ISOLATION AND CHARACTERIZATION OF MANNANASE PRODUCING MICROORGANISM

INTRODUCTION

The copra meal is abundant agricultural waste which is discharged by coconut industries. It can not be fully utilized as ingredients for monogastric animals feed due to high content of non-starch polysaccharides or soluble fiber (Mendoza *et al.,* 1994) which is a limitation of its utilization in high level (Purwadaria *et al*., 1995). Previously, samples of defatted copra meal by-product from industry was analyzed and found that 60-70% of the carbohydrate content was composed with beta-mannan. Beta-mannan is a linear polysaccharide composed of repeating β-1,4 mannose and α -1,6 galactose units attached to the beta-mannan backbone. These structures can be hydrolyzed to manno-oligosaccharides by beta-mannanase producing microorganism.

Beta-mannanase (Endo-1,4-β-D-mannanase, EC 3.2.1.78) catalyzes the random cleavage of β-D-1,4 mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galactoglucomannan and mannan (Stoll *et al.,* 1999). Mannanases from different organisms have been studied; bacteria, fungi, higher plants and animals (reviewed by Dekker and Richards, 1976 and Viikari, *et al.,* 1993). The interest in β-mannanase and other hemicellulose-degradinding enzymes has recently increased, because of their potential application in the food industries (Viikari *et al.,* 1993, 1994). The products from β-D-mannan hydrolysis by beta-mannanase is manno-oligosaccharides which was expected to be prebiotics, a non-digestible food ingredient (oligosaccharides), that beneficially affects the host by selective stimulation on the growth and activation of one or a limited number of bacteria in the colon (Gibson, 1999). Most potential prebiotics are carbohydrates, either polysaccharide such as inulin, or oligosaccharide including fructooligosaccharides (FOS) and galactooligosaccharides (GOS). The fermentation of those carbohydrates by the intestinal microbiota results in end products that are beneficial to the host for health promotion. To obtain the effective prebiotic, specific enzyme activities and their substrate are needed to be considered. Using manno-oligosaccharides, as an idea

or principle to produce prebiotic from copra meal, is expected to be useful to improve the growth performance of animal. It has also been reported that manno-oligosaccharides is a special nutrient or growth promoter for probiotics, such as *Bifidobacterium* sp. and *Lactobacillus* sp. Manno-oligosaccharides could also be effective in reducing colonization of type 1- fimbriated, α-D-mannose-sensitive agglutination, *Escherichia coli* and *Salmonella serovar* Enteritidis.

This thesis has researched on isolation and characterization of mannanase producing microorganism from soil. Thus, the mannanase treatment has been potential to improve the useful nutrients from the lower cost and quality feed ingredients such as copra meal that have high amounts of mannan content. The products of mannanase degradation might be specific oligosaccharide called prebiotic, which support the growth of probiotic of both human and animal. In addition, mannanases are also useful in other fields, including biobleaching of pulp, bioconversion of biomass wastes to fermentable sugar, reducing the viscosity of coffee extracts and detergent industry.

OBJECTIVES

The objectives of this study are:

1. To isolate, screen and identify mannanase producing microorganism

2. To characterize crude mannanase

LITERATURE REVIEW

1. Coconut tree

Coconut, one of the most valuable plant to man, is a poly-nuances plant. The tree can be found in most tropical climates, growing to 27 m or more tall and about 30-45 cm in diameter. The narrow, long, rigid leaflets compose the leaves, which are of great length 12 feet or more. The flowers are yellowish-white and the fruits, borne in clusters of 10-30 and weight about 1.2-2.0 kg (Taffin, 1999). In Thailand, the coconut palm is called "Ma-Praw". It can regenerate in sandy soils and areas of tropics, originated in tropical Asia or Polynesia and distribution of the coconut palm extends over most of the tropical islands. It tolerates at low-high pH, heat, insects, poor soil, salt, sand and slope that is between approximately 25° N and 25° S (reported from Indochina-Indonesia and Hindustani, 2001), which Asia is the major production region, with an estimated 91% of the total cultivated area of 11 million hectares (Taffin.1999). The three countries, Indonesia, Phillippine and India, have cultivated approximately 75% of the total production with 8 million hectares and also on some places outside the tropic zone such as: Africa, South America, and North America.

Palmae, the palm family to which the coconut belong to, is one of the oldest and most diverse of the plant families. Palms have many botanical characteristics such as woody trunk in many species, perennial growth, leaves which are folded like a fan and the production of a single seed. There have been sixty other species under the genus *Cocos*, but the coconut palm stands by itself that within the genus *Cocos* only one species, nucifera. The coconut tree (Coconut palm) "*Cocos nucifera L*", is in the palm family (Taffin, 1999).

There is a considerable number of varieties of coconut worldwide. Two major classes of coconuts are typically recognized on the basis of approximation: tall and dwarf (Table 1). The ones most commonly planted for commercial purposes are the tall tree varieties, which are slow to mature and first flower, six to ten years after

planting and height 27 m or so. They produce medium-to-large size nuts and have a life span of sixty to seventy years. The dwarf tree varieties may have originated as a mutation of tall tree types, which may grow to height 2 m and begin flower after three years, their life span is only about thirty years. They are normally self-pollinated, are early bearing and have a high annual bunch yield, so they are important in breeding program (Santos and Sangare, 1992).

Table 1 Basic characterization of Tall and Dwarf coconuts

Traits	Tall	Dwarf
Geographic distribution	Widely distributed and commercials value	Less widely distributed and generally of non Commercial
Stem circumference	Enlarged and with a bulbous base	Thin, with a cylindrical or tapering base
Mode of pollination	Mostly crossed	Mostly selfed
Pigmentation of nuts and petiole of leaves	Most are mixtures of green and brown	Either pure greens, or browns, yellows and reds
Height increment per year	Greater than 50 cm	Less than 50 cm
Years to beginning of productive maturity	Late (5-7 years)	Early (3-4 years)
Useful life span	More than 50 cm	Less than 50 cm

Table 1 (Continued)

Traits	Tall	Dwarf
Nut size (whole)	Very small to large	Very small to medium
Phenotyping variation		
- within cultivar	High	Low
- between cultivar	High	High
Root distribution	Generally more dense plentiful	Less dense and few
Reaction to adverse conditions	Generally less sensitive	Sensitive to hypersensitive
Culture requirement	Average	High input required
Leaf and bunch attachment	Very strong	Fragile

Source: Santos and Sangare (1992)

The report from Agricultural statistics of Thailand crop 2001/02 has shown that coconut tree are generally cultivated in Thailand (Table 2). The Southern part of Thailand, especially Nakhonsrithammarat province are the biggest cultivation area.

2. Copra and copra meal

The using of the coconut palm, and its products, are probably more extensive than those of any other plant. The coconut tree begins to yield fruit in 5-6 years on good soils, more like 7-9 years, and reachs full bearing in 12-13 years. Fruit set to maturity is 8-10 months from setting of female flowers, then the cell wall break down to form a liquid, the coconut milk, which containing actively multiplying cells (Taffin, 1999). After 12 months, the nut is followed by a hardening stage when the cell membranes are laid down and become attached to seed coat, while the liquid phase lose more and more organic matter, it means, the nut must be harvested fully ripe for making copra meal or desiccated coconut. In some areas nuts are allowed to fall naturally, and collected regularly that the copra meal has a white and 10-15 mm in thick (Taffin, 1999). Data on the composition of the fruits for Tall and Dwarf palm ecotypes in the plant collection of the Marc Delorme Station in Cote d'Ivoire are given in Table 3.

The copra meal may be cured by sun-drying, or by an oven-drying, or by a combination of both. Sun-drying requires 6-8 consecutive days of good bright sunshine and the copra meal reduces moisture content of the wet meat from 50-55% to 5-6% (Thampan, 1975). After that the copra is stored in well-ventilated, dry area. Good desiccated coconut should be white in color, crisp, with a fresh nutty flavor, and should contain less than 20% moisture and 68-72% oil. The major producing countries are the Indonesia and Phillipines (Choct, 2001). The yield of copra meal in the world in 2000 was given in Table 4.

Table 2 The coconut tree planted area in Thailand.

Provinces	Area of cultivation (rai)	Yield (kg/rai)
Songkla	26.906	89.17
Nakornsrithammarat	116.633	998.20
Pattharung	15.412	1.160
Stull	5.349	1.119
Chanthaburi	19.547	487
Trad	12.850	721
Sakaew	3.113	1.089

Source: Agricultural statistics of Thailand crop year (2001/02)

Table 3 Weight of different fruit components from various sources

Cultivar	Origin	Weight (g)				
		Fruit	Husk	Shell	Water	Endosperm
Tall	Thailand	1851	575	273	449	553
Tall	Cote d'Ivoire	1161	544	173	110	334
Dwarf	Sri Lankan Green	365	198	48	21	98
Dwarf	Malayan Yellow	743	262	102	132	246

Source: Taffin (1999)

Copra is the local south pacific name for dried sections of the meaty inner lining of the coconut seed. It is the principal commercial product derived from the coconut palm, and is used primarily as a source of coconut oil. To remove the oil, copra meal is pulverized between rollers, steamed and pressed at a pressure of about 500 kg per square cm (about 6500 lb per square inch). High-quality copra usually contains about 60 to 65 percent oil, so the coconut oil makes up about 20 percent of all vegetable oils used in the world. It is a common ingredient in margarine, vegetable shortening, salad oils, and confections. And also used in the manufacture of soaps, detergents, and shampoos because it has high levels of lauric acid, an ingredient that gives soap a quick-lathering property. The remaining residue, coconut oil cake, was used as a protein and energy rich cattle feed. In Thailand, the coconut meal is also used as food and animals feed.

Moreover, copra meal is not suitable for feed because it contains a rather large amount of fiber and its amino acid composition is not quite acceptable (Yoshikawa *et al.*,2000). Chemical composition of copra meal residue, which 91% is dry matter containing approximately 52% Nitrogen free extract and 19% crude protein content. And also the high fiber content in copra meal seriously limits its use in poultry diets. The fiber is high in polymer called mannans that have low digestibility and often have a laxative effect in poultry and swine (NRC, 1998).

Samples of defatted copra meal were obtained from Phillipines and analyzes for protein, carbohydrate, fat and fiber content by the analytical services of Woodson and Tanent Latbs. These samples of copra meal contained about 48% carbohydrate, 5% lignin, 21% protein and nearly 10% fat (Luis, 2000). The nutrient values of defatted copra meal were highly variable in quality as shown in Table 5. Additionally, analysis of copra meal showed that there was 60-70% of the carbohydrate in beta-mannan or a complex derivative of beta-mannan as galactomannans (Luis, 2000).

In Thailand, the copra meal contained low level of lysine and histidine (Outhai, 1986). Considering chemistry composition per 100 g(% dry matter), the copra meal contains 10.0-13.3% moisture, 6.0-41.6% fat, 14.3-19.8% protein, 32.8-

45.3% carbohydrates, 8.9-12.2% fibers, 1.0-5.7% ash and ME value 3080 kcal/kg, 10 mg Ca, 24 mg P, and 1.7 mg Fe. In United States of America, the nutrients in copra meal was, as dry matter, 83.7%, protein 50-73%, fat 100%, and total starch 94.1%. The digestibility of copra meal depended on processing in oil extraction especial temperature in production, while the digestibility of amino acids may be further reduced due to excessive temperature during processing. The amino acid composition of copra meal is inferior to many other protein sources and also it is still deficient in important essential amino acid such as lysine, methionine, threonine and histidine but high in arginine (NRC, 1998).

3. Mannans

Mannans are the major polysaccharide of softwood hemicellulose. Depending to the predominant sugar in their main chain and structure, accounting for 15-20% (dry basis) in softwood, 5% in hardwoods. Many mannan-based carbon sources have been used to cultivate microorganism. These included locust bean gum (Ademark *et al*., 1998 and Puchart *et al*., 2003), guar gum (McCutchen *et al*., 1996), and konjac flour (Oda *et al*., 1993). Mannans are main chain of galactomannan, glucomannan, galactoglucomannan and mannan (Stoll *et al*., 1999), and are β-1,4 backbone commonly with branched side chain such as α-1,6-linked galactosyl residues (Khanongnuch *et al*., 1999 and Abe *et al*., 1994). It is mostly found as heteroglycans, an important structural components part of cell walls in higher terrestrial plants, including the endosperm of coconut seeds (Ooi and Kikuchi, 1995 and Tamaru *et al*., 1995) and copra meal (Hossain *et al*., 1996). Therefore, mannan polysaccharides, a component of hemicellulose, was found mainly as storage carbohydrates in the bulbs and endosperm of some plants. For example, all legumes, guar gum, guar beans from *Cyamopsis tetragonolotus*, locust bean gum from *Ceratonia siliqua*, ivory palm nuts from *Phytephus macrocarpa*, coffee beans, coconut seed and the bulb of konjac (Ooi and Kikuchi, 1995).

Table 4 The yield of copra meal in the world by selected countries

Country	Yield (million tons)	% of Total
India	10.0	20
Indonesia	14.7	30
Phillipine	12.0	25
Thailand	1.44	3
Other	4.1	9

Source: Choct, 2001.

Table 5 Proximate composition (%) of coconut meal

Composition	Dried copra meal		Fresh copra meal[c]
	Thailand[a]	Unite State of America[b]	
Moisture	10	8.0	78.93
Crude protein	20-25	19.2	1.09
Crude fiber	12	14.4	2.93
Oil	6	2.1	7.17
Ash	7	2.5	0.21
Metabolizable energy, ME	3.080	1.525	-

Source: [a]Outhai, 1986.
[b]NRC, 1998.
[c]Jarurat, 1985.

4. Mannan degrading enzyme

Ivory-nut mannan has been hydrolysed and identified D-mannose in the hydrolysate (Matheson, 1996). A range of polysaccharides that contain D-mannose has been found in plant sources (Ademark *et al*. 1998), and these include polymer based on a (1→4)-β-linked mannan backbone. These polysaccharides could be divided chemically into mannan, glucomannan, galactomannan and galactoglucomannan.

The galactomannan structure is the major structure of copra meal as shown in Figure 1. The complete hydrolysis of the structure is required a number of different catalytic activities. Endo-mannanase or beta-D-mannanase cleaves between adjacent mannose resides of the mannan backbone (1,4-β-D-mannan mannohydrolase, EC.3.2.1.78) and exo-acting β-mannosidases (β-D-mannopyranoside hydrolase, EC.3.2.1.25) are necessary for the hydrolysis of mannobiose. The α-1,6-D-galactose side chains of galactomannan are removed by the action of α-galactosidase (α-galactoside galactohydrolase, EC.3.2.1.22) (Halstead *et al*., 2000).

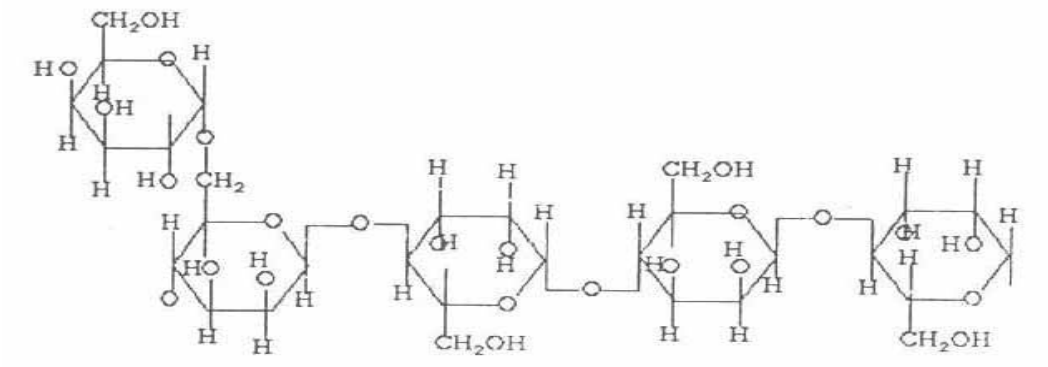

Figure 1 Structural features of galactomannan

Source: Duffaud *et al*., (1997)

4.1 Source of beta-mannanase

Beta-mannanase are widely distributed. They can be found in prokaryotes, eukaryotes and higher eukaryotes, including protozoa, insects, snails and germinating plant seeds. Beta-mannanase from various bacterial and fungal sources have been studied extensively as shown Table 6.

Beta-mannanases are useful in many fields including biobleaching of pulp in paper industry (Gübitz *et al.,* 1996), bioconversion of biomass wastes to fermentable sugars (Chandrakant and Bisaria, 1998), upgrading of animal feed stuff (Ray *et al.,* 1982) and reducing the viscosity of coffee extracts (Hashimoto and Fukumoto, 1969). They also have potential application in the production of mano-oligosaccharides, which are utilized selectively by intestinal *Bifidobacterium spp* and used as valuable food sweetener or additive (Tomotari, 1990).

Hossain *et al.,* (1996) has studied the beta-mannanase from *Bacillus* sp. KK01 which was isolated from soil. They found that the culture medium while using copra meal as carbon source contained 2 unit/ml in mannanase activity. This was corresponding to the study of Abe *et al.* (1994) who isolated a bacterium from soil using copra meal in crude mannan preparation. The results showed that the bacterium belonged to genus *Bacillus* and could produce mannanase effectively.

In 1996, fifty actinomycetes strains were screened for the production of mannanase activity during growth in both liquid and solid media containing locust bean gum (Montiel *et al.,* 1996). *Streptomyces scabies* CECT 3340 and *Stremtomyces ipomoea* CECT 3341 which had mannanase activity of 294.3 and 242.9 unit/ml, respectively, were selected.

Table 6 Beta-mannanase producing microorganism.

Sources	References
Bacteria	
Bacillus sp. AM-001	Akino *et al*., 1988
Bacillus sp. KK01	Hossain *et al*., 1996
Bacillus licheniformis	Feng *et al*., 2003
Bacillus stearothermophilus	Talbot *et al*., 1990
Bacillus subtilis	Ratto and Poutanen, 1988
Vibrio sp. MA-138	Tamaru *et al*., 1995
Vibrio sp. MA-128	Araki *et al*., 1992
Vibrio sp. MA-129	Araki *et al*., 1992
Cellulomonas fimi	Stoll *et al*., 1999
Cadocellum saccharolyticum	Bicho *et al*., 1991
	Gibbs *et al*., 1992
Caldicellulosiruptor sp. RT8B4	Gibbs *et al*., 1992
Clostridium butyricum strain Antonie Leewenhoek	Xiuzhu *et al*., 1991
Streptomyces sp.	Takahashi *et al*., 1984
Streptomyces scabies	Monitel *et al*., 1999
Streptomyces ipomoea	Monitel *et al*., 1999
Streptomyces lividans 66	Areand *et al*., 1993
Enterococcus casseliflavus FL 2121	Oda *et al*., 1993
Pseudomanas sp.	Yamamura *et al*., 1990
Pseudomanas fluorescens supsp. *Cellulose*	Braithwaite *et al*., 1993
	Bolam *et al*., 1996
	McCutchen *et al*., 1996
Thermotiga neapolitana 5068	Duffaud *et al*., 1997

Table 6 (Continued)

Sources	References
Thermotiga maritime	Brown *et al*., 1993
Thermomonospora fusca	Hilge *et al*., 1998
Thielavia terrestris	Araujo *et al*., 1990
Polyporus versicolor	Johnson and Ross, 1990
Acromonas sp.	Arald, 1983
Fungi	
Aspergillus niger	Tsujisaka *et al*., 1972
	Ademark *et al*., 1998
	Ademark *et al*., 2001
	Yamazaki *et al*., 1976
Aspergillus niger NCH-189	Lin and Chen, 2003
Aspergillus carbonarius	Ghareib and Nour-el-Dien, 1994
Penicillium simplicissimum	Luonteri *et al*., 1998
Penicillium ochrochloron	Dey *et al*., 1993
Rhodothermus marinus	Politiz *et al*., 2000
Aspergillus fumigatus	Puchart *et al*., 2003
Penicillium purpurogenum	Park *et al*., 1987
Penicillium sp. 23	Varbancts *et al*., 2001
Rhizopus niveus	Hashimoto *et al*., 1969
Trichoderma reesei RUT C-30	Zeilinger *et al*., 1993
Trichoderma reesei C-30	Arisan-atac *et al*., 1993

Table 6 (Continued)

Sources	References
Trichoderma harzianum E58	Torrie *et al*., 1990
Thermomyces lanuginosus	Puchart *et al*., 1999
Trichoderma reesei	Stalbrand *et al*., 1993
	Stalbrand *et al*., 1995
	Margolles-Clark *et al*., 1996
	Harjunpaa *et al*., 1995
	Tenkanen *et al*., 1997
	Buchert *et al*., 1993
Sporotichum cellulophilum	Araujo *et al*., 1991
Sclerotium rolfsii	Sachslehner and Haltrich, 1999
	Sachslehner and Haltrich, 1999
	Sachslehner *et al*., 1998
	Sachslehner *et al*., 1998
	Gubitz *et al*., 1996

Source: Chatchai Sa-nguansook (2002)

Wongkattiya (1998) has isolated 249 actinomycetes from roots and soil. Mannanase activity was detected from 38 isolates of actinomycetes by using gel diffusion assay with a substrate containing 0.1% (W/V) locust bean gum. Actinomycetes isolate number E2/22, identified as *Streptomyces* sp. E2/22 produced the highest mannanase activity in the medium using locust bean gum as carbon source.

Kataowa and Tokiwa (1998) isolated and characterized mannanase-producing anaerobic bacterium from soil and methanogenic sludges. They found that

Clostridium tertium KT-5A, from lotus soil had the highest mannanase activity. High yields of mannanase were obtained by inducing enzyme production with guar gum and bee extract/peptone as carbon and nitrogen sources, respectively.

Anyway, a new screening method for detection of beta-mannanase producing microorganism had been studied. The report of Kremnicky *et al.* (1996) using the 2 different dyed substrates, Ostazin Brilliant Red-galactomannan and Remazol Brilliant Blue xylan in the medium for screening 449 isolates of yeasts and yeast-like microorganism. The results showed that the frequency of mannanase activity was found within the genera *Stephanoascus* and *Aureobasidium*. The best producers of beta-mannanase were found to be the strain *Aurebasidium pullulans.*

4.2 Characteristic of beta-mannanase

Beta-mannanase from microorganism has been widely studied in fungi and bacteria but had less attention in yeast found. In 1996, Gübitz *et al*., had studied the purified beta-mannanase from *Sclerotium rolfsii*. This enzyme was purified to electrophoretic homogeneity by means of ultrafiltration, anion exchange chromatography and gel filtration. It has a molecular mass of 61.2 kDa and the pI value is in the acidic region at 3.5. The pH and temperature optima were 2.9 and 74˚C, respectively.

Ademark, *et al.,* 1998 purified beta-mannanase was achieved using only three steps by *A. niger*. The described method is simple and yields a high recovery of mannanase activity 46%, the specific activity was 3860 nkat ml^{-1} protein, which is six times the value obtained by McCleary (1988). The isoelectric point of the single beta-mannanase detected was 3.7 and the molecular weigh 40 kDa. However, optimal pH for β-mannanase activity was 3.5 and the enzyme proved to be stable in the pH 3.5-7 and optimal temperature at 50˚C.

Arisan-Atac *et al.* (1993) purified beta-mannanase, with the molecular mass of 46 kDa, from the *Trichoderma reesii* C-30. The enzyme has pI value of 5.2. It has an optimal pH at 5.0 and broad pH stability between 2.5 to 7.0. In addition, it is stable for 60 min at 55˚C and has an optimal temperature at 75˚C.

Besides, Stalbrand *et al.* (1993) has found two mannanases from *T. reesei.* The enzymes were purified by using ion-exchange chromatography, affinity chromatography and chromatofousing. Their molecular weights were determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and shown as 51 and 53 kDa while their pI did 4.6 and 5.4, respectively.

In addition, Lin and Chen (2004) enhanced mannanase production by submerged culture of *Aspergillus niger* NCH-189 and using defatted copra meal based media at 30˚C for pH 7.0.

Beta-mannanase from *Aspergillus niger* was purified to electrophoretic homogeneity in three steps using ammonium sulfate precipitation, anion-exchange and gel filtration chromatography (Ademark *et al.,* 1998). Its pI and Molecular weight 3.7 and 40 kDa, respectively.

Moreover, beta-mannanase from *Vibrio* sp. Strain MA-138 has been investigated by Tamaru *et al*. (1995). The enzyme was purified by ammonium sulfate precipitation, gel filtration, adsorption and ion-exchange chromatography. The molecular weight as determined on SDS-PAGE was 49 kDa while isoelectric point was 3.8. The purified enzyme exhibited maximal activity at pH 6.5 and 40˚C.

Akino *et al*. (1988) has characterized three beta-mannanase of an alkalophilic *Bacillus* sp AM-001. Molecular weight of the purified enzyme, M-I, M-II and M-III were 58, 59 and 42 kDa by SDS-PAGE while their pI were 5.9, 5.7 and 5.1, respectively. Michaelis constants (K_m) of the M-I of beta-mannan from copra, locust bean and konjac were 2.0, 3.8 and 7.7 mg ml^{-1} and their maximum velocities (V_{max})

corresponding to these saccharides were 730, 1470 and 1880 U mg^{-1} protein, respectively.

Puchart *et al*. (2003) has characterized two beta-mannanase of a thermotolerant fungus, *Aspergillus fumigatus* IMI 385708. Two types of MAN I and MAN II were characterized results in the molecular weight of 60 and 63 kDa, pI of 4.9-5.2 and 4.75-4.9, respectively. MAN I as well as MAN II showed highest activity at pH 4.5 and 60˚C and were stable in the pH range 4.5-8.5 and up to 55˚C.

4.3 Mode of action

Beta-mannanase (Endo-1,4-β-mannanase, EC.3.2.1.78) are hydrolytic enzymes which catalyze randomly of β-1,4 mannosidase linkages within the backbones of mannan, galactomannan, glucomannan and galactoglucomannan (Stoll *et al.,* 1999). As shown in the Figure 2, the several respects of the action pattern of the enzyme is very similar to that of beta-mannosidase, but there are differences in the nature of the preferred substrate and the anomeric configuration of the released D-mannose (McCleary, 1979 and 1988).

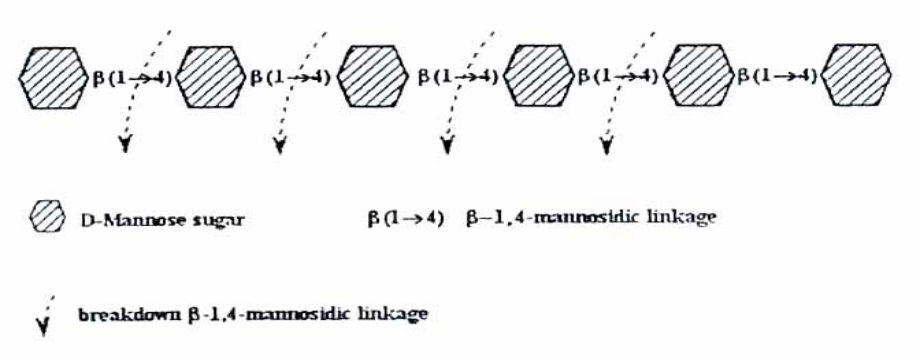

Figure 2 Mode of action of beta-mannanase and the 1,4-β-D-mannan chain

Source: McCleary, 1988.

The extent of hydrolysis of galactomannan by beta-mannanase depends on the degree of substitution and the distribution of the substituents, glucose in glucomannan and galactose in galactomannan (Stalbrand *et al.*, 1993).

Beta-mannanase has different mode of action on D-galactomannans and manno-oligosaccharides. The studies of McCleary, 1979 showed that the highly purified mannanase from *Bacillus subtilis* is able to hydrolyse coffee bean D-galactomannan to mannobiose, mannotriose and mannotetraose.

Corresponding the above studies, Sachslehner *et al*., 2000 reported coffee mannan, was isolated from green defatted Arabica beans, were efficiently hydrolyzed by the *Sclerotium rolfsii* mannanase. The results showed the significant reduction of viscosity of coffee extract and increased the reducing sugar content continuously due to the release of various manno-oligosaccharide including mannotetraose, mannotriose and mannobiose. Hydrolysis products were detected by TLC method.

In addition, the purified mannanase from *Sclerotium (Athelia) rolfsii*, with molecular weight of 46.5 kDa by SDS-PAGE exerts its activity on ivory nut. The results show that the main end-products mannotriose and to a lesser extent mannobiose inhibit its activity moderately (Sachslehner and Haltrich, 1999).

5. Benefits of beta-mannanase on diet improvement

In formulation of commercial poultry diets, roles of feed enzymes can be considered in two ways. First, the enzymes can be proved as addition to the normal formulation to give the benefit in feed efficiency. Second, enzyme inclusion allow an increasing in the metabolizable energy (ME) value, by increasing an availability of energy source from feed ingredients instead of more expensive oil. Research to define the effect of β-mannanase activity on digestion of galactomannans, reduced the anti-nutritional properties, found that they significantly improved the feed conversion of swine and broiler (Jackson *et al*., 1999, Teves *et al*., 1988) and also the absorption of nutrients such as starch, protein and fat. It was proposed that the enzymes acted by:

1. Benefits of β-mannanase come from the release of entrapped nutrients, by disrupting the cereal cell walls, allowing a greater digestion of encapsulation nutrients, which will improve the nutrient and energy utilization values.

2. Effects of enzymes in reducing digest viscosity from cause of the greater molecular mass of NSP, allowing increased movement of endogenous enzymes. Some experiments have shown significant improvements in nutrients digestibility in poultry after enzyme treatment.

3. Reducing the dry matter content of the excreted digest and the excreta output, which will improve litter quality, and possibly decrease the production of gas (NH_4, H_2S) in the poultry production.

4. β-Mannanase can reverse the negative impact caused by β-galactomannan interferes with glucose metabolism and insulin secretion rates in swine (Jackson *et al*., 1999) or blocking the inhibition function of β-mannanases. The suppression of insulin secretion can impair the intestinal uptake and untilization of glucose and amino acids in peripheral tissues by monogastric animals.

5. Making the nutrients component more available, resulting in a lower formula cost.

Information on the use of β-mannanase enzymes in diets containing copra meal (CM) is scarce, but from the ability of bacterial β-mannanase, many scientists attempted to used the enzyme from bacteria for animal feed production by trial as feed supplement in order to improved nutrient in the diets. Several experiments were conducted with broilers, and the data showed that the β-mannanase improved body weight and feed conversion ratio (FCR) by about 3% ($p<0.05$) (McNaughton *et al*., 1998). Other data form chicken studies also indicate that enzyme supplementation in corn/soybean diets improved ME by 143 kcal ME/kg feed. Since the content of mannose in soybean meal and corn is very low, it is difficult to understand the mechanism whereby mannanase can have such an effect. Some of promising and possible result have been recently obtained which related researches shown as follow:

Teves *et al*. (1988) reported on the nutritional value studies of the CM treated with bacterial β-mannanase in broiler diets, the result clearly indicate that mannanase treatment of CM improved the feed efficiency of broilers. This improvement is attributed to increased availability of nutrients and also apparent metabolizable enzyme in the mannanase treated CM and possibly due to improved amino acid balance.

Purwadaria *et al*. (1995) reported for an ability of β-mannanase to increase fiber digestibility *in vitro* after incubation of CM with *Aspergillus niger*. It is possible that a mannanase-based enzyme may improve the nutritive value of CM.

Dingle *et al*. (1997) attempted to use the β-mannanase supplementation of broiler diets containing 20% CM, three treatments consisted of corn/soy, corn/CM, corn/CM + enzyme added 1 kg/ton and corn/CM + enzyme added 2 kg/ton. They reported that body weight tended to increase with addition of both CM and enzyme at either level, however the increase was significantly only when comparing the enzyme supplemented CM diets with the corn/soy diet. And there was a trend forward improved FCR first with addition of CM then increasing level of enzyme addition.

James *et al*. (1998) discovered the improved use by turkeys of corn-soy diets with β-mannanase (Hemicell®). In turkeys, fed the 44% SBM had lower body weight (BW) and a higher average feed conversion ratio (FCR) than turkeys fed the 48% SBM (no enzyme). The β-mannanase can improve the BW and FCR ($p<0.05$) of the turkeys fed either 48% or 44% SBM for 1-18 weeks. It would appear that β-mannanase improves both energy and protein availability in lower quality feeds; an observation with major economic implications for turkey production.

Jackson *et al*. (1999) explained the capability of β-mannanase (Hemicell®) from *Bacillus lentus*, which is a fermentation product, for using in the diets. It contains high amounts of β-mannanase that degrade β-mannanase in feed, which claimed that a β-mannanase supplementation of a corn-soy type layer diet, in both low and high energy diets, increased egg weight, and hen-day and hen-house production in

hy-line birds. This study indicates that Hemicell® is capable of increasing egg production in laying hen, and the enzyme decreased feed consumption while maintaining performance of laying hen.

Odetallah *et al*. (2000) investigated the effects of β-mannanase, Hemicell®, and reported that some of the adverse effect of the soybean meal (SBM) anti-nutritional factors due to their highly viscous properties, which reduces feed conversion, in corn-SBM diets on turkey growth performance can be alleviated by dietary β-mannanase supplementation. Hemicell® supplementation generally improved performance of the hens, in these hens, Hemicell® improve body weight gain and FCR by 1% and 3%, respectively ($p<0.05$).

In the same year, Daskiran *et al*., 2000 studied on evaluation of a commercial β-mannanase (Hemicell®) supplementation on broiler performance in diets varying in β-mannan contain. Guar gum was used to alter the β-mannan level in a corn-soy based diets, increasing guar gum levels gradually decreased feed efficiency while enzyme supplementation improved feed efficiency. Enzyme efficiency on FCR declined with increasing enzyme concentration. These results indicate that the β-mannanase may improve the utilization of nutrients from diets high in β-mannan content.

In the light of a situation where fewer, if any, antibiotics will be allowed in poultry feed in the future. Perhaps the role of these oligosaccharides released from NSPs by enzymes hyhrolysis, needs to be investigated to assess their prebiotic effects as opposed to their anti-nutritive effects in poultry (Kocher *et al*., 1999). Because, the oligosaccharides have prebiotic properties, contribute to the maintenance of a healthy gut flora (Wallace and Chesson, 1995).

6. Prebiotics

A prebiotic is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health (Gibson and Roberfroid, 1995 and Van Loo *et al*, 1999). It is a substance selectively of the fermentation in the hindgut (Gibson, 1998), which modifies the composition of the colonic microflora in such a way that a few of the potentially health-promoting bacteria (especially lecto-bacilli and bifidobacteria) become predominant in numbers (Roberfroid, 1998). For example the oligosaccharides was prebiotics including fructo-oligosaccharides: FOS, Trans-galacto-oligosaccharides: TOS and Inulo-oligosaccharides: IOS (Jenkins *et al.,* 1999; Cruz *et al*., 1999; Van Laere *et al*., 2000; Bouhnik *et al*., 1997 and Yun *et al*., 1999).

There are various other oligomers that cannot be digested may also be prebiotics. These include some sugar alcohols, lactulose, as well as oligosaccharides that contain xylose, galactose, soya, maltose and mannose (Gibson, 1998). Recently, there are reported that manno-oligosaccharides produced from konjac glucomannan were found to be one of the best growth factors for lactic acid bacteria (Ooi and Kikuchi, 1995). And also there have been found to stimulate the growth of *Bifidobacterium* (Oda *et al*., 1993).

Important developments also include the use of prebiotics that have activities with multiple biological functions. Moreover, manno-oligosaccharides and oligosaccharides that have mannose side chains were also effective in reducing colonization of various bacteria including *Escherichia coli* and *Salmonella* sp. In intestinal tract of chicken (Gibson, 1998; Lyons, 1994 and Wallace and Chesson, 1995).

MATERIALS AND METHODS

1. Material

The coconut residual cakes, usually called copra meal, were collected from Pakkret Market, Nonthaburi, Thailand and used as a substrate from enzyme assay and 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.

2. 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.

3. Isolation and screening of microorganism producing mannanase

3.1 Isolation and enumeration

The sample, 1 g of solid sample or 1 ml of liquid sample, was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). The solution was mixed by reward Stomacher 80 Lab System with normal mode for 60 second. One percent (v/v) of the solution was transferred into 20 ml of sterilized isolated medium (IM); BIM, FIM and YIM (Abe *et al*., 1994) with 1% copra meal for bacteria, fungi and yeast isolation, respectively. The microbial cells were grown under aerobic condition by shaking at 150 rpm for 18-24 hr at 45˚C.

3.2 Primary screening

The culture broth from 3.1 were serial diluted and spread to isolate medium (BIM, FIM and YIM) containing locust bean gum instead of copra meal. Then, the cells were allowed to grow at 45˚C for bacteria, 18-24 hr and for fungi and yeast, 3-7 days. The colonies with a clear zone showed mannanase activity (Figure 3) and isolates were calculated ratio of diameter clear zone/diameter colony. The all of isolates were selected and kept in 20% glycerol at -87˚C for further study.

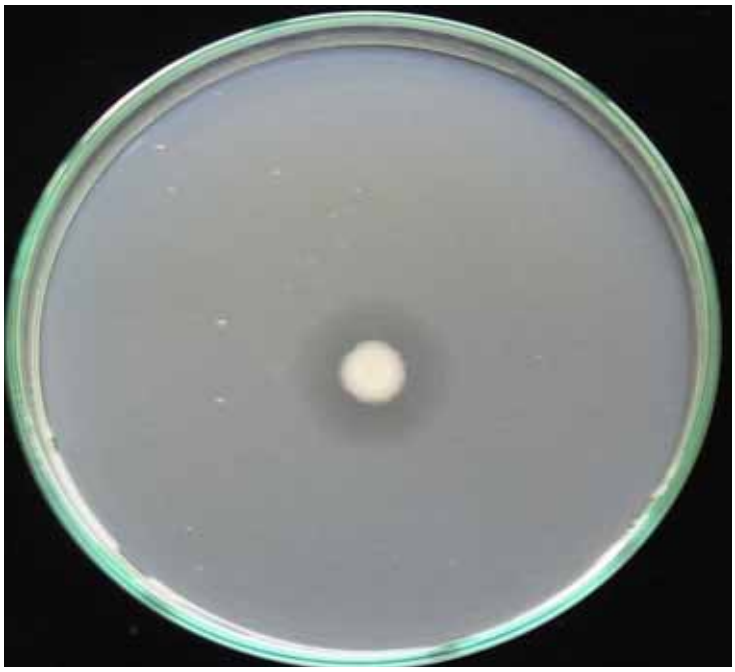

Figure 3 Clear zone of effective isolate in locust bean gum medium

3.3 Secondary screening

3.3.1 Mannanase activity

The positive colonies showing a clear zone was confirmed for mannanase activity. Enzyme production were performed in 500 ml Erlenmeyer flask containing 300 ml of producing enzyme medium (PM) (modified from Abe *et al.,* 1994). The composition was 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% Inoculum in pH 7.0. The flasks were shaken by incubator shaker at 150 rpm, 45˚C for 24 hr. Then, the culture broth was sampled daily, filtered by suction through Whatman No. 1 filter paper and centrifuged at 7.000 rpm, 4˚C for 15 min. The supernatant were collected and kept at -20˚C for further study.

Mannanase activity was assayed by performing the reaction mixture composing of sample, 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 experiment condition.

3.3.2 Effect of culture filtrate on lactic acid bacteria growth

The inoculum of *Lactobacillus reuteri* AC-5 was grown in 50 ml of MRS broth medium for 12-15 hr 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 medium and 5 ml of one plus 1% of the supernatant for 4 hr at 37˚C. After 4 hr of incubation, the cells number of their culture were determined by plate assay on MRS with 1.5% agar and grow at 37˚C for overnight. The isolate yielding culture filtrate

which showed promotion and inhibition of *Lactobacillus reuteri* AC-5 were selected for further study.

Enhance 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).

3.3.3 Effect of culture filtrate on pathogen *Salmonella serovar* Enteritidis S003 and *Escherichia coli* E010 growth

The inoculum of *Salmonella serovar* Enteritidis S003 or *Escherichia coli* E010 were cultivated on 50 ml of NB medium under aerobic condition by shaking at 150 rpm for 12-15 hr at 37˚C. After that, 1% of the inoculum, which was adjusted to Absorbance of 0.5 nm at 600 nm were transferred into 5 ml NB medium and 5 ml of NB plus 1% of the supernatant and grow at 37˚C by shaking at 150 rpm for 4 hr. The cell numbers of their culture were determined by plate assay on NB with 1.5% agar and grow at 37˚C for overnight. The isolate yielding culture filtrate showed inhibition and promotion of *Salmonella serovar* Enteritidis S003 and *Escherichia coli* E010. Then, they were kept on NB agar-plate for further study, as previously described (Nitisinprasert *et al.,* 2000).

Inhibition activity was determined as $\frac{[CB - SB]}{CB} \times 100$

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

4. Identification of bacteria producing mannanase

4.1 Determination of morphology and motility tests

The effective isolate was grown in 5 ml of NB medium under aerobic condition by shaking in 150 rpm for 18-24 hr at 45˚C. Then culture broth was streaked on NA medium and incubated at 45˚C for 24 hr.

Cell shape and gram stain of 24 hr culture broth were examined by phase contrast microscope to determined the cell morphology. The tests motility, catalase, growth, colony and spore-forming were investigated by the method described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975; Atlas, 1997; Forbe *et al*., 1998).

4.2 Determination of physical tests

The effective isolate was grown on NA plate by inoculating one colony from 24 hr of cell into 5 ml of NB medium under aerobic condition by shaking 150 rpm at temperatures of 5˚C, 10˚C, 30˚C, 45˚C, 55˚C and 65˚C, in pH of 5.7 and 6.8 with concentration NaCl of 2%, 5%, 7% and 10% for 18-24 hr, as described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975). 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).

4.3 Determination of biological tests

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 hr. The data put into the version 2.1 of MS DOS APILAB program.

5. Sequencing and analysis of the 16S rRNA gene

5.1 PCR of 16S rRNA gene of the effective isolate

Chromosomal DNA was isolated by a modified method of Anderson and Mckay (1983), in which the alkaline denaturation step was omitted. Briefly, the cells were re-suspended in SET buffer and treated with lysozyme and 20% SDS to break the cells and release their internal components. The cell lysate was deproteinized by successive extractions with organic solvents: phenol, phenol-chloroform-isoamyl and chloroform-isoamyl alcohol. After removing the RNA by treating with RNase A, the chromosomal DNA was precipitated with isopropanol followed by 70% ethanol. This DNA was used as a template for the polymerase chain reaction (PCR) that was conducted according to Newton and Graham (1997). Thus, the oligonucleotide primers 8 UA (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1407B (reverse primer: 5'-GACGGGCGGTGTGTAC-3'), previously utilized by Nitisinprasert *et al.* (2000), were used to amplify about 1.4 kb fragment from the 16S rRNA gene. PCR was carried out in a volume of 25 µl containing 10x buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosplate (dNTP), 1 µM of each primer (KU-Vector DNA Synthesis Unit, Kasetsart University), 1 U of *Taq* polymerase (Promega, USA) and about 200 ng of template DNA. All amplification reactions were carried out in a DNA thermal cycler (PCR machine, Touchdown Hybaid) and the program was performed as follows 6.7: 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 35^{th} cycle, the extension reaction was continued for another 15 min at 72˚C to ensure the completion of the final extension step. PCR products were analyzed by electrophoresis on 1.5% agarose gel using TBE buffer and stained with ethidium bromide at 2.5 µg/ml and visualized under UV-light in the gel doc/image analyzer.

5.2 Cloning and sequencing of the 16S rRNA gene of the effective isolate

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. The ligation reaction consisted of 2x Rapid Ligation buffer (5 µl), pGEM-T easy vector (1 µl), PCR product (1 µl), T4 DNA Ligase (1 µl) and ddH_2O (2 µl). The resulting ligation product was transformed into *E. coli* DH5α competent cells in accordance with the manufacturer protocol. Briefly, 2 µl of ligation product was added to 50 µl of competent cells and subjected to heat shock at 42˚C for 90 sec. After cooling on ice bath, 950 µl SOC medium was added to the cells and then incubated for 2 hr at 37˚C with shaking at 150 rpm. Then, 100 µl of the culture was plated on LB/Amp/IPTG/X-gal plates and incubated at 45˚C for 16-24 hr.

Selection of the positive clones with 1.4 kb PCR insert was based on the expressed blue/white phenotypes. White colonies were picked up and cultured in NB broth supplied with ampicillin (100 µg/ml) (Sigma), from which the recombinant plasmid DNA was isolated using the alkaline lysis procedure (Sambrook and Russell, 2001). Briefly, the cells was harvested and re-suspended in TEG buffer, into which lysis solution (NaOH and SDS) was added upon incubation on ice bath. The resulting suspension was neutralized with potassium acetate to remove the chromosomal DNA. The plasmid DNA was collected by precipitation with isopropanol followed by 70% ethanol. The precipitated DNA was treated with RNase and extracted with phenol-chloroform followed by chloroform-isoamyl alcohol. Further precipitation of the aqueous phase with NaOAC/glycogen/absolute ethanol provided the plasmid DNA. This plasmid DNA was subjected to restriction endonuclease analysis (REA). It was digested with *Eco*RI (Promega) that contained ddH_2O (23 µl), 10xbuffer H (3 µl), plasmid DNA (2 µl) and *Eco*RI (12 U/ml) (2 µl). The resulting reaction mixture was incubated for 1 hr at 37˚C and then analyzed by agarose gel electrophoresis in order to confirm the presence of insert corresponding to the size of the 16S rRNA gene.

Recombinant plasmid DNA templates 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.

6. Enzyme production

The positive colonies showing a clear zone was confirmed for mannanase activity. After the cultivation of organism and enzyme production were performed on 500 ml Erlenmeyer flask containing 300 ml of producing enzyme medium (PM) (modified from Abe *et al.,* 1994). The medium composition was 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 in pH 7.0. The medium was sterilized at 121˚C for 15-20 min.

6.1 Determination of growth temperatures

The effective isolate was cultivated in 5 ml of NB medium producing enzyme medium under aerobic conditions by shaking at 150 rpm for 24 hr at 45˚C. After 3% culture broth was transferred in 300 ml of producing enzyme medium (PM) by shaking 150 rpm at temperatures 30, 35, 37, 45 and 50˚C for collecting sample as: 0, 4, 8, 12, 16, 20 and 24 hr. And also the culture broth were diluted spread-plate for cells count, while the supernatant or filtrate culture was assayed for mannanase activity.

6.2 Determination of growth pH

The strain NT6.7 was cultivated in 5 ml of NB medium producing enzyme medium under aerobic conditions by shaking at 150 rpm for 24 hr at 45˚C. After 3% culture broth was transferred in 300 ml of producing enzyme medium (PM). The PM was adjusted pH: 4, 5, 6, 7 and 8 by shaking 150 rpm at temperature 45˚C for

collecting sample as: 12, 16, 20 and 24 hr. And also the culture broth were diluted spread-plate for cells count, while the supernatant or filtrate culture was assayed for mannanase activity.

6.3 Determination of carbon sources

The strain NT6.7 was cultivated in 5 ml of NB medium producing enzyme medium under aerobic conditions by shaking at 150 rpm for 24 hr at 45˚C. After 3% culture broth was transferred in 300 ml of producing enzyme medium (PM). The PM were used different carbon sources as: copra meal (CM), locust bean gum (LG), konjac flour (KF) and gaur gum (GG) by shaking 150 rpm at temperature 45˚C for collecting sample as: 8, 12, 16, 20 and 24 hr. And also the culture broth were diluted spread-plate for cells count, while the supernatant or filtrate culture was assayed for mannanase activity.

7. Enzyme characterization

7.1 Optimum pH

The mannanase activity was assayed as described in 3.3.1. Locust bean gum (LG) was used as a substrate for determination of pH effects. The reaction mixture containing 0.01 g of locust bean gum liquid, 0.5 ml pH buffer and 0.5 ml of enzyme extract was incubated water bath at 45 ºC for 30 min. The buffer systems used for pH optima study were: 0.1 M citric acid buffer (pH 3-6) and 0.2 M dibasic sodium phosphate buffer, 50 mM monobasic potassium phosphate buffer (pH 7-8) and 50mM dibasic potassium phosphate buffer, 0.2 M glycine buffer (pH 9-10) and 0.2 M sodium hydroxide buffer.

7.2 Optimum temperature

The mannanase activity was assayed as described in 3.3.1. Locust bean gum (LG) was used as a substrate for determination of temperature effects. The

temperature study, the reaction mixture containing 0.01 g of locust bean gum liquid, 0.5 ml of enzyme extract and 0.5 ml potassium phosphate buffer (pH 7.0) was incubated water bath at 30, 35, 40, 45, 50, 55 and 60 ºC for 30 min.

7.3 Temperature stability

The mannanase activity was assayed as described in 3.3.1. Locust bean gum (LG) was used as a substrate for determination of temperature stability effects. The temperature stability study, the reaction mixture containing 0.01 g of locust bean gum liquid, 0.5 ml of enzyme extract and 0.5 ml potassium phosphate buffer (pH 7.0) was incubated water bath at 40, 50, 60 and 70 ºC and collected sample: 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 hr, respectively. After was incubated at 50 ºC for 30 min and the reaction was assayed according to the above method.

8. Place

This study was conducted at the Molecular Biology Laboratory, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand and Department of Food Sciences and Technology, University of Natural Resources and Applied Life Sciences Vienna, Austria.

9. Duration

This study was carried out during June 2004 to September 2006.

10. Funding source

The financial expense of this study was covered by under Enzyme Technology for Sustainable Production of Health-Related Prebiotic Sugars: ASEAN-EU University Network Program (AUNP).

RESULTS AND DISCUSSIONS

1. Isolation and primary screening

Twenty-three soil samples were isolated for effective microbes. A total of the 23 isolates (19 bacterial isolates and 4 fungal isolates), showed clear zone of mannanase activity in bacteria isolate medium (BIM) or fungal isolate medium (FIM) by using locust bean gum as substrate at 45°C (Table 7 and Table 8). Isolate NT 6.3 and NT 6.4 showed the highest ratio of diameter of clear zone/ diameter of colony of 6 in locust bean gum medium and also isolates NT 6.5, NT 6.6 and NT 6.7 showed ratio of diameter of clear zone/ diameter of colony of 4 which was higher than other isolates.

Table 7 The sample sources and the effective isolates

Source	Sample No	Total colony (cfu/ml)	Effective colony (cfu/ml)
	Bacteria		
Soil area, waste fiber from coconut	NT 1	25	2
Soil area, waste coconut	NT 6	23	12
Waste oil in the tank	NT 16	65	1
Soil area, fermentable fertilizer	NT 17	25	3
	Fungi		
Waste copra meal	NT 2	13	4
Sludge of sugar from industry	NW 1	15	1

NT = Nakhon Pathom

NW = Nakhon Sawan

Table 8 Clear zone and mannanase activity of 23 isolates, expressed as ratio of diameter of clear zone/ diameter of colony

Isolate No	Diameter of colony (mm)	Diameter of clear zone (mm)	Ratio of diameter of clear zone/ diameter of colony
Bacteria			
NT 1.1	1.5	3	2
NT 1.2	2.5	4	1.6
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			
NW 1.1	4	6	1.5

2. Secondary screening

The secondary screening were conducted with twenty-three isolates from primary screening. They were grown in producing enzyme medium (PM) and then the culture filtrate were assayed for mannanase activity. The highest mannanase activity was 0.306 unit/ml for isolate NT 6.7 (Figure 4). All isolates were observed for their effective pathogen inhibition and promotion of lactic acid bacteria growth. The results of *Salmonella serovar* Eteritidis S003 inhibition and promotion were shown in Figure 5-6 and also the inhibition and promotion *Escherichia coli* E010 were shown in Figure 7-8, respectively. The promotion and inhibition of *Lactobacillus reuteri* AC-5 were shown in Figure 9-10. Therefore, the best isolate was isolate NT 6.7 because the isolate NT 6.7 showed the highest mannanase activity (0.306 unit/ml) for all bacteria. Mannanase activity of isolate NT 6.3 and NT 6.4 were 0.280 unit/ml and 0.240 unit/ml, respectively. Moreover, the isolate NT 6.7 could promote *Lactobacillus reuteri* AC-5 up to 49.593% and strongly inhibited *Salmonella serovar* Eteritidis S003 and *Escherichia coli* E010. However, the isolate NT 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* Eteritidis S003 and *Escherichia coli* E010.

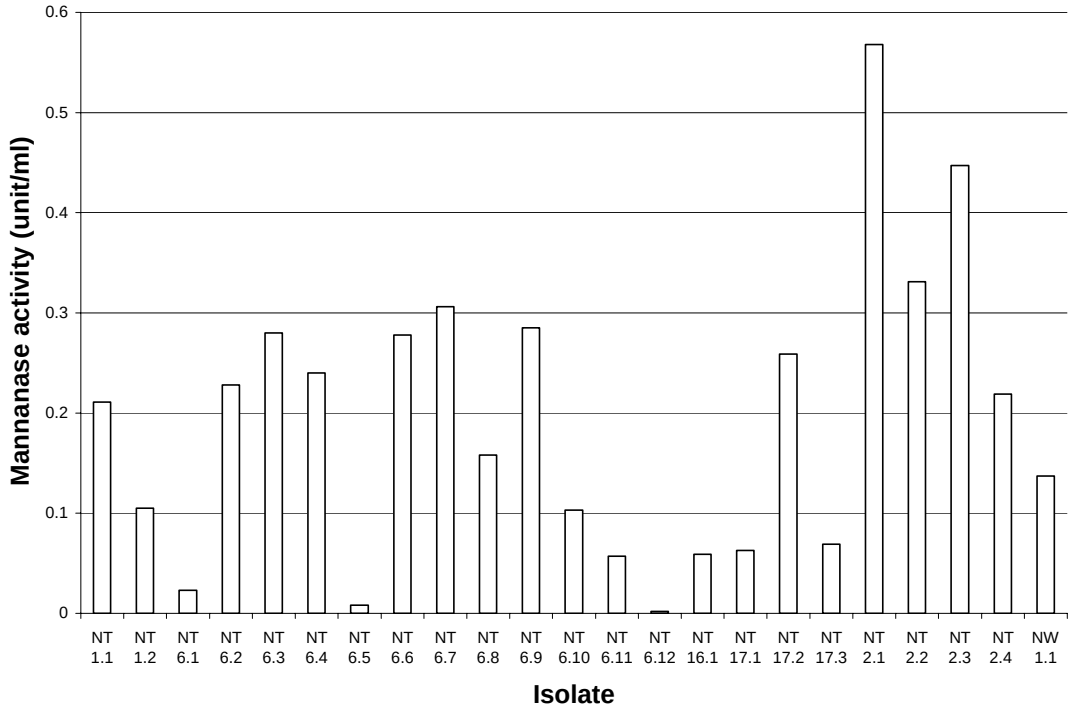

Figure 4 Mannanase activity of isolates by using copra meal (CM) as substrate

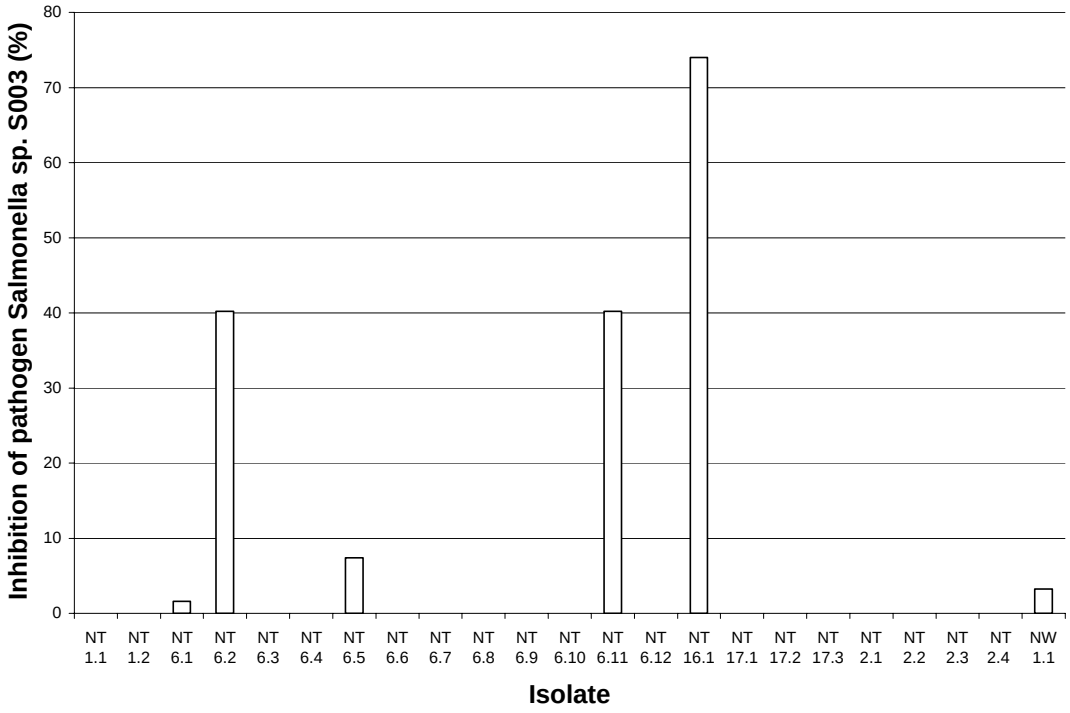

Figure 5 Inhibition of pathogen *Salmonella serovar* Eteritidis S003

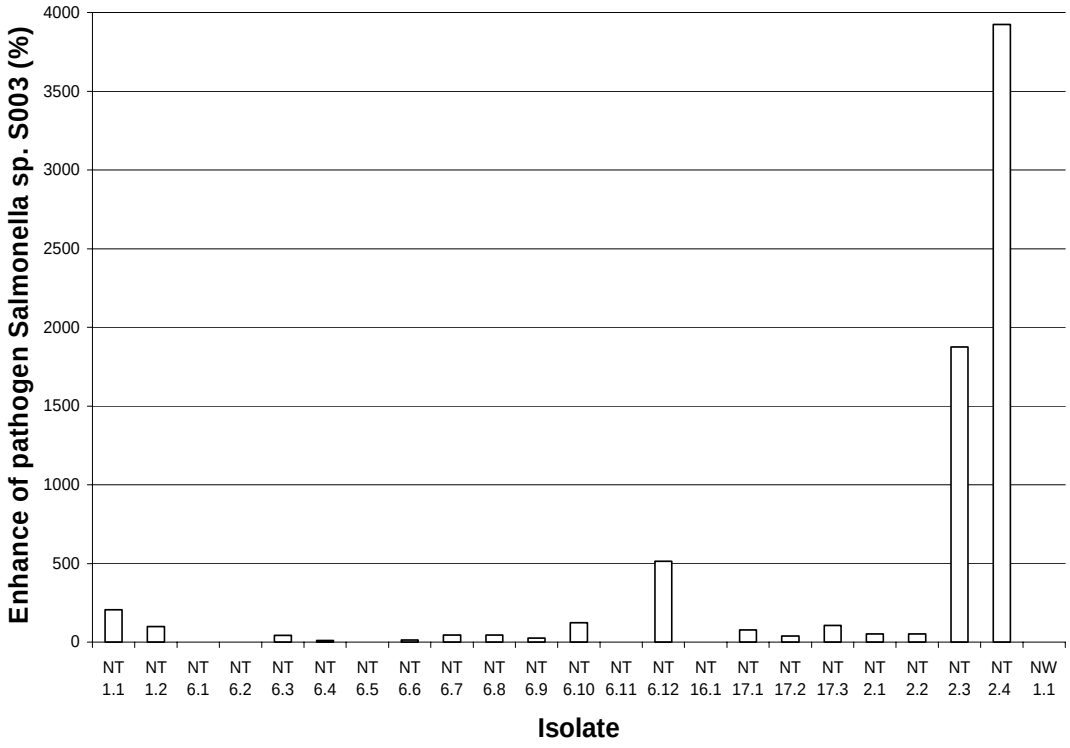

Figure 6 Enhance of pathogen *Salmonella serovar* Eteritidis S003

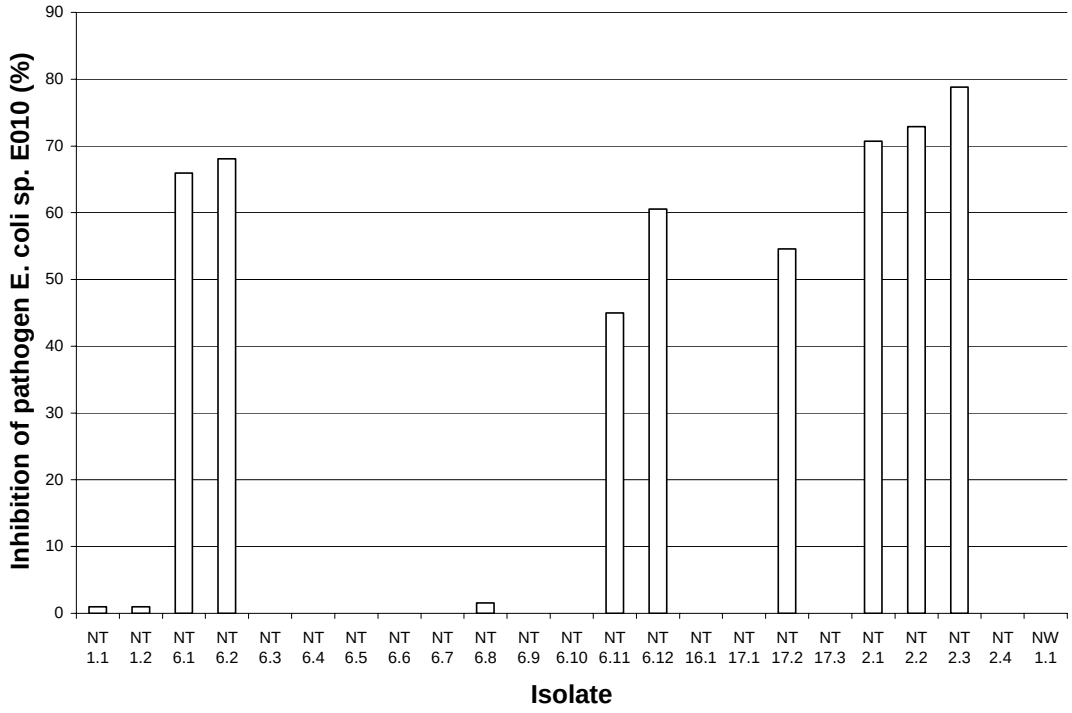

Figure 7 Inhibition of pathogen *Escherichia coli* E010

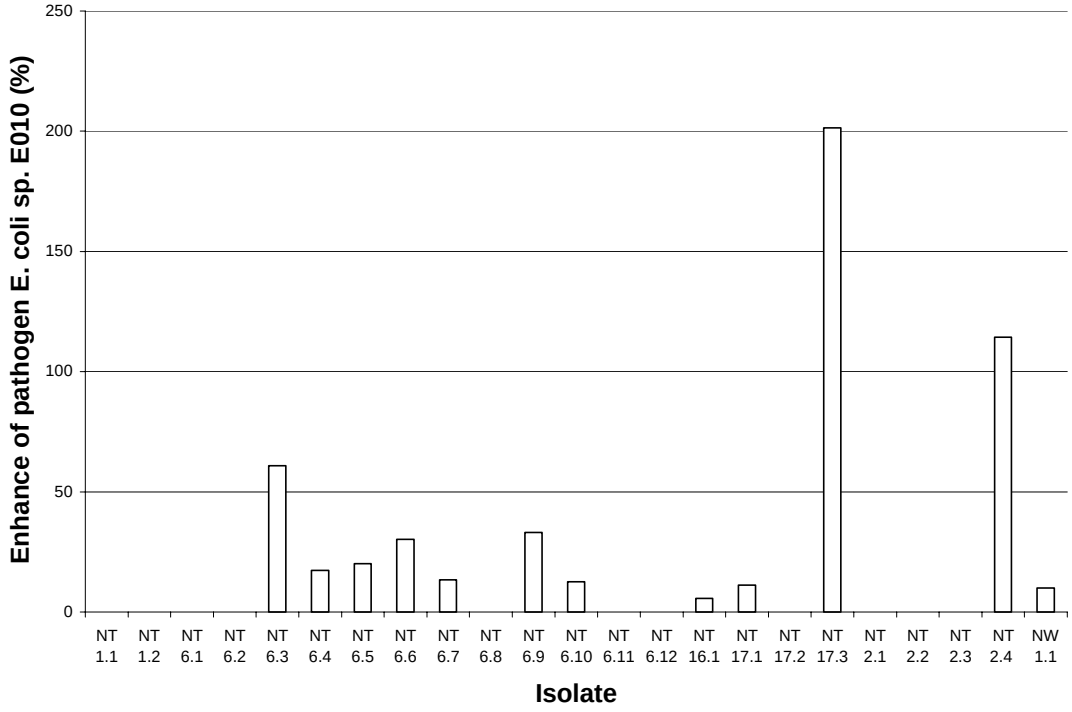

Figure 8 Enhance of pathogen *Escherichia coli* E010

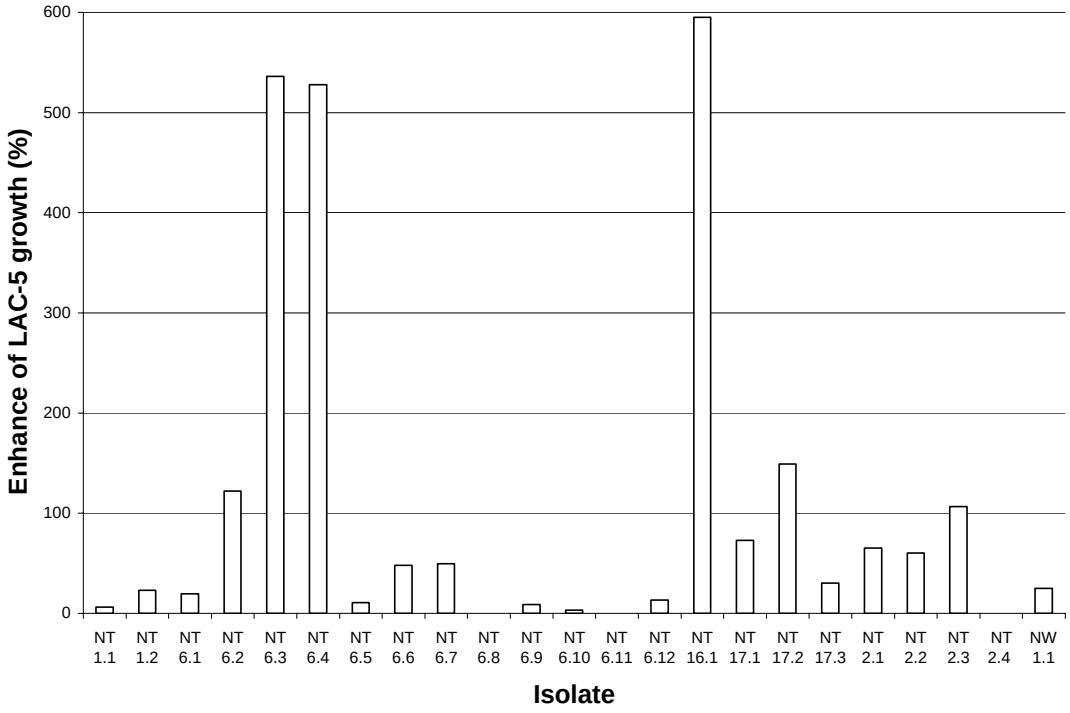

Figure 9 Enhance of *Lactobacillus reuteri* AC-5

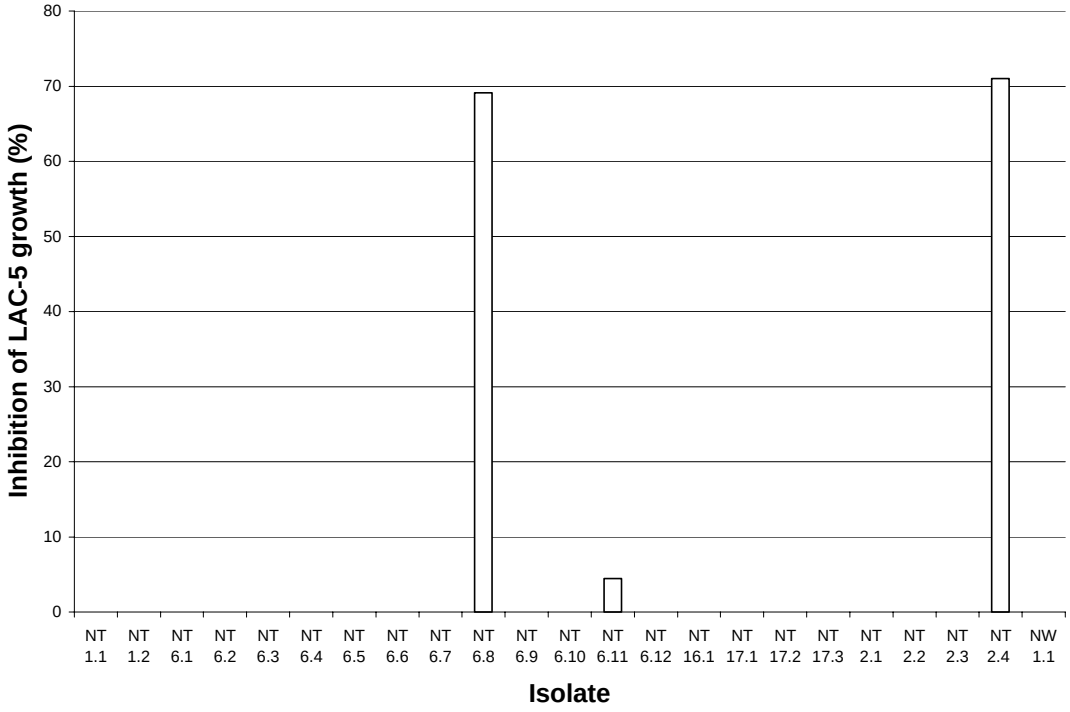

Figure 10 Inhibition of *Lactobacillus reuteri* AC-5

3. Bacterial identification of isolate NT 6.7

3.1 Morphology and motility

The isolate NT 6.7 was observed for its morphology and motility as shown in Table 9. The colony of isolate NT 6.7 on agar plate was punctiform, raised, entire and white. The isolate was Gram-positive, short rod, catalase-positive, growth-aerobe, non-motile and spore-center at 45°C for 24 hr as described in Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975).

Table 9 Morphology and motility of isolate NT 6.7.

Test	Morphology and motility	
	NT 6.7	*Bacillus circulans**
1. Morphology	Short rod	Short rod
2. Gram stain	+	+
3. Growth	Aerobe	Aerobe
4. Motility	Non-motile	Non-motile
5. Catalase	+	+
6. Spore	Central	Central
7. Colonies		
- Form	Punctiform	Punctiform
- Elevation	Raised	Raised
- Margin	Entire	Entire
- Colour	White	White

* data from Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975)

3.2 Physical test

As shown in Table 10, the isolate NT 6.7 was able to grow in NB broth, which supplemented with 2%, 5%, 7% and 10% of NaCl and this isolate could grow in NB broth adjusted to pH 5.7 and pH 6.8. Moreover, the isolate NT 6.7 grew at 30-55°C. All results were directed match database from Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975).

Table 10 Effective physiology of NT 6.7 strain and *Bacillus circulans*

Test	Characteristics and reaction	
	NT 6.7	*Bacillus circulans**
Growth at pH		
5.7	+	+
6.8	+	+
Growth at		
5°C	-	-
10°C	-	-
30°C	+	+
45°C	+	+
55°C	+	+
65°C	-	-
Growth in NaCl		
2 %	+	+
5 %	+	+
7 %	+	+
10 %	+	+

* data from Bergey's Manual of Systematic Bacteriology (Garvie, 1986, 1975)

3.3 Biochemical test

The isolate NT 6.7 were tested for carbohydrate fermentation and ability to utilize various carbon sources by using API 50 CBH kit, at 45°C for 24-48 hrs. Interestingly, it was able to metabolize carbon sources such as glycerol, inositol, sorbitol, esculin and D-raffinose (Table 11). With API kit database, the closet match for isolate NT 6.7 was proposed to be *Bacillus circulans* with 99.5 % identity. The carbohydrate fermentation pattern was similar to the mannanase producing *Bacillus circulans* from previous studies (Heck *et al.,* 2005).

Table 11 Carbohydrate fermentation patterns of isolate NT 6.7 and *Bacillus circulans*

Test	Strain	
	NT 6.7	*Bacillus circulans**
1. Glycerol	+	d
2. Erythritol	-	-
3. D-Arabinose	-	-
4. L-Arabinose	+	+
5. Ribose	+	+
6. D-Xylose	+	+
7. L-Xylose	-	-
8. Adonitol	-	-
9. β-Methyl-D-Xyloside	-	-
10. Galactose	+	+
11. D-Glucose	+	+
12. D-Fructose	+	+
13. D-Mannose	+	+
14. L-Sorbose	-	-
15. Rhamnose	+	+

Table 11 (Continued)

Test	Strain	
	NT 6.7	*Bacillus circulans**
16. Dulcitol	-	-
17. Inositol	+	d
18. Mannitol	+	+
19. Sorbitol	+	d
20. α-Methyl-D-Mannoside	-	-
21. α- Methyl-D-Glucose	+	+
22. N-Acetyl-D-Glucosamine	+	+
23. Amygdalin	+	+
24. Arbutin	+	+
25. Esculin	+	d
26. Salicin	+	+
27. Cellobiose	+	+
28. Maltose	+	+
29. Lactose	+	+
30. Melibiose	+	+
31. Sucrose	+	+
32. Trehalose	+	+
33. Inulin	+	+
34. Melezitose	-	-
35. D-Raffinose	+	d
36. Starch	+	+
37. Glycogen	+	+
38. Xylitol	+	+

Table 11 (Continued)

Test	Strain	
	NT 6.7	*Bacillus circulans**
39. β-Gentiobiose	+	+
40. D-Turanose	+	+
41. D-Lyxose	-	-
42. D-Tagatose	-	-
43. D-Fucose	-	-
44. L-Fucose	-	-
45. D-Arabitol	-	-
46. L-Arabitol	-	-
47. Gluconate	+	+
48. 2-Keto-gluconate	-	-
49. 5-Keto-gluconate	+	+

+, reaction production (positive); -, no reaction produced; d, 11-89 % of strains are positive; determined by API 50 CBH kit: isolate NT 6.7 was incubated at 45°C for 24-48 hr.

* data from Heck *et al.,* 2005.

3.4 PCR of 16S rRNA gene

The PCR amplification of the 16S rRNA gene gave a product of about 1.4 kb for the isolate NT 6.7. PCR product of the isolate NT 6.7 (Figure 11), were ligated with pGEM-T Easy vector and then transformed into competent cells of *E. coli* DH5α. The plasmid DNA was isolated and checked for the presence of insert by restriction endonuclease analysis. The recombinant plasmid DNA, containing the 16S rRNA gene, was sequenced on both strands by Bio Service Unit (BSU). Analysis of the DNA sequences by BLAST program (http://www.ncbi.nlm.nih.gov) revealed that the isolate NT 6.7 sequence (Figure 12) showed highest level of similarity (99 % identity) to *Bacillus circulans*. This result was consisted with the conventional method, morphological test (Table 9), physical test (Table 10), and biochemical test (Table 11) of API 50 CBH and confirmed that the isolate NT 6.7 was *Bacillus circulans*.

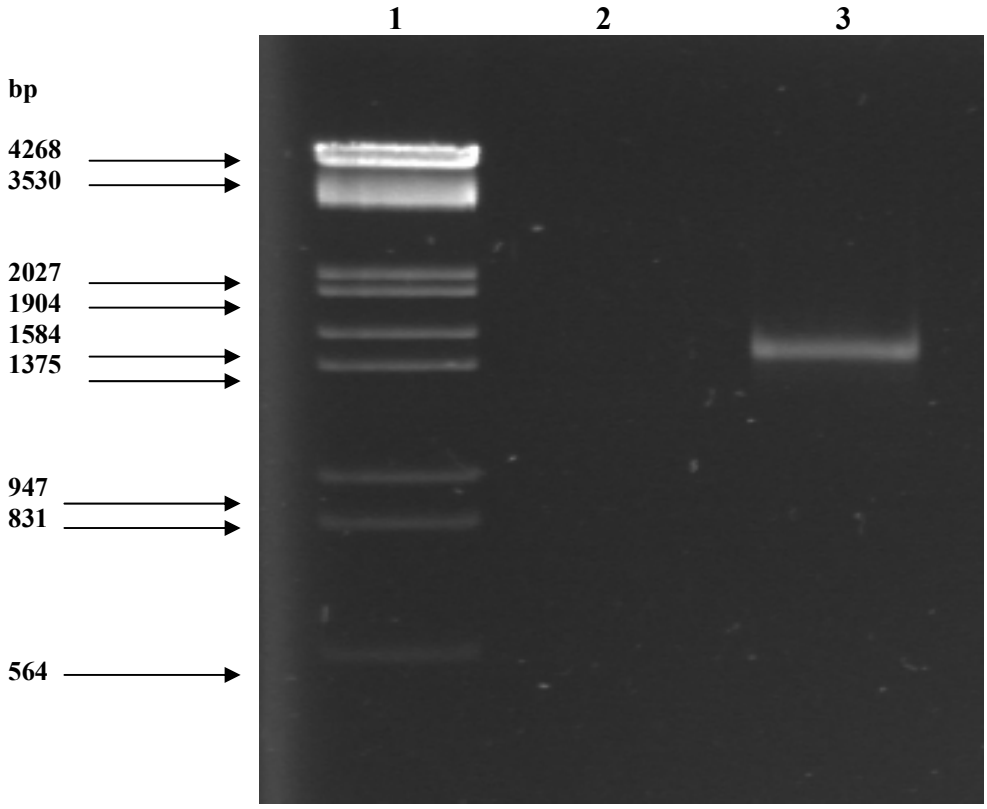

Figure 11 PCR amplification of the 16S rRNA gene of isolate NT 6.7

Lane 1 = 100 bp Marker

Lane 2 = Negative control

Lane 3 = PCR product of isolate NT 6.7

GACGGGCAGGTGTGTACAAGGCCCGGGNAACGTATTCACCGCGGCATGCTGAT
CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCG
AACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTACCCTTTGT
TCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGA
CGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAAC
TGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGA
AGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTC
TTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCG
TTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTA
CGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTC
AGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCT
CTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCATCGT
TTACGGCGNGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCC
TCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACAT
CTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAG
TTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACT
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CCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGT
ACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAG
CTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCG
TCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTG
AGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAG
CCGAAGCCACCTTTTATGTTTGAACCATGCGGTTTAAACAACCATCCGGTATTAG
CCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTC
ACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCCTCGACTTGCA
TGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTCT

Figure 12 The 16S rDNA sequence of isolate NT 6.7

4. Enzyme production

4.1 Effect of various temperature to enzyme production

The *Bacillus circulans* NT 6.7 was cultivated on locust bean gum medium (LG), pH 7.0 at different temperatures of 40, 45 and 50ºC, respectively. The result was showed in Figure 13, the highest enzyme activity was detected at 45°C (2.350 unit/ml), the yield of production from cell was 0.6206 unit/cfu and the specific growth rate was 0.2307 hr^{-1} (calculated by viable cell count). The result of *Bacillus circulans* NT 6.7 was similar to *Bacillus subtilis* 5H which produced mannanase at 45°C (Khanongnuch *et al.,* 1998), but it was higher than mannanase production from *Bacillus* sp. KK01 at 30°C (Hossain *et al.,* 1996) and *Aspergillus niger* NCH- 189 at 30°C (Lin and Chen, 2004).

4.2 Effect of various pH to enzyme production

The *Bacillus circulans* NT 6.7 was grown on locust bean gum medium (LG) at 45ºC, in different pH of 4.0, 5.0, 6.0, 7.0 and 8.0, respectively. The *Bacillus circulans* NT 6.7 could not grow at pH 4.0 and 5.0. At 20 hours, the highest mannanase activity was 2.410 unit/ml and the cell count was $8.5x10^7$ cfu/ml at pH 6.0 (Table 12). The pH 6.0 was the best pH for mannanase production from *Bacillus circulans* NT 6.7. The result was similar to mannanase production from *Sclerotium rolfsii*, pH 6.0 (Gübitz *et al.,* 1996), but lower than that reported for mannanase production from *Aspergillus niger*, pH 7.0 (Ademark *et al.,* 1998) and *Bacillus subtilis* KU- 1, pH 7.0 (Zakaria *et al.,* 1998).

Table 12 Effect of pH on growth of *Bacillus circulans* NT 6.7 at 45°C

pH	Viable cell count (cfu/ml)	Enzyme activity (unit/ml)
6	$8.5x10^7$	2.4101
7	$7.5x10^7$	1.4969
8	$8.9x10^7$	1.9038

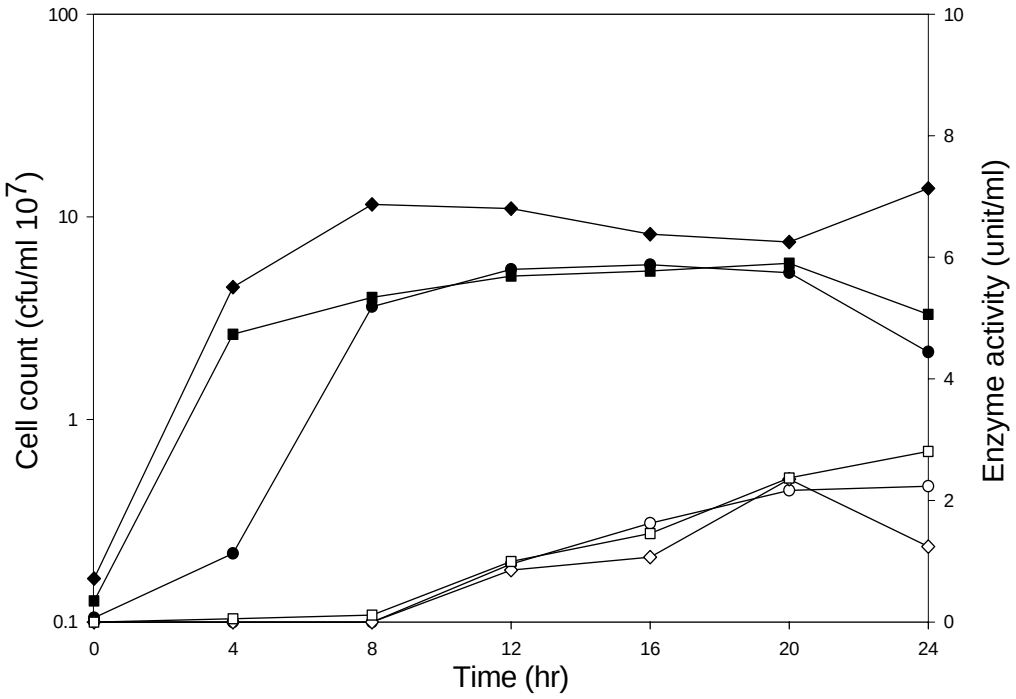
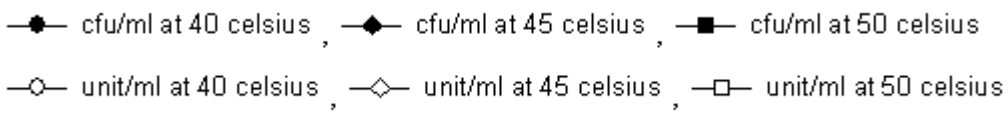


Figure 13 Profile of growth temperatures of *Bacillus circulans* NT 6.7 strain on locust bean gum medium (LG), at pH 7.0



4.3 Effect of various carbon sources to enzyme production

The cultivation of *Bacillus circulans* NT 6.7 was performed on PM medium with four various mannans as carbon sources; copra meal (CM), locust bean gum (LG), konjac flour (KF) and guar gum (GG) in pH 6.0. The flask was shaken by incubator shaker at 150 rpm, 45°C for 24 hr. The results were shown in Figure 14, but the data of copra meal did not show in Figure 14, because the *Bacillus circulans* NT 6.7 grew very slow in copra meal medium. The best carbon sources was locust bean gum. In locust bean gum, konjac flour and guar gum medium, cell grew rapidly in 0-8 hr after inoculation, but they had no enzyme activity and their enzyme activity could be detected after 8-24 hr. The highest enzyme activity was 1.567 unit/ml, the yield of production from cell was 0.665 unit/cfu and specific growth rate was 3.050 unit in locust bean gum medium. For other carbon sources, konjac flour and guar gum, the enzyme activity, yield of production from cell and specific growth rates were low. Previously, comparison five various mannans as carbon sources of copra meal (CM), defatted copra meal (dCM), locust bean gum (LG), konjac flour (KF) and guar gum (GG) have been reported at 30°C in pH 7.0 for 3 days. The best carbon source was defatted copra meal with highest mannanase activity 27.4 unit/ml. However, if four various carbon sources: CM, LG, KF and GG were compared, the best carbon source was locust bean gum with highest enzyme activity 7.8 unit/ml (Lin and Chen, 2004).

Therefore, all studies of the effect of temperature, carbon source and pH on fermentation revealed that the suitable carbon source was locust bean gum (LG), optimum temperature was at 45°C and optimum pH was 6.0. This condition was used for mannanase production by *Bacillus circulans* NT 6.7 strain in further study.

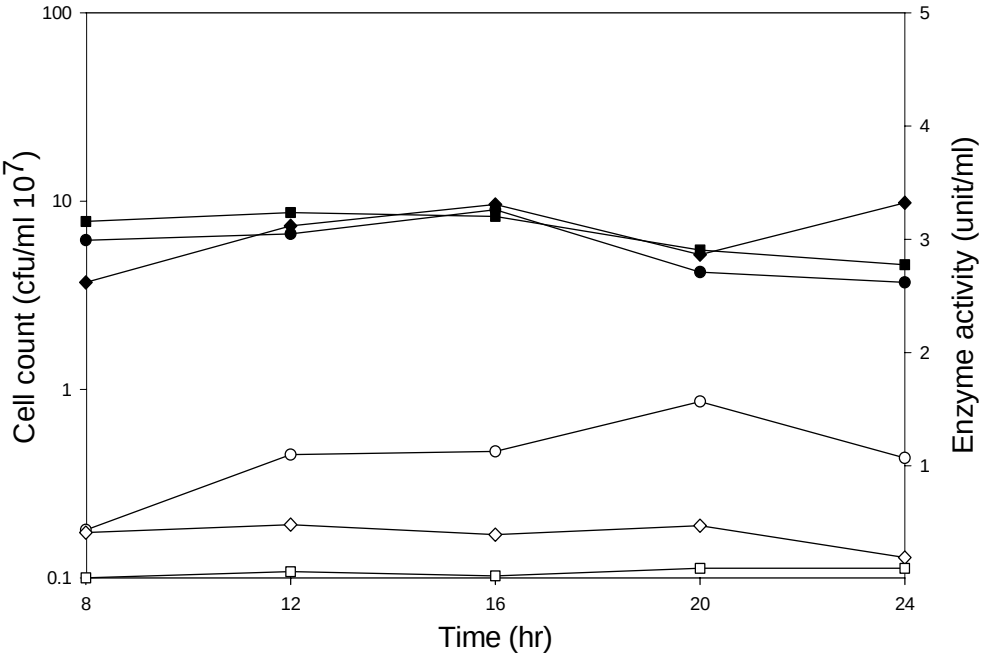


Figure 14 Profile of growth carbon sources of *Bacillus circulans* NT 6.7 strain at 45°C in pH 6.0

LG = Locust Bean Gum

KF = Konjac Flour

GG = Guar Gum

5. Enzyme characterization

5.1 Optimal pH

The *Bacillus circulans* NT 6.7 exhibiting high mannanase activity was studied for the enzyme properties. The effect of pH and temperature on mannanase activities were determined from the culture supernatant of *Bacillus circulans* NT 6.7. The optimal 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 by using locust bean gum as substrate. Thus, the *Bacillus circulans* NT 6.7 exhibited broad pH optimum from 6.0, 7.0, 8.0 and 9.0 (higher than 90 % of highest enzyme activity). Relative enzyme activities were lower than 50% at pH 5.0 and 10.0 (Figure 15). The optimal pH of this mannanase was pH 6-9. Moreover, the optimal pH of *Bacillus circulans* NT 6.7 was similar to that reported for *Bacillus subtilis* KU-1 strain at pH 7.0 (Zakaria *et al*., 1998) and *Aspergillus niger* NCH-189 strain at pH 7.0 (Lin and Chen, 2004). Thus, the optimal pH of *Bacillus circulans* NT 6.7 was higher than that reported for *Trichoderma harzianum* T4 at pH 3.0 (Ferreira and Filho, 2004) and *Sclerotium (Athelia) rolfsii* at pH 3.0 (Sachslehner and Dietmar, 1999).

5.2 Optimal temperature

The optimal temperature of mannanase from *Bacillus circulans* NT 6.7 was observed by incubating enzyme assays at various temperatures of 30, 35, 40, 45, 50, 55 and 60°C, in buffer pH 7.0 by using locust bean gum as substrate. The optimal temperature of *Bacillus circulans* NT 6.7 was 50°C as shown in Figure 16. Moreover, the optimum temperature of *Bacillus circulans* NT 6.7 was similar to optima temperature of *Aspergillus niger* at 50°C (Ademark *et al.*,1998) and *Bacillus subtilis* KU-1 at 50°C (Zakaria *et al*., 1998). However, the *Bacillus circulans* NT 6.7 was lower than *Trichoderma harzianum* T4 at 55°C (Ferreira and Filho, 2004) and *Bacillus* sp. KK01 at 60°C (Abe *et al*., 1996).

5.3 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 17. The *Bacillus circulans* NT 6.7 was stable at 40-50°C for 6 hr. Enzyme activity was not stable at 60 and 70°C. Compared with other mannanases, the stable of temperature range was quite wide and the remaining activity at the extremes (40 and 50°C) was satisfactorily high. Moreover, The thermal stability of mannanase for *Bacillus circulans* NT 6.7 was similar to *Bacillus subtilis* KU-1 strain at 50°C (Zakaria *et al*., 1998) and *Sclerotium (Athelia) rolfsii* at 50°C (Sachslehner. A and H. Dietmar. 1999). However, the *Bacillus circulans* NT 6.7 was lower than that reported for *Bacillus* sp. KK01 at 55°C (Abe *et al*., 1996) and *Trichoderma harzianum* T4 at 55-70°C (Ferreira and Filho, 2004).

Therefore, the mannanase from *Bacillus circulans* NT 6.7 was characterized; optimum pH 6-9, optimum temperature at 50°C and thermal stability at 40 - 50°C.

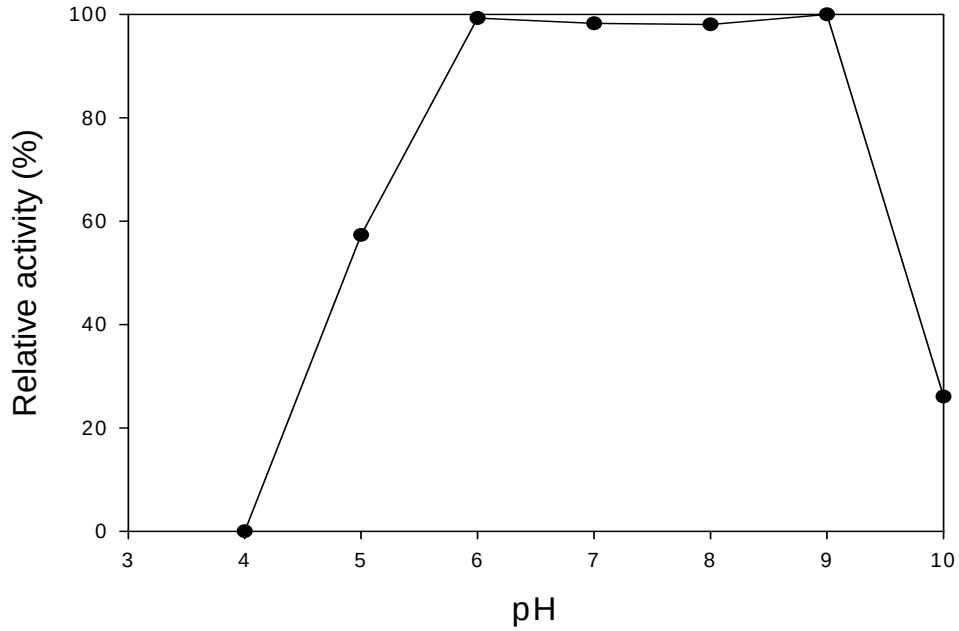


Figure 15 Optimum pH of mannanase from *Bacillus circulans* NT 6.7

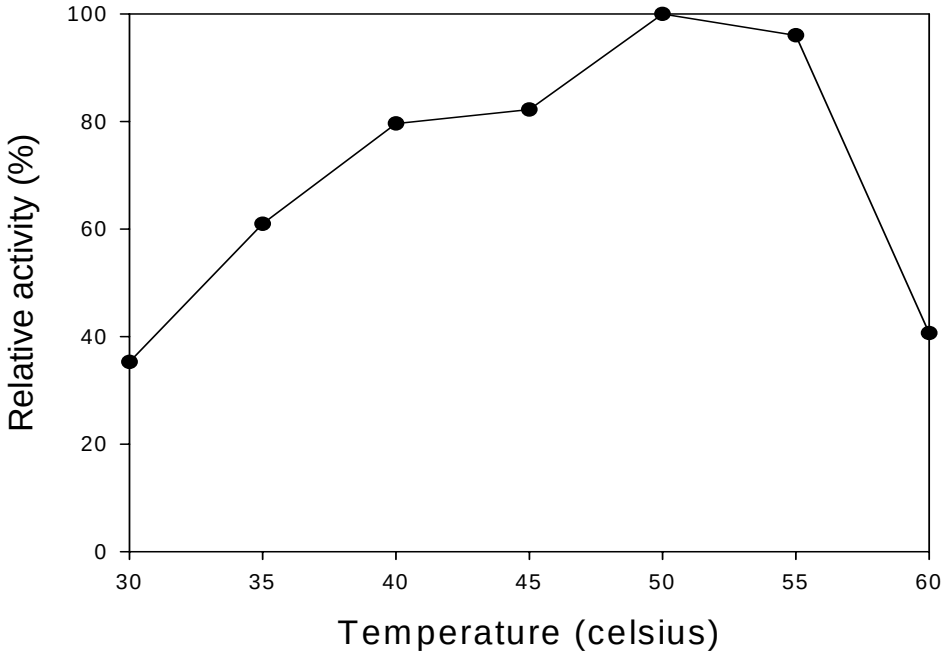


Figure 16 Optimum temperature of mannanase from *Bacillus circulans* NT 6.7

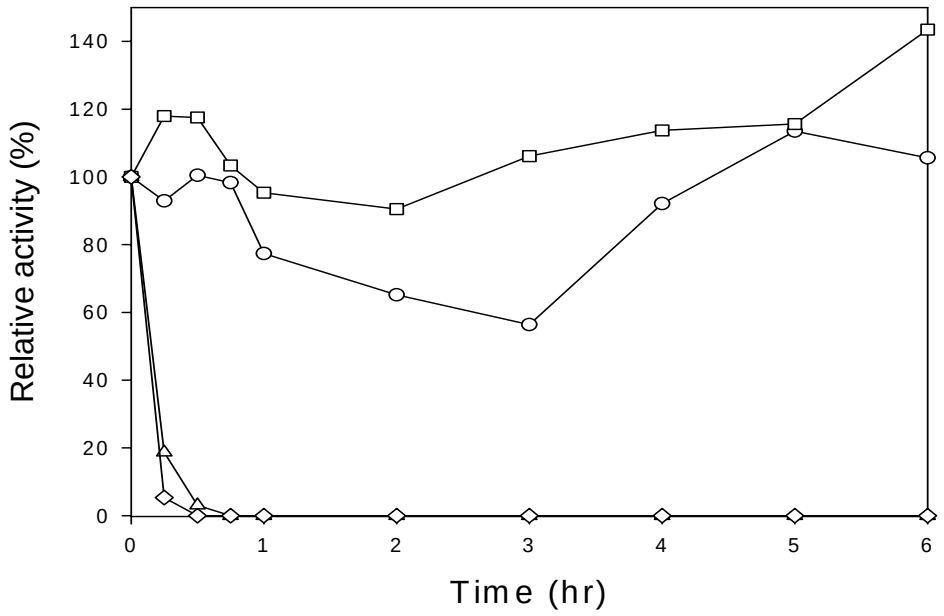
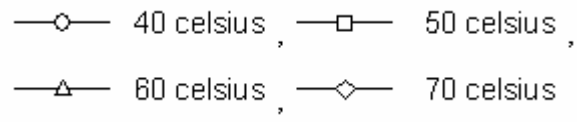


Figure 17 The thermal stability of mannanase from *Bacillus circulans* NT 6.7



CONCLUSION AND RECOMMENDATION

Twenty-three sources of soils were collected from 4 areas in Thailand. 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). The isolate NT 6.7, demonstrating broad inhibition pathogens of *Salmonella serovar* Eteritidis 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 optimum condition for mannanase production from *Bacillus circulans* NT 6.7 was pH 6.0, temperature at 45ºC and locust bean gum (LG) as carbon source. The *Bacillus circulans* NT 6.7 showed high activity (0.306 unit/ ml) with optimum pH 6-9, optimum temperature at 50ºC and thermal stability at 40-50ºC.

The products from copra-mannan (CM) hydrolysis by beta-mannanase is mannooligosaccharides (MOS), which is expected to be one kind of prebiotic, a non-digestible food ingredient (oligosaccharides) that beneficially affects the host by selectively stimulation on the growth and action of microorganisms using the prebiotics oligosaccharides. Yielding MOS, as an idea or principle to produce prebiotic from CM, is expected to be useful to improve the growth performance of animal. Action of prebiotic MOS was improved as follow; first, it is a special nutrient or growth promoter for probiotics, such as *Bifidobacteria* and *Lactobacillus*. Second, it can inhibit or block colonization of pathogen such as *Escherichia coli* or *Salmonella*. Preliminary results have also been positive with the addition of MOS to poultry diets. We have been interested in quality improvement of CM by beta-mannanase for using as feed ingredient. This research provided basic data to determine the capable of beta-mannanase on MOS preparation from CM in the future.

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APPENDIX

Appendix Table 1 Mannanase activity of isolates by using copra meal (CM) as substrate at 45°C.

Isolate	Mannanase activity (unit/ml)
NT 1.1	0.211
NT 1.2	0.105
NT 6.1	0.023
NT 6.2	0.228
NT 6.3	0.280
NT 6.4	0.240
NT 6.5	0.008
NT 6.6	0.278
NT 6.7	0.306
NT 6.8	0.158
NT 6.9	0.285
NT 6.10	0.103
NT 6.11	0.057
NT 6.12	0.002
NT 16.1	0.059
NT 17.1	0.063
NT 17.2	0.259
NT 17.3	0.069
NT 2.1	0.568
NT 2.2	0.331
NT 2.3	0.447
NT 2.4	0.219
NW 1.1	0.137

Appendix Table 2 Inhibition of pathogen *Salmonella serovar* Eteritidis S003 at 37°C.

Isolate	Inhibition activity (%)
NT 1.1	0
NT 1.2	0
NT 6.1	1.5
NT 6.2	40.2
NT 6.3	0
NT 6.4	0
NT 6.5	7.3
NT 6.6	0
NT 6.7	0
NT 6.8	0
NT 6.9	0
NT 6.10	0
NT 6.11	40.2
NT 6.12	0
NT 16.1	74.0
NT 17.1	0
NT 17.2	0
NT 17.3	0
NT 2.1	0
NT 2.2	0
NT 2.3	0
NT 2.4	0
NW 1.1	3.2
	0

Appendix Table 3 Enhance of pathogen *Salmonella serovar* Eteritidis S003 at 37°C.

Isolate	Enhance activity (%)
NT 1.1	205.0
NT 1.2	100.0
NT 6.1	0
NT 6.2	0
NT 6.3	42.7
NT 6.4	11.8
NT 6.5	0
NT 6.6	12.6
NT 6.7	46.3
NT 6.8	46.3
NT 6.9	26.8
NT 6.10	123.6
NT 6.11	0
NT 6.12	515.0
NT 16.1	0
NT 17.1	77.4
NT 17.2	37.9
NT 17.3	105.0
NT 2.1	52.2
NT 2.2	52.2
NT 2.3	1875.0
NT 2.4	3925.0
NW 1.1	0

Appendix Table 4 Inhibition of pathogen *Escherichia coli* E010 at 37°C.

Isolate	Inhibition activity (%)
NT 1.1	0.9
NT 1.2	0.9
NT 6.1	65.9
NT 6.2	68.0
NT 6.3	0
NT 6.4	0
NT 6.5	0
NT 6.6	0
NT 6.7	0
NT 6.8	1.5
NT 6.9	0
NT 6.10	0
NT 6.11	45.0
NT 6.12	60.5
NT 16.1	0
NT 17.1	0
NT 17.2	54.592
NT 17.3	0
NT 2.1	70.6
NT 2.2	72.8
NT 2.3	78.8
NT 2.4	0
NW 1.1	0

Appendix Table 5 Enhance of pathogen *Escherichia coli* E010 at 37°C.

Isolate	Enhance activity (%)
NT 1.1	0
NT 1.2	0
NT 6.1	0
NT 6.2	0
NT 6.3	60.9
NT 6.4	17.2
NT 6.5	20.1
NT 6.6	30.2
NT 6.7	13.3
NT 6.8	0
NT 6.9	33.0
NT 6.10	12.5
NT 6.11	0
NT 6.12	0
NT 16.1	5.7
NT 17.1	11.2
NT 17.2	0
NT 17.3	201.4
NT 2.1	0
NT 2.2	0
NT 2.3	0
NT 2.4	117.3
NW 1.1	10.0

Appendix Table 6 Enhance of *Lactobacillus reuteri* AC-5 at 37°C.

Isolate	Enhance activity (%)
NT 1.1	6.2
NT 1.2	22.9
NT 6.1	19.4
NT 6.2	122.2
NT 6.3	536.1
NT 6.4	527.7
NT 6.5	10.5
NT 6.6	47.9
NT 6.7	49.5
NT 6.8	0
NT 6.9	8.8
NT 6.10	3.3
NT 6.11	0
NT 6.12	13.3
NT 16.1	595.0
NT 17.1	72.9
NT 17.2	148.9
NT 17.3	30.0
NT 2.1	65.2
NT 2.2	60.1
NT 2.3	106.5
NT 2.4	0
NW 1.1	25.0

Appendix Table 7 Inhibition of *Lactobacillus reuteri* AC-5 at 37°C.

Isolate	Inhibition activity (%)
NT 1.1	0
NT 1.2	0
NT 6.1	0
NT 6.2	0
NT 6.3	0
NT 6.4	0
NT 6.5	0
NT 6.6	0
NT 6.7	0
NT 6.8	69.1
NT 6.9	0
NT 6.10	0
NT 6.11	4.4
NT 6.12	0
NT 16.1	0
NT 17.1	0
NT 17.2	0
NT 17.3	0
NT 2.1	0
NT 2.2	0
NT 2.3	0
NT 2.4	74.0
NW 1.1	0

Appendix Table 8 Optimum pH of mannanase from *Bacillus circulans* NT 6.7 at 45°C

pH	Relative activity (%)
3.0	0
4.0	0
5.0	57.3
6.0	99.2
7.0	98.2
8.0	98.0
9.0	100
10.0	26.0

Appendix Table 9 Optimum temperature of mannanase from *Bacillus circulans* NT 6.7 at pH 7.0

Temperature (°C)	Relative activity (%)
30	35.2
35	60.9
40	79.6
45	82.2
50	100
55	96.0
60	40.6

Appendix Table 10 The thermal stability of mannanase from *Bacillus circulans* NT 6.7

Temperature (°C)	Time (hr)	Relative activity (%)
40	0	100
	0.25	92.8
	0.5	100.4
	0.75	98.2
	1	77.3
	2	65.1
	3	56.3
	4	92.1
	5	113.5
	6	105.6
50	0	100
	0.25	117.9
	0.5	117.5
	0.75	103.3
	1	95.3
	2	90.5
	3	106.1
	4	113.7
	5	115.6
	6	143.4

Appendix Table 10 (Continued)

Temperature (°C)	Time (hr)	Relative activity (%)
60	0	100
	0.25	18.7
	0.5	3.0
	0.75	0
	1	0
	2	0
	3	0
	4	0
	5	0
	6	0
70	0	100
	0.25	5.3
	0.5	0
	0.75	0
	1	0
	2	0
	3	0
	4	0
	5	0
	6	0

Appendix Table 11 Profile of growth temperatures of *Bacillus circulans* NT 6.7 Strain on locust bean gum medium (LG) at pH 7.0

Temperature (°C)	Time (hr)	Yp/x (unit/cfu)	μ_{cfu} (hr^{-1})
40	0	0	
	4	0	
	8	0	
	12	0.0429	0.1918
	16	0.0684	
	20	0.1019	
	24	0.4063	
45	0	0	
	4	0	
	8	0	
	12	0.3046	0.2307
	16	0.0813	
	20	0.1241	
	24	0.6206	
50	0	0	
	4	0.0081	
	8	0.0083	
	12	0.0521	0.1873
	16	0.0705	
	20	0.1023	
	24	0.2663	

Appendix Table 12 Profile of growth carbon sources of *Bacillus circulans* NT 6.7 strain at 45°C in pH 6.0

Carbon source	Time (hr)	Yp/x (unit/cfu)	$Yp/_{OD}$ (unit)
Locust bean gum	8	0	0
	12	0.6652	0.4105
	16	0.1237	0.1678
	20	0.5667	1.4405
	24	0.3179	3.0509
Konjac flour	8	0	0
	12	0.0094	0.0703
	16	0.0015	0.1674
	20	0.0201	0.2634
	24	0.0181	1.6382
Gaur gum	8	0	0
	12	0.0306	0.0444
	16	0.0170	0.1073
	20	0.0419	0.4028
	24	0.0071	0.0043

Appendix 13 Components of bacteria isolate medium (BIM) for per liter

Copra meal	10.0	g
Yeast Extract	1.0	g
Poly peptone	1.0	g
NH_4NO_3	1.0	g
KH_2PO_4	1.4	g
$MgCl_2$	0.2	g
pH	6.9	

Appendix 14 Components of bacteria isolate medium (BIM) for per liter, clear zone.

Locust bean gum	10.0	g
Yeast Extract	1.0	g
Poly peptone	1.0	g
NH_4NO_3	1.0	g
KH_2PO_4	1.4	g
$MgCl_2$	0.2	g
Agar	15.0	g
pH	6.9	

Appendix 15 Components of fungi isolate medium (FIM) for per liter.

Copra meal	10.0	g
Yeast Extract	14.3	g
$(NH4)_2SO_4$	2.1	g
$MgSO_4 . 2H_2O$	0.3	g
$CaCl_2 . 2H_2O$	0.3	g
$FeSO4 . 7H_2O$	0.5	g
KH_2PO_4	10.0	g
Glucose	20.0	g
pH	5.2, 5.25	

Appendix 16 Components of fungi isolate medium (FIM) for per liter, clear zone

Locust bean gum	10.0	g
Yeast Extract	14.3	g
$(NH4)_2SO_4$	2.1	g
$MgSO_4 . 2H_2O$	0.3	g
$CaCl_2 . 2H_2O$	0.3	g
$FeSO_4 . 7H_2O$	0.5	g
KH_2PO_4	10.0	g
Glucose	20.0	g
Agar	15.0	g
pH	5.2, 5.25	

Appendix 17 Components of yeast isolate medium (YIM) for per liter.

Copra meal	10.0	g
Yeast Extract	2.0	g
KH_2PO_4	2.0	g
$(NH4)_2SO_4$	2.0	g
pH	5.5	

Appendix 18 Components of yeast isolate medium (YIM) for per liter, clear zone.

Locust bean gum	10.0	g
Yeast Extract	2.0	g
KH_2PO_4	2.0	g
$(NH4)_2SO_4$	2.0	g
Agar	15.0	g
pH	5.5	

Appendix 19 Components of producing enzyme medium (PM) for per liter, CM.

Copra meal	10.0	g
Poly peptone	30.0	g
KH_2PO_4	15.0	g
$MgSO_4 . 7H_2O$	0.6	g
Corn steep liquor (v/v)	25.0	g
pH	7.0	

Appendix 20 Components of producing enzyme medium (PM) for per liter, LG.

Locust bean gum	10.0	g
Poly peptone	30.0	g
KH_2PO^4	15.0	g
$MgSO_4 . 7H_2O$	0.6	g
Corn steep liquor (v/v)	25.0	g
pH	7.0	

Appendix 21 Components of isolate medium (IM) for per liter.

Beef Extract	3.0	g
Poly peptone	5.0	g
pH	6.9	