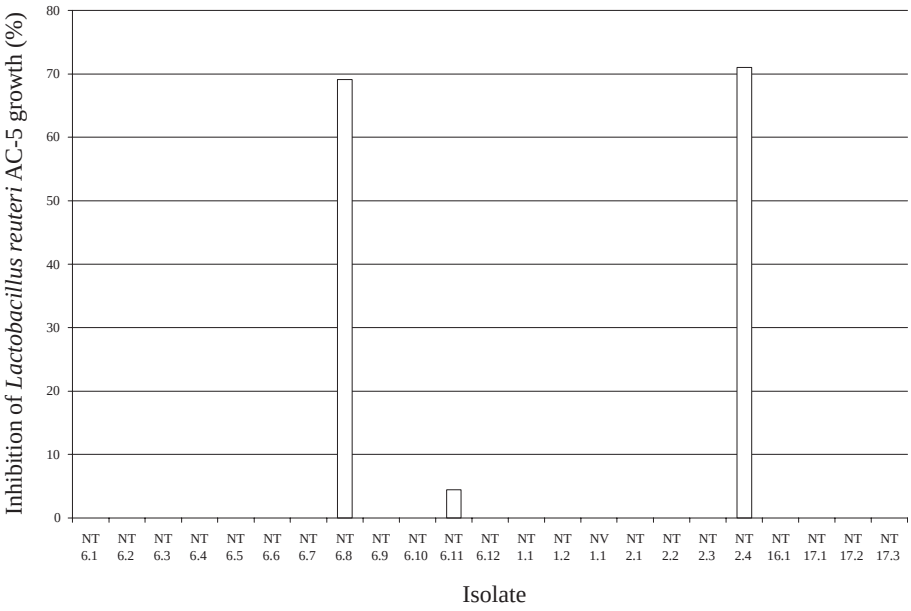

Figure 7 Inhibition of *Lactobacillus reuteri* AC-5

reported in *Trichoderma harzianum* T4 (Ferreira and Filho, 2004) and *Sclerotium rolfsii* (Sachslehner and Haltrich, 1999), in which optimal pH was at pH 3.0.

Optimal temperature The optimum temperature of mannanase from *Bacillus circulans* NT 6.7 was observed by assaying enzyme at various temperatures of 30, 35, 40, 45, 50, 55 and 60∂C, at pH 7.0 using locust bean gum as substrate. The optimal temperature of *Bacillus circulans* NT 6.7 mannanase was at 50∂C, as shown in Figure 9, similar to that of *Aspergillus niger* (Ademark et al., 1998) and *Bacillus subtilis* KU-1 (Zakaria et al., 1998) but lower than that reported in *Trichoderma harzianum* T4 (Ferreira and Filho, 2004) and *Bacillus* sp. KK01 (Abe et al., 1994), of which the optimum temperature was 55 and 60∂C, respectively.

Thermal stability The thermal stability of mannanase from *Bacillus circulans* NT 6.7 was studied at 40, 50, 60 and 70∂C. The result was shown in Figure 10. The *Bacillus circulans* NT 6.7 mannanase was stable at 40-50∂C for 6 hrs. Enzyme activity rapidly decreased at 60 and 70∂C. Compared with other mannanases, the range of thermal stability of *Bacillus circulans* NT 6.7 enzyme was quite wide and the remaining activity at the extremes (40 and 50∂C) was satisfactorily high. Interestingly, the enzyme activities gradually increased after 3 hr of incubation at 40 and 50∂C. This might be the effects of crude enzyme, inhibitors, etc. Moreover, the thermal stability of mannanase from *Bacillus circulans* NT 6.7 was similar to that of *Bacillus subtilis* KU-1 (Zakaria *et al*., 1998) and *Sclerotium rolfsii* (Sachslehner and Haltrich 1999). However, it was lower than that reported for *Bacillus* sp. KK01, 55∂C (Abe *et al*., 1994) and *Trichoderma harzianum* T4, 55-70∂C (Ferreira and Filho, 2004).

CONCLUSION

Twenty-three soil samples from 4 areas in Thailand were isolated and screened for mannanase producing microorganism. A total of 23 bacteria and fungi isolates were grown in copra meal medium broth (CM) at 45∂C and 19 bacteria and 4 fungi showed clear zones in locust bean gum medium (LG) of agar plate. Isolate NT 6.7, demonstrating broad inhibition pathogens of

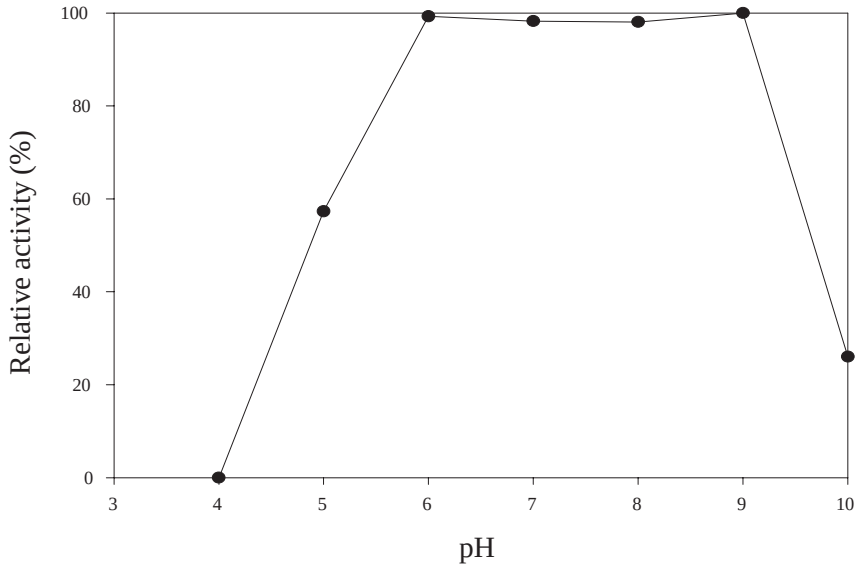

Figure 8 Optimal pH of mannanase from *Bacillus circulans* NT 6.7

Salmonella serovar Enteritidis S003 and *Escherichia coli* E010 with promotion *Lactobacillus reuteri* AC-5 were selected. Based on morphological, physiological, biochemical and molecular methods, this bacterium was identified as *Bacillus circulans*. The *Bacillus circulans* NT 6.7 showed high activity with optimum pH 6.0-7.0, optimum temperature at 50∂C and thermal stability at 40-50∂C. As the products from copra-mannan (CM) hydrolysis by beta-mannanase, mannooligosaccharides (MOS) have been be expected to be one kind of prebiotic, the effective mannanase and its host have been studied. This research could provide basic data to the capability

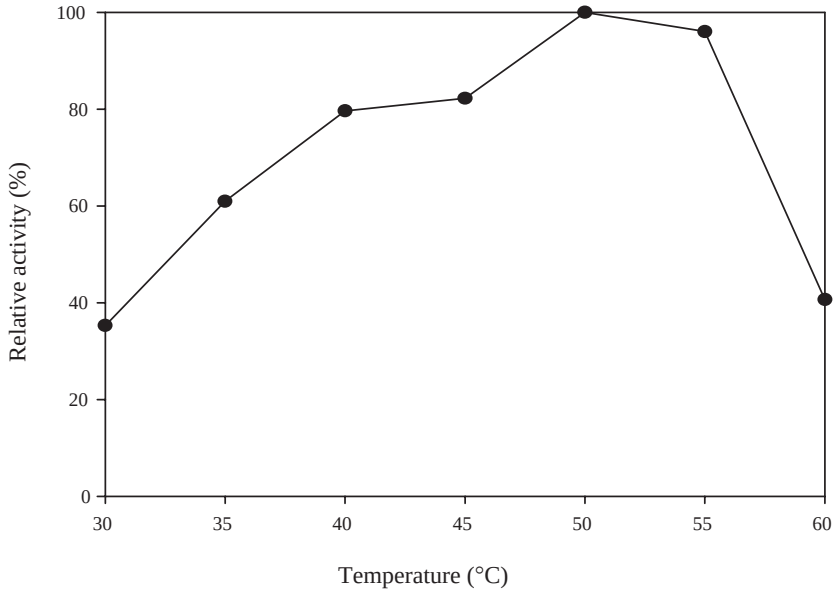

Figure 9 Optimal temperature of mannanase from *Bacillus circulans* NT 6.7

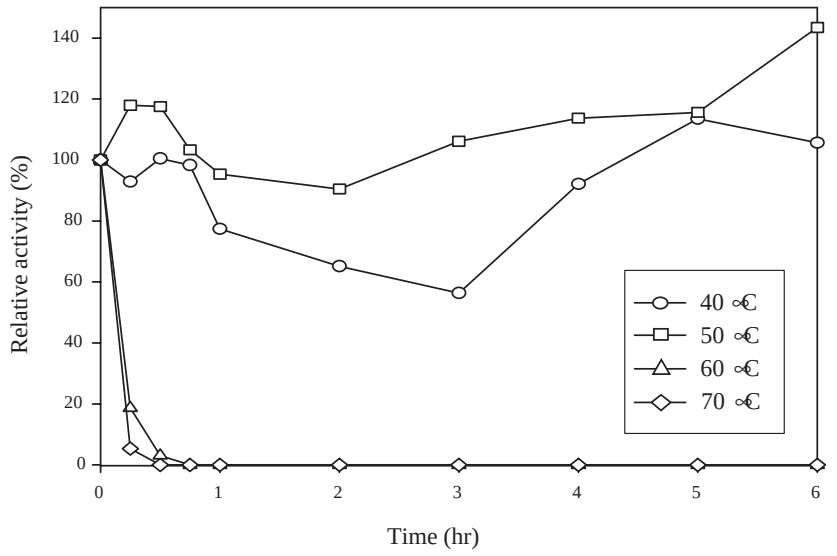

Figure 10 Thermal stability of mannanase from *Bacillus circulans* NT 6.7

of this mannanase application on MOS preparation and improvement the nutrient values, metabolized energy and the digestibility of CM in future.

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