# CHAPTER 4

# Results and Discussions

## 4.1 Enzyme production

### 4.1.1 Enzyme production in various nitrogen sources

In this research, *Bacillus subtilis* M7, a lipase defective mutant strain obtained by X-ray mutagenesis of *B. subtilis* MR10, was investigated for the capability of β-mannanase production for application in the MOS production from β-mannan in copra meal. The result of β-mannanase production in the medium containing various inorganic and organic nitrogen sources was shown in Fig. 4.1 and 4.2, respectively. The best inorganic nitrogen source was ammonium sulfate. The highest enzyme activity was 10.4 U/ml. However, β-mannanase activity from ammonium sulfate was insignificant different compared to others.

In addition, the best organic nitrogen source was yeast extract but it was highly expensive source. However, β-mannanase activity from yeast extract was insignificant different compared to others. Therefore, soybean meal, low-priced organic nitrogen source containing galactomannan as the main β-mannan (Mullin and Xu, 2001), was more interesting for β-mannanase production. The enzyme activity was 10.98 U/ml.

The activity of β-mannanase produced by *B. subtilis* M7 was superior to *Penicillium occitanis* Pol6 on sodium nitrate and yeast extract as inorganic and organic nitrogen sources, respectively (Blibech *et al*., 2010), and *Paenibacillus cookii* on ammonium sulfate as an inorganic nitrogen source, as well as, soybean meal and tryptone as organic nitrogen sources (Yin *et al*., 2012), but inferior to alkaliphilic *Bacillus* sp. N16-5 on peptone and yeast extract as organic nitrogen sources (Lin *et al*., 2007), and *Bacillus* sp. MSJ-5 on yeast extract and soybean meal as organic nitrogen sources (Zhang *et al*., 2009).

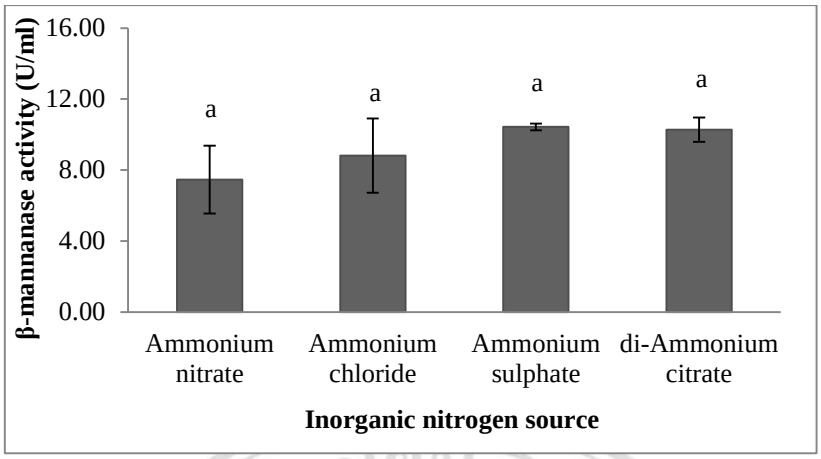


**Figure 4.1** Effect of inorganic nitrogen source on β-mannanase production by *B. subtilis* M7 at 37°C with 180 rpm.

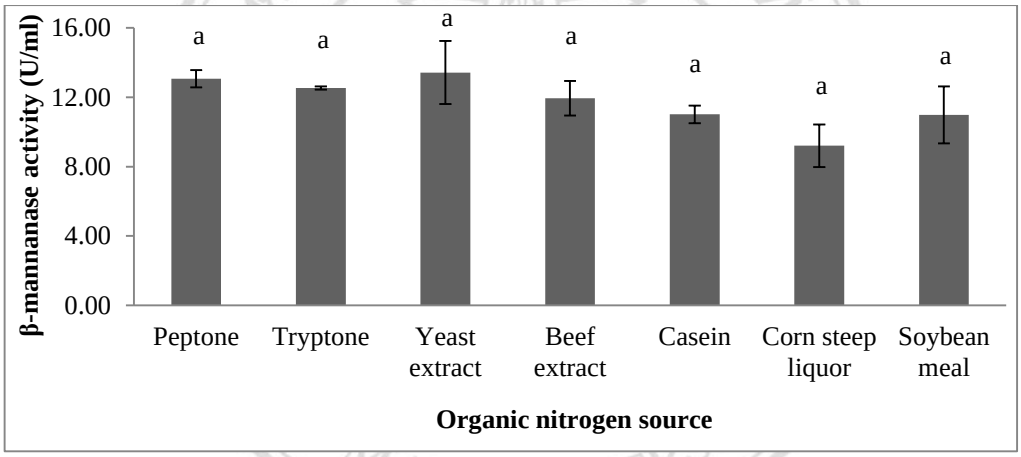


**Figure 4.2** Effect of organic nitrogen source on β-mannanase production by *B. subtilis* M7 at 37°C with 180 rpm.

### 4.1.2 Screening for the factors influenced on β-mannanase production

The effect of nutritional factors on β-mannanase production by *B. subtilis* M7 was determined by regression analysis (Table 4.1). The *P*-value less than 0.05 showed the significant effect on the β-mannanase production. The plus and minus values mean the positive and negative effects on β-mannanase production, respectively. Only copra meal was found to be the positive significant factor affecting on β-mannanase production in the similar way when locust bean gum and konjac flour were used as the carbon source (Table 4.2 and 4.3). The highly positive factors as copra meal, locust bean gum and konjac flour were further investigated to find the optimal quantity for β-mannanase production.

**Table 4.1** The least square linear regression analysis of Plackett and Burman statistical design for screening of the important factors affecting on β-mannanase production while copra meal is a carbon source.

| Variables | Coefficient | *P*-value |
|---|---|---|
| Model | 6.48 | 0.0731 |
| Soybean meal | 0.98 | 0.3759 |
| $K_2HPO_4$ | -0.59 | 0.5836 |
| $KH_2PO_4$ | 1.68 | 0.1564 |
| $CaCl_2.2H_2O$ | 1.53 | 0.1895 |
| $ZnSO_4.7H_2O$ | -1.86 | 0.1249 |
| $(NH_4)_2SO_4$ | 1.52 | 0.1935 |
| $MgSO_4.7H_2O$ | -1.68 | 0.1575 |
| Copra meal | 4.15 | 0.0093 |
| $R^2$ = 0.8636 | | |

**Table 4.2** The least square linear regression analysis of Plackett and Burman statistical design for screening of the important factors affecting on β-mannanase production while locust bean gum is a carbon source.

| Variables | Coefficient | *P*-value |
|---|---|---|
| Model | 7.85 | 0.0892 |
| Soybean meal | 0.93 | 0.4236 |
| $K_2HPO_4$ | -0.36 | 0.7509 |
| $KH_2PO_4$ | 1.77 | 0.1570 |
| $CaCl_2.2H_2O$ | 1.56 | 0.2017 |
| $ZnSO_4.7H_2O$ | -1.58 | 0.1969 |
| $(NH_4)_2SO_4$ | 1.31 | 0.2732 |
| $MgSO_4.7H_2O$ | -2.00 | 0.1191 |
| Locust bean gum | 4.17 | 0.0111 |
| $R^2$ = 0.8505 | | |

**Table 4.3** The least square linear regression analysis of Plackett and Burman statistical design for screening of the important factors affecting on β-mannanase production while konjac flour is a carbon source.

| Variables | Coefficient | *P*-value |
|---|---|---|
| Model | 8.23 | 0.0484 |
| Soybean meal | 1.23 | 0.1936 |
| $K_2HPO_4$ | -0.52 | 0.5543 |
| $KH_2PO_4$ | 0.69 | 0.4385 |
| $CaCl_2.2H_2O$ | 1.12 | 0.2279 |
| $ZnSO_4.7H_2O$ | -1.47 | 0.1324 |
| $(NH_4)_2SO_4$ | 1.33 | 0.1663 |
| $MgSO_4.7H_2O$ | -1.31 | 0.1695 |
| Konjac flour | 4.14 | 0.0039 |
| $R^2$ = 0.8869 | | |

### 4.1.3 Effect of carbon source on β-mannanase production

To investigate the effect of carbon source on β-mannanase production, various concentrations of copra meal was added in the optimal medium. The results are shown in Fig. 4.3a. The presence of copra meal resulted higher β-mannanase activity obtained than the control and the optimal β-mannanase production (11.97 U/ml) was found from the culture with 6 g/l copra meal. Moreover, when locust bean gum and konjac flour were the carbon source, the optimal enzyme activities were 29.49 U/ml obtained from the culture with 10 g/l locust bean gum (Fig. 4.3b), and 30.90 U/ml obtained from that with 4 g/l konjac flour (Fig. 4.3c). The β-mannanase production from locust bean gum and konjac flour was higher than that from copra meal. However, the β-mannanase production by this bacterial strain on copra meal was higher than *Penicillium occitanis* Pol6 on carob seed flour (Blibech *et al*., 2010), *Thermomyces lanuginosus* and *Sclerotium rolfsii* on locust bean gum (Gubitz and Steiner, 1995).

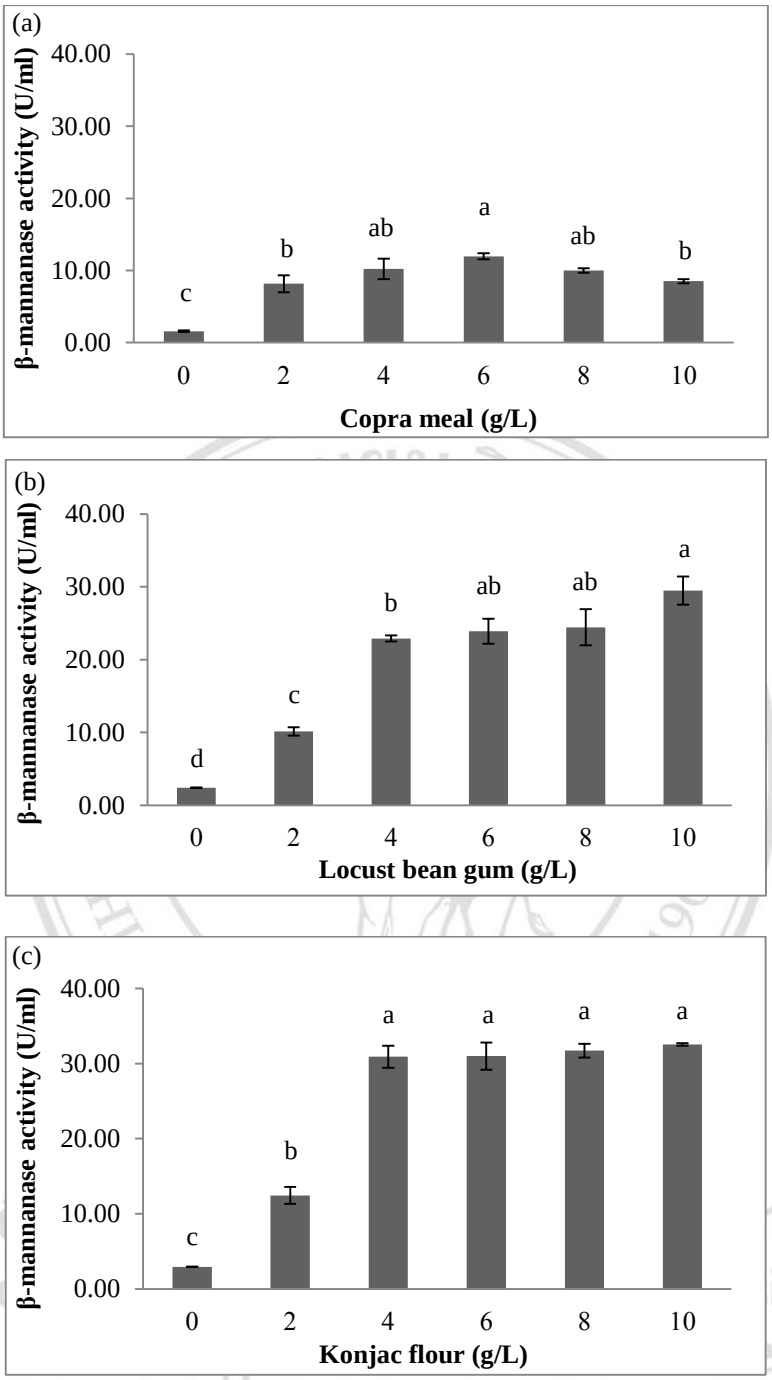


**Figure 4.3** Effect carbon source of on β-mannanase production by *B. subtilis* M7. (a) copra meal, (b) locust bean gum and (c) konjac flour.

### 4.1.4 Time course of β-mannanase production

Fig. 4.4a presents the β-mannanase activity from the optimized medium containing gram per liter of 5 soybean meal, 0.3 $K_2HPO_4$, 1 $KH_2PO_4$, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$ and 6 copra meal with initial pH 6.8 during 30 h cultivation. It was found that *B. subtilis* M7 maintained the β-mannanase producing capability almost the same level with the parent strain, *B. subtilis* MR10. The β-mannanase of 12.65 U/ml was obtained at 30 h cultivation at 37ºC. However, the highest value of the enzyme activity of 13.63 U/ml was found at 48 h of cultivation.

In the same way, the β-mannanase activity from the optimized medium with 10 g/l locust bean gum and from that with 4 g/l konjac flour was presented in Fig. 4.4b and 4.4c, respectively. The enzyme activities of *B. subtilis* M7 were 28.89 U/ml obtained from the optimized medium with locust bean gum at 30 h (Fig. 4.4b), and 29.05 U/ml obtained from that with konjac flour at 30 h (Fig. 4.4c). However, the copra meal production in some parts of the world is plentiful and available worldwide (Sundu *et al.*, 2009; FAO, 2011). Moreover, copra meal is more cost-effective than locust bean gum and konjac flour. Thus, alternative cheaper copra meal was selected for further study.

### 4.1.5 Effect of various temperatures on β-mannanase production

The *B. subtilis* M7 was cultivated on optimized copra meal medium, pH 6.8 at different temperatures of 30, 37 and 45ºC, respectively. The result is presented in Fig. 4.5, the highest β-mannanase activity of 12.58 U/ml was achieved at 37°C. However, β-mannanase activity at 37°C was insignificant different compared to others. The β-mannanase production of *B. subtilis* M7 was higher than that obtained from *Bacillus* sp. KK01 at 30°C (Hossain *et al.*, 1996) and *Aspergillus niger* NCH-189 at 30°C (Lin and Chen, 2004).

### 4.1.6 Effect of pH on β-mannanase production

The *B. subtilis* M7 was cultivated on optimized copra meal medium at 37ºC with a pH range of 4.0-8.0. The optimal pH on β-mannanase production was obtained with a pH range of 6.8-7.0. The maximal β-mannanase activity was obtained as 11.73 U/ml at pH 6.8 as shown in Fig. 4.6. The result was higher than β-mannanase production by *Sclerotium rolfsii*, pH 6.0 (Gübitz *et al.*, 1996).

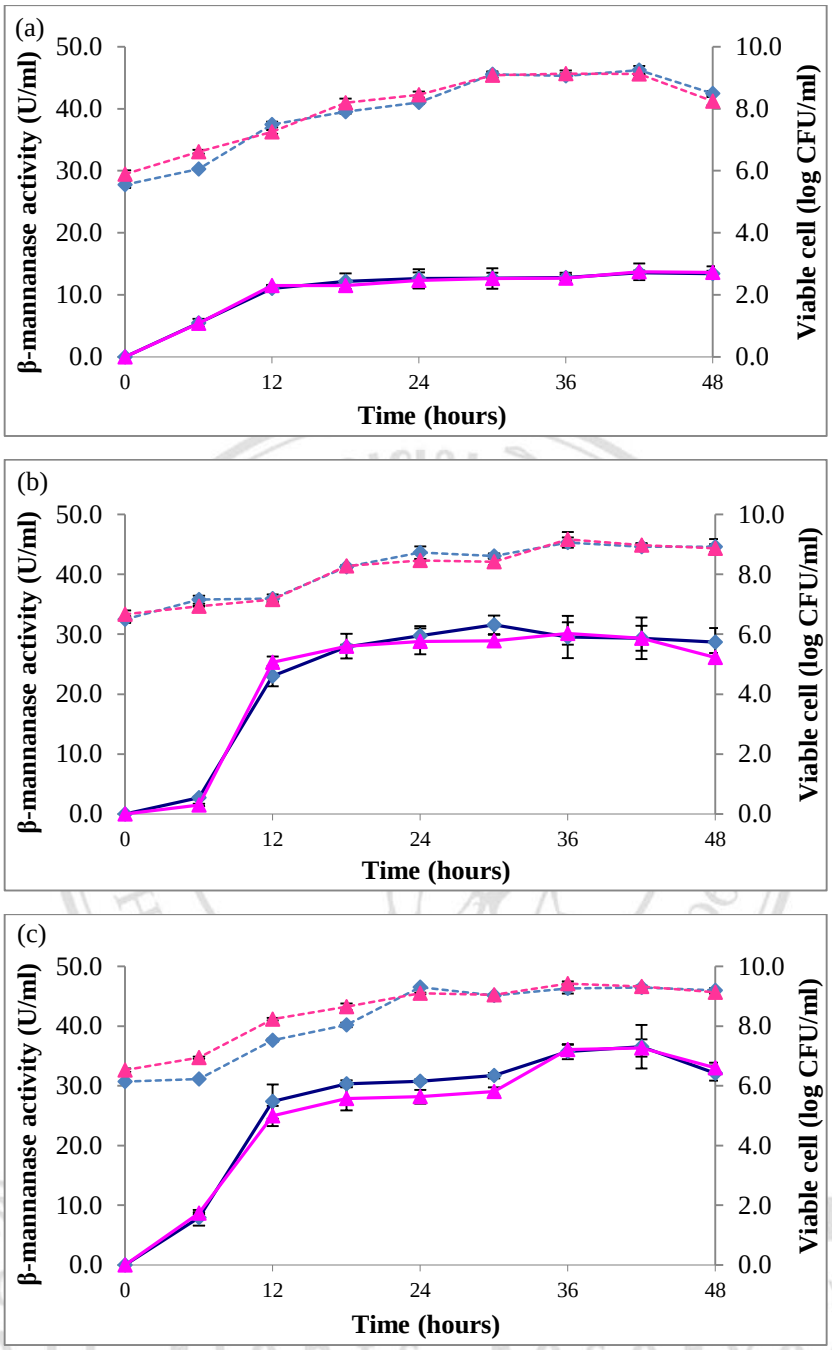


**Figure 4.4** Time course of β-mannanase production of *B. subtilis* MR10 (♦) and *B. subtilis* M7 (▲) cultivated in the optimal medium with (a) copra meal, (b) locust bean gum and (c) konjac flour as a sole carbon source. - - -, viable cell (log CFU/ml); ——, β-mannanase activity (U/ml).

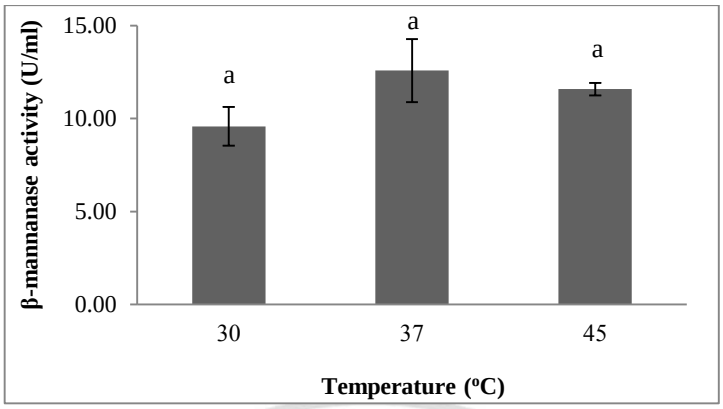


**Figure 4.5** Effect of growth temperatures on β-mannanase production by *B. subtilis* M7 at 180 rpm.

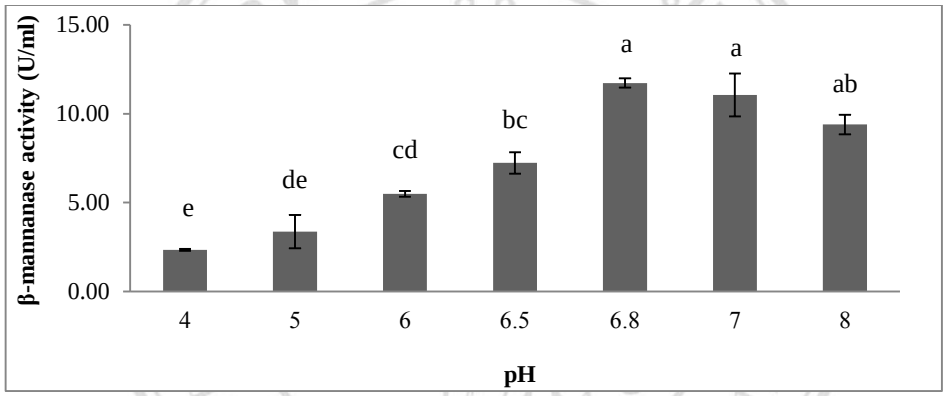


**Figure 4.6** Effect of initial pH on β-mannanase production by *B. subtilis* M7 at 37ºC with 180 rpm.

### 4.1.7 Scale up β-mannanase production

The *B. subtilis* M7 was cultivated on optimal copra meal medium, pH 6.8 at 37ºC with various agitation rates of 200 and 400 rpm. The result is presented in Fig. 4.7, the highest β-mannanase activity of 16.51 U/ml was achieved at 400 rpm for 30 h because higher agitation rate increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. It led to the better growth and higher enzyme activity (Feng *et al.*, 2003). However, the result was lower than β-mannanase production that from *B. subtilis* TJ-102 cultivated at 37ºC and 200 rpm (Wang *et al.*, 2013) and from *B. licheniformis* NK-27 cultivated at 30ºC and 600 rpm (Feng *et al.*, 2003).

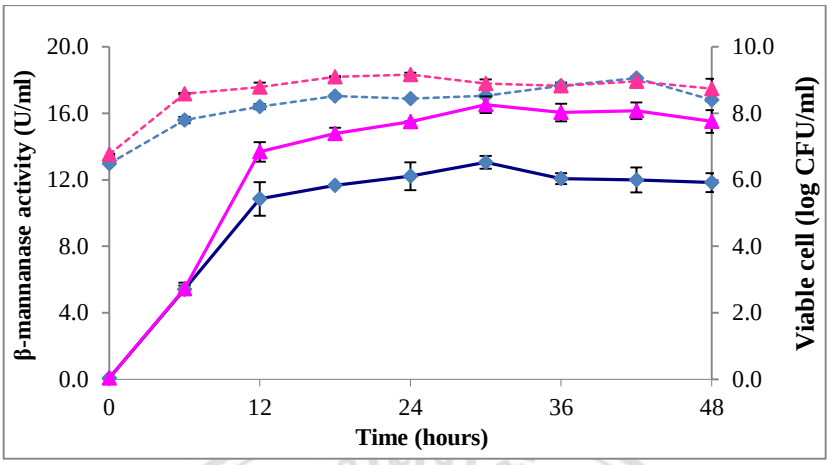


**Figure 4.7** Time course of β-mannanase production of *B. subtilis* M7 cultivated in the optimal medium at 37ºC with different agitation rates. ♦, 200 rpm; ▲, 400 rpm; - - -, viable cell (log CFU/ml); ——, β-mannanase activity (U/ml).

### 4.1.8 Effect of different additives on stability of β-mannanase

The most appropriate stabilizer for β-mannanase from *B. subtilis* M7 was investigated among 5 stabilizers (Figure 4.8). It was found that there were 4 additives including sorbitol, sodium chloride, ammonium sulfate and starch capable of stabilizing of β-mannanase activity within 42 days when compared to control (enzyme without additive). Particularly, sodium chloride acted the best stabilizer because the residual activity was still stable at 94% when stored crude enzyme at 4 ºC for 42 days. While those of ammonium sulfate, sorbitol and starch still remained 87%, 85% and 84%, respectively. However, those of these additives were not quite different when compared to that of control, since the β-mannanase production medium containing ammonium sulfate as stabilizer. Using additives is the most popular method of enzyme stabilization to enhance enzyme storage stability. However, some of the stabilizing additives might interfere with the final use of the enzyme due to inconsistency with the reaction system (Iyer *et al.*, 2008). Furthermore, sodium chloride demonstrated to be the best stabilizer for crude β-mannanase from this study. This data was relevant to that reported for the malic enzyme from *Halobacterium cutirubrum* (Cazzulo and Vidal, 1972). The results supported the proposal of Hubbard and Miller (1969) that the salts may play different roles in the stabilization and activation of enzymes.

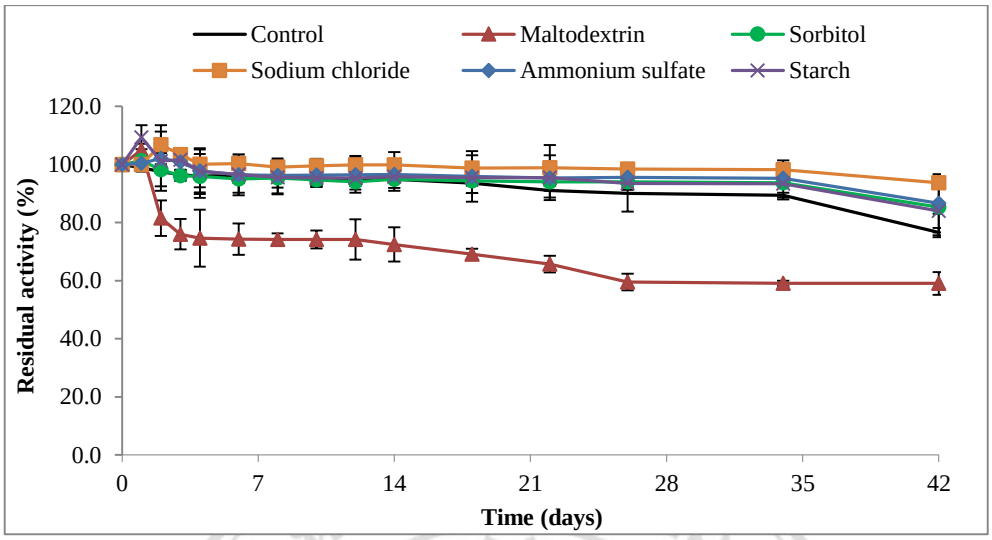


**Figure 4.8** Storage stability of β-mannanase from *B. subtilis* M7 with different stabilizers at 4°C.

## 4.2 Enzyme purification

Finally, the β-mannanase was purified to homogeneity after ion exchange chromatography (Fig. 4.9) and gel filtration chromatography (Fig. 4.10). The chromatogram of gel filtration chromatography showed only one peak of β-mannanase activity which corresponded to the main protein peak. The result of the enzyme purification is summarized in Table 4.4. The enzyme was purified 23.5-fold with a final yield of 4% of the activity in the culture supernatant. Then, fractions No. 81-84 of gel filtration chromatography, the enzyme fractions showing the highest enzyme activity, were analyzed by SDS-PAGE. The result was shown in Fig. 4.11. SDS-PAGE revealed that both the purified enzyme from ion exchange and gel filtration chromatography showed the same single band of protein (data not shown) with a molecular mass of approximately 42 kDa.

Only one single β-mannanase protein was presented in the culture of *B. subtilis* M7 cultivated in the medium containing copra meal. It is a monomeric protein of 42 kDa. The molecular mass of the enzyme is similar to those obtained from *Bacillus* spp. (37-40 kDa) (Jiang *et al.*, 2006; Zakaria *et al.*, 1998; Mendoza *et al.*, 1994) and from *Trichoderma* sp. (32-46 kDa) (Ferreira and Filho, 2004). However, the purified β-mannanase from *Bacillus* sp. strain JAMB-750 was reported a molecular mass of 130 kDa (Takeda *et al.*, 2004), similar to β-mannanases from *Aspergillus* spp. which ranging from 30-110 kDa (Regalado *et al.*, 2000).

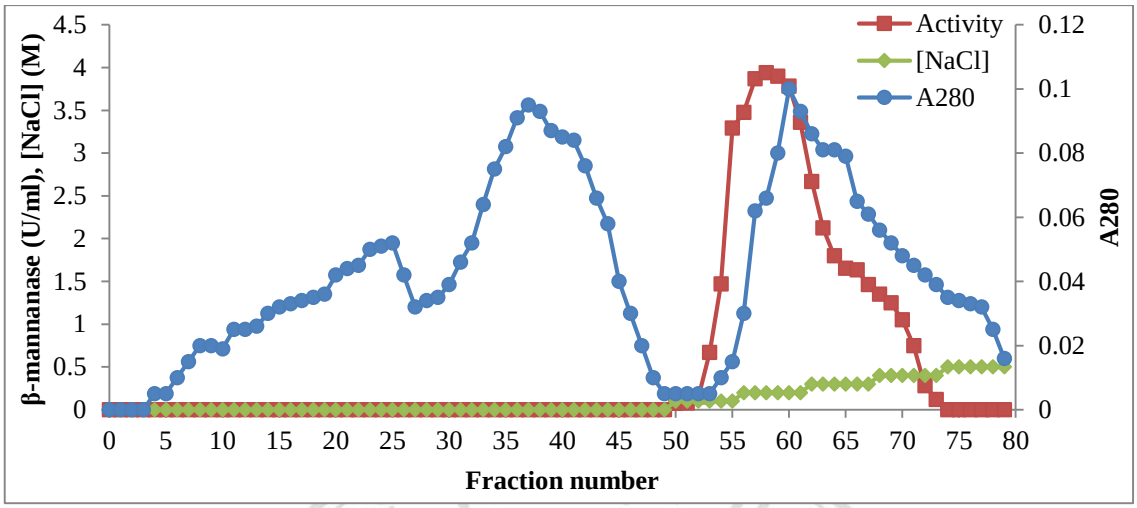


**Figure 4.9** Elution profiles of β-mannanases from *B. subtilis* M7 on DEAE-Sephadex A-50 column chromatography pH 7.5.

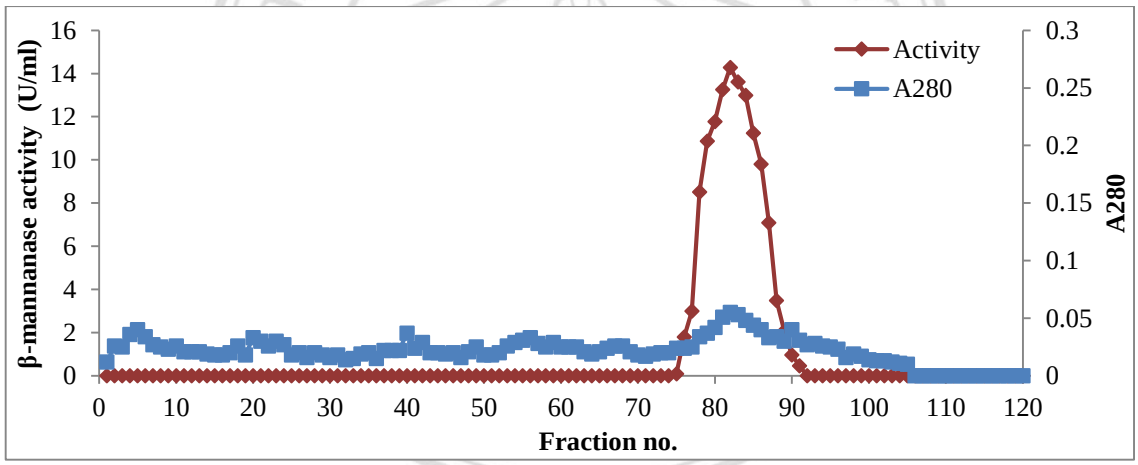


**Figure 4.10** Elution profiles of β-mannanases from *B. subtilis* M7 on Sephacryl S-100 gel filtration chromatography pH 7.5.

**Table 4.4** Purification of β-mannanase from *B. subtilis* M7.

| Step of purification | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification (fold) | Discovery (%) |
|---|---|---|---|---|---|
| Crude enzyme | 18865 | 66 | 286 | 1.0 | 100 |
| Precipitation by $(NH_4)_2SO_4$ | 12534 | 19.2 | 653 | 2.3 | 66 |
| DEAE-Sephadex A-50 | 3795 | 0.75 | 5060 | 17.7 | 20 |
| Sephacryl S-100 | 671.5 | 0.1 | 6715 | 23.5 | 4 |

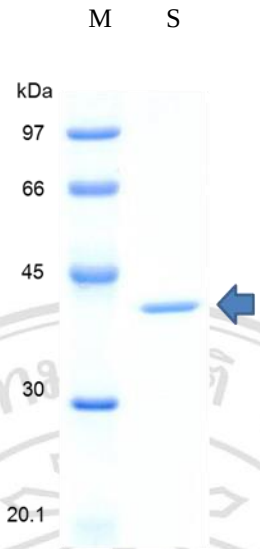


**Figure 4.11** SDS-PAGE of purified β-mannanase from *B. subtilis* M7. Lane M: molecular weight marker; Lane S: purified β-mannanase.

## 4.3 Characterization of purified β-mannanase

### 4.3.1 Effects of pH and temperature on enzyme activity

The purified β-mannanase showed optimal activity at pH 5.0-7.0 (Fig. 4.12a). The same pH effecting result was found for β-mannanase from *Acinetobacter* sp. ST 1-1 (Titapoka *et al.*, 2008), *B. amyloliquefaciens* I0A 1 (Mabrouk and Ahwany, 2008) and *B. circulans* M-21 (Mou *et al*., 2011). The enzyme was stable over a broad pH range of 4.0 to 9.0 (Fig. 4.13a). β-Mannanase from *Acinetobacter* sp. ST 1-1 was stable in the pH range of 3 to 10 (Titapoka *et al.*, 2008). Nevertheless, the enzyme from *Bacillus* sp. MG-33 was not stable under alkaline conditions (Meenakshi *et al*., 2010).

The enzyme exhibited the maximum activity at 50-60ºC (Fig. 4.12b) while β-mannanase from *Bacillus* sp. N16-5 showed a higher activity at 70°C (Lin *et al*., 2007). The enzyme retained more than 90% of its maximum activity at 60°C. Temperature above 60°C presented a more sudden inactivation of the enzyme. The thermostability of the enzyme up to 60ºC for 1 h (Fig. 4.13b), was similar to that of mannanases reported for *Bacillus* sp. (Zhang *et al*., 2009; Meenakshi *et al*. 2010) and higher than that from *Bacillus* sp. (Hossain *et al*., 1996; McCleary, 1979).

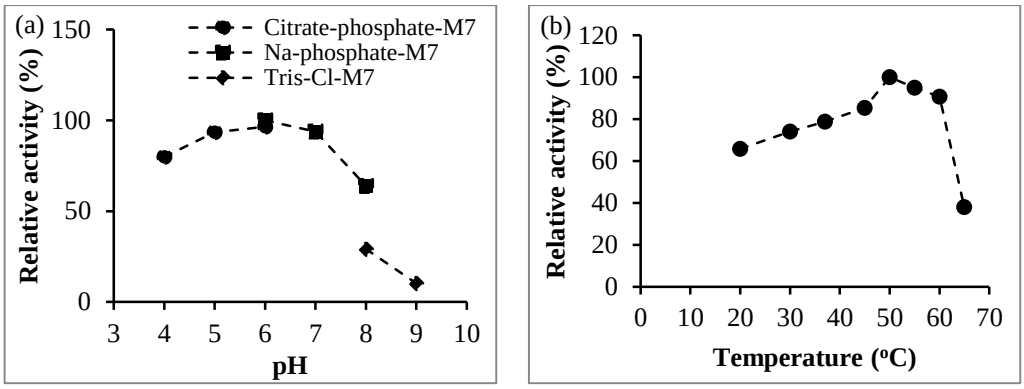


**Figure 4.12** Effect of pH (a) and temperature (b) on the activity of β-mannanase from *B. subtilis* M7.

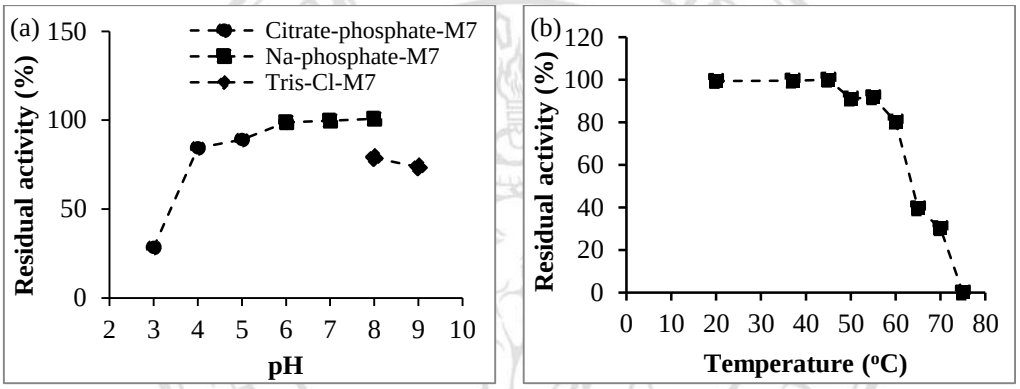


**Figure 4.13** pH stability at 4 °C for 24 h (a) and thermostability for 1 h (b) of β-mannanase from *B. subtilis* M7.

### 4.3.2 Substrate specificity

Table 4.5 showed the relative activities of β-mannanase for various polysaccharide substrates. β-Mannanase exhibited the highest activity against locust bean gum, which was taken as 100% relative activity. It also hydrolysed konjac flour, guar gum and copra meal with lower relative activities of 61.3, 21.7 and 7.9%, respectively.

This result is similar to those from *B. subtilis* WY34, having high activity against locust bean gum (Jiang *et al*., 2006). Nevertheless, the lower β-mannanase activity is assayed in the presence of copra meal as substrate compared with the enzyme activity towards locust bean gum. This may be due to the difference of β-mannan content in both substrates. Locust bean gum was reported to consist of approximately 80% β-mannan (Bouzouita *et al*., 2007; Andrade *et al*., 1999) while only 20% β-mannan was found in copra meal (Khanongnuch *et al*., 2006).

**Table 4.5** Substrate specificity of β-mannanase from *B. subtilis* M7.

| Substrate | Relative activity (%) |
|---|---|
| Locust bean gum | 100.0±0.1 |
| Guar gum | 21.7±0.1 |
| Konjac flour | 61.3±0.1 |
| Copra meal | 7.9±0.1 |

### 4.3.3 Effect of various metal ions and reagents

The effect of various metal ions and chemical reagents on β-mannanase activity was shown in Table 4.6. The enzymes were significantly activated by $Co^{2+}$, $Mn^{2+}$, $Fe^{3+}$, $Al^{3+}$ and mercaptoethanol. In contrast, other metal ions acted as inhibitors in decreasing activity of the purified β-mannanase. The *B. subtilis* WY34 mannanase was also inhibited by $Li^{+}$ and $Cu^{2+}$ (Jiang *et al*., 2006). The metal ions could interact with -SH group to form mercaptide, or with carboxyl and imidazole groups. The effect of metal ion ensures that the active site of the enzyme consists of -SH group (Yin *et al*., 2012). The chemical reagent SDS and EDTA inhibited β-mannanase activity by about 60 and 90%, respectively. The *B. subtilis* WY34 β-mannanase was also inhibited by EDTA (Jiang *et al*., 2006). EDTA, a chelating agent, could chelate or sequester the active site metal ion of enzyme. This leads to deactivate metal-dependent enzymes (Dominguez and Ward, 2009). In contrast, mercaptoethanol, a reducing agent, can activate -SH group on the enzyme molecules to enhance the enzyme activity (Yin *et al*., 2012). Therefore, the enzyme was concluded to be the metal-dependent enzyme that required a metal ion for activation of the enzyme.

### 4.3.4 Kinetics of the β-mannanase reaction

The effect of various concentrations of substrates on β-mannanase activity was examined (Fig. 4.14). Lineweaver-Burk double reciprocal plots were used to calculate the $K_m$ value for locust bean gum. The $K_m$ value of the purified enzyme was 30.34 mg/ml. Maximum velocities ($V_{max}$) were 1347.76 μmole/min/ml. β-mannanase from *B. subtilis* M7 exhibited both higher obvious $K_m$ and $V_{max}$ values than β-mannanases from *B. subtilis* WY34, *Thermotoga neapolitana* 5068 and *Aspergillus aculeatus* (Jiang *et al*., 2006; Duffaud *et al*., 1997; Setati *et al*., 2001).

**Table 4.6** Effect of cations and reagents on the activity of β-mannanase from *B. subtilis* M7.

| Cation | Relative activity (%) |
|---|---|
| KCl | 96.0±0.02 |
| NaCl | 98.8±0.02 |
| LiCl | 95.3±0.03 |
| $CoCl_2$ | 118.2±0.03 |
| $MgCl_2$ | 95.6±0.07 |
| $CuSO_4$ | 70.4±0.02 |
| $BaCl_2$ | 90.1±0.03 |
| $MnCl_2$ | 104.8±0.03 |
| $CaCl_2$ | 89.0±0.02 |
| $Pb(NO_3)_2$ | 82.1±0.03 |
| $FeCl_3$ | 114.3±0.05 |
| $AlCl_3$ | 102.5±0.02 |
| **Reagents** | |
| EDTA | 13.1±0.08 |
| SDS | 39.1±0.24 |
| 2-mercaptoethanol | 138.0±0.06 |

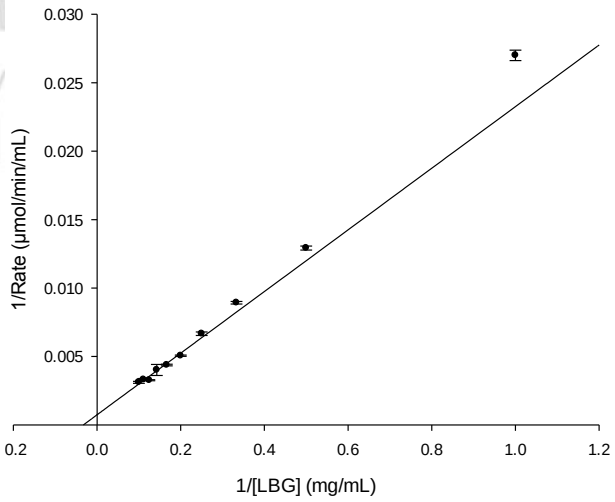


**Figure 4.14** Linweaver Burk plots of β-mannanase from *B. subtilis* M7.

### 4.3.5 Analysis of hydrolysis product

MOS generated from locust bean gum and copra meal hydrolysis by purified *B. subtilis* M7 β-mannanase and separated by thin layer chromatography were presented in Fig. 4.15. The pattern of MOS from copra meal hydrolysis was similar to MOS obtained from locust bean gum hydrolysis. However, the density of MOS products indicated that the purified enzyme reacted with locust bean gum and generated MOS better than the reaction using copra meal as substrate. This might be due to the difference in β-mannan content in both substrates. Locust bean gum was reported to comprise approximately 80% β-mannan (Bouzouita *et al*., 2007; Andrade *et al*., 1999) while only 20% β-mannan was found in copra meal (Khanongnuch *et al*., 2006). The pattern of MOS from hydrolysis of locust bean gum and copra meal by β-mannanase of *B. subtilis* M7 was similar to that from hydrolysis of copra mannan and locust bean gum by β-mannanase of *B. subtilis* WY34 (Jiang *et al*., 2006). MOS hydrolyzed by β-mannanase of *Streptomyces* sp. strain no. 17 and *Bacillus* sp. strain AM-001 was larger than mannobiose (Takahashi *et al*., 1984; Akino *et al*., 1988) and that of *B. subtilis*, *Aeromonas* sp. strain F-25, *Pseudomonas* sp. strain PT-5 and *Enterococcus casseliflavus* FL2121 was larger than mannotriose for many oligosaccharides production (Emi *et al*., 1972; Araki, 1983; Yamaura *et al*., 1990; Oda *et al*., 1993). From our result, the β-mannanase from *B. subtilis* M7 is possible to apply for MOS production and the MOS products are also expected to be used as functional food additives for promoting the growth of beneficial intestinal bacteria affecting on human health as same as the suggestion from the previous reports.

From this research, when compared to the previous research, it was found that the activity of β-mannanase produced by *B. subtilis* M7 using copra meal as substrate was higher than that from *B. subtilis* 5H using locust bean gum as substrate (Sa-nguansook, 2002). Moreover, the β-mannanase from *B. subtilis* M7 was more thermostable and stable over a broad pH range. This was an essential property for applications in the industries, especially β-mannan hydrolysis for MOS production.

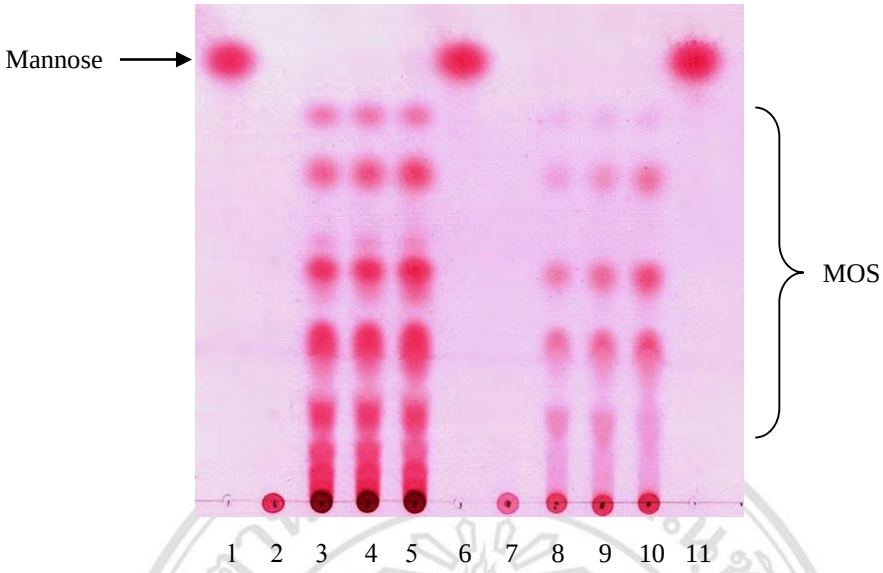


**Figure 4.15** Thin layer chromatography of locust bean gum and copra meal hydrolysates digested by the purified β-mannanase from *B. subtilis* M7 after 6 h incubation. Lane 1, 6 and 11 are standard mannose; Lane 2, 3, 4 and 5 are locust bean gum hydrolysis products from 0, 0.5, 1 and 6 h of reaction time, respectively; Lane 7, 8, 9 and 10 are copra meal hydrolysis products from 0, 0.5, 1 and 6 h of reaction time, respectively**.**