CHARACTERIZATION OF MANNANASE FROM EFFECTIVE BACTERIAL STRAINS AND THEIR APPLICATION FOR PREBIOTIC PRODUCTION

INTRODUCTION

Coconut are grown in most South-Asian countries in the tropical zone (Hossain *et al*, 1996) including Thailand, and its products are probably more extensive than those of any other plants. Moreover, to date, about 30-40% coconut oil extracted from copra was used as a source of fuel due to a worldwide oil crisis. In 2005, the oilseed produced from copra meal in South-East Asia was estimated for 4.39 million Metric Tons [www.mindbranch.com]. About 1.67 million Metric Tons were obtained from Thailand (Center for Agricultural Information, Thailand 2005).

Copra meal is an agricultural waste composing of 45-60% non-starch polysaccharides which cannot be utilized by monogastric animals. The non-starch polysaccharides in dry copra meal mainly consist of 65% galactomannan, 5% lignin, 21% protein and nearly 10% fat (Choct, 2001; Luis *et* al, 2000; Purwadaria *et al*, 1995). The copra meal contains a large amount of galactomannan which consists of repeating β-1,4 mannose and α -1,6 galactose units attached to the β-1,4 mannose backbone. These structures can be hydrolyzed to manno-oligosaccharides by beta-mannanase producing microorganisms.

Beta-D-mannanases (mannan-endo-1,4-β-mannohydrolase, E.C.3.2.1.78) are hydrolytic enzymes which catalyze randomly β-1,4 mannosidic linkages within the backbones of mannan, galactomannan, glucomannan and galactoglucomannan (Stoll *et al,* 1999). The products from β-D-mannan hydrolysis by beta-mannanases are mannooligosaccharides which are prebiotics. It has also been reported that manno-oligosaccharides are useful for the host by selective stimulation on the growth of gut microflora including *Bifidobacterium* sp. and *Lactobacilli* sp. (Gibbson, 1999). In addition, mannooligosaccharides are also effective in reducing colonization of type

1- fimbriated, α-D-mannose-sensitive agglutination, *Escherichia coli* and *Salmonella* sp., leading to elimination of these bacteria with the digesta flow instead of binding to mucosal receptor (Parks *et al*, 2001 and Fermandez *et al*, 2002).

The aim of this study was to isolate and characterize effective beta-mannanase from bacterial source for the production of a manno-oligosaccharide to be used as prebiotics.

OBJECTIVES

1. Isolation, screening and identification of microorganisms producing mannanase
2. Purification and characterization of mannanase

LITERATURE REVIEW

1. Coconut tree

1.1 Source

The discovery of fossilized nuts of the coconut palm in New Zealand and India shows that the coconut palm has existed for several million years. The origin of the species is not precisely known, but the coconut probably originated in either the Pacific or the Far East and crossed the Indian Ocean to Africa (Harries, 1978).

Coconuts are cultivated throughout the humid intertropical zone, between approximately 25°N and 25°S. Asia is the major production region, with an estimated 91% of the total cultivated area of 11 million hectares (Taffin *et al,* 1999). Two countries, The Phillipines and Indonesia have cultivated approximately 65% of the world's copra meal production (Sunda and Dingle, 2006). In Thailand, the coconut palm, called 'Ma-Praw', is a polyannual plant. The report from Agricultural statistics of Thailand crop 2001 has shown that coconut trees are generally cultivated in Thailand (in Table 1). The Southern part of Thailand, especially Nakornsrithammarat province, is the biggest cultivation area.

The coconut tree is one of the most valuable plants because every part of the tree is most useful. However, the fruit is the major part used for coconut oil production of approximately 4.39 million Metric Tons from South East Asia [www.mindbranch.com]. In Thailand, these products were up to approximately 1.67 million Metric tons in 2005 (Center for Agricultural Information, Thailand 2005).

Table 1 Area of cultivation and yield of coconut tree in Thailand in 2001

Provinces	Area of cultivation (rai)	Yield (kg/rai)
Songkla	26,906	869.17
Nakornsrithammarat	116,633	998.20
Patthalung	15,412	1,160.80
stull	5,349	1,119.51
Chanthaburi	19,547	487
Trad	12,850	721
Sakaew	3,113	1,089

Source: modified from Agricultural statistics of Thailand crop year 2001/02

1.2 Physical characteristics

The coconut tree, *Cocos nucifer L.*, is classified as the Plame family (Taffin *et al*, 1999). The tree is found in tropical climate, with the height to 27 m or more and about 30-45 cm in diameter. The flowers are yellowish-white and the fruits or nuts are typically 20-30 cm and 1.2-2.0 kg in length and weight, respectively (Taffin *et al*, 1999). The mature fruit of 3.4 kg, is composed of about 35% husk, 12% shell, 22% meat and 25% water (Guarte *et al*, 1996) (Figure 1). The fruit becomes ripe for making copra meal after 12 months. The copra meal has a white color and 10-15 mm in thickness (Taffin *et al*, 1999).

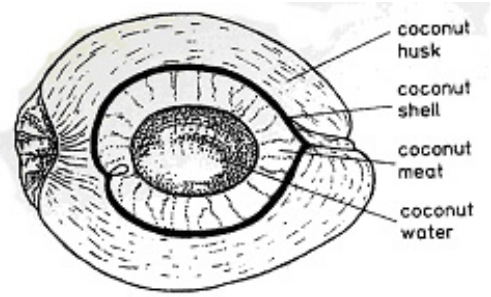

Figure 1 Longitudinal section showing the different parts of coconut
Source: Guarte *et al*. (1996)

Coconut palms are classified into two main groups, Tall palms and Dwarf palms (in Table 2). For Tall palms, they are cross-pollinated and grow slow. Tall palms bear many nuts which are of medium size and with a low copra meal yield. While, Dwarf palms, are shorter with thinner stems. They are normally self-pollinated, are early bearing and have a high annual bunch yield. Dwaf palms are therefore important in breeding programs (Santos and Sangare, 1992). As an example, data on the composition of the fruits of Tall and Dwarf palm ecotypes in the plant collection of the Marc Delorme Station in Cote d'Ivoire are given in Table 3.

Table 2 Basic characteristics of Tall and Dwarf coconuts

Traits	Tall	Dwarf
Geographic distribution	Widely distributed and commercial grown	Less widely distributed and generally of non commercial value
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 years	Less than 50 years
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 and plentiful	Less dense and few
Reaction to adverse conditions	Generally less sensitive	Sensitive tohypersensitive
Culture requirements	Average	High input required
Leaf and bunch attachment	Very strong	Fragile

Source: Santos and Sangare (1992)

Table 3 Weight of different components of fruits from various sources

Cultivar	Origin	Weight in 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 *et al.* (1999)

2. Copra meal and its chemical characteristics

Copra meal is the local south pacific name for dried sections of the meatly inner lining of the coconut seed. It is obtained from the kernel of coconut fruit which may be cured by sun-drying or drying machines. The essential requirement of copra meal drying is to bring down the moisture content of the wet meat from 50-55% to 5-6% (Thampan, 1975). Good desiccated coconut should be white in color, crisp with a fresh nutty flavor. It should contain 68-72% oil and less than 20% moisture.

As shown in Table 4, the nutrient values of defatted copra meal were very variable in quality. The residue, in the form of dried cake chunks, is further reduced by grinding to copra meal. Dried copra meal produced by the expeller process has a residual oil content of about 6% (Table 4). This is sometimes reduced further by solvent extraction depending on the market demand for oil which at the present time is quite high. When it contains high residual level of oil in copra meal, it is a valuable source of energy for swine and poultry. Coconut oil is composed predominantly of short chain saturated fatty acids (50% C12:0; 15% C14:0) that are easily digested by swine and poultry (NRC, 1998).

Table 4 Proximate composition (%) of coconut meal

Composition	Dried copra meal		Fresh copra meal[c]
	Thailand[a]	United 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 (1994)

[c] Jarurat (1985)

Moreover, copra meal contains a rather large amount of crude protein and crude fiber (Table 4). Thus, copra meal can be utilized as ingredients for monogastric animal feed. However, the high fiber content and deficiency in important essential amino acids, such as lysine, methionine, threonine and histidine but high in arginine, in copra meal (Sunda and Dingle, 2006; Yoshikawa *et al*, 2000; NRC, 1994) limits the use of copra meal in feed. The fiber is high in a polymer called galactomannan that has low digestibility and often has a laxative effect in poultry and swine (NRC, 1998).

The structure of galactomannan, the major component of copra meal is given in Figure 2. It consists of a beta-D-1,4-mannopyranose backbone with branchpoints from their 6-positions linked to alpha-D-galactose (Burhanudin and Dingle 2006).

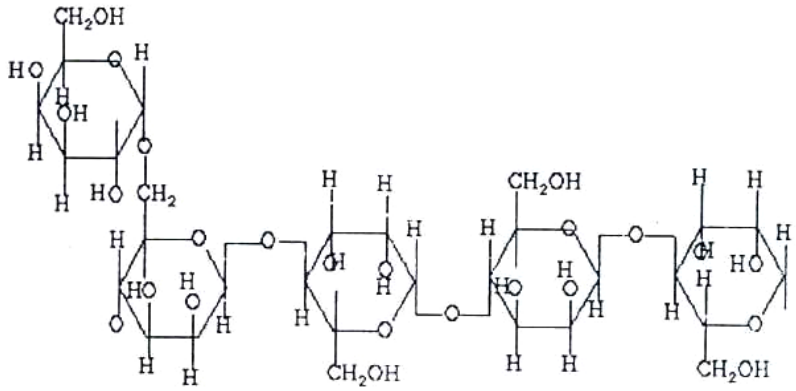

Figure 2 Structural features of galactomannan
Source: Duffaud *et al*. (1997)

3. Copra mannan degrading enzyme

The galactomannan which is the main structure of copra meal could be hydrolyzed by a number of different catalytic activities. Endo-mannanase or beta-D-mannanase cleaves between adjacent mannose residues of the mannan backbone (1,4-β-D-mannan mannohydrolase, E.C.3.2.1.78) and exo-acting β-mannosidases (β-D-mannopyranoside hydrolase, E.C. 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, E.C. 3.2.1.22) (Halstead *et al*, 2000).

3.1 Beta-mannanase

3.1.1 Sources of beta-mannanase and its isolation methods

Beta-mannanase is widely distributed 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 in Table 5 and 6, respectively.

Table 5 Beta-mannanase producing bacteria

Sources	References
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)
Bacteroides ovatus	Gherardimi *et al*. (1987)
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)
Caldocellum saccharolyticum	Bicho *et al*. (1991)
	Gibbs *et al*. (1992)
Caldicellulosiruptor sp. RT8B4	Gibbs *et al*. (1996)
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)
Pseudomonasfluorescens supsp.*Cellulose*	Braithwaite *et al*. (1993)
	Bolam *et al*. (1996)
Thermotiga neapolitana 5068	McCutchen *et al*. (1996)
	Duffaud *et al*. (1997)
Thermotoga maritime	Brown *et al*. (1993)
Thermomonospora fusca	Hilge *et al*. (1998)
Polyporus versicolor	Johnson and Ross (1990)

Table 6 Beta-mannanase producing fungi

Sources	References
Aspergillus niger	Ademark *et al*. (1998)
Aspergillus niger NCH-189	Lin and Chen (2003)
Aspergillus carbonarius	Ghareib and Nour-el-Dien (1994)
Trichoderma reesei	Stalbrand *et al*. (1993)
	Stalbrand *et al*. (1995)
	Harjunpaa *et al*. (1995)
	Tenkenso *et al*. (1997)
	Tenkanen *et al*. (1997)
	Buchert *et al*. (1993)
Trichoderma reesei C-30	Arisan-atac *et al*. (1993)
Trichoderma harzianum E58	Torrie *et al*. (1990)
Thermomyces lanuginosus	Puchart *et al*. (1999)
Sporotichum cellulophilum	Araujo *et al*. (1991)
Sclerotium rolfsii	Sachslehner and Haltrich (1999)
	Sachslehner *et al*. (1998)
	Gubitz *et al*. (1996)
Penicillium purpurogenum	Park *et al*. (1987)
Rhodothermus marinus	Politiz *et al*. (2000)

The previous studies showed that mannanase producing bacteria had been isolated from various sources. For instance, Hossain *et al*. (1996) and Abe *et al*. (1994), isolated bacteria from soil using copra meal as carbon source for cultivation. The results showed that the bacterium which secreted mannanase to culture fluid belonged to genus *Bacillus*.

Moreover, in 1999, fifity 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*, 1999). *Streptomyces scabies* CECT 3340

and *Streptomyces ipomoea* CECT 3341 which had mannanase activities of 294.3 and 242.9 U/l, respectively, were selected for the biobleaching of pine kraft pulps.

While, Wongkattiya (1998) isolated 249 actinomycetes from roots and soil. Mannanase activity was detected in 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 mananase activity of 149.92 U/ml 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 galactomannan guar gum and beef extract/peptone as carbon and nitrogen sources, respectively.

A screening method for detection of beta-mannanase producing microorganism had been studied. Kremnicky *et al*. (1996) incorporated 2 different dyed substrates, Ostazin Brilliant Red-galactomannan and Remazol Brilliant Blue-xylan into the medium for screening 449 isolates of yeasts and yeast-like microorganism. The highest frequency of mannanase activity was found within the genera *Stephanoascus* and *Aureobasidium*. The best producers of beta-mananase were found to be the strain *Aureobasidium pullulans*.

A new assay for quantifying mannanase activity using Congo Red dye was studied by Downie *et al*, 1994. One percent (w/v) Congo Red dye resulted in fast staining times (15 min) and good contrast. The edges of zones of clearing on gels comprised of 0.7% (w/v) Phytagel were most distinct at 0.1% (w/v) galactomannan. The detection sensitivity for the enzyme was similar to that of the viscometric assay.

3.1.2 Mode of action

Beta-D-mannanase (mannan endo-1,4-β-mannohydrolase, E.C. 3.2.1.78) are hydrolytic enzymes which hydrolyze randomly β-1,4 mannosidic linkages within the backbones of mannan, galactomannan, glucomannan and galactoglucomannan (Stoll *et al*, 1999) (Figure 3).

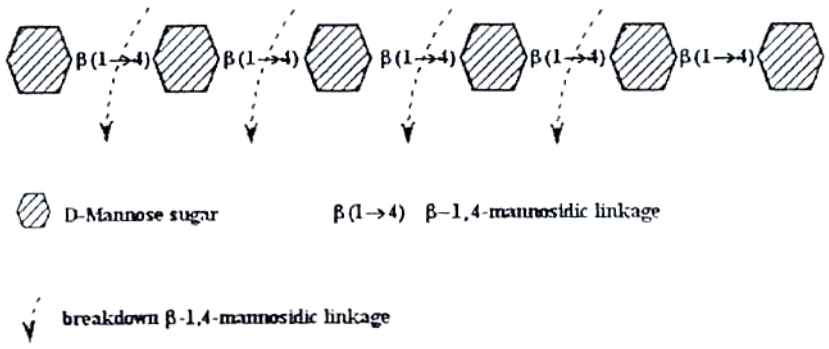

Figure 3 Mode of action of beta-mannanase and the 1,4 -β-D-mannan chain
Source: McCleary (1988)

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 galactomannnan (Stalbrand, 1990).

Beta-D-mannanase has different modes of action on different anomeric configuration of the released D-mannose (McCleary, 1988). McCleary (1979) showed that the purified mannanase from *Bacillus subtilis* is able to hydrolyse coffee bean D-galactomannan to mannobiose, mannotriose and mannotetraose. Corresponding to the studies of Sachslehner *et al*. (2000) who reported that coffee mannans isolated from green defatted Arabica beans were efficiently hydrolysed by the *Sclerotium rolfsii* mannanase. The results showed the significant reduction of viscosity of coffee extract and increase of the reducing sugar content continuously due to the release of various manno-oligosaccharides including mannotetraose, mannotriose and mannobiose.

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

In addition, the purified mannanase from *Tyromyces palustris* could hydrolyze glucomannan which was isolated from larch holocellulose. The products were fractionated by gel filtration on a polyacrylamide gel in water, and partition chromatography on ion exchange resins in 80% ethanol. The resulting products showed that the purified mannanase was capable of hydrolyzing β-(1,4)-mannosidic and glucosidic linkages of glucomannan, and exhibited strong affinity for -β-D-Man*p*-(1,4)- β-D-Glc*p*- than ; β-D-Glc*p*-(1,4)-β-D-Man*p*:.

3.1.3 Characteristics of beta-mannanase

Beta-mannanase has been widely studied in fungi and bacteria but less so in yeast. Various inducers had been used for improving mannanase production. Gubitz *et al*. (1995) showed that *Sclerotium* rolfsi, plant pathogen basidiomycete secreted beta-mannanase into the medium containing *Amorphophallus konjak* glucomannan as an inducer. While, Lin and Chen (2003) enhanced mannanase production by submerged culture of *Aspergillus niger* NCH-189 using defatted copra meal based media.

The physical properties of mannanases vary depending on types of microorganisms. The pH optimum was exhibited over a broad pH range of 2.9-7.5 (Hossain *et al*, 1996; Kremnicky *et al*, 1996; Araujo and Ward 1990b; Oda *et al*, 1993b), and temperature optimum had been reported over the range at 40-74°C (Zhang *et al*, 2000; Zakaria *et al*, 1998; Tamaru *et al*, 1995; Mendoza *et al*, 1994; Khanongnuch *et al*, 1988).

Moreover, the pulified mannanase from various sources presented molecular weight and isoelectric point in the range of 40-59 kDa and 3.5-5.9,

respectively. For example, Gubitz *et al*. (1996) studied the purified beta-mannanase from *Sclerotium rolfsii*. This enzyme was purified to electrophoretic homogeniety by means of ultrafiltration, anion exchange chromatography and gel filtration. It had a molecular mass of 61.2 kDa and the pI value was in the acidic region at 3.5. The pH and temperature optima were 2.9 and 74°C, respectively.

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

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

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). The purified enzyme had an isoelectric point of 3.7 and molecular mass of 40 kDa.

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 and isoelectric point was 3.8. The purified enzyme exhibited maximal activity at pH 6.5 and 40°C.

Akino *et al*. (1988) characterized three beta-mannanases of an alkalophilic *Bacillus* sp AM-001. Molecular weight of the purified enzymes, 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 β-mannan from copra,

locust bean and konjak were 2.0, 3.8 and 7.7 mg/ml and their maximum velocities (V_{max}) corresponding to these saccharides were 730, 1470 and 1880 U/mg protein, respectively.

3.2 Beta-mannosidase

3.2.1 Sources of beta-mannosidase

Beta-mannosidase has been reported to occur in a wide range of plant and animal tissues and several microorganisms (McCleary, 1983). However, only a few were reported, due to high activity and stability of the enzyme from these sources (Table 7).

Table 7 Sources of beta-mannosidase

Sources	References
Bacteria	
Thermotoga neapolitana 5068	Duffaud *et al*. (1997)
Thermotoga maritima	Brown *et al*. (1993)
Bacillus sp.	Akino *et al*. (1988)
Enterococcus casseliflavus	Oda *et al*. (1993)
Streptomyces sp. E2/22	Wongkattiya (1998)
Pyrococcus furlosus	Bauer *et al*. (1996)
Fungi	
Aspergillus sp.	Galkwad *et al*. (1995)
Aspergillus niger	Ajisaka *et al*. (1995)
Aspergillus awamori	Neustroev *et al*. (1991)
Sclerotium rolfsii	Gubtiz *et al*. (1996)

3.2.2 Mode of action

Beta-mannosidase (β-D-mannosidase mannohydrolase, E.C. 3.2.1. 25) catalyzes the hydrolytic cleavage of β-1,4 linkage of mannobiose and mannotriose. Moreover, beta-mannosidase cleaves the beta-mannosidic linkages in various sources of beta-mannans, for example, copra meal, konjak, locust bean and guar bean to D-mannose as shown in Figure 4 (Akino *et al*, 1988). It could play synergism action with beta-mannosidase and alpha-galactosidase to increase hydrolysing extracellular galactomannans to monosaccharides (Duffaud *et al*, 1997).

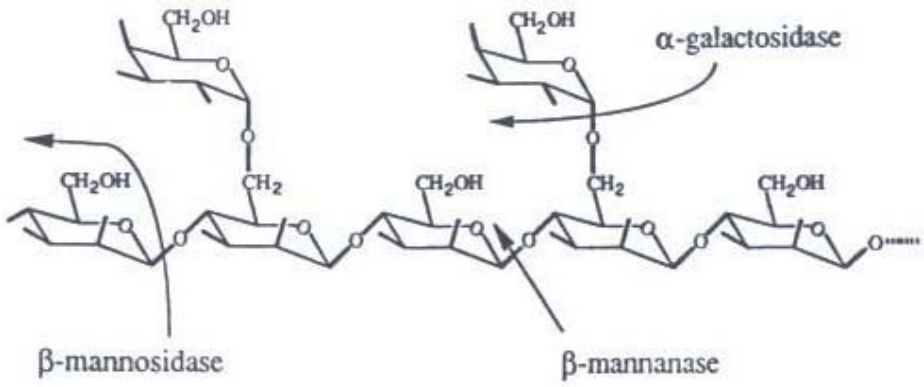

Figure 4 Mode of action of beta-mannosidase on galactomannan molecule
Source: Duffaud *et al*. (1997)

However, attempts to demonstrate the presence of the enzyme have met with limited success. Many of the problems appeared to be due to a relatively specific interaction of these enzymes with other relatively insoluble protein materials presenting in the organism and also to the unstability of the enzyme (McCleary, 1988).

3.2.3 Characterization of beta-mannosidase

Beta-mannosidase activity can be assayed by using β-D-manno tetraitol or β-D-mannopentaitol as substrate and measuring the release of D-mannose

by a suitable reducing sugar. A more convenient assay, beta-mannosidase could be detected by using *p*-nitrophenyl β-D-mannoside and the rate of cleavage of glycosidic bonds measured as the rate of release of *p*-nitrophenol.

McCleary (1983) found that the beta-mannosidase from the gut solution of *Helix pomatia* was not only specific to the (1,4)-linked β-D-mannosyl residues but also (1,3)- and-(1,6)-linked β-D-mannosyl residues.

Gubitz *et al.* (1996a) had studied beta-mannosidase purified from *Sclerotium rolfsii*, a plant pathogenic Basidiomycete, by precipitation with ammonium sulfate and ethanol, and hydrophobic interaction chromatography. The molecular weight determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric point for beta-mannosidase were 57.5 kDa and 4.5, respectively.

Furthermore, the purified beta-mannosidase from the culture fluid of alkalophilic *Bacillus* sp AM-001 had molecular weight and pI of 94 kDa and 5.5, respectively. The enzyme was most active at pH 6.0 and 50°C and was inhibited by some metal ions and chemical reagents as follows: Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+}. But it was not inhibited by D-mannose, D-manitol or D-mannonic acid-γ-lactone (Akino *et al*, 1988).

3.3 Alpha-galactosidase

3.3.1 Sources of alpha-galactosidase

Many microorganisms have been shown to produce alpha-galactosidase (Table 8-9). The alpha-galactosidases have been found to be both extracellular and intracellular enzymes. Pederson and Goodman (1980) reported that alpha-galactosidase from *Bacillus stearothermophilus* AT-7 was an intracellular,

tetrameric isozyme of approximately 300 kDa. In addition, alpha-galactosidase was found in plant as well (McCleary, 1988; Dey and Pridham, 1972).

Table 8 Alpha-galactosidase producing bacteria

Sources	References
Bacteria	
Thermotoga neapolitana 5068	Duffaud *et al*. (1997)
	McCutchen *et al*. (1996)
Thermotoga neapolitana	Vielle *et al*. (1995)
	Dakhova *et al*. (1993)
Thermotoga maritima	Gabelsberger *et al*. (1993)
	Winterhalter and Liebl (1995)
Mortierella vinacea	Shibuya *et al*. (1997)
Bacillus stearothermophilus	Fridijonsson *et al*. (1999)
Bacillus stearothermophilus NUB 3621	Fridijonsson *et al*. (1999)
Pseudomonas fluorescens subsp. *Cellulose*	Halstead *et al*. (2000)
	Hazlewood *et al*. (1998)
	Braithwaite *et al*. (1995)
	Rixon *et al*. (1992)
Phanerochaete chrysosporium	Brummer *et al*. (1999)
Streptococcus mutums	Aduse-Opokn *et al*. (1991)
Bifidobacterium adolescentis	Van den Broek *et al*. (1999)
Bacteroides ovatus	Gherardimi *et al*. (1985)
Clostridium thermocellum	Clarke *et al*. (2000)

Table 9 Alpha-galactosidase producing Fungi and Yeast

Sources	References
Fungi	
Aspergillus niger	Ademark *et al*. (2001)
Aspergillus tamarii	Civas *et al*. (1984)
Aspergillus nidulans	Rios *et al*. (1993)
Aspergillus ficuum	Zapater *et al*. (1990)
Penicillium sp.23	Varbanets *et al*. (2001)
Penicillium simplicissimum	Luonteri *et al*. (1998)
Penicillium ochrochloron	Dey *et al*. (1993)
Trichoderma reesei	Stalbrand *et al*. (1993)
	Margolles-Clark *et al*. (1996)
Trichoderma reesei RUT C-30	Zeilinger *et al*. (1993)
Sporotichum cellulophilum	Araujo and Ward (1991)
Yeast	
Candida guilliermondii H404	Hashimoto *et al*. (1993)

3.3.2 Mode of action

Alpha-galactosidase (alpha-D-galactoside galactohydrolase, E.C. 3.2. 1.22) or melibiase catalyzes the cleavage of terminal α-1,6-linked galactosyl residues (in Figure 4) from a wide range of substrates. Polysaccharides, linear and branched oligosaccharides for example, melibiose, raffinose, stachyose, and verbascose (Mccleary, 1988), and synthetic substrates such as *p*-nitrophenyl-α-D-galactopyrano-side (PNGP) and methyl -α-D-galactopyranoside (Ademark *et al*, 2001) had been used as substrate for α-galactosidase.

Ademark *et al*. (2001) showed that multiple alpha-galactosidases from *Aspergillus niger* also degraded galactomanno-oligosaccharides and released 66% of the galactose residues from polymeric locust bean gum galactomannan.

Previous studies showed that the main products from the action of alpha-galactosidase could be different depending on both types of substrate and sources of the enzyme used. Purified alpha-galactosidase from *Penicillium* sp. 23 was specific for hydrolysis, link-type at varying rates of the terminal galactose from disaccharides, attached by α-1,2-, α-1,3- and α–1,6-links. These enzymes were ineffective towards disaccharides with α-1,4-links (Varbanets *et al*, 2001).

3.3.3 Characterization of alpha-galactosidase

Halstead *et al*. (2000) showed that the alpha-galactosidase A from *Pseudomonas fluorescens* subsp. *cellulosa* had a molecular weight of 45.9 kDa, designated alpha-galactosidase 27A (Aga27A).

While, McCutchen *et al*. (1996) reported the properties of alpha-galactosidase from the hyperthermophilic bacterium, *Thermotoga neapolitana* 5068 (TN5068). The purified enzyme showed an optimum temperature at 100-105°C and optimum pH at 7.5. Half-life of the enzyme at 90°C and 100°C were 130 and 3 minutes, respectively.

Pederson and Goodman (1980) studied isozymes of alpha-galactosidase from *Bacillus stearothermophilus* strain AT-7. Alpha-galactosidase I had a pH optimum of 6 and a half-life at 65°C of > 2 h at low protein concentration. While, alpha-galactosidase II has a pH optimum of 7 and a half-life at 65°C of about 3 minutes.

Alpha-galactosidases are used in many industries, especially food processing. They are used for hydrolysis of raffinose in beet sugar syrup (Kobayashi *et al*, 1972), to break down galacto-oligosaccharides in soy bean milk and other legume-derived foods (Cristofaro *et al*, 1974) and to improve the gelling properties of galactomannans to be used as food thickeners (Bulpin *et al*, 1990).

4. Prebiotics

4.1 Definition

Prebiotic is a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth or limiting a number of bacteria in the colon, that can improve the host health (Gibson and Roberfroid, 1995). Thus, the prebiotic approach advocates administration of non-viable entities and therefore overcomes survival problems in the upper gastrointestinal tract. The prebiotic concept considers that many potentially health-promoting microorganisms, such as bifidobacteria and lactobacilli, are already resident in the colon (Gibson *et al*, 2000).

Any dietary material that enters the large intestine is a candidate prebiotic. This includes carbohydrates such as resistant starch and dietary fiber as well as proteins and lipids. However, it is the case that current prebiotics are confined to non-digestible oligosaccharides.

The non-digestible oligosaccharides contain mixtures of oligomers of different chain lengths and are characterized by the average number of osyl moieties. The non-digestible oligosaccharides are oligomeric carbohydrates, the bonds of which are in a configuration that allow resistance to hydrolytic activities of intestinal digestive enzymes. But they are fermented by the colonic bacteria (Roberfroid, 2002).

4.2 Type of prebiotics

4.2.1 Polysaccharides

Inulin type fructans had been reported that could produce effect on intestinal bifidobacteria (Gibson *et al*, 2000). Inulin naturally occurs in several foods, such as, garlic, onion, asparagus, chicory, artichoke, banana and wheat (Gibson *et al*, 2000). Moreover, starch that is resistant to upper gut digestion is metabolized by bifidobacteria and lactobacilli (Gibson *et al*, 2000).

4.2.2 Oligosaccharides

Oligosaccharides are sugars consisting of between approximately 2-20 saccharide units. Various other oligomers that cannot be digested may be also prebiotics.These include lactulose, fructooligosaccharides (FOS), galactooligo-saccharides (GOS), soybean oligosaccharide, lactosucrose, isomaltooligosaccharides (IMO), glucooligosaccharides (GOS), xylooligosaccharides (XOS), and mannooligosaccharides (MOS) (Gibson *et al*, 2000). Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides or through enzymatic transfer reactions from lower molecular weight sugars.

The sugars recently studied are yeast cell wall oligosaccharides that stimulate immunity and modify gut microflora. Mannose-containing polysaccharides are a major constituent of the cell wall of baker's yeast, *Saccharomyces cerevisiae*. The component consists of mannan 30%, glucan 30% and protein 12% (Lyon, 1994). Glucan forms the matrix of the cell wall which are covered by mannose sugar (Figure 5). These mannose are arranged in a highly branched chain of mannopyranoside residues. Its backbone has the chain of alpha-1,6, with side chains bound by alpha-1,2 and alpha-1,3. It is known that the immunodominant side chains are of four types, mannotetraose, mannotriose, mannobiose and mannose. They have powerful antigenic stimulating properties, which involve processing of phagocytosis, a diverse family of white blood cell.

For example, when a tissue is infected, it will express selectins which protrude from cell surface. Passing of phagocytes attached to these selectins occur by the lectin-carbohydrate mechanism. From experiment, it was found that the phagocytic activities were increased when peripheral blood from three-month-old Wistar male rats contained manno-oligosaccharides (Lyon, 1994). Moreover, oligosaccharides containing mannose may also affect the immune system by stimulation of the liver to secrete mannose binding protein. This structure can also bind to the bacterial capsules (Newman, 1994).

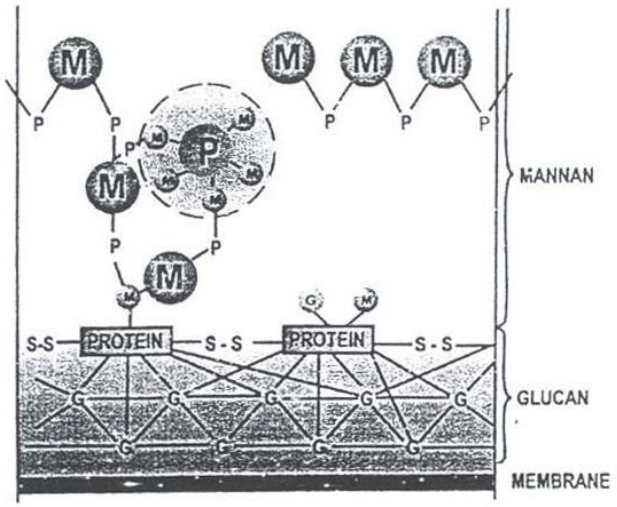

Figure 5 Structure of the yeast cell wall

Source: Sa-nguansook (2002)

4.2.3 Sugar alcohols

Sugar alcohols such as lacitol, sorbitol and maltitol may also be candidate prebiotics.

In addition, prebiotics have been incorporated into many functional foods and drinks in Japan as shown in Table10.

Table 10 Application of oligosaccharides in 'Foods for Specified Health Use' (FOSHU) in Japan

Oligosaccharide	Product type	Product	Manufacturer
Fructo-oligosaccharides	Table-top sweetener	Meioligo granules and syrup	Meiji Seika
		and MS Meioligo syrup	
		Fructo-oligo-saccharide	Nippon Oligo
		Oligo 55 and Oligosugar 39	Hakubun
	Coffee drink	Oligo coffee	Meiji Seika
	Custard dessert	Meioligo Purin	Meiji Milk Products
			Meiji Seika
	Candies	Oligo Yogurette and	
		Oligo candy	
		Maiasa Sokaina	
Lactulose	Soft drink	Cup Oligo Sweet Extra	Morinaga milk Industry
Transgalacto-		Oligomate HP	
oligosaccharides	Table-top sweetener	Oligotop	Nissin Sugar
		Power gold	Yakult Honsha
		Ecolife	Ito Kampo Seiyaku
Isomalto-oligosaccharide	Soft drink	Oligo time	Asahi Beverage
	Lactic acid bacteria drink	Oligo CC	Miru Sohonsha
	Table-top sweetener	Sutto and Pisesu	Showa Sangyo
Soybean oligosaccharides	Soft drink	Soy oligosaccharide syrup	Calpis Co.
		Eitos (syrup and granules)	Joban Yakuhin Kogyo
	Table-top sweetener	Bifiup	Calpis Co.
		Yogurina	Taisho Pharmaceutical
		Morishige Genskissu	Calpis Co
Xylo-oligosaccharide	Lactic acid drink	Sukkiri Kaicho and	Suntory
	Flavoured vinegar	Sukkiri Kaicho Jouka	Morushige Ueda
	Chocolate and Candy	One a Night	Lotte
		Oligo 2400 (Apple, Carrot,	
Lactosucrose	Soft drink	Grape)	Otsuka Pharmaceutical
		Oligo No Asahi, Nyuka	Taisho Shokuhin Kogyo
		Oligo, Nyuka Oligo	
	Table-top sweetner	Graduated, Oligo No Okage	Ensuiko Sugar
		and Oligo No Okage EX	
		Frozen yogurt	
		Piku Oligo Candy	
		Piku Oligo biscuit	
	Frozen yogurt		Ezaki Glico
	Candy		Ezaki Glico
	Biscuit		Ezaki Glico

Source: Gibson *et al*. (2000)

4.3 Production of prebiotics

There are many oligosaccharides used as prebiotics. These molecules are manufactured using a range of extraction and chemical and enzymatic synthesis methods.

4.3.1 Extraction

The simplest approach to the manufacture of prebiotics is to extract them from a biological material. This is currently commercially performed with inulin extracted from chicory and with raffinose and stachyose extracted from soy beans or chicory chips extracted by hot water (Gibson *et al*, 2000).

4.3.2 Chemical methods

Lactulose is the only prebiotic manufactured using chemical rather than enzymatic technology (Timmermans, 1996). The reaction is a base-catalysed isomerisation of the glucosyl moiety of the lactose to form fructose by the Lobry de Bruyn-Alberda van Ekenstein reaction (in Figure 6) (Gibson *et al*, 2000).

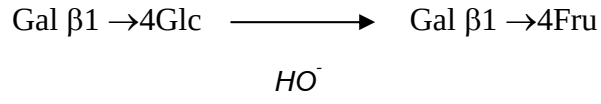

Figure 6 Lobry de Bruyn-Alberda van Ekenstein reaction
Source: Gibson *et al*. (2000)

The reaction produces pyranose and furanose forms of the lactulose. Sodium hydroxide can be used as the base in the reaction, which needs to control the conversion rate.

4.3.3 Enzyme technology

Although chemical methods can be used, they are generally inferior to enzyme-mediated approaches. Chemical methods usually produce unwanted colour and flavour compounds, which need to be removed in additional steps. Therefore, enzyme technology could be used to produce specific product.

Harjunpaa *et al*. (1995) had studied the mannanase from *Trichoderma reesei*. Attention was focused on the degradation pattern and stereochemical course of the hydrolysis. The results showed that the final products of reaction were mannotriose which has transglycosylation.

5. Mannooligosaccharides, MOS

5.1 Characterization of MOS

Important developments also include the use of prebiotics that have activities with multiple biological functions. Moreover, mannose, mannooligo-saccharides and oligosaccharides that have mannose side chains were also effective in reducing colonization of various bacteria in intestinal tract of animal. (Lyons, 1994). The function and characteristic of manno-oligasaccharide had been studied.

Mannooligosaccharides (MOS) not only have activities with multiple biological functions but can also modulate the immunoreactivity of the intestinal associated lymphoid tissue, providing protection against bacterial adhesion (Huyghebaert, 2003). Mannose, mannooligosaccharides and oligosaccharides that have mannose side chains were also effective in reducing colonization of various bacteria including type 1-fimbriated, α-D-mannose-sensitive agglutination, *Escherichia coli* and *Salmonella* sp. It leads to elimination of these bacteria with the digesta flow instead of binding to mucosal receptors (Parks *et al*, 2001 and Fermandez *et al*, 2002). In contrast Clostridia do not express type-1-fimbriae involved other mechanisms such as: aggregation and modulation of the immune responses.

Pathogens with the mannose-specific Type-1 fimbriae adsorb to the manno-oligosaccharides instead of attaching to intestinal epithelial cells and, therefore, move through the intestine without colonization (Ofek *et al*, 1977). Mannose was shown by Oyofo *et al*. (1989) to inhibit the *in vitro* attachment of *Salmonella typhimurium* to intestinal cells of the day old chicken. Then, they provided evidence that dietary D-mannose was successful at inhibiting the intestinal colonization of *S. typhimurium* in broilers.

The type-1 fimbria is a thin, 7 nanometer wide and approximately 1 micrometer long, rod-shaped surface organelle. It is a heteropolymer consisting of four different subunits (Stentebjerg *et al*, 1999). Approximately 1,000 copies of the major building element, FimA, are polymerized into a right-handed helical structure also containing small percentages of the minor components, FimF, FimG and FimH (Brinton, 1965; Klemm and Christiansen (1987); Krogfelt and Klemm (1988)). It has been shown that the receptor-recognizing element of type 1 fimbriae is the 30 kDa FimH protein. Type 1 fimbriae are found on the majority of *E. coli* strains and are widespread among other members of *Enterobacteriaceae* (Klemm and Krogfelt, 1994).

Ooi and Kikuchi (1995) reported that mannooligosaccharides produced from konjak glucomannan were one of the best growth factors for lactic acid bacteria and have been found to stimulate the growth of *Bifidobacterium* sp. (Oda *et al*, 1993).

Mannooligosaccharides affected to recognition that cell surface carbohydrates have an important role as receptors for bacterial and viral pathogenic microorganisms, blood cells and antibodies. These glycobiology can bring some control to intestinal pathogens (Perry, 1995). While the response of animal to these complex sugars is not entirely understood, two possible modes of action have been proposed. These include: (1) Blocking colonization of pathogenic microorganism (2) The pathogenic microorganism cannot use MOS as the nutrients (Lyons, 1994).

5.1.1 Blocking colonization of pathogens

Another possible mode of action for mannan-based oligosaccharides involves interference with colonization of intestinal pathogens. Bacteria have lectins on the cell surface that recognize specific sugars and allow the cell to attach to that sugar (Newman, 1994). To colonize the mucosal surfaces, bacteria must first bind to receptors containing D-mannose epithelial cells with bacterial lectins in these tissues. Therefore, if mannose or manno-oligosaccharides are present in the intestinal tract in sufficient concentration, it may be possible to block the mannose specific receptors (lectin) on the surface of pathogenic bacteria including coliforms, *E. coli* and *Salmonella* sp. and to reduce intestinal epithelium colonization (Martin (1994); Ishihara (2000); Spring (2000)).

Mannose-specific adhesive have also been found in a variety of gram-negative bacteria, including members of the family *Enterobacteriaceae*, for instance, *Shigella, Enterobacter* and *Klebsiella* sp. (Adlerberth, 1996) and *E.coli* strain Nissle 1917 (Stentebjerg-Olesen *et al*, 1999).

5.1.2 The pathogenic microorganism cannot use MOS as the nutrients

The ability of mannanoligosaccharides to resist bacterial digestion may also explain some of the observed effects. It has been demonstrated that when selected pathogens are incubated with mannan, it does not support beneficial species growth such as lactobacilli and bifidobacteria (Lyons, 1994).

5.2 Production of MOS and its application

The role of oligosaccharides released from nondigestible oligosaccharides by enzyme hydrolysis had been investigated to assess their prebiotic effects. In the recent years, beta-mannanase had been used in feed because beta-mannanase could produce MOS as major product of hydrolysis reaction. Moreover, Choct and Kocher,

(2001) are more interested in using this mannanase to improve the nutritive value of non-conventional ingredients including copra meal.

In addition, beta-mannanase from *Bacillus subtilis* 5H are able to improve the quality of coconut meal. Twenty percents of enzymatic treated coconut meal which mixed in the broiler diets decreased total number of *E. coli* and *Salmonella* sp. to the lowest after 4 weeks.

MATERIALS AND METHODS

1. Material

The coconut residual cakes, usually called copra meal, bought from Pakkret Market Thailand was used as a substrate for enzyme assay and a carbon source for medium formulation. The residual was dried at 60°C for 4 h. After that, the residual was blended and milled by Hammer mill (Janke & Kunkel IKA Labortechnik) to obtain the product with the particle size of 0.5 mm.

2. Sample sources

Forty-nine samples of soils, coconut waste and fermented coconut collected from various sources in Thailand were used as sources for isolation of mannanase producing microorganism.

3. **Microorganisms and culture condition**

Lactobacillus reuteri KUB-AC5, *Escherichia coli* E010 and *Salmonella* sero Enteritidis S003 from the culture collection of the Department of Biotechnology, Kasetsart University, Thailand, were used as target strains for secondary screening. The strain KUB-AC5 was grown in MRS broth (Merck, Darmstadt, Germany) at 37°C for 12-15 h while the strain E010 and S003 were grown in Nutrient broth, NB, (Pronadisa, Madrid, Spain) under the same condition with shaking at 150 rpm.

4. Medium

4.1 Isolation medium

For enrichment step, 1 % of copra meal was added to Isolation medium. Bacteria isolation medium (BIM) (modified from Abe *et al*, 1994) consisted of (w/v) 0.1% yeast extract, 0.1% polypeptone, 0.1% NH_4NO_3, 0.14% KH_2PO_4 and 0.02%

$MgCl_2$. Fungi isolation medium (FIM) (modified from Vladimir *et al*, 1999) consisted of (w/v) 1.43% yeast extract, 0.21% $(NH_4)_2SO_4$, 0.03% $MgSO_4$, 0.03% $CaCl_2$, 0.05% $FeSO_4$, 1% KH_2PO_4 and 2% glucose. While, yeast Isolation medium (YIM) (modified from Kremnicky *et al*, 1996) consisted of (W/V) 0.2% yeast extract, 0.2% KH_2PO_4 and 0.2% $(NH_4)_2SO_4$.

4.2 Enzyme production medium (PM)

Enzyme production medium (PM) (modified from Mohammad *et al*, 1996) consisted of (w/v or v/v) 1% copra meal, 1.5% KH_2PO_4, 3% polypeptone, 0.06% $MgSO_4$ and 2.5% corn steep liquor.

5. Screening mannanase producing microorganism

5.1 Enrichment

The sample, 1 g of solid sample or 1 ml of liquid sample, was suspended in 10 ml of sterilized 0.8% normal saline. The solution was mixed by stomacher with normal mode in 60 second. One percent (v/v) of the solution was transferred into 20 ml of sterilized IM under aerobic condition by shaking at 150 rpm for 18 h at 37°C for bacteria and 30°C for fungi and yeast.

5.2 Primary screening

The appropriate dilution of the culture broth from 5.1 was spreaded on BIM agar medium. After incubation at 37°C for 18 h, the mannanase activity of each isolate was determined by the ratio of diameter of clear zone to diameter of colony. The effective colonies were collected and maintained as frozen stocks at -87° C in the presence of 20% glycerol for further study.

The culture broth from BIM was spreaded on BIM containing 1.5% agar and 1% locust bean gum and allowed the growth of bacteria, fungi and yeast at 37, 30,

and 30°C for 16-24 h, respectively. The colonies with a clear zone showing mannanase activity against locust bean gum (MAL) were selected and kept at -87°C for further study.

To confirm mannanase activity against copra mannan, each active isolates was prepared as an inoculum by growing in 20 ml of NB under aerobic conditions by shaking at 150 rpm for 18 h at 37°C. Then, one percent (v/v) of each preparative culture solution was transferred into 100 ml of PM in a 250 ml flask and grown at 37°C for 24 h. The preparative culture was centrifuged at 4°C, 9000 rpm for 15 min. The supernatant was collected to determine mannanase activity against copra mannan and to perform secondary screening.

5.3 Secondary screening

The secondary screening was based on the effect of supernatant containing copra mannan hydrolysate (CM-hydrolysate) to the growth of target bacteria, *L. reuteri* KUB-AC5, *S.* Enteritidis S003 and *E. coli* E010. One percent of culture solution of each target strains, adjusted to absorbance of 0.5 at 600 nanometer, and 1% of the CM-hydrolysate was transferred into 5 ml of MRS and NB to determine the growth of KUB-AC5 and S003 as wells E010, respectively. The mixture was incubated at 37°C for 4 h. After that, the culture was spreaded on either MRS or NB containing 1.5% agar and grown at 37°C for overnight.

The enhancing activity of KUB-AC5 growth was determined as equation of [SF-CF]. Where SF and CF were the amount of cell number in MRS with CM-hydrolysate (log cfu/ml) and the amount of cells in MRS with PM (log cfu/ml), respectively.

While the inhibition activity of S003 and E010 were determined in equation of [CF-SF] where SF and CF were the amount of cells in NB with CM-hydrolysate (log cfu/ml) and the amount of cells in NB with PM (log cfu/ml).

6. Identification of Isolate ST1-1 and CW2-3

The inoculum of effective isolates were grown in 20 ml of NB under aerobic condition by shaking at 150 rpm for 24 h at 37°C. After incubation, the culture broth of each isolate was used as a sample for further study.

6.1 Determination of morphology and other physical characteristics

Cell shape and Gram-stain of 24 h culture broth were examined by phase contrast microscope (Olympus CH 30). The Gram stain was investigated by the method of Beishier (1991). The test of motility, catalase, oxidase, spore-forming, capsule-forming and Voges-Proskauer (VP) reaction methyl red, ability to utilize citrate, indole reaction, gelatin hydrolysis, glucose fermentation and ability to produce hydrogen peroxide were investigated using the methods described by Brown (2005).

6.2 Determination of growth at various temperatures and sodium chloride concentrations

The growth of the selected strains at temperature of 10, 37 and 45°C were determined by inoculating one colony of an overnight culture into NB medium. The growth of each strain was examined by observing the turbidity of the broth. Salt tolerance was tested in NB medium supplemented with 5% (w/v) sodium chloride. The abilities to grow under all these conditions were evaluated spectro-photometrically by measuring the turbidity at 600 nm after 24h incubation time as previously described by Noel (1984).

6.3 Determination of carbohydrate fermentation patterns

API 50CH Rapid fermentation strips in CHB medium (API Laboratory Products Ltd. Biomerieus Saa, France) were used to determine the biochemical and carbohydrate fermentation patterns. The experiment was performed at 37°C.

The results of biochemical test and carbohydrate fermentation were determined after 24 and 48 h.

6.4 Nucleotide sequence analysis of the 16S rDNA

6.4.1 PCR amplification of 16S rDNA gene

Preparation of chromosomal DNA of each active strains as a template and their 16S rDNA were performed according to Alemu *et al*, 2006. Briefly, the cells were resuspended in SET buffer and treated with lysozyme and 20% SDS to break the cells and release their intracellular components. The cell lysate was deproteinized by successive extractions with organic solvents: phenol, phenol-chloroform 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).The oligonucleotide primers, 8UA (forward primer: 5'-AGAGTTTGATCC TGGCTCAG-3') and 1407B (reverse primer: 5'- GACGGGCGGTGTGTAC-3'), were used to amplify about 1.4 kb fragment from 16S rDNA gene.

PCR was carried out in a volume of 25 μl containing 10xbuffer, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 1 μM of each primer (KU-Vector DNA Synthesis Unit, Kasetsart University), 1U of *Taq* polymerase (promega, USA) and about 200 ng of template DNA. All amplification reactions were investigated 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 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% agarose gel using TBE buffer.

6.4.2 Cloning and sequencing of 16S rDNA

The amplified products of 16S rDNA were purified by using QIAEX II Gel Extraction Kit (QIAGEN Inc, USA) and ligated into pGem-T Easy vector by the method of the manufacturer (Promega Co.Ltd). 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 competent cell of *E. coli* DH5α was prepared by calcium chloride method according to the method of Rodrigues and Tait, 1983. The resulting ligation products were transformed into *E. coli* DH5α competent cells in accordance with the manufacturer protocol. Briefly, 2 μl of ligation products were 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 incubated for 2 h at 37°C with shaking at 150 rpm. Then, 100 μl of the culture was spreaded on LB/Amp/IPTG/ X-gal agar medium and incubated at 37°C for 18h.

White colonies were picked up and cultured in LB broth plus ampicillin (100 μg/ml) (Sigma). These colonies were isolated by alkaline lysis procedure (Sambrook and Russell, 2001). Briefly, the cells were 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. After that, the plasmid DNA was collected by removing the aqueous phase (upper phase). This plasmid DNA 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 reaction mixture was incubated for 1 h 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.

The sequence of amplified product 16S rDNA was analyzed by Bioservice Unit (BSU), Thailand. The resulting sequence was compared with the non-redundant nucleotide database from GenBank by using the BLAST program.

7. Enzyme production

The innoculum of effective strain was prepared in 20 ml of NB under aerobic conditions by shaking at 150 rpm for 18-24 h at 37°C. One percent (v/v) of inoculum was transferred into 100 ml of PM containing 1% of copra meal in a 250 ml flask and grown at 37°C for 24 h. Then, the culture was centrifuged at 4°C, 8000 rpm for 20 min and the supernatant was stored at -20°C for further study.

8. Determination of mannanase activity

Mannanase activity was measured by incubating the reaction mixture of 0.5 ml of sample and 0.5 ml of 50 mM potassium phosphate buffer pH 7.0 with 0.5% copra meal at 50°C for 60 min. Amount of reducing sugar released was determined by dinitrosalicylic acid (DNS) method (Miller 1959).

One unit of enzyme activity was defined as amount of enzyme producing 1 micromole mannose per minute under the experimental condition.

9. Protein determination

Protein concentration was determined by the method of Lowry *et al*. (1951). Bovine serum albumin was used as a standard.

10. Enzyme purification

10.1 Ammonium sulfate precipitation

The concentrated mannanase was prepared by saturated ammonium sulfate precipitation. One hundred ml of solution consisting of 30 ml cell free supernatant and 70 ml saturated ammonium sulfate solution was centrifuged at 4°C, 7000 g for 30 min. The protein precipitate was suspended in 10 mM Bis-Tris buffer, and stored at -20°C for further study.

10.2 Anion exchange chromatography

10.2.1 Q Sepharose chromatography

Two ml of concentrated enzyme from 10.1 was applied to Q Sepharose column (1.6x20 cm) equilibrated with 10 mM Bis-Tris buffer (Buffer A) (Fluka, USA) pH 7.0, with flow rate 2 ml/min. The column was washed extensively with buffer A until no eluting protein was detected by absorbance measurement at 280 nm. The column was then eluted with a linear 0 to 1 M sodium chloride in 10 mM Bis-Tris buffer (Buffer B), and 5 ml fractions were collected. The fractions were assayed for mannanase activity as described in 8. The fractions which contained mannanase activity were pooled and then concentrated approximately 10-fold by ultrafiltration with 10,000-molecular-weight-cutoff membranes on a 180 ml stirring cell (Amicon, Beverly, Mass).

10.2.2 Q Source Chromatography

Fractions (5 ml) of the concentrated mannanase preparation from the previous step were applied to a Q Source column equilibrated with the same Buffer A, with flow rate 2 ml/min. The column was washed extensively with buffer A until no eluting protein was detected by absorbance measurements at 280 nm. The column was then eluted with a linear 0 to 1 M sodium chloride in Buffer B, and 5 ml fractions were collected. The fractions were assayed for mannanase activity as described in 8. The fractions with mannanase activity were pooled for further step.

10.3 Ultrafiltration

The enzyme from 10.2.2 was further purified by ultrafiltration using 50,000-molecular-weight-cutoff membrane on a 180 ml stirring cell (Amicon, Beverly, Mass). Both filtrate and retentrate were then separated and assayed for mannanase activity as described in 8.

11. Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of the purified enzyme was determined by SDS-PAGE according to Laemnli (1970) using 10% acrylamide separating gel (as described in Appendix A) and stained with silver stain plus kit (Bio-Rad, USA). The pre-stained marker (Bio-Rad, USA) used composed of myosin (195,755), β-galactosidase (107,181), bovine serum albumin (59,299), ovalbumin (41,220), carbonic anhydrase (27,578), soybean trypsin inhibitor (20,514), lysozyme (15,189) and aprotinin (6,458).

12. Native- polyacrylamide gel electrophoresis (Native-PAGE)

The native purified enzyme was determined by 7.5% acrylamide in separating gel (as described in Appendix A) and stained with silver stain plus kit (Bio-Rad, USA). The high molecular weight native marker kit (Amersham Bioscience, Sweden) used consisted of thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000) and albumin (66,000).

13. Isoelectric focusing

Isoelectric focusing was performed on Ampholine^{TM} PAGplate pH 3.5-9.5 (Amersham Biosciences, Sweden) according to instructions of the manufacturer. The broad range pI calibration kits (Amersham Biosciences, Sweden) used were: amyloglucosidase (pI 3.50), methyl red dye (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human

carbonic anhydrase B (pI 6.55), horse myoglobin-acidic band (pI 6.85), horse myoglobin-basic band (pI 7.35), lentil lectin-acidic band (pI 8.15), lentil lectin-middle band (pI 8.45), lentil-lectin basic band (pI 8.65) and trypsinogen (pI 9.30).

14. Determination of optimum pH and pH stability

The optimum pH of enzyme activity was examined at pH 3.0-10.0 under standard assay conditions. Fifty mM of various buffers: citrate (pH3.0-6.0), phosphate (pH6.0-8.0) and glycine-NaOH (pH 8.0-10.0) were used. The enzyme reaction was incubated at 50°C for 60 min in the presence of 0.5% LBG dissolved in the buffers. The effect of pH on enzyme stability was determined using the same buffer system in the range of 3.0-10.0. After incubation of the enzyme solution at various pH values for 24h at 4°C without the substrate, the remaining enzyme activity was measured at pH and temperature optimum for 60 min.

15. Determination of optimum temperature and temperature stability

The effect of temperature on enzyme activity was performed at temperatures ranging from 30-60°C in 50mM buffer at optimum pH for 60 min. Thermal stability of the enzyme was determined at various temperatures in 50mM buffer at optimal pH for 30 min. Then, the remaining enzyme activity was measured.

16. Determination of kinetic parameters, K_m, V_{max} and K_{cat}

The Michaelis-Menten kinetic parameters, K_m, V_{max} and K_{cat} were calculated for mannanase. The K_m, V_{max} and K_{cat} values were estimated by using either LBG and Konjak mannan substrate. These reactions were performed with various concentrations of substrate from 0.1-1% (w/v) under the optimum conditions and followed their activities every 5 min for 30 min.

17. Determination of effect of metal ions

The effect of various metal ions; EDTA, Li^{+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Co^{+} and mercaptoethanol on the enzyme activity were determined by measuring the activity of the enzyme in the presence of 1 mM of each ion in the reaction mixture using 10 mM citrate buffer pH 4.0 under the optimum conditions.

18. Determination of substrate specificity

The activity of purified enzyme on various substrate 0.5% (w/v): LBG, α-mannan, ivory nut mannan, konjac glucomannan, xylan (from oat spelts), CMC, avicel and copra meal was determined as described in 8 under the optimum conditions.

19. Determination of amino acid sequence

The amino acid sequence of purified enzyme was analyzed at Research and Research Training Services, University of Newcastle, Australia. N-terminal amino acid sequence homology was analyzed using the BLAST database.

20. Thin-layer chromatography (TLC) analysis

The action pattern of purified enzyme to both LBG and copra meal were determined by TLC modified from the method of Sachslehner *et al*, (2000). Glucose, maltose, galactose, mannose butylate and mannooligosaccharide M2-M7 were used as standards. The solvent used as mobile phase was composed of butanol:isopropanol:ethanol: deionized water in the ratio of 2:3:3:2, respectively. Two μl of each degradation products were applied on Kieselgel 60 (Merck) and developed for 90 min in developing solvent. The brown spots of sugars were developed by dipping in 0.2% (w/v) orcinol in 10% (v/v) sulfuric acid in ethanol and incubated at 100°C for 15 min.

21. Determination of prebiotic properties

For determination of prebiotic properties of copra meal hydrolysate (CM-hydrolysate), the purified mannanase from *Klebsiella oxytoca* CW2-3, S1, was incubated with 0.5% copra meal in 10 mM citrate buffer pH 4.0. The reaction mixture were composed of 0.5 ml, 0.05 U/ml of S1 and 0.5 ml of 0.5% copra meal in 10 mM citrate buffer pH 4.0. The reaction mixtures were incubated at 40°C, for 2 h. Then, the reaction was stopped at 70°C for 10 min. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). The amount of reducing sugar in CM-hydrolysate were performed at the concentration of 0.03, 0.06 and 0.09 mg/ml, defined as 1n, 2n and 3n, respectively and tested for prebiotic properties study as described in 5.3. The ratio of the CM-hydrolysate combination added to reaction mixture are as shown in Table 11.

Table 11 Ratio of the CM-hydrolysate combination added to reaction mixture

Reducing sugar (mg/ml)	Volume (μl)	
	CM-hydrolysate	Buffer
0.03 ;1n	66.66	133.33
0.06 ;2n	133.33	66.66
0.09 ;3n	200	-

22. Place

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

23. Duration

This study was carried out during June 2003 up to December 2006.

RESULTS AND DISCUSSION

1. Isolation and screening of microorganisms

1.1 Primary screening

Forty-nine samples of soil, coconut waste and fermented coconut in Thailand were used as sources for isolation of mannanase producing bacteria. The primary screening was determined by a colony with a clear zone surrounding. Copra meal was target substrate to screen for the bacterial strains possessing mannanase activity. However, it could not dissolve well in BIM, FIM and YIM. Therefore, LBG was used as a substrate instead for this primary screening. Forty-eight bacterial isolates from 3,055 isolates exhibited the activities of 1.33-3.0. The isolate CW2-3 showed the maximum activity of 3.0. While, only one fungi isolate from 1,079 isolates, SN2-1, could display the activity of 1.62 and no yeast isolate having mannanase activity was detected. The primary screening activities were shown in Figure 7.

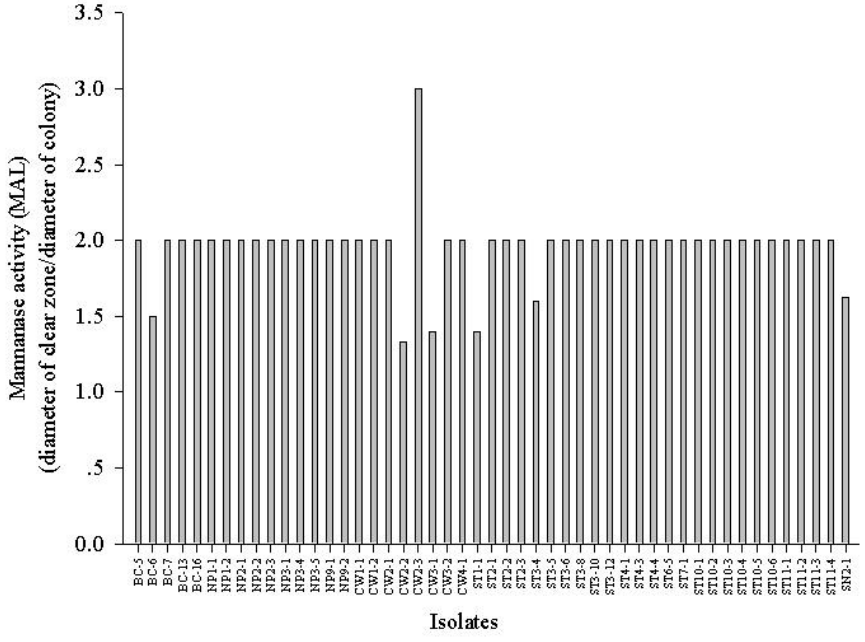

Figure 7 Mannanase activity of 49 active isolates on IM agar medium containing locust bean gum

The frequency and occurrence of the active isolates found were only 1.57%. It seemed that mannanase producing bacteria hardly occurred in these sources of Thailand. However, this study aims to look for the specific mannanase which was active against copra meal. Thus, all 49 isolates were further tested for their activities against copra meal. The results showed that only 10 isolates CW1-2, CW2-1, CW2-3, ST1-1, ST2-2, ST3-4, ST3-10, BC-5 BC-7 and SN2-1 showed mannanase activity of 0.086 – 0.870 U/ml as shown in Figure 8.

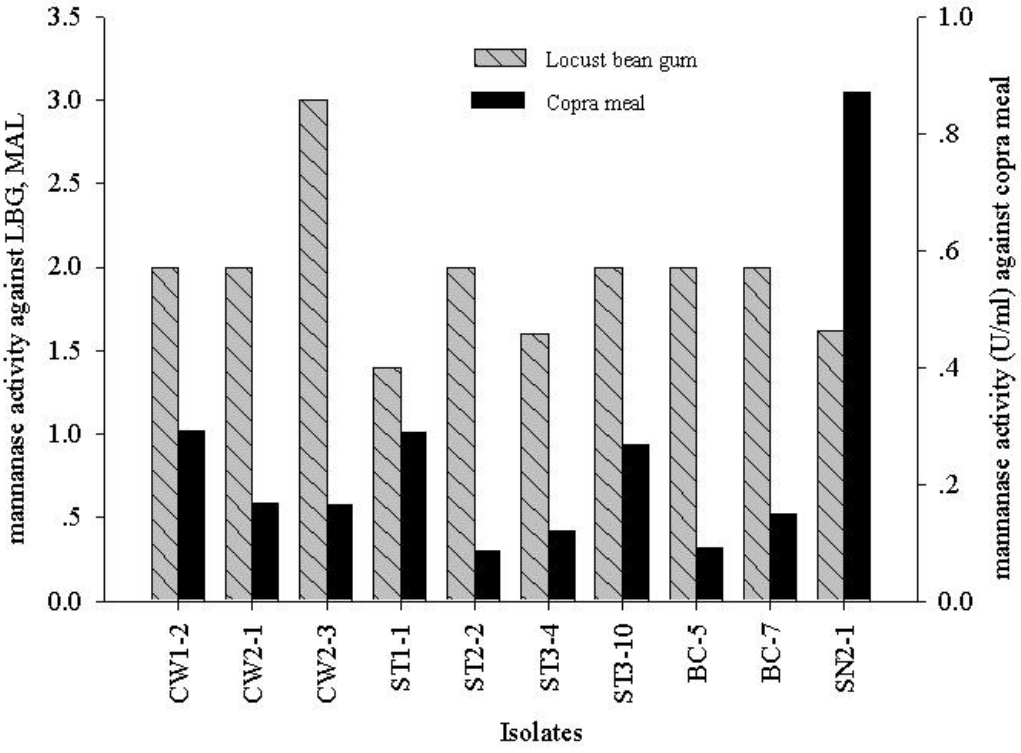

Figure 8 Comparison on mannanase activities of 10 active isolates against locust bean gum and copra meal

The fungal isolate SN2-1, exhibited the highest mannanase activity of 0.87 U/ml whereas the isolate ST2-2 gave the lowest of 0.086 U/ml. The mannanase activity obtained were not correlated to the MAL as shown in Figure 8. The structure of both LBG and copra meal are composed of mannose and galactose. However, they were different in the number of galactose substitutions. Locust bean gum contained higher galactose number than copra mannan did (Burhanudin and

Dingle, 2006). This may lead to different substrate specificity of mannanase from different sources.

Among mannanase producing bacterial isolates, the isolate ST1-1 showed the highest mannanase activity of 0.290 U/ml while the isolate ST2-2 had the lowest mannanase activity of 0.054 U/mg protein. By the results of MAL, the isolate BC5, BC7, CW1-2, CW2-1, CW2-3, ST1-1, ST2-2, ST3-4 and ST3-10 showed the relative activity of 66.7, 66.7, 66.7, 66.7, 100, 46.7, 66.7, 53.3 and 66.7% of the highest activity obtained from the isolate CW2-3, respectively. While, the isolate BC5, BC7, CW1-2, CW2-1, CW2-3, ST1-1, ST2-2, ST3-4 and ST3-10 displayed the relative mannanase activity against copra meal of 31.7, 51.37, 99.6, 57.9, 56.9, 100, 29.6, 41.0 and 92.4%, respectively.

Three separate groups were defined according to the relative activity against both substrates as: (group I) similar activity, CW2-1; (group II) higher activity of mannanase against LBG, CW2-3, ST2-2, ST3-4, BC5 and BC7; and (group III) higher activity of mannanase against copra meal, CW1-2, ST1-1, and ST3-10. The equal activity against both substrate from only one isolate of group I indicated that only one step of primary screening by activity assay on BIM with LBG as a substrate could be used. While, the other isolates produced mannanase activity specific to the substrate type. Therefore, detection of mannanase producing bacteria would need two steps for effective primary screening.

1.2 Secondary screening

Generally, the method to screen microorganism relating to prebiotic production would observe from the growth of target microorganism in the medium containing prebiotic oligosaccharides such as fructo-, gluco-, galacto- and manno-oligosaccharide (Kitova *et al*, 2006). These microbial targets could grow in the medium by utilising the degraded oligosaccharide prebiotic as a carbon source (Gibson *et al*, 2000; Mitterdorfer *et al*, 2001). In this experiment, the supernatant obtained from cultivation in PM medium called CM-hydrolysate was expected to

produce oligosaccharides obtained from degradation of copra meal added. Therefore, 10 active isolates were secondary screened according to the effect of CM-hydrolysate on the promotion or inhibition of the growth of *L. reuteri* KUB-AC5 and *E. coli* E010 as well as *S.* Enteritidis S003, respectively. In order to remove the background of the growth activity due to PM medium, growth of KUB-AC5 or E010 and S003 in MRS or NB containing PM medium were studied in the same manner as secondary screening. The results showed that the cell number of KUB-AC5 decreased to about 4.5 times while those of E010 and S003 increased to about 4.2 and 3 times. Therefore, the growth activity due to PM medium was taken into account to determine the enhancing activity and inhibition activity as mensioned elsewhere.

Considering the effect of CM-hydrolysate on the growth of KUB-AC5, 10 isolates showed the enhancing activity of 0.09-2.15 log cfu/ml as shown in Figure 9A. The isolate CW2-3 displayed the highest enhancing activity of 2.15 log cfu/ml. The CM-hydrolysate from 10 isolates exhibited low inhibition activity of 0.41-1.72 log cfu/ml against S003 (Figure 9B). When the effect of each hydrolysates on the growth of E010 were carried out, only low inhibition activity of 0.46-1.78 log cfu/ml from CW1-2, CW2-1, ST1-1, ST3-10, BC5 and BC5 were obtained (Figure 9B). While the CM-hydrolysate from the isolate CW2-3, ST2-2 and ST3-4 enhanced the growth of E010. Moreover, the CM-hydrolysate from the isolate SN2-1 enhanced the growth of both S003 and E010 as shown in Figure 9B.

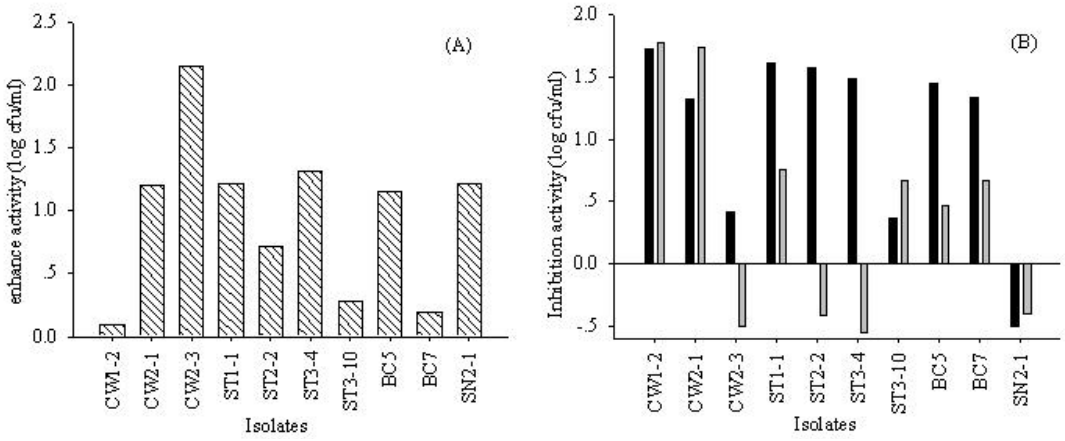

Figure 9 Secondary screening by 10 effective isolates. (A) enhancing activity on *L. reuteri* KUB-AC5; (B) inhibition activity on *S.* Enteritidis S003 and *E. coli* E010

The mannanase activity of isolate CW2-3 obtained from CM-hydrolysate was lower than that of isolate ST1-1. However, its CM-hydrolysate showed higher enhancing activity to KUB-AC5 about 10 times. The results implied that the hydrolysate from this isolate may have prebiotic property to promote the growth of probiotic KUB-AC5 and limit the number of pathogenic bacteria like S003. Manno-oligosaccharides have been reported to reduce incidence of *S.* Enterica and pathogenic *E. coli* in pig and chicken (Naughton *et al*, 2000; Oyofo *et al*, 1989). However, the hydrolysate from isolate CW 2-3 did not inhibit growth of E010. Few studies have investigated non-digestible oligosaccharides as prebiotic to reduce *E. coli* in animals. Generally, fructo-oligosaccharides were successful to reduce *E. coli* in animal more than MOS (Naughton *et al*, 2000; Rao, 1999).

Up to date the method to screen mannanase producing microorganism for prebiotic formation expectation was only single step of mannanase activity determination (Abe *et al*, 1994; Araujo and Ward. 1990a,b; Oda *et al*, 1993). In this experiment, the screening were based on both mannanase activity and the prebiotic properties. This would provide the advantage to obtain the effective strain for prebiotic production. However, this method proposed was only a preliminary step of screening. The metabolites existing in CM-hydrolysate may or may not affect the

growth of target strains. Since mannanase activity against copra meal and enhancing activity to KUB-AC5 are preferred, only two isolate, ST1-1 and CW2-3 were, therefore, selected for further study.

2. Identification of Isolate ST1-1 and CW2-3

2.1 Morphology and biochemical study

According to isolate ST1-1, as shown in Table 12, the isolate ST1-1 was aerobic, non-sporeforming, Gram negative, short rod and pale yellow in color. Twenty four hour cell grown in NB medium was 0.8-1.0 μm in diameter and 1.2-2.0 μm in length. This indicated that the isolate ST1-1 belonged to a group of Gram negative aerobic rods and cocci bacteria. According to Bergey's Manual of systematic of bacteriology (1984), the group of Gram negative aerobic rods and cocci bacteria have 8 families of *Pseudomonadaceae*, *Azotobacteraceae*, *Rhizobiaceae*, *Methylococcaceae*, *Halobacteriaceae*, *Acetobacteraceae*, *Legionellaceae* and *Neisseriaceae*. According to the test of API 20 and API 50 kits, the isolate ST1-1 could produce catalase and utilized citrate, ethanol, acetate, lactate, L-arabinose, ribose, D-xylose, glucose, mannose, rhamnose, cellobiose, maltose and lactose. Based on its morphology and biochemical properties mensioned above, this isolate could belong to family *Neisseriaceae* genus *Acinetobacter* as shown in Table 12. However, the taxonomy of *Acinetobacter* did not possess any unique characteristics (Bergey's Manual of systematic of bacteriology 1984) because they shared many similarities in morphology and physiology with the member of genera *Moraxella*, *Branhamella*, *Neisseria* and *Acinetobacter* (Wiedmann-Al-Ahmad *et al*, 1994).

While, isolate CW2-3 was facultative anaerobe, non-sporeforming, Gram negative, rod and white color. Cell grown in NB for 24h was 0.7-0.9 μm in diameter and 1.2-2.5 μm in length. This strain could produce indole and catalase but not oxidase as shown in Table 13. According to Bergey's Manual of systematic of bacteriology (1984), these characteristics belonged to 3 families consisting of *Enterobacteriaceae*, *Vibrionaceae* and *Pasturellaceae*. However, the negative oxidase

test could be taken as a key criterion for the differentiation indicating the family of *Enterobacteriaceae*. Therefore, the isolate CW2-3 should belong to the family *Enterobacteriaceae* in the group of facultatively anaerobic Gram negative rods. The isolate CW2-3 could utilize citrate, produce gas from glucose and ferment glucose, rhamnose, arabinose, fructose, lactose and raffinose (Table13). The isolate CW2-3 could grow at 10-45°C and exhibited indole production. Comparison of these properties with the differential characteristics in this Family, the Bergey's Manual would suggest that these isolate be classified *Klebsiella oxytoca* as shown in Table 13. However, this isolate could not utilize inulin which would show opposite result to character of *K. oxytoca*. Therefore, further identification based on molecular genetic was recommended.

Table 12 Characteristics of isolate ST1-1

Property	*Acinetobacter* sp.	ST1-1
Cell diameter, μm	0.9-1.6	0.8-1.0
Cell length, μm	1.5-2.5	1.2-2.0
Gram staining	-	-
Oxidase	-	-
Catalase	+	+
Growth temperature, °C	20-30	10-45
Growth on NaCl	-	-
Glucose fermentation	-	+
Utilization of citrate	+	+
Ethanol	+	+
L-arabinose	+ [a]	-
Ribose	+ [a]	+
D-Xylose	+ [a]	+
Galactose	+ [a]	+
Glucose	+ [a]	+
Mannose	+ [a]	+
Rhamnose	+ [a]	-
Cellobiose	+ [a]	+
Maltose	+ [a]	-
Lactose	+ [a]	+

+, positive result; -, negative result; + [a] , 11-89% of strains positive

Table 13 Characteristics of isolate CW2-3 comparing to different species of *Klebsiella*

Property	*pneumoniae*	*oxytoca*	*terrigena*	*planticola*	CW2-3
Indole production	-	+	-	d	+
Growth at 10°C	-	+	+	+	+
Methyl red test	-	-	+	+	-
Vp reaction	+	+	+	+	+
Oxidase test	-	-	-	-	-
Fermentation of:					
Melezitose	-	d	+	-	-
Sorbose	d	+	+	+	+
L-arabinose	+	+	+	+	+
Inulin	-	+	d	d	-
Inositol	+	+	+	+	+
Lactose	+	+	+	+	+
Mannitol	+	+	+	+	+
Rhamnose	+	+	+	+	+
Sucrose	+	+	+	+	+
Glucose	+	+	+	+	+
Raffinose	+	+	+	+	+
Sorbitol	+	+	+	+	+
Adonitol	d	+	+	d	+
Dulicitol	d	d	-	d	d
Utilization of citrate	+	+	+	+	+
Arginine dehydrolase	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	-
Ornithine decarboxylase	-	-	-	-	-
Gelatin hydrolysis	-	d	-	-	-
Production of H_2S	-	-	-	-	-
2-keto-gluconate	-	d	-	-	-

+, positive result; -, negative result; d, some (less than 90%) strains positive

2.2 16S rDNA gene analysis

Analysis of 16S rDNA sequence of both isolates, ST1-1 and CW2-3, were performed to confirm the conventional identification. The PCR products of the 16S rDNA gene gave a product of about 1400 bp for both isolates as shown in Figure 10. The PCR products, lane 4 and 5, were ligated with pGEM-T Easy vector system and transformed to competent cell of *E. coli* DH5α (lane 6 and 7). The plasmid DNA were isolated (lane 8 and 9) and checked for the presence of insert by *Eco* RI (lane 10 and 11). The size of the recombinant DNA were about 4400 bp. By *Eco* RI digestion three major fragments with sizes of about 750, 750 bp and 3000 bp were obtained. The combination of these three fragments gave about 4500 bp by calculation close to the recombinant size of 4400 bp.

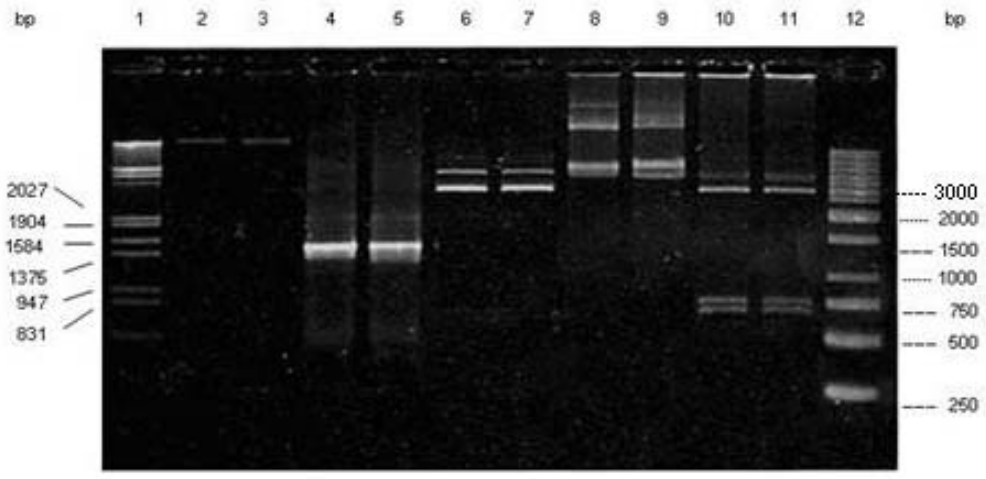

Figure 10 Summary of 16S rDNA gene analysis of the isolate ST1-1 and CW2-3. Lane 1: Lamda DNA/*Eco* RI+*Hind III* Marker (Fermentas), Lane 2: Chromosomal DNA of ST1-1, Lane 3: Chromosomal DNA of CW2-3, Lane 4: PCR product of ST1-1, Lane 5: PCR product of CW2-3, Lane 6: Recombinant plasmid of ST1-1, Lane 7: Recombinant plasmid of CW2-3, Lane 8,9: pGEM^{R}-TEasy Vector (Promega), Lane 10: *Eco* RI digested products of 8, Lane 11: *Eco* RI digested products of 9, Lane 12: 1 kb DNA Ladder (Fermentas) (1% agarose gel)

The recombinant DNA, containing the 16S rDNA gene, was sequenced on both strands. Analysis of the DNA sequences by BLAST program comparing to the database of the GenBank (http://www.ncbi.nlm.nih.gov) revealed that isolate ST1-1 was 98% identity to *Acinetobacter* sp. TUT1001, whereas isolate CW2-3 exhibited 100% identity to *Klebsiella oxytoca* strain NG-14 (Figure 11 and 12).

Genus *Acinetobacter* are ubiquitous organism and clinical importance member (Carr *et al*, 2003). Moreover, the ecology of the species is not well elucidated. The habitats occupied by *Acinetobater* species is activated sludge (Carr *et al*, 2003; Wiedmann-Al-Ahmad *et al*, 1994; Beacham *et al*, 1990; Knight *et al*, 1993), cotton (Nishimura *et al*, 1988), and oil-degrading (Di Cello *et al*, 1997).

While, *K. oxytoca* has the native ability to metabolize cellobioses, cellotriose, xylobiose, xylotriose, sucrose, and all monomeric sugars which are constituents of lignocellulose (Zhou and Ingram, 1999; Quian *et al*, 2003). To dates, *K. oxytoca* was developed as a biocatalyst for cellulose fermentation of ethanol production (Moniruzzaman *et al*, 1997; Wood and Ingram 1992; Golias *et al*, 2002; Lynd *et al*, 2005). So far mannanase producing bacteria were from *Bacillus* sp. (Zakaria *et al*, 1998; Zhang *et al*, 2000; Ethier *et al*, 1998; Araujo and Ward, 1990a; Araujo and Ward, 1990b; Akino *et al*, 1988), *Streptomyces* sp. (Takahashi *et al*,. 1984); *Enterococcus* sp. (Oda *et al*, 1993b) and *Vibrio* sp. (Tamuru *et al*, 1995).

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599
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Figure 11 Alignment of the 16S rDNA gene sequence of ST1-1 with *Acinetobacter* sp. TUT1001

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719

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1198
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Figure 11 (Continued) Alignment of the 16S rDNA gene sequence of ST1-1 with *Acinetobacter* sp. TUT1001

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Query  1197  TTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGAT
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Sbjct  1379  TGTACACACCGCCCGTC  1395
```

Figure 11 (Continued) Alignment of the 16S rDNA gene sequence of ST1-1 with *Acinetobacter* sp. TUT1001

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Query  940   CACCGCCCGTCA  951
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Sbjct  1279  CACCGCCCGTCA  1290
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Figure 12 Alignment of the 16S rDNA gene sequence of CW2-3 with *Klebsiella oxytoca* strain NG-14

3. Characterization of crude enzyme from ST1-1 and CW2-3

3.1 The pH effect on mannanase activity

The effects of pH on mannanase activities were determined from the supernatant of both isolates ST1-1 and CW2-3 as shown in Figure 13. The pH optima of mannanase activity were determined by conducting the activity assays on LBG at 50°C in various pH values of 3-10. The pH optimum of ST1-1 and CW2-3 were 6 and 7 as shown in Fig 13 A and B, respectively. On either side of this pH optimum, the activity declined sharply. They both exhibited a narrow pH range which were similar to mannanase activity from the other *Bacillus* mannanase (pH 6.5-6.8) (Araujo and Ward 1990b; Jiang *et al*, 2006). The pH optimum of these isolates were similar to the other bacterial mannanase of 5.5 –7.5 (Hossain *et al*, 1996; Kremnicky *et al*, 1996; Araujo and Ward 1990b; Oda *et al*, 1993b). While, they were different from *Bacillus* sp. JAMB-750 (Takeda *et al*, 2004) and *Bacillus* sp. (Akino *et al*, 1988) having pH optimum in the alkaline range at 7-9. Therefore, mannanase from both ST1-1 and CW2-3 exhibited pH optimum at acidic to neutral range.

The stability of mannanase from CW2-3 exhibited a broad pH range of 3-6 while the activities rapidly decreased at pH 7.0 -10.0 with the remaining activity about 45-20% (Figure 13B). These results suggested that the mannanase from CW2-3 was rather stable in the acidic region similar to the other fungal mannanase (Puchart *et al*, 2004; Sachslehner and Haltrich, 1999). The pH stability of mannanases from the isolate ST1-1 displayed a wide pH range of 3-10 with more than 80% remaining activity after a long period (24h) of incubation at 4°C (Figure 13A).

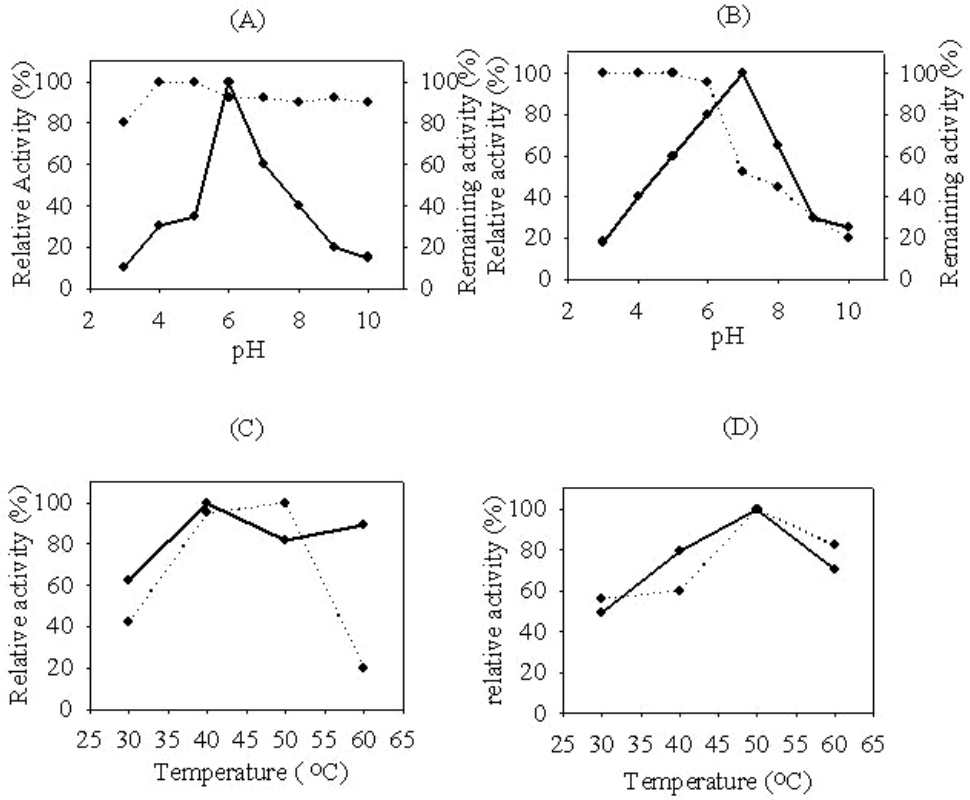

Figure 13 The effect of temperature and pH on mannanase activity of the isolate ST1-1 (A, C) and the isolate CW2-3 (B, D). (—●—) optimum; (·····●·····) stability.

3.2 The temperature effect on mannanase activity

The optimum temperature of mannanases were observed at 30 - 60°C. As shown in Figure 13, the temperature optima of mannanase from the isolate ST1-1 and CW2-3 were 40°C and 50°C, respectively (Figure 13 C and D). However, 82 and 89% of mannanase activity from ST1-1 was still remained at 50 and 60°C, respectively while those from CW2-3 were 79 and 70% at 40 and 60°C, respectively. Generally, the bacterial mannanase was optimally active at 50-60°C (Zakaria *et al*, 1998; Zhang *et al*, 2000; Khanongnuch *et al*, 1988; Mendoza *et al*, 1994). The mannanase properties from both CW 2-3 and ST1-1 being active at wide temperature range up to high temperature were rather similar to the other bacterial mannanases.

Considering mannanase stability, the stabilities of the enzyme from isolate CW2-3 showed wide temperature range of 40-60°C. Eighty-two percents of mannanase activity still remained at high temperature up to 60°C (Figure 13D). It can clearly be concluded that mannanase from CW2-3 was thermostable similar to several mannanases from thermophilic fungi and other bacterial strains (Akino *et al*, 1988; Jiang *et al*, 2006; Puchart *et al*, 2004). The mannanase from isolate ST1-1 was different in thermostability. The mannanase from ST1-1 was stable at 40-50°C for 30 min with relative activity around 95-100%, but it was more thermally unstable, the relative activity was decreased rapidly to 42% and 20% at 30 and 60°C, respectively (Figure 13C). However, the main interest of this experiment were to find effective strains which could produce mannanase whose mannooligosaccharide product showed a good prebiotic characteristics. Therefore, *K. oxytoca* CW2-3 which not only could produce mannanase but also exhibited prebiotic properties was selected for further studies.

4. Purification of mannanase from *Klebsiella oxytoca* CW2-3

4.1 Purification of mannanase by ammonium sulfate precipitation

The crude supernatant was subjected to 40-80 ml of saturated ammonium sulfate solution, in a total mixture volume of 100 ml. The precipitated protein collected by centrifugation (7000g) at 4°C for 30 min was dissolved in 10 mM Bis-Tris buffer, pH 7.0, and used for mannanase activity assay. The optimum condition for ammonium sulfate precipitation was shown in the mixture of crude enzyme:saturated ammonium sulfate solution of 30:70 (v/v), respectively (Figure 14). The results are shown in Table 14. Although, the protein recovery of this method was very low (8.60%), but it did not affect the mannanase activity. Therefore, this step could be used only for concentrating the enzyme but a disadvantage for purification.

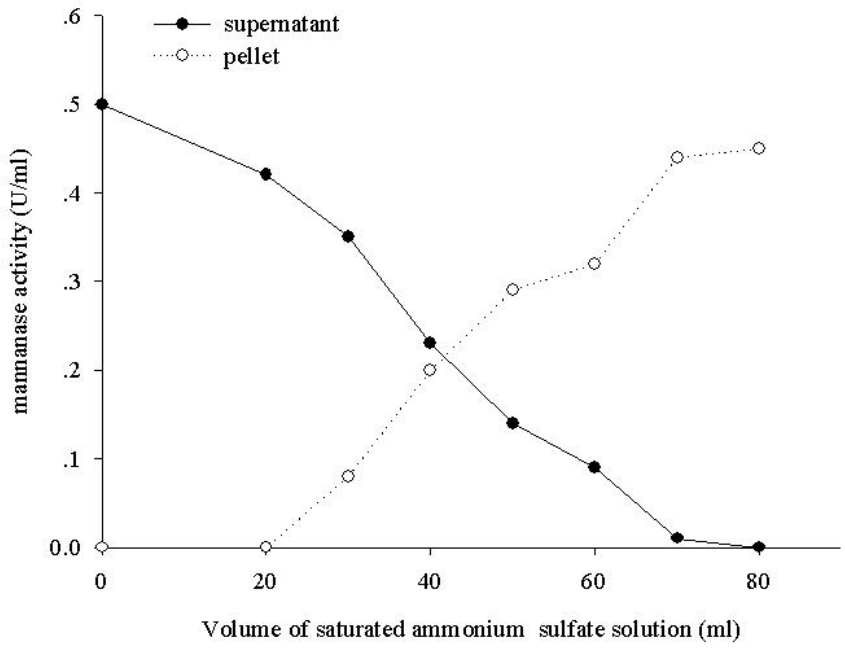

Figure 14 Ammonium sulfate precipitation of crude enzyme from *Klebsiella oxytoca* CW2-3

4.2 Purification of mannanase by anion exchange chromatography

Before loading sample to Q Sepharose column, the samples from ammonium sulfate precipitation were desalted by dialysis against distilled water, and the pooled sample was used for further purification.

4.2.1 Purification of mannanase by Q Sepharose chromatography

Two ml of enzyme (44.3 U, 288.4 mg protein) was loaded onto the column, unbound proteins were washed with 2 bed volume of 10 mM Bis-Tris buffer, pH 7.0 and then the enzyme was eluted with a linear gradient of 0-1 M sodium chloride in the same buffer. As shown in Figure 15, one peak of mannanase activity was obtained, fractions 11-14 were pooled, and named mannanase S1. The mannanase S1 was concentrated and desalted by using ultrafiltration with molecular weight cut off of 10,000 dalton. The purification is summarized in Table 14. The mannanase S1 was purified 32.62-fold.

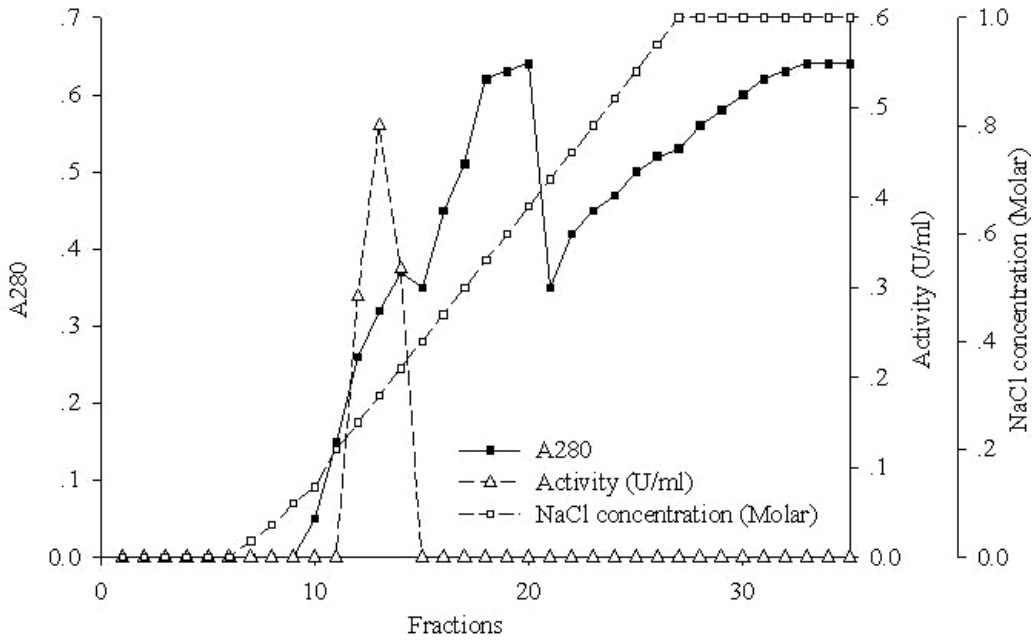

Figure 15 Anion exchange chromatography of mannanase on Q Sepharose I (1.6 x 20 cm column, equilibrated with 10 mM Bis-Tris buffer pH 7.0, with flow rate 2 ml/min, eluted with a linear gradient of 0-1.0 M sodium chloride in the same buffer)

The enzyme S1 was re-loaded onto Q Sepharose chromatography column, called Q Sepharose II, with the same method as above. As shown in Figure 16, one peak of mannanase activity was examined, fractions 10-14 were combined. The mannanase S1 was concentrated and desalted by using ultrafiltration with molecular weight cut off 10,000 dalton. Table 14 indicated that the mannanase S1 was purified 128.19-fold.

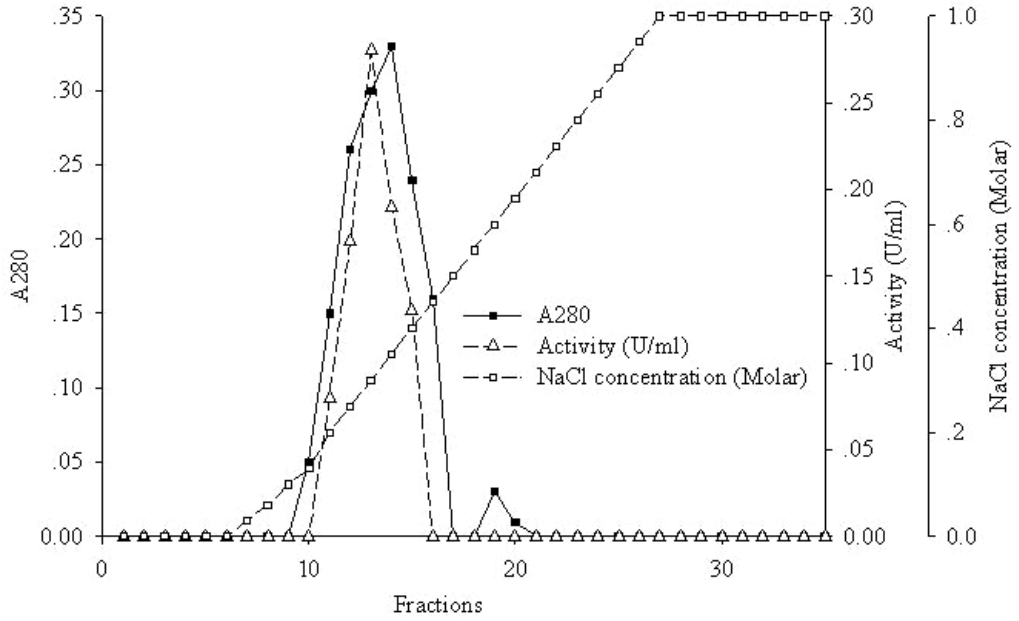

Figure 16 Anion exchange chromatography of mannanase on Q Sepharose II (1.6 x 20 cm column, equilibrated with 10 mM Bis-Tris buffer pH 7.0, with flow rate 2 ml/min, eluted with a linear gradient of 0-1.0 M sodium chloride in the same buffer)

4.2.2 Purification of mannanase by Q Source chromatography

By Q Source chromatography the active fractions 10-14 (Figure 17) were pooled and concentrated by using ultrafiltration with molecular weight cut off 50,000 dalton filter membrane, the activity of S1 was detected from retentrate. The specific activity of S1 decresed to 26 U/mg protein. Purification and yield of 100-fold, and 0.51% were obtained, respectively. The low yield was mainly due to loss of enzyme protein in the ammonium sulfate precipitation.

Table 14 Purification of mannanase from *Klebsiella oxytoca* CW2-3

Steps	Total Volume (ml)	Total activity (Units)	Total protein (mg)	Sp. Activity (U/mg protein)	Purification fold	Yield (%)
crude	1000	515	1962	0.26	1	100.00
Precipitate+dialysis	100	44.3	288.4	0.15	0.58	8.60
Qsepharose I+UF	20	7.8	0.92	8.48	32.62	1.51
Qsepharose II+UF	20	4	0.12	33.33	128.19	0.77
Qsource+UF	20	2.6	0.10	26	100	0.51

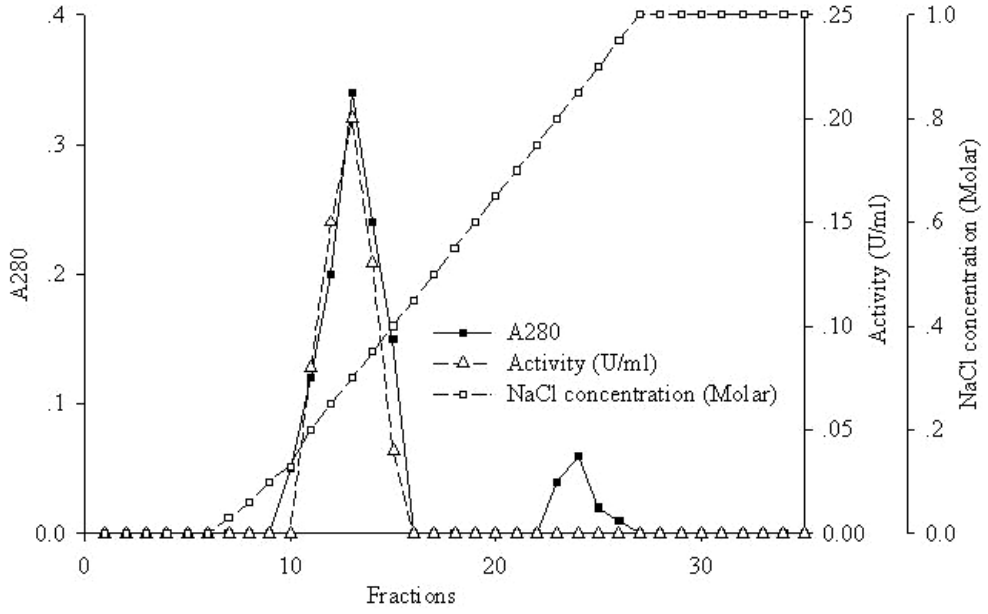

Figure 17 Anion exchange chromatography of mannanase on Q Source (1.6 x 20 cm column, equilibrated with 10 mM Bis-Tris buffer pH 7.0, with flow rate 2 ml/min, eluted with a linear gradient of 0-1.0 M sodium chloride in the same buffer)

5. Characterization of mannanase S1

5.1 Determination of the molecular weight

The mannanase S1 was detected from ultrafiltration retentrate fraction. Molecular weight determination on SDS-PAGE resulted in a single band of approximately 165 kDa, as shown in Figure 18. This band was cut and dissolved in buffer to re-nature mannanase S1. Determination of mannanase activity against locust bean gum showed mannanase activity of 0.09 U/ml.

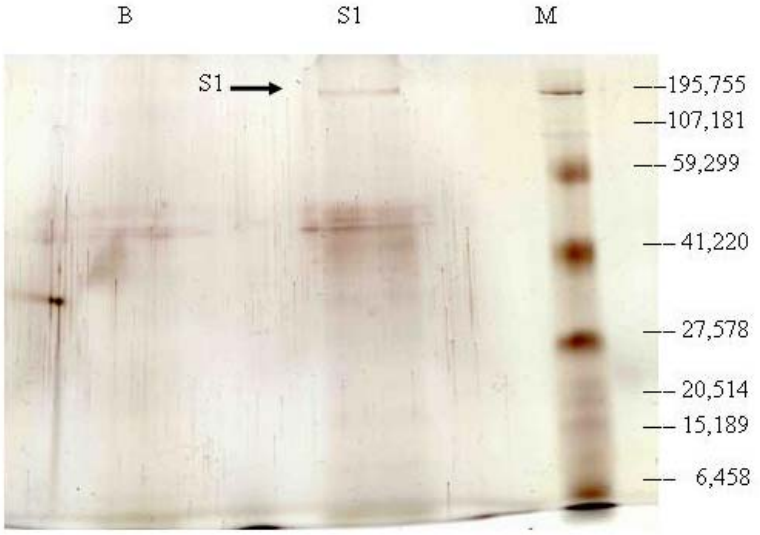

Figure 18 SDS-PAGE of purified S1; B: Sample buffer, S1: purified S1, M: prestained marker

To confirm the purity and size of S1, native-PAGE was also performed revealing single band of high molecular weight by GeneTools version 3.06.04 (Syngene, USA) as shown in Figure 19.

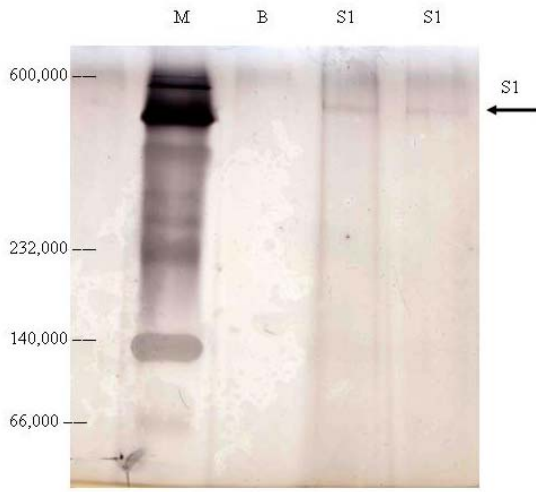

Figure 19 Native-PAGE of purified S1; M: native marker, B: sample buffer, S1: purified S1

5.2 Determination of the isoelectric point

To confirm the purity of mannanase S1, pI value was determined using isoelectric focusing. The pI value of 3.5 was found from S1 which was analyzed by GeneTools version 3.06.04 (Syngene, USA), as shown in Figure 20.

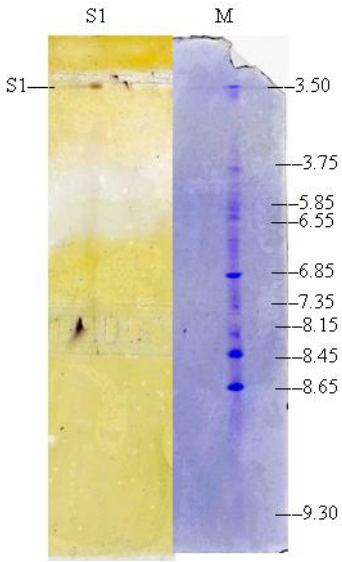

Figure 20 Isoelectric focusing electrophoresis of purified S1; S1: purified S1, M: broad pI calibration kit (Amersham, Sweden)

In conclusion, only one mannanase S1 was detected from effective *Klebsiella oxytoca* CW2-3. The mannanase S1 was a single protein of 165 kDa with pI of 3.5. The molecular weight of mannanase S1 obtained here was higher than those obtained from other microorganisms. Bacterial mannanase, especially *Bacillus* sp, had the molecular weight in the range of 37 to 40 kDa (Jiang *et al*, 2006; Zakaria *et al*, 1998; Mendoza *et al*, 1994). Besides, many fungal mannanases, including the Genus *Aspergillus*, which range from 30 to 110 kDa (Regalado *et al*, 2000), Genus *Trichoderma*, which range from 32 to 46 kDa (Ferreira H.M., and E.X.F. Filho, 2004; Arisan-Atac *et al*, 1993). However, Takeda *et al.* (2004) reported that the purified mannanase from *Bacillus* sp. strain JAMB-750 has molecular mass of 130 kDa.

The pI value obtained of 3.5 is quite low when compared to other bacterial mannanases (Zakaria *et* al, 1998; Mendoza *et al*, 1994). The pI value of mannanase S1 is similar to fungal mannanase which is typically in the acidic region of 2.75-5.5 (Sachslehner and Haltrich, 1999; Ademark *et al*, 1998; Gubitiz *et al*, 1996; Mendoza *et al*, 1994; Arisan-Atac *et al*, 1993). While, the purified mannanase which expressed in ripening tomato fruit showed the single mannanase of pI 9.0 for the mature protein.

5.3 The effect of pH on mannanase S1

The optimum pH of mannanase was determined by conducting the activity assay on LBG at 40°C at various pH values from 3.0-10.0. The highest activity of S1 was shown at pH 4.0 (Figure 21). Corresponding to the pH optimum of both bacterial and fungal mannanase, they were in the range of 3.0 -6.0 (Sachslehner and Haltrich, 1999; Ademark *et al*, 1998; Gubitiz *et al*, 1996; Mendoza *et al*, 1994; Arisan-Atac *et al*, 1993). However, some from *Bacillus subtilis* KU-1, *Bacillus* sp. KK01, *Bacillus* sp. strain JAMB-750, *Thermotoga neapolitana* 5068 and *Streptomyces ipomoea* showed higher optimum pH in the range of 7.0–10.0 (Takeda *et al*, 2004; Montiel *et al*, 2002; Zakaria *et al*, 1998; Hossain *et al*, 1996; Duffaud *et al*, 1997). Although these have generally acidic pH optima, it is

noteworthy that the S1 mannanase retains approximately 60% of its maximal activity at acidic region. Thus, the purified enzyme might be useful for industry at low working pH.

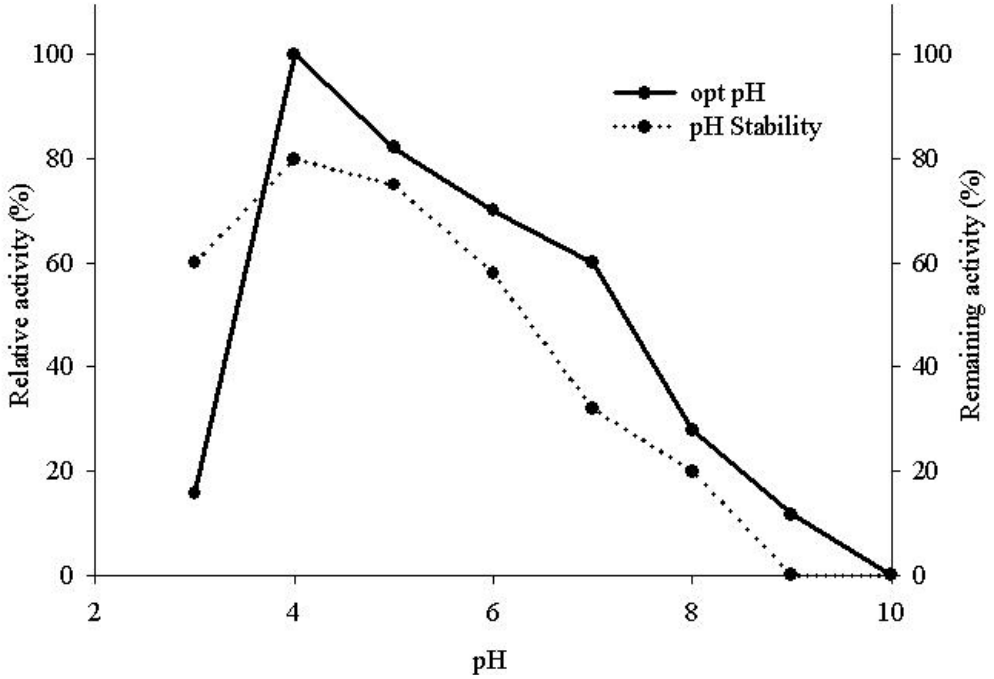

Figure 21 The pH effect on mannanase S1 from *Klebsiella oxytoca* CW2-3. The assays were carried out at 40°C for 30 min. (10mM: citrate (pH3.0-6.0), potassium phosphate (pH6.0-8.0) and glycine-NaOH (pH 8.0-10.0). The pH stability was determined in the same buffer system in the range 3.0-10.0 without the substrate for 2h at 4°C. Remaining activity was assayed at pH 4.0, 40°C for 30 min.

Furthermore, the stability of mannanase S1 exhibited a broad pH range of 3-5 while the activities rapidly decreased at pH 6.0-8.0 with the remaining activity of about 58-20%, respectively (Figure 21). The activity of mannanase S1 was completely lost at pH 9.0. The enzyme was relatively less stable at high than at low pH. These results suggested that the mannanase S1 was rather stable in the acidic region similar to the other fungal mannanases (Puchart *et al*, 2004; Sachslehner and Haltrich, 1999). Moreover, these results were similar to the pH stability of crude enzyme. The crude enzyme was also stable in the acidic pH region of 3-6 at 4°C for 24h with remaining activity of more than 90% (Figure 13).

5.4 The effect of temperature on mannanase S1

The optimum temperature for mannanase S1 was investigated by conducting the activity assay on LBG in citrate buffer, pH 4.0 at various temperatures from 30-70°C for 30 min. The maximum activity of S1 was at 40°C as shown in Figure 22, which was different from the crude enzyme. The temperature optimum of the crude enzyme was at 50°C. The relative activity of mannanase was 60% at 70°C for 30 min. Generally, the bacterial mannanase was optimally active at 50-60°C (Zakaria *et al*, 1998; Zhang *et al*, 2000; Khanongnuch *et al*, 1988; Mendoza *et al*, 1994). However, thermostable mannanase from *Bacillus subtilis* WY34 and *Thermotoga neapolitana* 5068, showed temperature optimum of 65°C and 90-92°C, respectively. (Jiang *et al*, 2006; Duffaud *et al*, 1997).

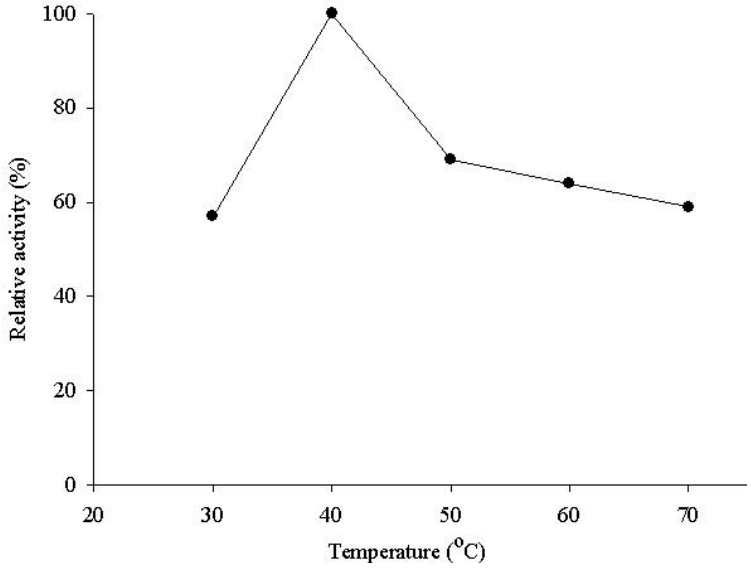

Figure 22 The effect of temperature on mannanase S1 from *Klebsiella oxytoca* CW2-3. The assays were carried out in 10mM citrate buffer pH 4.0.

Considering stability of mannanase S1 at various temperatures, half life of remaining activity of mannanase S1 at 40, 50, 60, 70°C was 4, 3, 3, and 1h, respectively. These results suggested that S1 was quite stable at high temperature corresponding to mannanase from *Sclerotium rolfsii* which displayed stablity at 50°C for 3h (Gubitz *et al*, 1996), and also mannanase from *Trichoderma harzianum* which

was stable at 70°C for 1h (Ferreira and Filho, 2004). Besides, the stability of mannanase S1 was similar to that of the crude enzyme. The 82% of the crude enzyme activity still remained at the high temperature up to 60°C (Figure 13D). Therefore, it was concluded that mannanase from *Klebsiella oxytoca* CW2-3 was thermostable.

5.5 The effect of metal ions

Metal ions and other agents may affect conformational changes and consequent loss of enzyme activity (Ferreira and Filho, 2004). The effects of various metal ions and chemical reagents on mannanase activities are shown in Table 15. The mannanase activity was completely inhibited by EDTA and Zn^{2+}, strongly inhibited by Ca^{2+}(12.90%), Cu^{2+} (16.13%), Fe^{2+}(16.13%), Mn^{2+}(22.58%), Ni^{2+}(32.26%), Mg^{2+}(35.49%), and Li^{+} (45.16%), and was slightly inhibited by mercaptoethanol (83.87%). The mannanase activity was activated by Co^{2+} (129%) similar to mannanase from *Bacillus subtilis* WY34 (Jiang *et al*, 2006) which was also activated by Co^{2+} (126.4%). The result may suggest that the tryptophan residues play an important part at the site or the substrate binding site of mannanase activity.

Table 15 Effect of metal ions on S1 from *Klebsiella oxytoca* CW2-3

Ion	Relative activity (%)
Control	100
EDTA	0
$ZnSO_4$	0
$CaCl_2$	12.90
$CuSO_4$	16.13
$FeSO_4$	16.13
$MnSO_4$	22.58
$NiSO_4$	32.26
$MgSO_4$	35.49
LiCl	45.16
Mercaptoethanol	83.87
$CoCl_2$	129

5.6 Substrate specificity

The relative activities of the mannanase S1 on various substrates were shown in Table 16. The enzyme hydrolyzed LBG, ivory nut mannan, konjak glucomannan, and copra meal with relative activities of 100, 60, 54, and 20, respectively. The mannanase S1, exerted activity on only different β-1,4 mannosidic linkages of mannans (ivory nut), galactomannan (locust bean gum) and glucomannan (konjak mannan). On the other hand, it showed no hydrolysis activity against alpha-mannan, xylan (from oat spelts), carboxymethylcellulose, and Avicel. It might be proposed that the mannanase S1 from *Klebsiella oxytoca* CW2-3 exhibited high specificity for galactomannan substrate, with no xylanase and cellulase activities detected.

Table 16 Substrate specificity of S1 from *Klebsiella oxytoca* CW2-3 toward various substrates

Substrate	Relative activity (100%)
Locust bean gum (galactomannan)	100
Ivory nut mannan (mannan)	60
Konjak glucomannan (glucomannan)	54
Alpha-mannan (yeast)	<1
Xylan (from oat spelts)	<1
Carboxymethylcellulose	<1
Avicel	<1
Copra meal	20

5.7 Enzyme kinetics

The Michaelis-Menten constants (K_m), maximum velocity (V_{max}) and the catalytic constant (K_{cat}) were determined for locust bean gum and konjak mannan. Results are summarized in Table 17.

Table 17 Characteristic constants of S1 from *Klebsiella oxytoca* CW2-3

Characteristic constant	Value	
	Locust bean gum	Konjak mannan
Michaelis-Menten constant (K_m) (mg/ml)	1.056	1.038
Maximum velocity (V_{max}) μU/ml.min	6.149	6.183
Catalytic rate constant (k_{cat}) (sec^{-1})	0.047	0.047

The K_m, V_{max} and k_{cat} values of mannanase S1 from *Klebsiella oxytoca* CW2-3 on locust bean gum were 1.056 mg/ml, 6.149 μU/ml.min, and 0.047 sec^{-1}, respectively. The K_m value is a numerical value which is specific for the source of enzyme. The K_m value in this work corresponds to K_m value of *Bacillus subtilis* KU-1 of 0.91 mg/ml (Zakaria *et al*, 1998), but differs from the K_m value of *Bacillus* sp. KK01 of 3.95 mg/ml (Hossain *et al*, 1996). On the other hand, the K_m, V_{max} and k_{cat} values of S1 from *Klebsiella oxytoca* CW2-3 on konjak mannan were 1.038 mg/ml, 6.183 μU/ml.min, and 0.047 sec^{-1}, respectively. This means that the kinetic values for LBG and konjak mannan were the same. It seems that the mannanase S1 could be applied for various substrate types of β-1,4 mannosidic linkages. However, they were quite different in their mannanase activity of about 2 folds as shown in Table 16. It might be possible that the kinetic values reported in this work were determined by commercial substrates which may contain other interfering substances affecting mannanase activity and provide equally appearent kinetic values as summarized in Table 18.

Table 18 The summarized properties of purified mannanase, S1, from *Klebsiella oxytoca* CW2-3

Molecular weight	SDS-PAGE	165 kDa
Isoelectric point	Isoelectric focusing, IEF	3.5
pH optimum	40°C, 30 min	pH 4.0 (10 mM citrate)
Temperature optimum (°C)	30 min, pH 4.0	40°C
K_m	Galactomannan from LBG	1.056 mg/ml
	Glucomannan from Konjac	1.038 mg/ml
V_{max}	Galactomannan from LBG	6.149 μU/ml.min
	Glucomannan from Konjac	6.183 μU/ml.min
K_{cat}	Galactomannan from LBG	0.047 Sec^{-1}
	Glucomannan from Konjac	0.047 Sec^{-1}
Half life	40°C, pH 4.0	4 h
	50°C, pH 4.0	3 h
	60°C, pH 4.0	3 h
	70°C, pH 4.0	1 h

5.8 Amino acid sequence

The results were shown in Appendix Figure A1. N-terminal amino acid sequence homology was analyzed using the BLAST database. The first 15 N-terminal amino acid sequence (GRVGEAGPHGPHGPH) of the mannanase S1 showed no matching with the N-terminal region of any bacterial mannanase in Protein-Protein BLAST (blastp) (http://www.ncbi.nlm.nih.gov; 04/04/2007).

6. Degradation pattern

To determine the degradation products from the action of mannanase S1 on locust bean gum and copra meal, each reaction was performed and followed at 0, 1, 2, 3, and 4 h.

6.1 Locust bean gum degradation by mannanase S1

The mannanase S1 degraded LBG and produced various sizes of oligosaccharides as shown in Figure 23. This indicates that the mannanase is an endomannanase. The action of the enzyme on mannooligosaccharides were also analyzed by TLC. As shown in Figure 23, the enzyme hardly hydrolyzed mannotriose and mannobiose. Considering the product mobilities compared to the standard oligosaccharides, the product were galactose, intermediate between mannotriose and mannobiose, defined as ITB, and molecules larger than mannotetraose, defined as LMT. It is in good agreement with the results of Ademark *et al*, (1998) that *Aspergillus niger* mannanase was able to degrade mannotriose and mannotetraose, but not mannobiose. Furthermore, Sugiyama *et al*, (1973) reported that hydrolysis products from mannan existed as various kinds of unknown oligosaccharides and unknown substances. The ITB, therefore, compared to those standard oligosaccharides, would be trisaccharide and oligosaccharides, whose molecules were larger than mannotetraose. However, the effect of metal ions as shown in Table 15 indicated that the Co^{2+}ion could activate the mannanase activity. Therefore, the next experiment was to look for the effect of various Co^{2+}ion concentrations on mannanase activity as shown in Figure 24. The highest mannanase activity of 0.055 U/ml was observed at 100 mM Co^{2+}ion. Therefore, the degradation products from the action of 100 mM $CoCl_2$ treated mannanase S1 on locust bean gum, each reaction was performed and followed at 0, 1, 2, 3, and 4h are as shown in Figure 25.

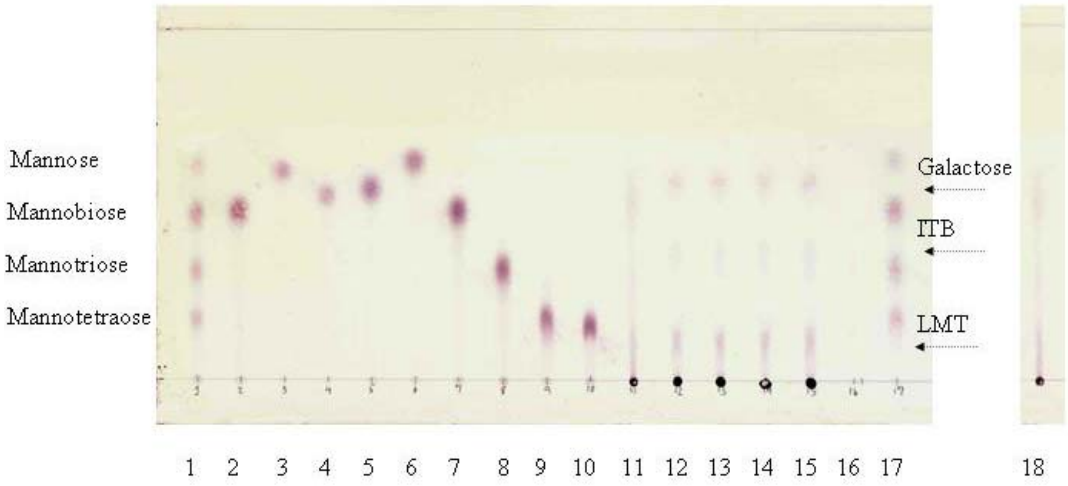

Figure 23 Locust bean gum degradation by S1 from *Klebsiella oxytoca* CW2-3 at various times of 0, 1, 2, 3, and 4h. Lane 1: standard mannooligosaccharides, Lane 2: mannose-7-butyrate, Lane 3: Glucose, Lane 4: Maltose, Lane 5: galactose, Lane 6: mannose, Lane 7: mannobiose, Lane 8: mannotriose, Lane 9: mannotetraose, Lane 10: mannoheptaose, Lane 11: 0h, Lane 12: 1h, Lane 13: 2h, Lane 14: 3h, Lane 15: 4h, Lane 16: S1, Lane 17: standard mannooligosaccharides, Lane 18: locust bean gum.

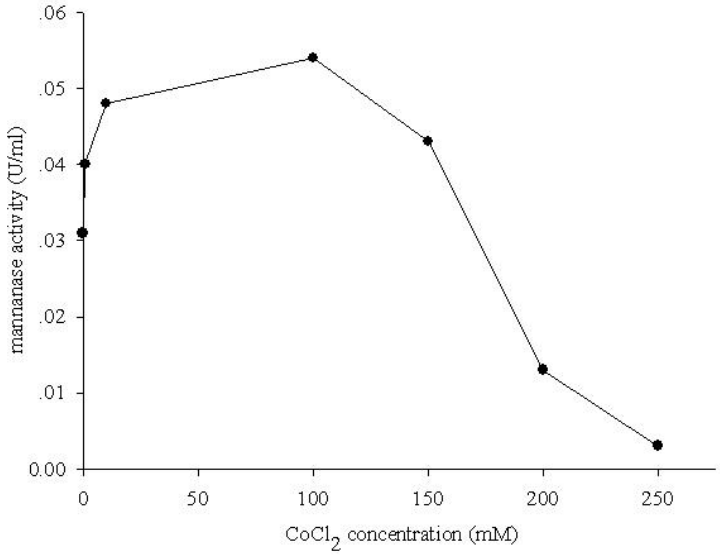

Figure 24 Effect of $CoCl_2$ concentration (mM) on S1 mannanase activity.

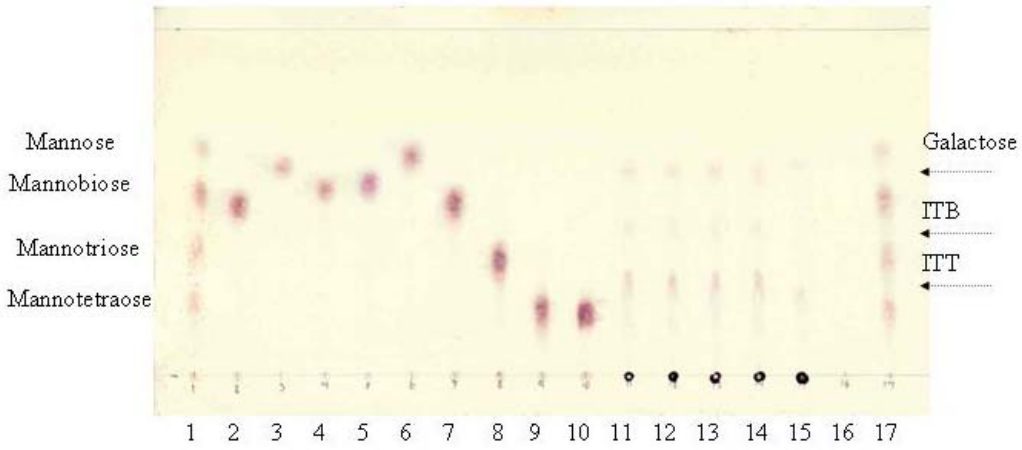

Figure 25 Locust bean gum degradation by S1 with 100mM $CoCl_2$ at various times of 0, 1, 2, 3, and 4h. Lane 1: standard mannooligosaccharides, Lane 2: mannose-7- butyrate, Lane 3: Glucose, Lane 4: Maltose, Lane 5: galactose, Lane 6: mannose, Lane 7: mannobiose, Lane 8: mannotriose, Lane 9: mannotetraose, Lane 10: mannoheptaose, Lane 11: 0h, Lane 12: 1h, Lane 13: 2h, Lane 14: 3h, Lane 15: 4h, Lane 16: S1, Lane 17: standard mannooligosaccharides.

The product mobilities were intermediate between standard mannooligosaccharide (Figure 25). According to the product mobilities comparing to those standard oligosaccharides, the products were galactose, ITB, and intermediate between mannotriose and mannotetraose, defined as ITT, which were different from the action of mannanase S1 alone. It indicated that the 100 mM $CoCl_2$ treated mannanase S1 could degrade LBG and produce galactose, mannotriose and mannotetraose. It might be possible that the action of mannanase S1 was typically randomized in galactomannan structure. To confirm this result, the LBG degradation profile at 2h with various $CoCl_2$ concentrations was investigated, as shown in Figure 26, and the quantity of the oligosaccharide products were determinined under standard condition and summarized in Table 19. The results indicated that higher amount of oligosaccharide was produced at high cobalt concentration.

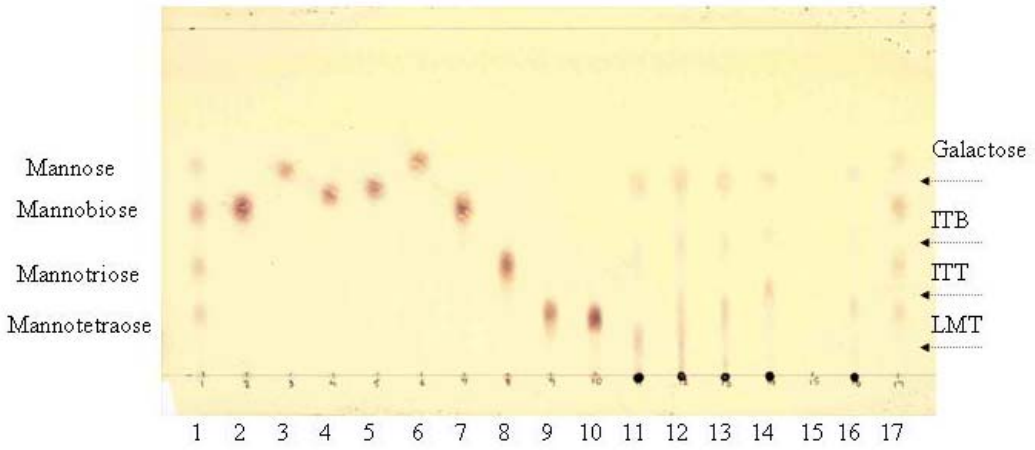

Figure 26 Locust bean gum degradation by S1 at 2h with various $CoCl_2$ concentration. Lane 1: standard mannooligosaccharides, Lane 2: mannose-7-butyrate, Lane 3: Glucose, Lane 4: Maltose, Lane 5: galactose, Lane 6: mannose, Lane 7: mannobiose, Lane 8: mannotriose, Lane 9: mannotetraose, Lane 10: mannoheptaose, Lane 11: no added, Lane 12: 1mM $CoCl_2$, Lane 13: 10mM $CoCl_2$, Lane 14: 100mM $CoCl_2$, Lane 15: S1, Lane 16: 0h, Lane 17: standard mannooligosaccharides.

Table 19 Reducing sugar of degraded products from S1 with various $CoCl_2$ concentration on LBG at 2 h, 40°C

Sample	Reducing sugar (mg/ml)
Control	0.03
1 mM $CoCl_2$	0.06
10 mM $CoCl_2$	0.16
100 mM $CoCl_2$	0.31

6.2 Copra meal degradation by mannanase S1

Copra meal hydrolysis profile by mannanase S1 was shown in Figure 27. Comparing to the standard oligosaccharides, only galactose appeared since 0h. It might be possible that the copra meal already contained galactose molecules. It is different from the reports of Jiang *et al*. (2006) that when copra mannan were

incubated with the enzyme from *Bacillus subtilis* WY34, mannotetraose, mannotriose and mannobiose were produced. However, the single position of the products mobolities detected might be due to low amount of reducing sugar, less than 0.05 mg/ml, undetectable by TLC method.

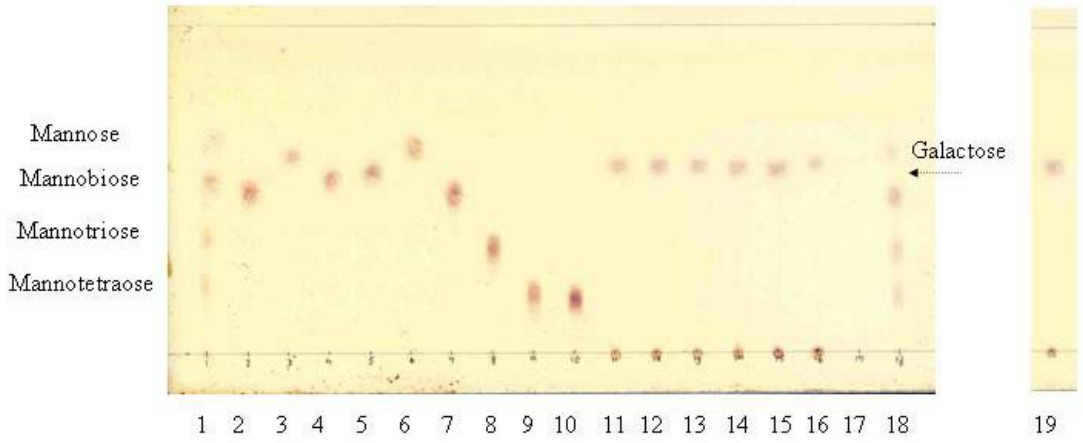

Figure 27 Copra meal degradation by S1 at various times of 0, 1, 2, 3, 4 and 24h. Lane 1: standard mannooligosaccharides, Lane 2: mannose-7- butyrate, Lane 3: Glucose, Lane 4: Maltose, Lane 5: galactose, Lane 6: mannose, Lane 7: mannobiose, Lane 8: mannotriose, Lane 9: mannotetraose, Lane 10: mannoheptaose, Lane 11: 0h, Lane 12: 1h, Lane 13: 2h, Lane 14: 3h, Lane 15: 4h, Lane 16: 24h, Lane 17: S1, Lane 18: standard mannooligosaccharides, Lane 19: copra meal.

Therefore, the optimum condition for copra meal degradation of 100 mM $CoCl_2$ treated mannanase S1 was investigated and followed at 0, 1, 2, 3, 4, and 24h as shown in Figure 28. In addition, the amount of reducing sugar was determined under the standard condition as shown in Table 20.

Table 20 Reducing sugar of degraded products from S1 with 100 mM $CoCl_2$ on copra meal at 40°C

Sample (h)	Reducing sugar (mg/ml)
0	< 0.05
1	0.18
2	0.2
3	0.2
4	0.21
24	0.21

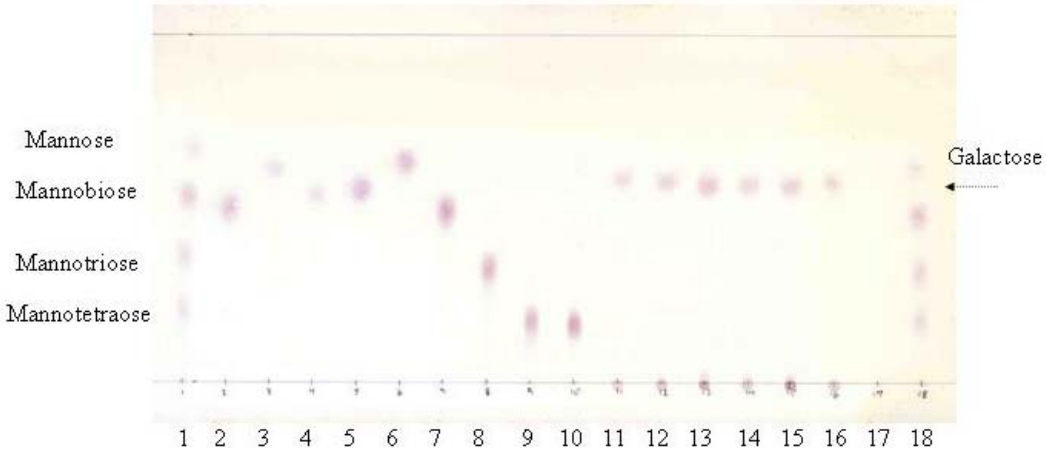

Figure 28 Copra meal degradation by S1 with 100 mM $CoCl_2$ at various times of 0, 1, 2, 3, 4 and 24h. Lane1: standard mannooligosaccharides, Lane 2: mannose-7- butyrate, Lane 3: Glucose, Lane 4: Maltose, Lane 5: galactose, Lane 6: mannose, Lane 7: mannobiose, Lane 8: mannotriose, Lane 9: mannotetraose, Lane 10: mannoheptaose, Lane 11: 0h, Lane 12: 1h, Lane 13: 2h, Lane 14: 3h, Lane 15: 4h, Lane 16: 24h, Lane 17: S1, Lane 18: standard mannooligosaccharides.

According to Figure 28, the product mobilities are also detected at the same position as standard galactose similar to the previous experiment as shown in Figure 27. Thus, it could be proposed that the mannanase S1 is highly specific for the mannose molecule near to the galactose molecule in galactomannan structure. Compare to the LBG hydrolysis, mannotriose, mannotetraose and galactose were detected (Figure 23, 25-26). Since locust bean gum contained higher galactose number than copra meal did (Burhanudin and Dingle 2006), this may lead to higher amount of mannotriose, mannotetraose and galactose produced by mannanase S1 that could be detected by TLC method. However, the detection of galactose from both LBG and copra meal degradation by mannanase S1 might suggest the presence in mannanase S1 of both mannanase and alpha-galactosidase as well.

7. Determination of prebiotic properties

Since mannanase S1 from *Klebsiella oxytoca* CW2-3 could hydrolyze LBG and copra meal and produced mannooligosaccharides which was a kind of prebiotics. The ability to use copra meal hydrolysate (CM-hydrolysate) as prebiotic was investigated. And since the acitivity of the mannanase S1 could be activated by Co^{2+} ion thus, both CM-hydrolysate obtained from 100 mM $CoCl_2$ treated (Co-CM-hydrolysate) and untreated (CM-hydrolysate) mannanase S1 were tested. The different concentration of reducing sugar of 0.03, 0.06, and 0.09 mg/ml from CM-hydrolysate defined as 1n, 2n, and 3n, respectively and of 0.31, 0.62 and 0.93 mg/ml from Co-CM-hydrolysate defined as 1c, 2c, and 3c, respectively were tested for prebiotic properties.

7.1 The effect of copra meal hydrolysate (CM-hydrolysate)

The effect of CM-hydrolysate from mannanase S1 on *L. reuteri* KUB-AC5 *E.coli* E010 as well as *Salmonella* serovar Enteritidis S003 were investigated. All samples in the CM-hydrolysate group had no effect on the growth of KUB-AC5 ($p > 0.05$) as shown in Table 21. However, 3n of CM-hydrolysate showed maximum enhancing activity of 0.11 log cfu/ml (Figure 29). However, the CM-hydrolysate

enhanced *E. coli* E010 growth. These results corresponded to the results of CM-hydrolysate from crude enzyme (Figure 9). Moreover, all samples of the CM-hydrolysate group had greater S003 growth inhibition by 0.91-1.90 log cfu/ml ($p < 0.05$) (Table 21). The 0.09 mg/ml of reducing sugar of CM-hydrolysate exhibited the highest inhibition activity of 1.90 log cfu/ml (Figure 29).

Table 21 The effect of CM-hydrolysate on *L. reuteri* KUB-AC5, *Salmonella* serovar Enteritidis S003, and *E. coli* E010 growth.

Treatment	Survival cell (log cfu/ml)		
	KUB-AC5	S003	E010
Control	9.68^{ab}	12.92^{a}	10.08^{c}
0.03 ;1n	9.59^{b}	12.01^{b}	10.50^{b}
0.06 ;2n	9.64^{b}	11.86^{c}	10.97^{a}
0.09 ;3n	9.79^{a}	11.02^{d}	10.10^{c}

Superscript alphabet in row presents difference in statistic with $P < 0.05$.

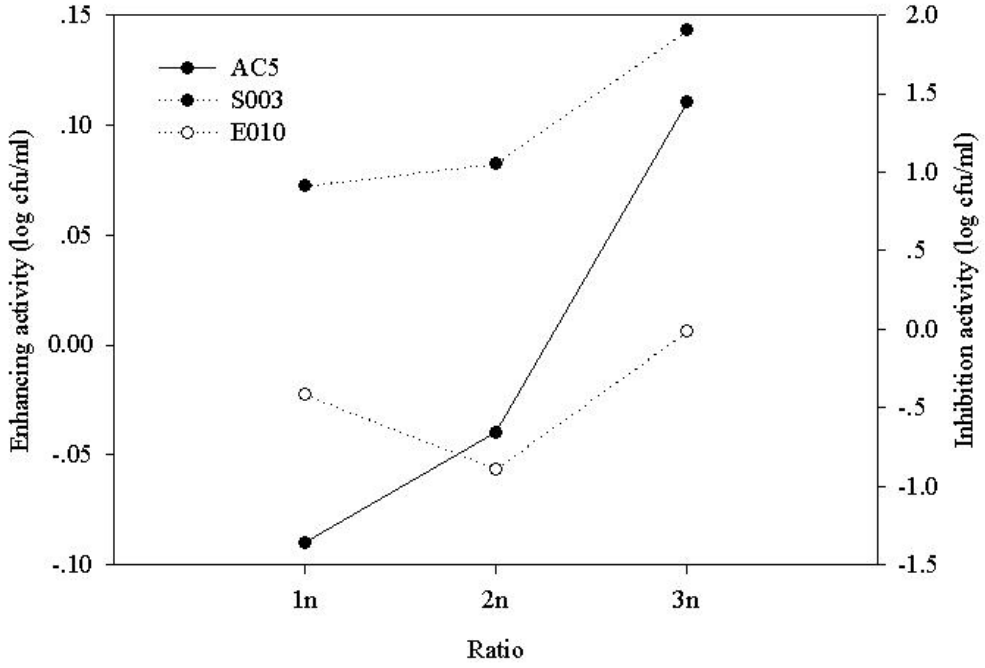

Figure 29 Effect of CM-hydrolysate on the enhancing activity of *L. reuteri* KUB-AC5 and inhibition activity of *Salmonella* serovar Enteritidis S003 and *E. coli* E010 .

7.2 The effect of Cobalt ion treated copra meal hydrolysate (Co-CM-hydrolysate).

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All treatments with the Co-CM-hydrolysate had effects on the growth of KUB-AC5, S003 and E010 growth ($p < 0.05$). The results are shown in Table 22. The effect of 2c and 3c of Co-CM-hydrolysate were not significantly different to KUB-AC5 growth. They exhibited the enhancing activity of 1.41-1.87 log cfu/ml (Figure 30). The 3c of Co-CM-hydrolysate showed the highest enhancing activity of 1.87 log cfu/ml. Moreover, the 3c of Co-CM-hydrolysate showed the highest inhibition activity to S003 of 1.27 log cfu/ml as well (Figure 30). Few studies have investigated the effect of mannooligosaccharides (MOS) on *Salmonella* sp. Spring *et al*. (2000) determined the effect of MOS on cecal fermentation parameters, cecal microflora, and enteric pathogen in chicks. Cecal *Salmonella typhimurium* 29E concentrations decreased from 5.40 to 4.01 log cfu/g ($P<0.05$) after received 4000 ppm dietary MOS. Moreover, *Salmonella typhimurium* challenged turkeys fed by

MOS had a decresed incidence of fecal contamination, whereas broilers fed by MOS reduced fecal counts of *Salmonella Dublin* and *E. coli* (Spring *et al*, 1996).

However, the Co-CM-hydrolysate enhanced E010 growth (Table 22). These results corresponded to the results of CM-hydrolysate. Few studies have investigated non-digestible oligosaccharides as prebiotic to reduce *E. coli* in animals. Generally, fructo-oligosaccharides were successful to reduce *E. coli* in animal more than MOS (Naughton *et al*, 2000; Rao, 1999). It might be conclude that both CM-hydrolysate and Co-CM-hydrolysate produced from *K. oxytoca* CW2-3 are not suitable to use for E010 growth inhibition. However, the hydrolysate did enhance the growth of *L. reuteri* KUB-AC5 and inhibit the growth of *Salmonella* serovar Enteritidis S003.

Table 22 The effect of Co-CM-hydrolysate on *L. reuteri* KUB-AC5, *Salmonella* serovar Enteritidis S003, and *E. coli* E010 growth.

Treatment	Survival cell (log cfu/ml)		
	KUB-AC5	S003	E010
Control	8.20^{c}	12.39^{b}	8.12^{d}
0.31 mg/ml;1c	9.61^{b}	12.75^{a}	10.50^{b}
0.62 mg/ml;2c	9.97^{a}	11.95^{c}	10.85^{a}
0.93 mg/ml;3c	10.07^{a}	11.12^{d}	10.17^{c}

Superscript alphabet in row presents difference in statistic with $P < 0.05$

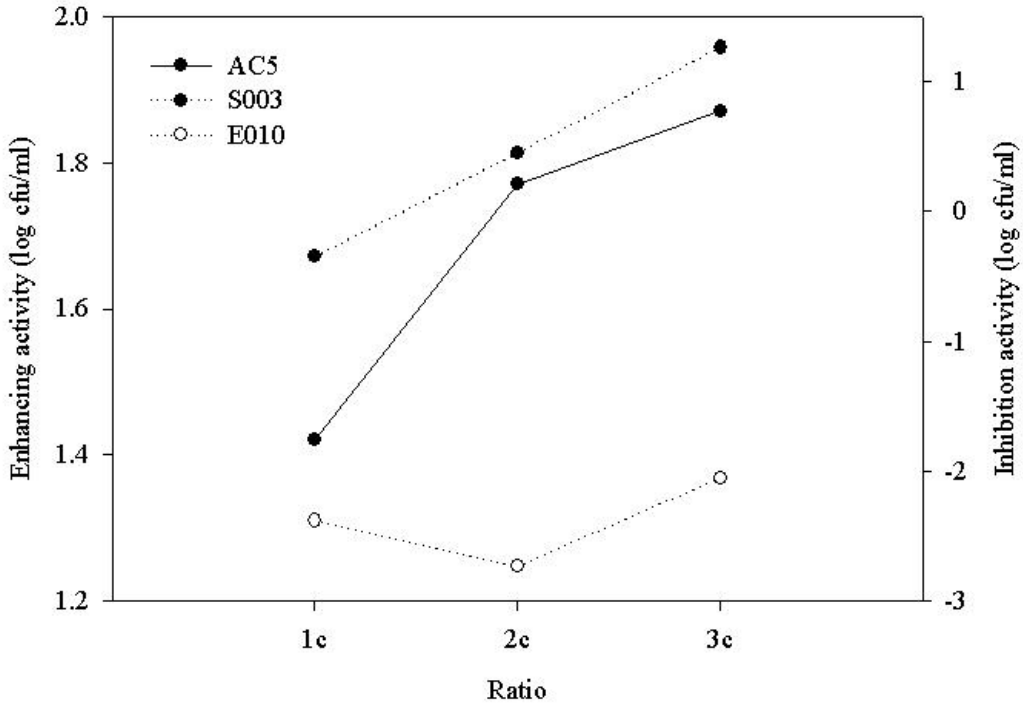

Figure 30 Effect of Co-CM-hydrolysate on enhancing activity of *L. reuteri* KUB-AC5 and inhibition activity of *Salmonella* serovar Enteritidis S003 and *E. coli* E010 .

CONCLUSION

In order to select for effective mannanase-producing bacterial strains which can produce a mannooligosaccharide to be used as a prebiotic, two steps of screening process were proposed. The enriched culture in isolation medium containing copra meal were primary screened on BIM with 1% locust bean gum (LBG) resulting in 48 mannanase producing bacterial isolates from 3,055 isolates showing the activities of 1.33 – 3.0. The isolate CW2-3 showed the maximum activity of 3. While, only one fungi isolate from 1,079 isolates, SN2-1, could display the activity of 1.62. However, only 10 isolates CW1-2, CW2-1, CW2-3, ST1-1, ST2-2, ST3-4, ST3-10, BC-5 BC-7 and SN2-1 possessed mannanase activity of 0.086 – 0.870 U/ml against copra meal.

Considering secondary screening based on the effect of CM-hydrolysate on the growth of *L. reuteri* KUB-AC5, 10 isolates showed the enhancing activity of 0.09-2.15 log cfu/ml. The isolate CW2-3 displayed the highest enhancing activity of 2.15 log cfu/ml. The CM-hydrolysate from 10 isolates exhibited low inhibition activity of 0.41-1.72 log cfu/ml against *S.* Enteritidis S003. When the effect of each hydrolysates on the growth of *E. coli* E010 were tested, only low inhibition activity of 0.46-1.78 log cfu/ml from CW1-2, CW2-1, ST1-1, ST3-10, BC5 and BC5 were obtained. While the CM-hydrolysate from the isolate CW2-3, ST2-2 and ST3-4 enhanced the growth of *E. coli* E010.

Based on morphological, physical, biochemical and genetic properties, the isolate CW2-3 and ST1-1 were identified as *Klebsiella oxytoca* and *Acinetobacter* sp, respectively. The pH optima of mannanase activity was determined by conducting the activity assays on LBG at 50°C in various pH values of 3-10. The pH optimum of ST1-1 and CW2-3 were 6 and 7.

Mannanase from CW2-3 exhibited stability at a broad pH range of 3-6 while the activities rapidly decreased at pH 7.0-10.0 with remaining activity about 20-45%. The pH stability of mannanases from the isolate ST1-1 also displayed a wide pH

range of 3-10 with more than 80% remaining activity after a long period (24h) of incubation at 4°C.

The optimum temperature of mannanases were observed at 30 - 60°C. The temperature optima of the isolate ST1-1 and CW2-3 were 40°C and 50°C, respectively. However, 82 and 89% of mannanase activity from ST1-1 still remained at 50 and 60°C, respectively while that from CW2-3 at 40 and 60°C was 79 and 70%, respectively. The mannanase properties from both CW 2-3 and ST1-1 being active at wide temperature range up to high temperature was rather similar to the other bacterial mannanases.

Considering mannanase stability, the enzyme from isolate CW2-3 showed wide temperature stability range of 40-60°C. Eighty-two percents of mannanase activity still remained at high temperature up to 60°C. It can clearly be concluded that mannanase from CW2-3 was thermostable. The mannanase from the isolate ST1-1 was diffirent in thermostability. The enzyme was stable at 40-50°C for 30 min with relative activity around 95-100%, but it was more thermally unstable, the relative activity decreased rapidly to 42% and 20% at 30 and 60°C, respectively.

Purification of mannanase from *K. oxytoca* CW2-3 was accomplished by using anion exchange chromatography. Q sepharose and Q source chromatography were carried out under specified condition at pH 7.0 to obtain mannanase S1 of 99.23-fold purification with 3.15% recovery. Homogeneity of the mannanase S1 was determined by 10% SDS-PAGE and 7.5% native-PAGE. It was concluded that the mannanase S1 was a single protein approximately of 165 kDa with pI of 3.5.

The optimum pH of mannanase S1 was determined by conducting the activity assay on LBG at 40°C at various pH values from 3.0-10.0. The highest activity of mannanase S1 was shown at pH 4.0. The mannanase S1 exhibited a broad pH stability range of 3-5 while the activities rapidly decreased at pH 6.0-8.0 with the remaining

activity about 20-58%. The mannanase S1 activity was completely inhibited at pH 9.0. The enzyme was relatively less stable at alkaline than at acidic pH.

The optimum temperature for S1 was studied by conducting the activity assay on LBG in citrate buffer, pH 4.0 at various temperatures from 30-70°C for 30 min. The maximum activity of S1 was evident at 40°C. This mannnanase showed 60% relative activity at 70°C for 30 min. Considering stability of S1 at various temperatures, remaining activity of 50% of S1 was stable at 40, 50, 60, and 70°C for 4, 3, 3, and 1h, respectively. These results suggested that S1 was quite stable at high temperature. In addition, the mannanase activity was completely inhibited by EDTA and Zn^{2+}, but activated by Co^{2+} (129%). Moreover, the mannanase S1 exhibited activities against LBG, ivory nut mannan, konjak glucomannan, and copra meal, but not against α-mannan, xylan (from oat spelts), carboxymethylcellulose, and avicel.

The Michaelis-Menten constants (K_m), maximum velocity (V_{max}) and the catalytic constant (K_{cat}) were determined for locust bean gum and konjak mannan. The K_m, V_{max} and k_{cat} values of S1 from *Klebsiella oxytoca* CW2-3 on locust bean gum were 1.056 mg/ml, 6.149 μU/ml.min, and 0.047 sec^{-1}, respectively. On the other hand, the K_m, V_{max} and k_{cat} values of S1 from *Klebsiella oxytoca* CW2-3 on Konjak mannan were 1.038 mg/ml, 6.183 μU/ml.min, and 0.047 sec^{-1}, respectively. The first 15 N-terminal amino acid sequence (GRVGEAGPHGPHGPH) of the mannanase S1 showed no matching with the N-terminal region of any bacterial mannanase in protein database.

The degradation products from LBG hydrolysis of S1 are considered to be galactose, triose, tetraose and the oligosaccharides larger than mannotetraose. While, the copra meal hydrolysis product contained higher oligosaccharides.

The effect of CM-hydrolysate on the growth of *L. reuteri* KUB-AC5, *E. coli* E010 and *S.* Enteritidis S003 were determined by using mannanase S1, called CM-hydrolysate as compared to 100 mM $CoCl_2$ treated mannanase S1, called

Co-CM-hydrolysate. The Co-CM-hydrolysate exhibited enhancing activity of 1.41-1.87 log cfu/ml on the growth of KUB-AC5. The reducing sugar 0.93 mg/ml of Co-CM-hydrolysate showed the highest enhancing activity was 1.87 log cfu/ml.

The effect of CM-hydrolysate on the growth of S003, showed the inhibition activity of 0.91-1.90 log cfu/ml. Moreover, all samples in the CM-hydrolysate group were greater significant difference in S003 growth (p <0.05). While, the effect of Co-CM-hydrolysate on the growth of S003, showed the highest inhibition activity of 1.27 log cfu/ml at 0.93 mg/ml of reducing sugar in Co-CM-hydrolysate and were significant differences in S003 growth compare to control group ($p < 0.05$).

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APPENDIX

APPENDIX

1. Reducing sugar determination

Reducing sugar was determined by DNS method (Miller, 1959).

1.1 Reagents

1.1.1 DNS solution was prepared by dissolving 10g 3,5-Dinitrosalicylic acid in 1N NaOH 200 ml. 300g sodium potassium tartrate was added, mixed until it dissolved well and made volume to 1,000 ml with distilled water.

1.1.2 Mannose stock solution 2 mg/ml.

1.2 Method

1.2.1 200 μl of sample was mixed with 200 μl DNS solution in test tube.

1.2.2 Boiled the test tube containing reaction mixture in boiling water for 5 min.

1.2.3 After 5 min boiling, cool down the test tube immediately in ice-bath.

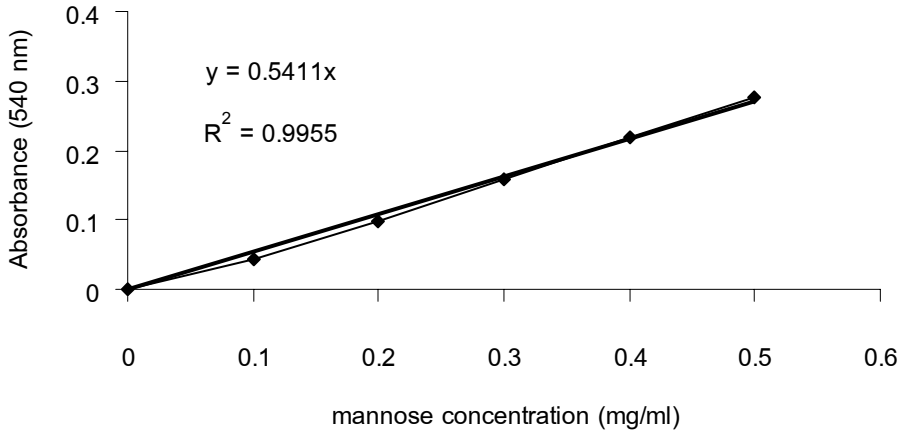
1.2.4 Added 2 ml of distilled water and mixed well.

1.2.5 Absorbance was measured by spectrophotometer at 540 nm.

1.3 Standard curve of reducing sugar

Standard mannose solution was prepared in the concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The reactions were carried out using the same method as

described in 1.2. The standard curve was plotted as absorbance against standard mannose concentration as in Appendix Figure A1



Appendix Figure A1 Standard curve of mannose concentration.

2. Protein determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

2.1 Reagents

2.1.1 Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

2.1.2 Reagent B: 0.5% $CuSO_4.5H_2O$

2.1.3 Reagent C: 1% Sodium potassium tartrate

2.1.4 Reagent D: A mixture of 100 ml of Reagent A, 1 ml of Reagent B and 1 ml of Reagent C. The solution should be freshly prepared before use.

2.1.5 Folin-ciocalteu phenol reagent: diluted 1:1 with distilled water to the final concentration of 1 N.

2.1.6 Bovine serum albumin stock solution (200 μg/ml): dissolved 20 mg of bovine serum albumin with distilled water and the solution was made up to 100 ml in volumetric flask.

2.2 Method

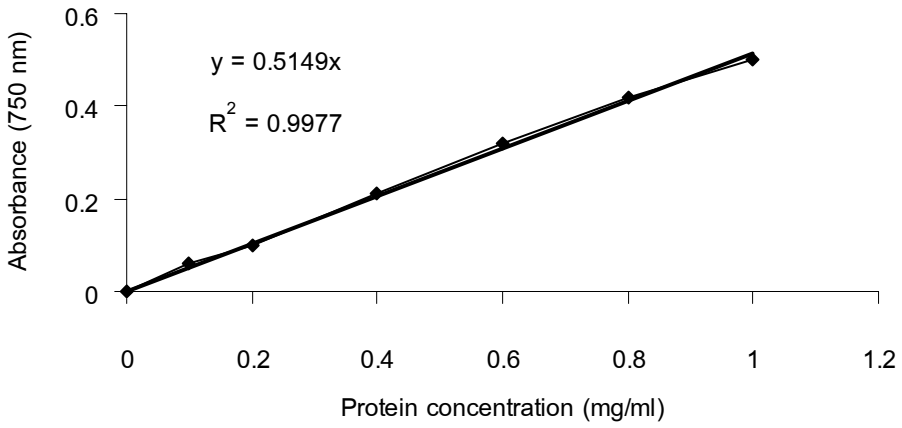
2.2.1 50 μl of sample was mixed with 1.5 ml Reagent D and incubated at room temperature for 10 min.

2.2.2 After 10 min incubation, 150 μl of 1 N Folin-ciocalteu phenol reagent was added to the sample and vortexed immediately and further incubated at room temperature for 30 min.

2.2.3 Absorbance was measured by spectrophotometer at 750 nm.

2.3 Standard curve of protein

Standard bovine serum albumin solution was prepared in the concentration of 100, 200, 400, 600, 800 and 1,000 μg/ml. The reactions were carried out using the same method as described in 2.2. The standard curve of protein was plotted as absorbance against standard bovin serum albumin concentration as in Appendix Figure A2.



Appendix Figure A2 Standard curve of protein using Lowry method.

3. Electrophoresis

3.1 Reagents

3.1.1 Acrylamide/Bis (30%T, 2.67%C): 14.6 g acrylamide and 0.4 g N'N'-bis- methylene-acrylamide were dissolved in 50 ml of distilled water (keep at 4°C).

3.1.2 1.5 M Tris-HCl, pH 8.8: 18.15 g Tris base dissolved in 50 ml of distilled water, adjusted pH to 8.8 with 6N HCl before made volume to 100 ml (keep at 4°C).

3.1.3 0.5 M Tris-HCl, pH 6.8: 6 g Tris base dissolved in 50 ml of distilled water, adjusted pH to 6.8 with 6N HCl before made volume to 100 ml (keep at 4°C).

3.1.4 10% Sodium dodecyl sulfate (SDS),: 10 g SDS dissolved in 50 ml of distilled water and made volume to 100 ml (keep at room temperature).

3.1.5 5X electrode (Running) buffer, pH 8.3: 6 g Tris base, 43.2 g Glycine and 3 g SDS dissolved in 600 ml of distilled water (keep at 4°C).

3.1.6 Sample buffer: (keep at room temperature) was prepared as listed in Appendix Table A1.

Appendix Table A1 A Sample buffer preparation

Component	SDS-PAGE (ml)	PAGE (ml)
Deionized water	9.5	14.5
0.5 M Tris-HCl, pH 6.8	2.5	2.5
Glycerol	2.0	2.0
10% SDS	4.0	-
2-Mercaptoethanol	1.0	-
1%(w/v) bromophenol blue	1.0	1.0
Total	20	20

3.2 Separating gel and stacking gel preparation

Separating gel and stacking were prepared by 10% and 4% acrylamide gel, respectively as shown in Appendix Table A2.

Appendix Table A2 Acrylamide gel electrophoresis preparation

Component	SDS-PAGE		PAGE	
	10% (ml)	4% (ml)	10% (ml)	4% (ml)
Deionized water	3.52	12.2	3.72	12.4
0.5 M Tris-HCl, pH8.8	5.0	-	5.0	-
0.5 M Tris-HCl, pH6.8	-	5.0	-	5.0
10% SDS	0.2	0.2	-	-
Acrylamide/bis	4.9	2.66	4.9	2.66
TEMED	0.01	0.02	0.01	0.02
10% ammonium persulfate	0.05	0.20	0.05	0.2

4 Buffer preparation

4.1 Citrate buffer

4.1.1 Stock solution

4.1.1.1 Solution A: 0.1M citric acid

4.1.1.2 Solution B: 0.1M sodium citrate

4.1.2 Buffer preparation

X ml of solution A and Y ml of solution B, diluted to a total of 100 ml.

Appendix Table A3 Citrate buffer preparation

X (ml)	Y (ml)	pH
46.5	3.5	3
33.0	17.0	4
20.5	29.5	5
9.5	41.5	6

4.2 Phosphate buffer

4.2.1 Stock solutions

4.2.1.1 Solution A: 0.2 M monobasic sodium phosphate

4.2.1.2 Solution B: 0.2 M dibasic sodium phosphate

4.2.2 Buffer preparation

X ml of solution A and Y ml of solution B, diluted to a total of 200 ml.

Appendix Table A4 Phosphate buffer preparation

X (ml)	Y (ml)	pH
87.7	12.3	6
39.0	61.0	7
5.3	94.7	8

4.3 Glycine-NaOH buffer

4.3.1 Stock solution

4.3.1.1 Solution A: 0.2 M glycine

4.3.1.2 Solution B: 0.2 M NaOH

4.3.2 Buffer preparation

50 ml of solution A and Y ml of solution B, diluted to a total of 200 ml.

Appendix Table A5 Glycine-NaOH buffer preparation

Y (ml)	pH
8.8	9
32.0	10

4.4 TE buffer

4.4.1 Stock solution

4.4.1.1 Solution A: 1 M Tris – HCl, pH 8.0

4.4.1.2 Solution B: 0.5 M EDTA, pH 8.0

4.4.2 Buffer preparation

One ml of solution A and 0.2 ml of solution B, diluted to a total of 100 ml.

4.5 Tris-borate buffer (TBE)

TBE buffer: 10.8 g of Tris base, 0.93 g of EDTA, and 5.5 g of boric acid were dissolved in 1000 ml of distilled water, adjusted pH to 8.0-8.2.

4.6 TEN buffer

4.6.1 Stock solution

4.6.1.1 Solution A: 1M Tris-HCl, pH 8.0

4.6.1.2 Solution B: 0.5 M EDTA, pH 8.0

4.6.1.3 Solution C: 5 M NaCl

4.6.2 Buffer preparation

With 0.1 ml of solution A, 0.02 ml of solution B and 0.02 ml of solution C, diluted to a total of 10 ml with sterilized distilled water.

4.7 SET buffer

4.7.1 Stock solution

4.7.1.1 Solution A: 1M Tris-HCl, pH 8.0

4.7.1.2 Solution B: 0.5 M EDTA, pH 8.0

4.7.1.3 Solution C: 25% sucrose

4.7.2 Buffer preparation

With 2.5 ml of solution A, 5 ml of solution B and 40 ml of solution C, diluted to a total of 50 ml with sterilized distilled water.

4.8 TEG buffer

4.8.1 Stock solution

4.8.1.1 Solution A: 1M Tris-HCl, pH 8.0

4.8.1.2 Solution B: 0.5 M EDTA, pH 8.0

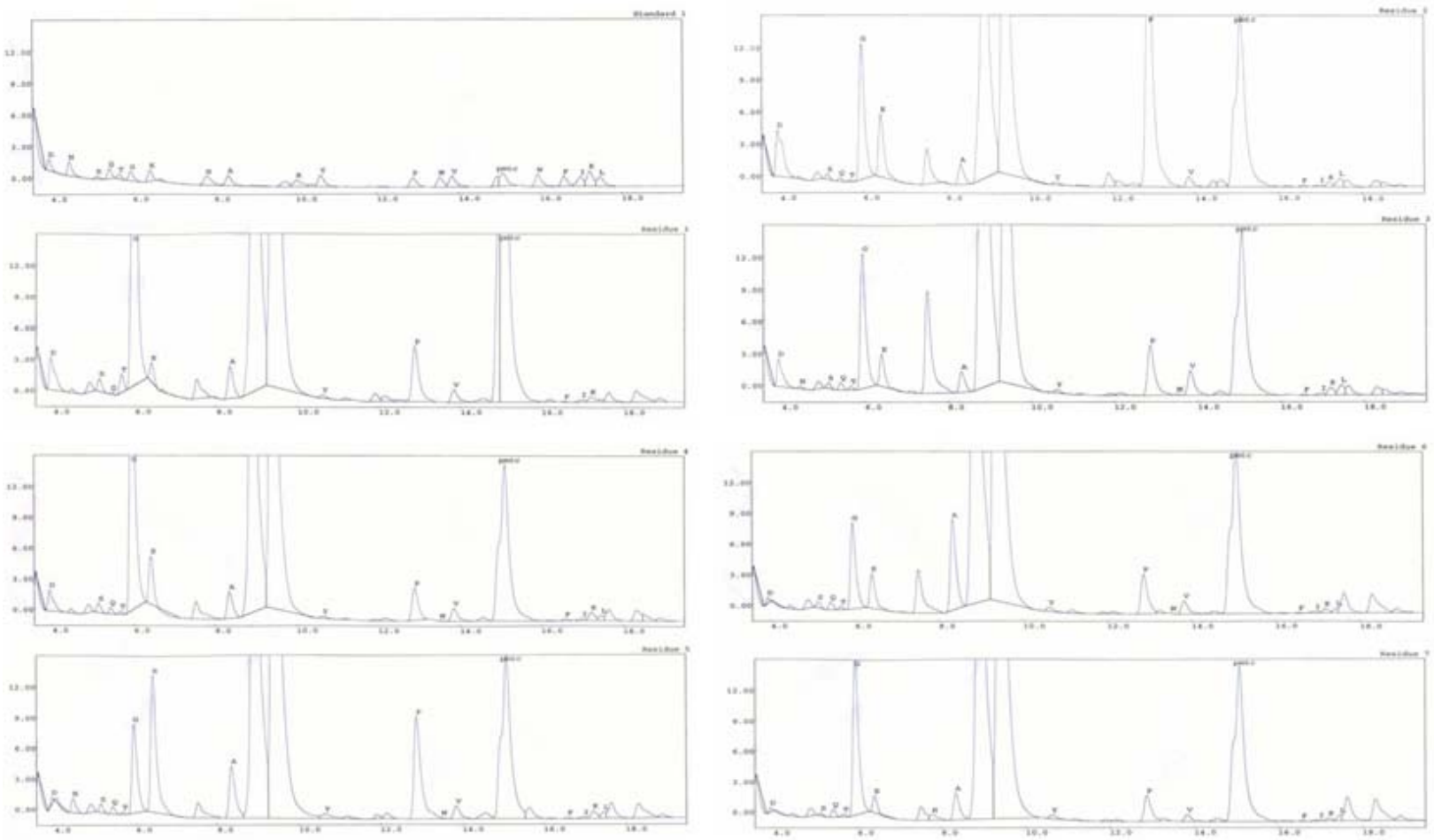
4.8.1.3 Solution C: 2 M sucrose

4.8.2 Buffer preparation

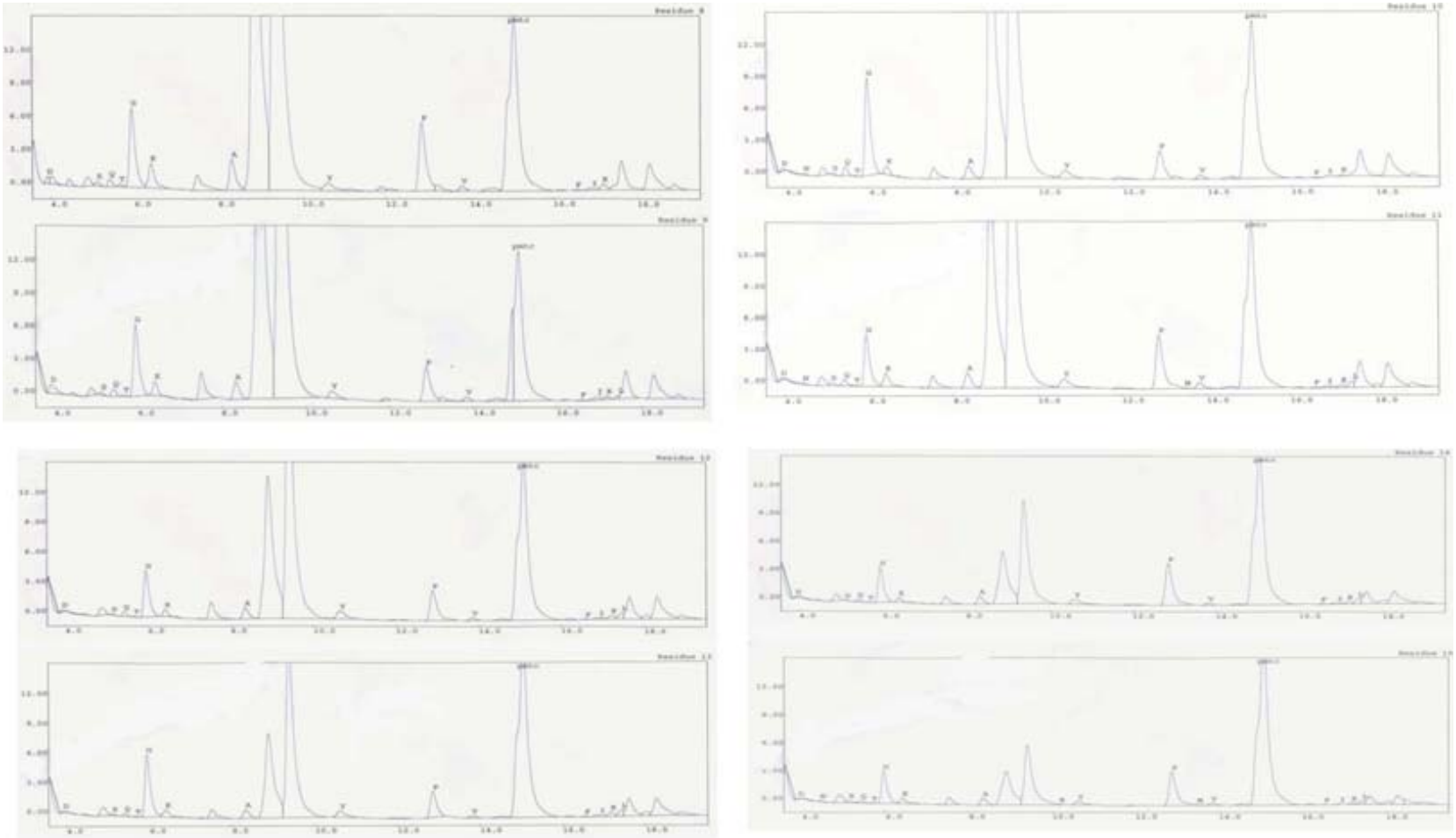
With 2.5 ml of solution A, 2.5 ml of solution B and 2 ml of solution C, diluted to a total of 100 ml with sterilized distilled water.

5. N-terminal amino acid sequence.

Protein sequencing of the purified mannanase S1 was analyzed at Research and Research Training Services, University of Newcastle, Australia. The results are shown in Appendix Figure A3-A4.



Appendix Figure A3 N-terminal amino acid sequence; standard amino acid sequence, residue 1-7



Appendix Figure A4 N-terminal amino acid sequence; residue 8-15

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