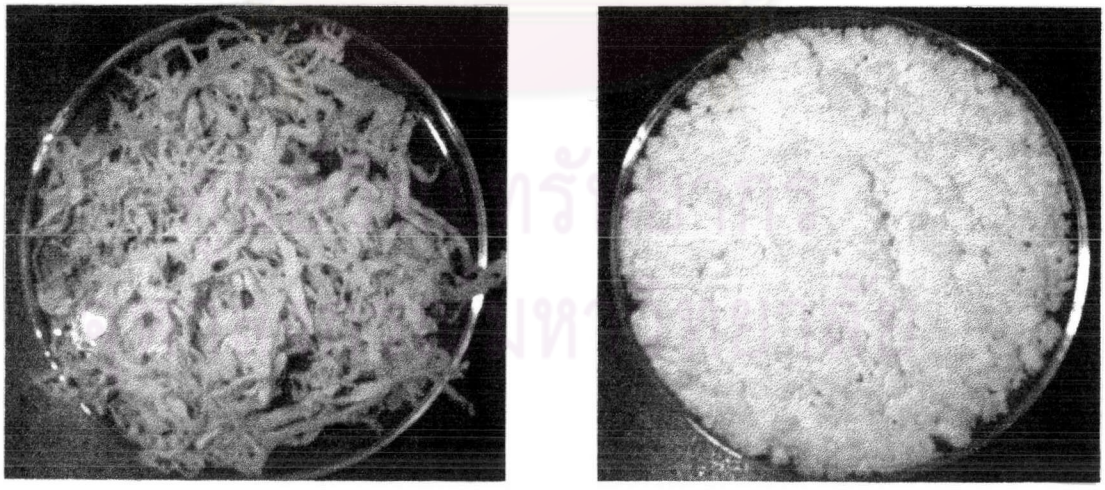
# CHAPTER III

# RESULTS AND DISCUSSIONS

## 3.1 Substrates and enzymes

3.1.1 Fibrous squid pen chitin

The fibrous squid pen chitin was prepared as a substrate by grinding strip chitin with an ultracentrifugal mill (Rector 970) to give fibrous chitin in two batches. The first batch the fibers had dimension ~ 50 μm in diameter and ~ 100 μm in length. The later batch used finer grinding set up to give fibrous chitin with ~ 25 μm in diameter and ~ 50 μm in length **(Figure 3.1)**. Most of our hydrolysis reactions were carried out by using fibrous β-chitin substrate with dimension about 25 μm in diameter and about 50 μm in length unless stated otherwise.



**(a)** **(b)**

**Figure 3.1** The squid pen chitin, (a) flake chitin, (b) fibrous β-chitin 100x50 μm (1x)

3.1.2 Colloidal chitin

The coloidal chitin was prepared under acidic condition according to the literature procedure.[62] In the final step, white slurry chitin was obtained. The concentration of colloidal chitin was 33.8 mg/mL after drying in vacuo. This colloidal chitin was stored at 4 °C for further use in all experiments throughout this thesis.

3.1.3 Protein assaying of enzyme

The analysis of protein of enzyme cellulase *Ac* (lot. Aus-0301) from Meiji Seika Kaisha, Ltd., Japan showed that the crude enzyme contained 70% protein with endo- and exo-chitinolytic activity of 18 mU and 0.25 mU per milligram of protein, respectively.[44] The crude enzyme was stored as a solid in a refrigerator at 4 °C for future use.

The enzyme chitinase *Serratia sp.* cloned (Chi 60) provided by Dr. Ruth Pichyangkura, Department of Biochemistry, Chulalongkorn University, contained 98.5 μg protein per 1 mL of crude enzyme peparation. The crude enzyme was stored as a yellow liquid in a refrigerator at 4 °C for future use.

3.1.4 Chitinase activity assaying

The chitinolytic activity of cellulase *Ac* (lot. Aus-0301) was 26 mU/mg of enzyme and the chitinolytic activity of Chi 60 was 214 mU/mL where one unit (U) of enzyme activity was defined as the amount of an enzyme able to produce the product with reducing ability equal to 1 μmole of GlcNAc per minute.

## 3.2 Study of hydrolytic products by HPLC

The calibration lines for *N*-acetyl-D-glucosamine (GlcNAc) and *N,N'*-diacetylchitobiose were showed in appendix A, Figure A1-A2. The factor of 353.29 and 495.54 from the slope of the calibration lines for of GlcNAc and $(GlcNAc)_2$ were used to calculate the amount of the GlcNAc and $(GlcNAc)_2$ from the peak areas in the HPLC chromatogram, respectively.

## 3.3 Study for the optimum condition in the enzymatic hydrolysis with cellulase *Acremonium cellulolyticus*

### 3.3.1 Concentration of chitin

- Powder chitin (3.0 μm)

The effect of the concentration of powder chitin on the production of $(GlcNAc)_2$ and GlcNAc was investigated in this experiment. The concentration of chitin was varied as 10, 20, 30, 40, 50, 60, 80, 100 and 200 mg/mL while the concentration of enzyme was fixed at 260 mU/mL. The results showed that the yield of $(GlcNAc)_2$ increased when the chitin concentration was increased from 10 mg/mL to 30 mg/mL **(Figure 3.2)** and almost constant when the chitin concentration was increased to 80 mg/mL. On the other hand, the yield of GlcNAc gradually decreased with the increasing chitin concentration. The mole ratio of $(GlcNAc)_2$/GlcNAc was thus increased with the increasing chitin concentration. The yield of $(GlcNAc)_2$ droped by about 30% and 56% at chitin concentration of 100 mg/mL and 200 mg/mL, respectively. Due to the high content of the solid chitin (> 80 mg/mL), the stirring became ineffective. The concentration of chitin at 80 mg/mL was thus an obvious choice for further optimization for $(GlcNAc)_2$ production.

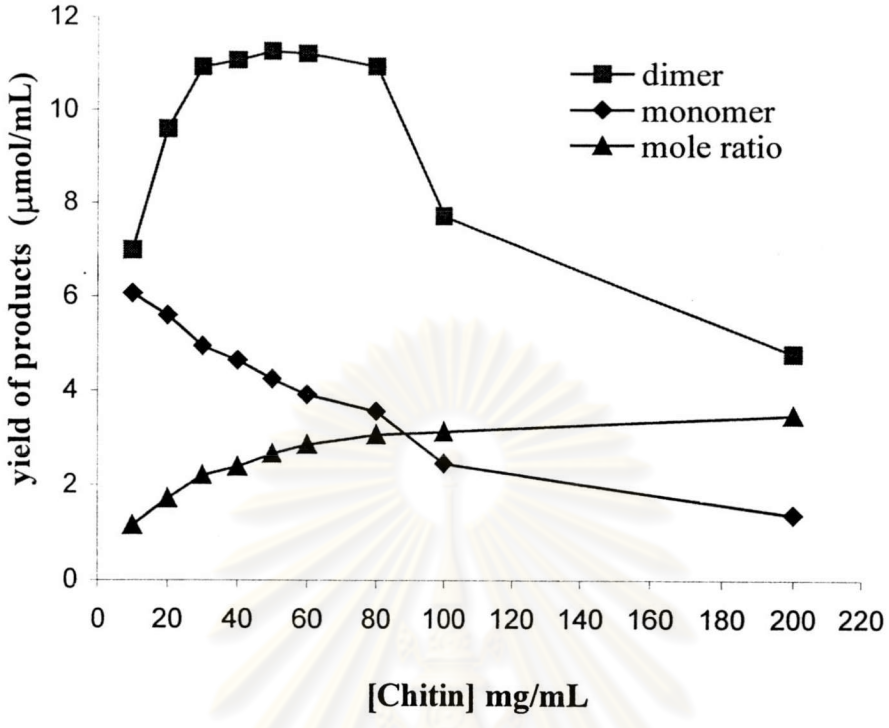



**Figure 3.2** The effect of chitin concentration on the chitinolysis of powder chitin with cellulase *Ac*. Conditions; [cellulase *Ac*] = 260 mU/mL; citrate-phosphate buffer pH 3.0 (0.1 M); 37 °C; 2 days.

- Fibrous chitin

For fibrous chitin, the concentration of chitin was varied as 10, 20, 30, 50 and 60 mg/mL with fixed enzyme concentration at 260 mU/mL. The results were similar to the results of powder chitin in certain respects. The yield of $(GlcNAc)_2$ and the mole ratio of $(GlcNAc)_2$/GlcNAc increased while the yield of GlcNAc decreased when the concentration of chitin was increased **(Figure 3.3)**. At chitin concentration of 60 mg/mL, the highest yield of $(GlcNAc)_2$ and the mole ratio of products were obtained. When the chitin concentration was increased more than 60 mg/mL the suspension became too thick to be stirred with a magnatic bar and the accurate sampling of the hydrolysate for HPLC analysis was not possible. Therefore, the concentration of fibrous chitin at 60 mg/mL was chosen for further study.

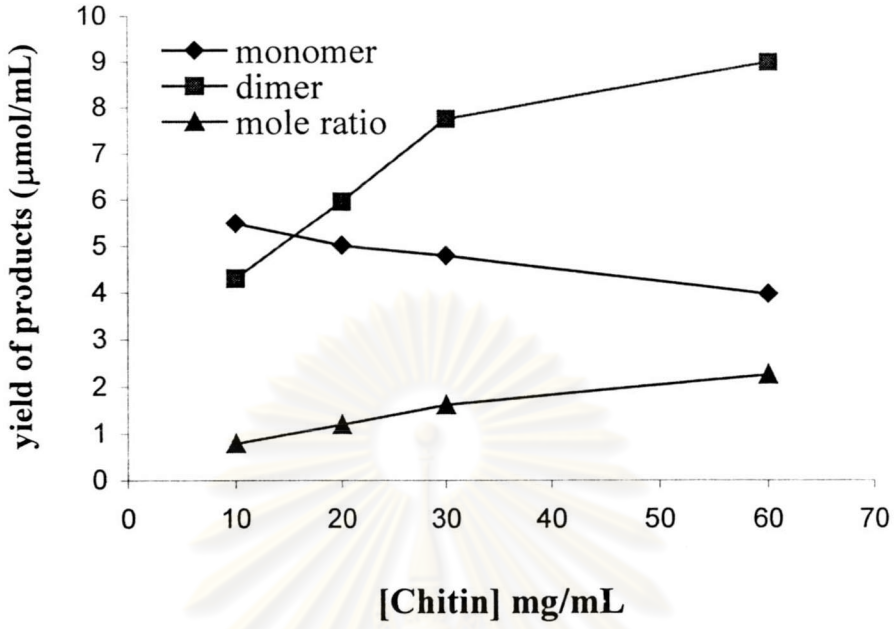


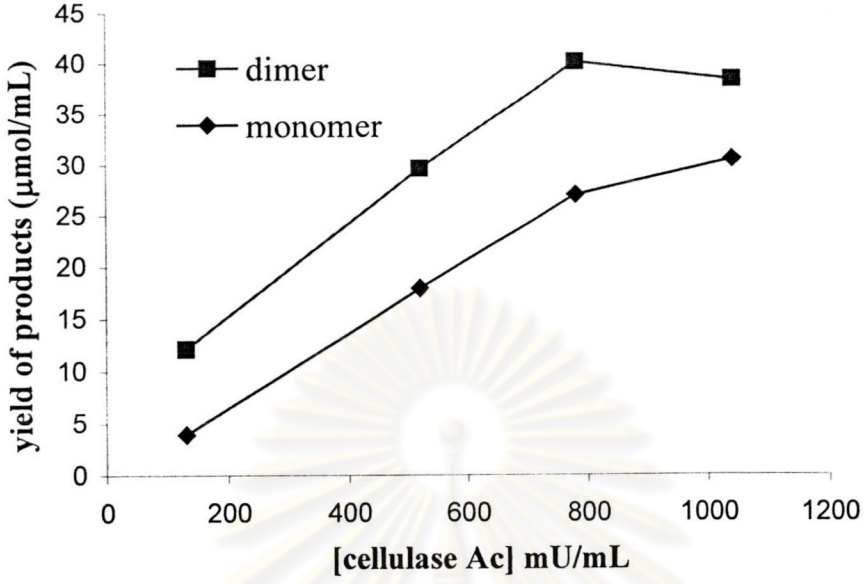

**Figure 3.3** The effect of chitin concentration on the chitinolysis of fibrous chitin with cellulase *Ac*. Conditions; [cellulase *Ac*] = 260 mU/mL; citrate-phosphate buffer pH 3.0 (0.1 M); 37 °C; 2 days.

3.3.2 Concentration of cellulase Ac

- Powder chitin

In this experiment, the concentration of enzyme was varied from 130 mU/mL to 1040 mU/mL while the concentration of powder β-chitin 3.0 μm was fixed at 80 mg/mL to find the optimum concentration of the enzyme. From the results, the yields of both saccharides went up with the increasing concentration of enzyme up to 780 mU/mL **(Figure 3.4)** while the mole ratio gradually decreased. Therefore, the optimum concentration of enzyme for 80 mg/mL of β-chitin was 780 mU/mL.

(a)

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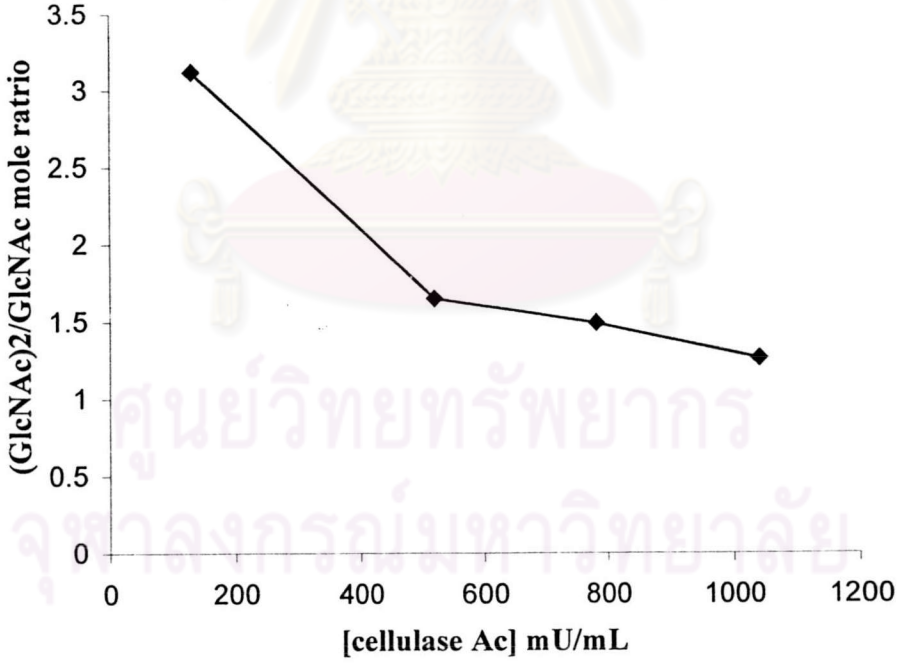


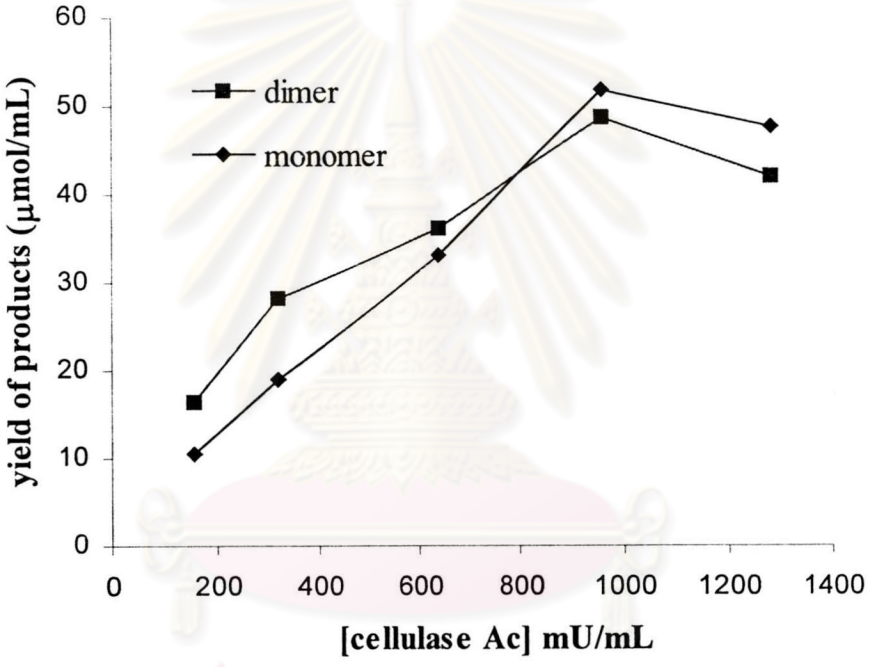



**Figure 3.4** The effect of enzyme concentration on (a) yield of $(GlcNAc)_2$ and GlcNAc and (b) product mole ratio. Condition; [chitin 3.0 µm] = 80 mg/mL; citrate-phosphate buffer pH 3.0 (0.1 M); 37 °C; 5 days.

- Fibrous chitin

For the fibrous β-chitin, the concentration of enzyme was varied from 159.5 mU/mL to 1276 mU/mL while the concentration of the fibrous chitin was fixed at 60 mg/mL. The results were similar to the powder chitin as the yield of $(GlcNAc)_2$ increased and reached the maximum yield when the concentration of enzyme used was 957 mU/mL **(Figure 3.5)**. Therefore, the concentration of enzyme at 957 mU/mL was chosen for further study.

**(a)**



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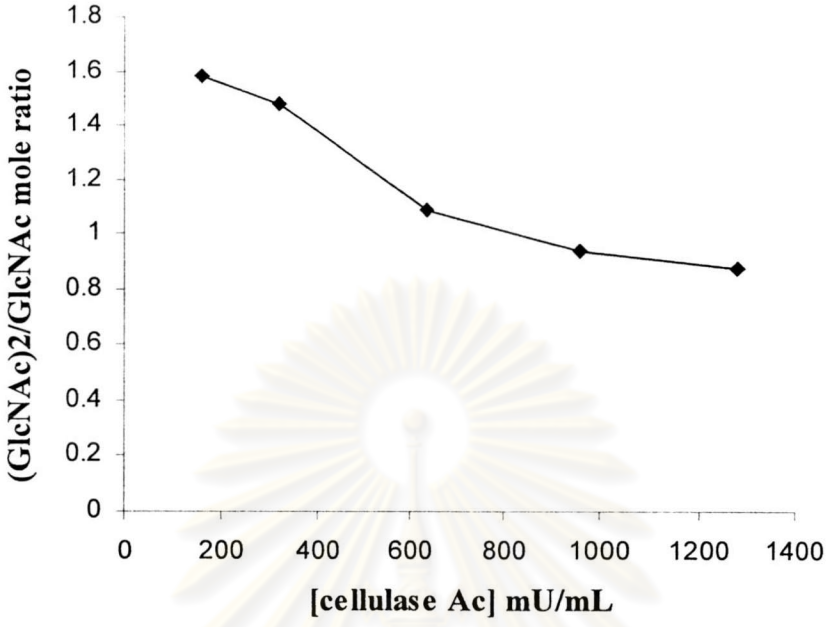
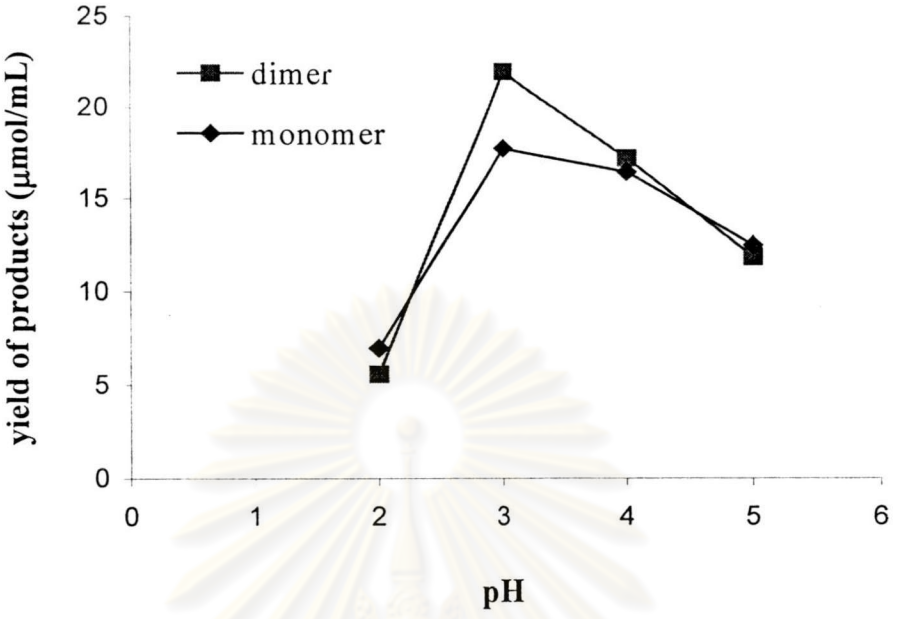



**Figure 3.5** The effect of enzyme concentration on (a) yield of $(GlcNAc)_2$ and GlcNAc and (b) product mole ratio . Condition; [fibrous chitin] = 60 mU/mL; citrate-phosphate buffer pH 3.0 (0.1 M); 37 °C; 5 days.

3.3.3 pH

The pH dependence of chitinolytic enzyme was investigated. The hydrolysis reactions were incubated at 37 °C in buffer solutions with pH ranging from 2.0 to 5.0 at chitin concentration of 60 mg/mL and enzyme concentration of 957 mU/mL. The optimum pH for cellulase *Acremonium cellulolyticus* found in this work was pH 3 same as reported in the literature .[44] The subsequent experiments were thus carried out in citrate-phosphate (McIlvaine) buffer pH 3.

(a)

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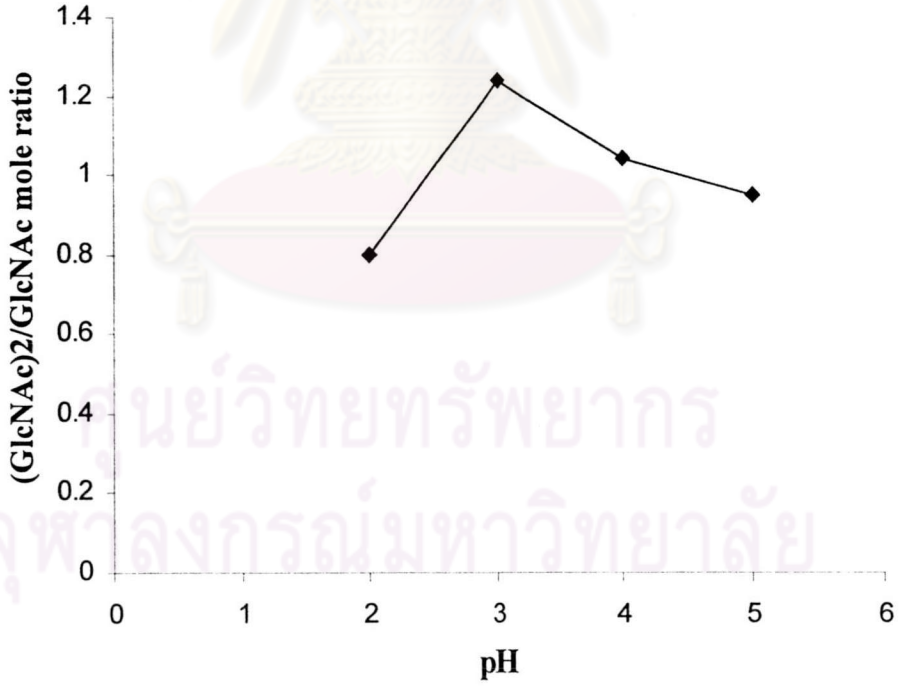


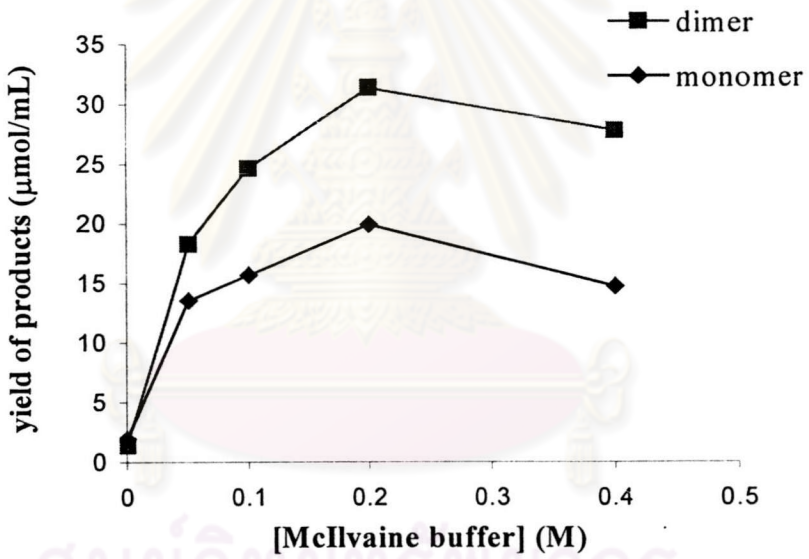



**Figure 3.6** The effect of pH on (a) yields of $(GlcNAc)_2$ and GlcNAc and (b) product mole ratio. Conditions; [β-chitin 25x50 μm] = 60 mg/mL, [cellulase *Ac*] = 957 mu/mL, 37 °C. The pH of solution were 2.0 (citrate buffer), 3.0-5.0 (citrate-phosphate buffer), 3 days.

3.3.4 Concentration of buffer

The concentration of buffer was varied from 0.0 M to 0.4 M at the optimum pH. The concentration of both $(GlcNAc)_2$ and GlcNAc products increased when the concentration of buffer was increased from 0.0 M to 0.2 M and become relatively constant at the concentration of the buffer solution above 0.2 M **(Figure 3.7)**. The results suggested that 0.2 M was the minimum buffer concentration required for maintaining the pH throughout the hydrolysis. It is also interesting to note that the $(GlcNAc)_2$/GlcNAc mole ratio also increased gradually with the increasing buffer concentration.

**(a)**



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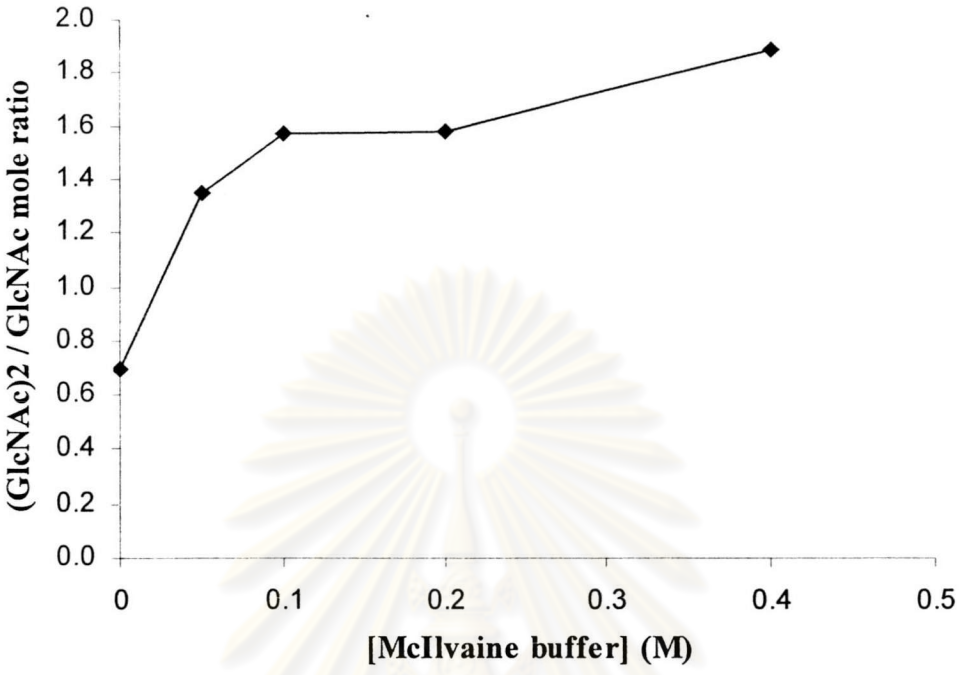
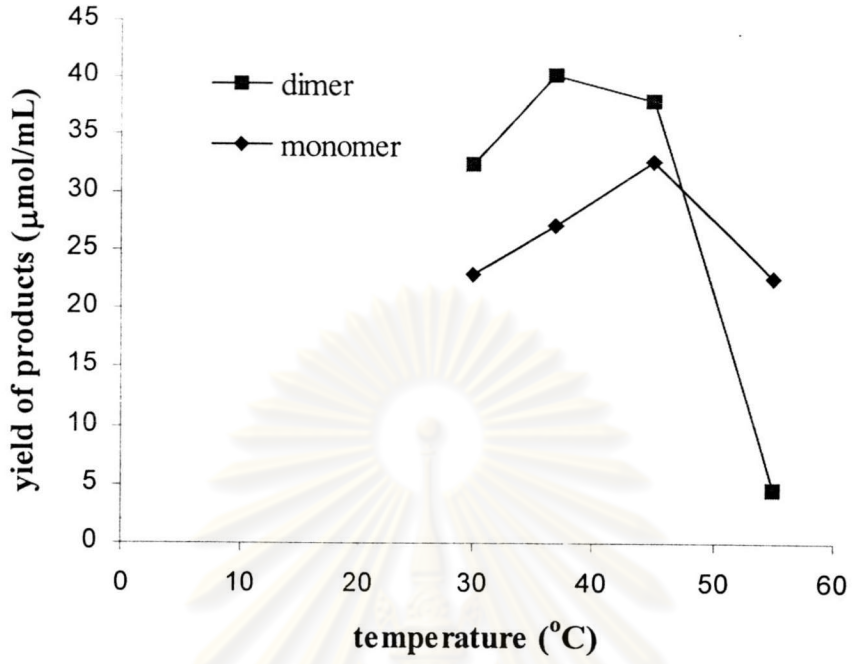



**Figure 3.7** The effect of concentration of buffer on (a) yields of $(GlcNAc)_2$ and GlcNAc and (b) product mole ratio. The buffer concentrations were 0.0 M to 0.4 M, [fibrous chitin] = 60 mg/mL, [cellulase *Ac*] = 957 mU/mL, McIlvaine buffer pH 3.0, 37 °C, 3 days.

3.3.5 Temperature

The study for optimum temperature for the hydrolysis of chitin with the enzyme cellulase *Ac* was carried out at 30, 37, 45 and 55 °C in McIlvaine buffer pH 3 (0.1 M) with chitin concentration of 80 mg/mL and enzyme concentration of 780 mU/mL. The yield of $(GlcNAc)_2$ peaked at 37 °C while the yield of GlcNAc reached the maximum at 45 °C **(Figure 3.8)**. At the temperature above 45 °C the yields of both products dropped considerably indicating the enzyme denature by heat. The temperature at 37 °C was thus chosen for further optimization in the production of $(GlcNAc)_2$.

**(a)**

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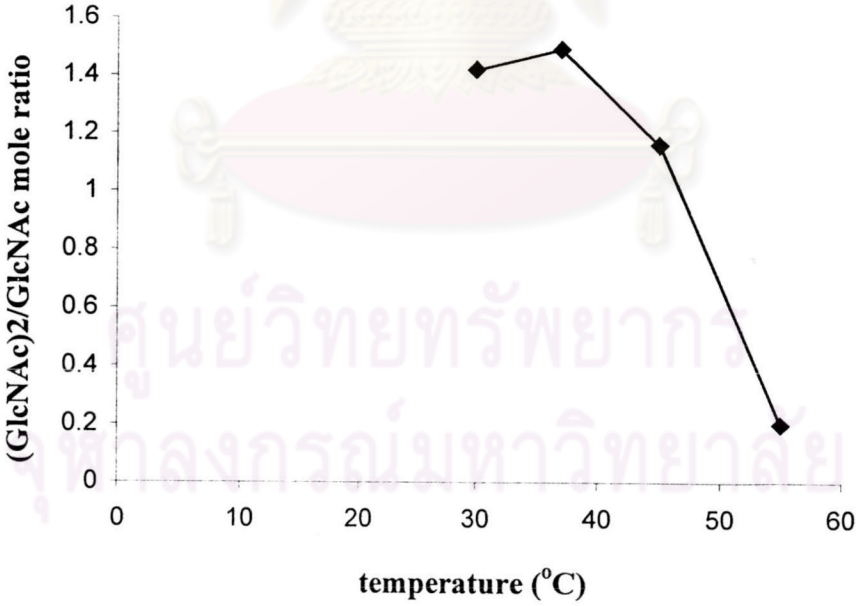



**Figure 3.8** The effect of temperature on (a) yields of $(GlcNAc)_2$ and GlcNAc and (b) product mole ratio. Condition; [fibrous chitin] = 60 mg/mL, [cellulase *Ac*] = 957 mU/mL, McIlvaine buffer pH 3.0, 37 °C, 5 days.

### 3.3.6 Enzyme affinity technique

To increase the $(GlcNAc)_2$/GlcNAc mole ratio, an enzyme adsorption or enzyme affinity technique was investigated. In this technique a mixture of enzyme and chitin substrate in cold buffer (4 °C) was kept in a refrigerator for 0, 1, 4 and 24 h. The mixture was then centrifuged and the buffer solution was replaced by the fresh buffer solution before incubation. The results showed that the adsorption was completed almost immediately as the yields of both $(GlcNAc)_2$ and GlcNAc did not vary significantly with the chilling time **(Figure 3.9)**. This enzyme adsorption technique increased the $(GlcNAc)_2$/GlcNAc mole ratio significantly from ~1 to over 3 **(Table 3.1)**, representing selectivity of over 75% by mole and over 85.2% by weight of $(GlcNAc)_2$ product. The higher $(GlcNAc)_2$/GlcNAc ratio was the result of higher yield of $(GlcNAc)_2$ and lower yield of GlcNAc. The results suggested that cellulase *Ac* contained both chitinase and chitobiase, and the former enzyme had greater affinity to the chitin surface. The chitobiase was washed away during the centrifugation and the replacement of the buffer solution, resulting in the lower conversion of $(GlcNAc)_2$ to GlcNAc.

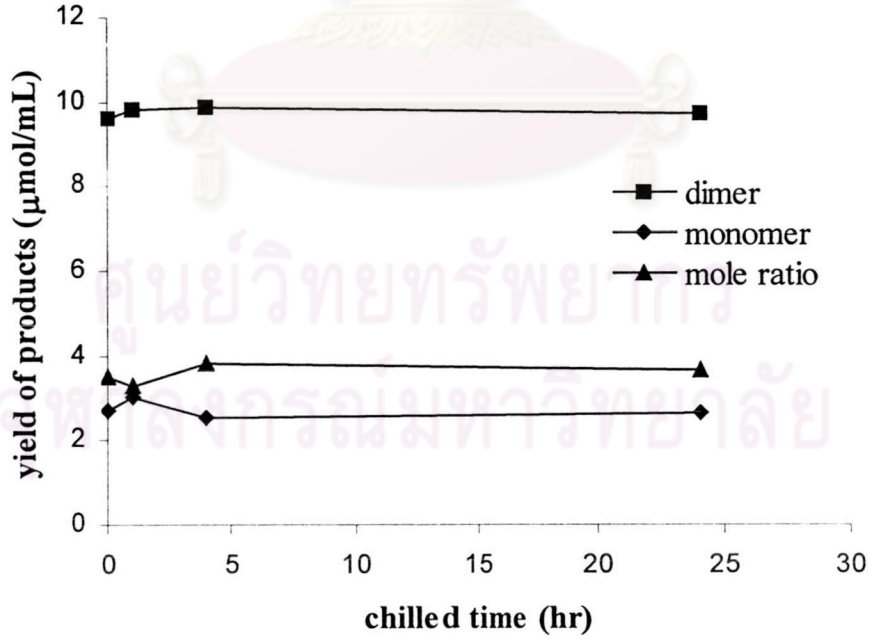



**Figure 3.9** The effect of the chilling time on the chitinolysis of powder chitin with cellulase *Ac* using enzyme adsorption technique. Conditions; [chitin] = 30 mg/mL, [cellulase *Ac*] = 260 mU/mL, McIlvaine buffer pH 3.0 (0.1 M), 37 °C, 3 days.

**Table 3.1** The effect of enzyme adsorption on the chitinolysis of fibrous chitin with cellulase *Ac* in comparison with non adsorption.

| | Days | Yield of $(GlcNAc)_2$ (μmole/mL) | Yield of GlcNAc (μmole/mL) | Mole ratio of $(GlcNAc)_2$/GlcNAc |
|---|---|---|---|---|
| Non ads. | 1 | 13.32 | 11.17 | 1.19 |
| | 2 | 16.86 | 15.56 | 1.08 |
| | 3 | 20.05 | 17.80 | 1.13 |
| **Ads.** | **1** | **16.64** | **4.60** | **3.62** |
| | **2** | **21.31** | **6.68** | **3.19** |
| | **3** | **25.56** | **8.41** | **3.04** |

Condition; [fibrous chitin] = 60 mg/mL, [cellulase *Ac*] = 936 mU/mL, McIlvaine buffer pH 3.0 (0.1 M), 37 °C.

### 3.4 Study for the optimum condition in the enzymatic hydrolysis with chitinase *Serratia sp.* cloned (Chi 60)

#### 3.4.1 The concentration of chitin

The concentration of chitin was varied from 10-50 mg/mL with a fixed concentration of enzyme at 19.1 mU/mL. At chitin concentration at 50 mg/mL, the reaction mixture was difficult to be stirred and sampled due to the high content of chitin. Therefore, only the results from the reaction with chitin concentration of 10-40 mg/mL are reported here. The results showed that the yield of $(GlcNAc)_2$ did not change significantly when the chitin concentration was increased from 10 mg/mL to 40 mg/mL **(Figure 3.10)** suggesting that 10 mg/mL of chitin was already in excess for 19.1 mU/mL of the enzyme. In contrast, the mole ratio drastically increased with the chitin concentration. The chitin concentration at 30 mg/mL was chosen for further study because it gave the high yield of $(GlcNAc)_2$ and also a high $(GlcNAc)_2$/ GlcNAc mole ratio.

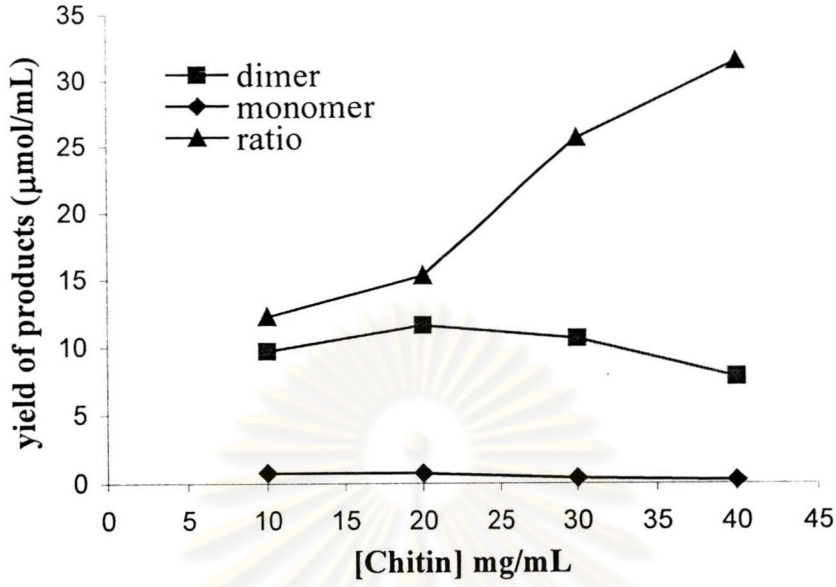



**Figure 3.10** The effect of chitin concentration on the chitinolysis. Conditions; [Chi 60] = 19.1 mU/mL, citrate-phosphate buffer pH 6.0 (0.1 M), 37 °C, 7 days.

3.4.2 Concentration of chitinase

The concentration of enzyme was varied from 4 mU/mL to 350 mU/mL. As expected, the yield of $(GlcNAc)_2$ increased with the increasing concentration of enzyme up to 150 mU/mL **(Figure 3.11)**, and the yield became relatively constant when the concentration of enzyme was over 150 mU/mL. This enzyme produced $(GlcNAc)_2$ as a major product when the small amount of enzyme was used. When high concentration of Chi 60 was used, the GlcNAc was observed. The yield of GlcNAc increased with the increasing of enzyme concentration and consequently the $(GlcNAc)_2$/GlcNAc mole ratio was decreased. From the results, it may be speculated that Chi 60 contained one or more enzymes which have endo- and exo-chitinolytic activities. Later enzyme affinity study (section 3.4.6) showed that Chi 60 was likely to contain only one enzyme which had both endo- and exo-chitinase activities. The exo-chitinase activity of this enzyme became more important at higher enzyme concentration. The concentration of enzyme at 150 mU/mL was thus used for a subsequent experiment where the concentration of chitin was 30 mg/mL.

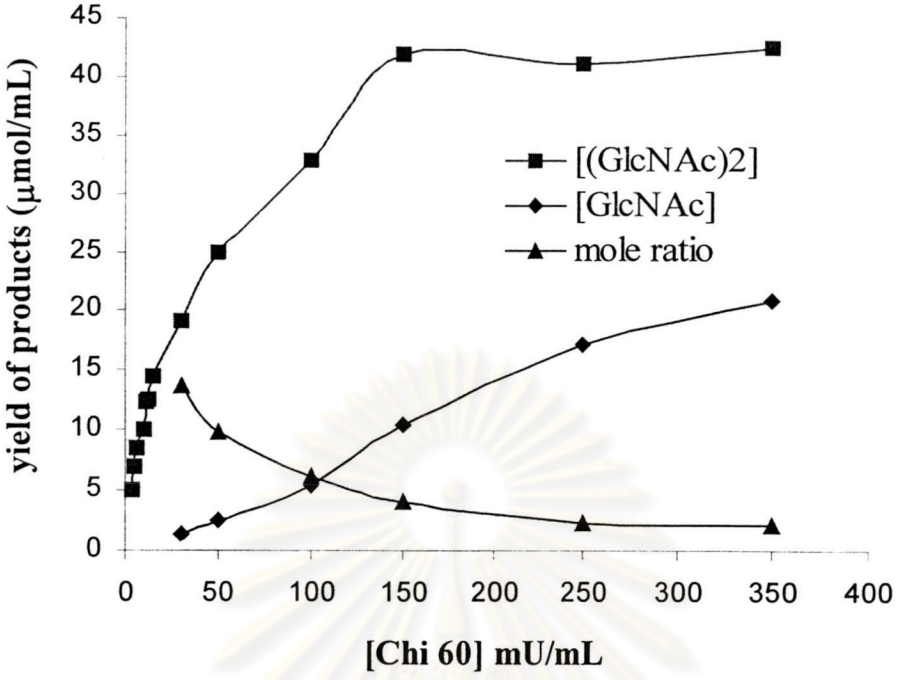



**Figure 3.11** The effect of the concentration of enzyme on the chitinolysis of fibrous chitin with Chi 60. Conditions; [fibrous chitin] = 30 mg/mL, citrate-phosphate buffer pH 6.0 (0.1 M), 37 °C, 2 days.

3.4.3 pH

The hydrolysis reactions were incubated at 37 °C in a citrate-phosphate buffer with pH ranging from 3.0 –7.0. It was apparent that the yield of $(GlcNAc)_2$ reached the maximum at pH 6.0, representing the optimum pH for this enzyme **(Figure 3.12)**. This result agreed with the results reported earlier on the bacterial chitinase.[46]

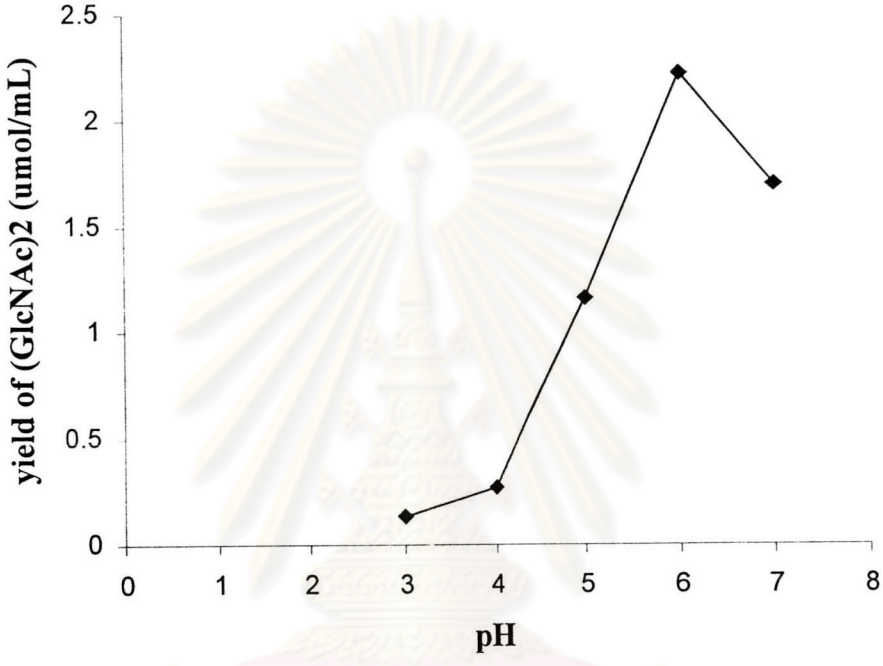



**Figure 3.12** The effect of pH on the chitinolysis of fibrous chitin with Chi 60. Conditions; [fibrous chitin] = 10 mg/mL, [Chi 60] = 4.77 mU/mL, 37 °C. 3 days.

3.4.4 Concentration of buffer

The concentration of buffer was varied from 0.05-0.4 M at the optimum pH. The results showed that the highest yield of $(GlcNAc)_2$ was obtained when the concentration of buffer was 0.05-0.1 M **(Figure 3.13)**. At higher buffer concentration (0.2-0.4 M), the yield of $(GlcNAc)_2$ was decreased significantly while the yield of GlcNAc was relatively constant. The $(GlcNAc)_2$/GlcNAc mole ratio was thus also varied according to the concentration of $(GlcNAc)_2$ product. Therefore, the optimum concentration range of buffer for Chi 60 was 0.05 to 0.1 M.

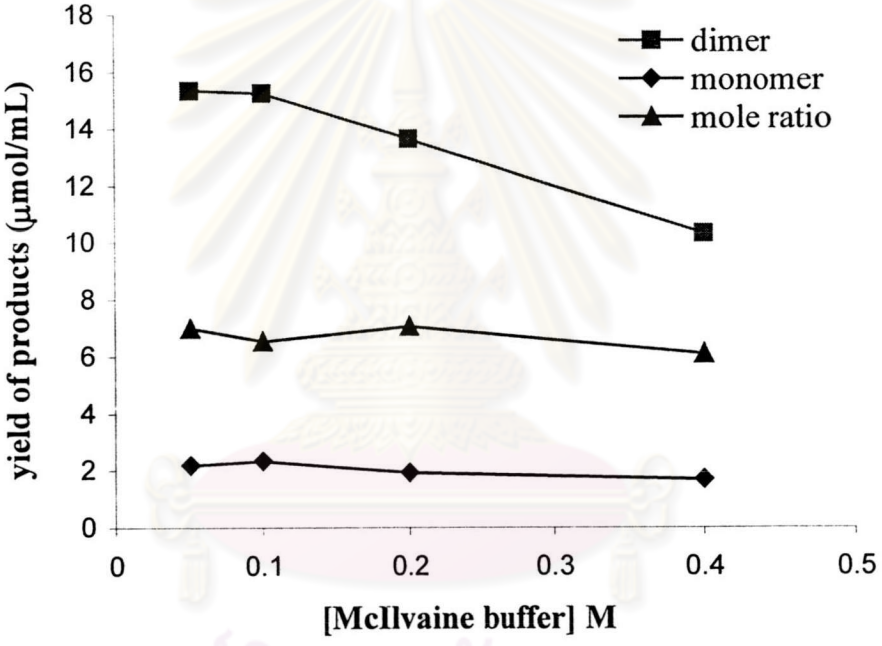
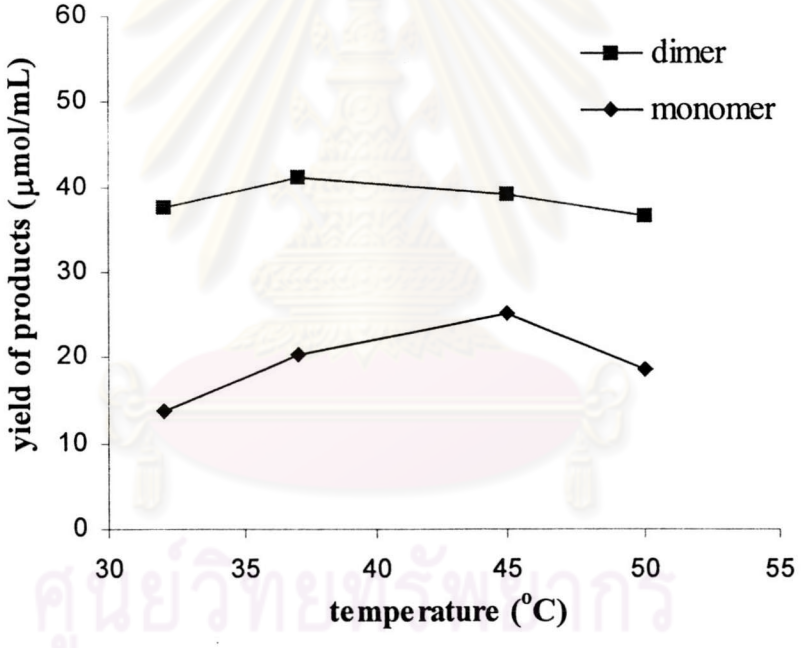



**Figure 3.13** The effect of concentration of buffer on the chitinolysis of fibrous chitin with Chi 60. Conditions; [fibrous chitin] = 30 mg/mL, [Chi 60] = 50 mU/mL, McIlvaine buffer pH 6.0, 37 °C. 3 days.

3.4.5 Temperature

From the analysis of hydrolytic products from the reaction with Chi 60, the result was quite similar to the reaction with cellulase *Ac*. The yield of $(GlcNAc)_2$ did not vary significantly with the temperatures but it peaked at 37 °C. The yield of GlcNAc varied more pronouncedly with the temperature reached the maximum at 45 °C. The $(GlcNAc)_2$ / GlcNAc mole ratio at 37 °C was moderate**(Figure 3.14)**. The temperature at 37 °C was thus the most suitable temperature for production of $(GlcNAc)_2$ by using this enzyme.

**(a)**



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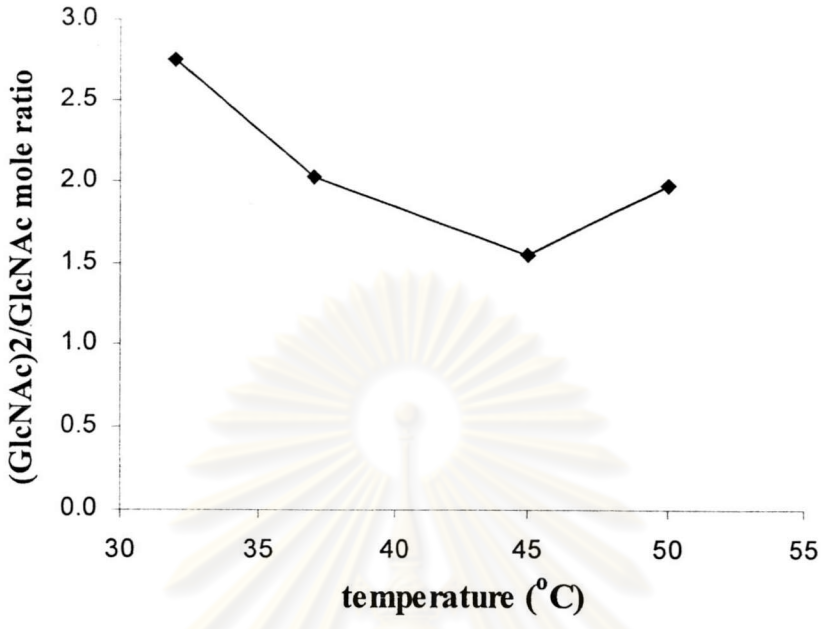


**Figure 3.14** The effect of temperature on (a) yields of products and (b) product mole ratio. Conditions; [fibrous chitin] = 30 mg/mL, [Chi 60] = 150 mU/mL, 3 days.

3.4.6 The enzyme affinity technique

Like the results when using cellulase *Ac*, the chilling time did not affect to both yield and mole ratio **(Figure 3.15)**. In comparison to the non adsorption results, the yields of both $(GlcNAc)_2$ and GlcNAc decreased over 50% whereas the $(GlcNAc)_2$/GlcNAc mole ratio increased. but the mole ratio increased. As the yields of both $(GlcNAc)_2$ and GlcNAc dropped significantly, the increase of mole ratio was not likely due to the difference in affinity of chitinase versus β-N-acetylhexosaminidase as in the case of cellulase *Ac*. It was more likely that the enzyme Chi 60 consisted of only one enzyme which had both endo- and exo-chitinolytic activities. It is worth noting here that the increase of $(GlcNAc)_2$/GlcNAc mole ratio was simply due to the lower concentration of enzyme remained in the reaction after the adsorption process. The results agreed with the previous study on the effect of enzyme concentration (section 3.4.2)

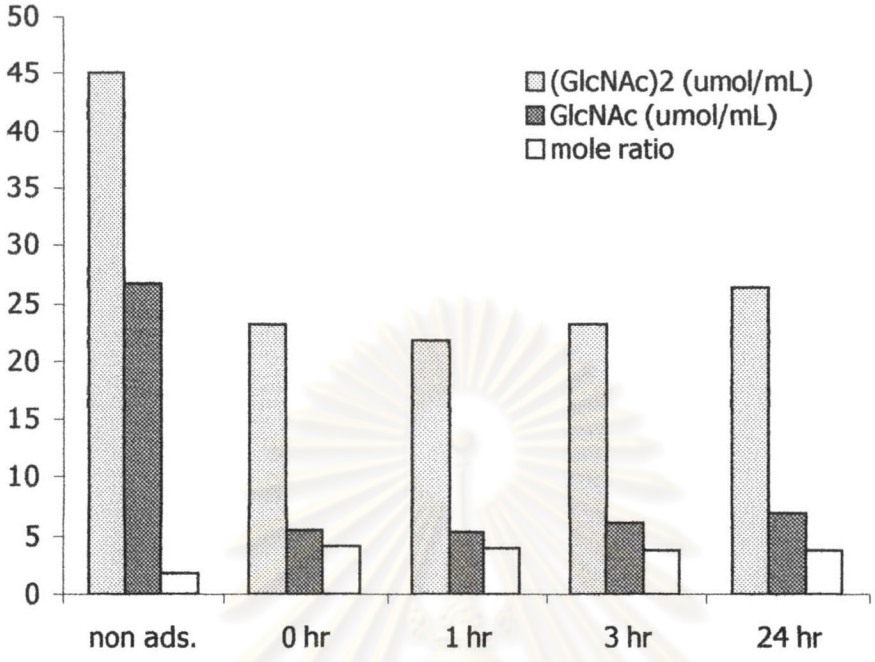


**Figure 3.15** The effect of enzyme adsorption on the chitinolysis of the fibrous chitin with Chi 60. Condition; [fibrous chitin] = 30 mg/mL, [Chi 60] = 150 mU/mL, 8 days.

3.4.7 Gram scale preparation of N,N′-diacetylchitobiose

The fibrous chitin 2 g was hydrolyzed with Chi 60 at the optimum concentration without using enzyme affinity technique. The yield of $(GlcNAc)_2$ and GlcNAc obtained in 6 days of incubation time was 0.89 and 0.07 g, respectively. This result could be translated into 42% yield of $(GlcNAc)_2$ and 93:7 selectivity of $(GlcNAc)_2$ over GlcNAc.

### 3.4.8 Hydrolysis of $(GlcNAc)_2$

From the results in the enzyme affinity experiment, it was proposed that cellulase *Ac* consisted of two enzymes: chitinase and β-N-acetylhexosaminidase which digest $(GlcNAc)_2$ to GlcNAc and Chi 60 consisted of only one enzyme: chitinase. To investigate this hypothesis, The $(GlcNAc)_2$ was hydrolyzed by these enzymes to test the β-N-acetylhexosaminidase activity. Indeed, cellulase *Ac* slowly digested $(GlcNAc)_2$ to GlcNAc while Chi 60 did not digest $(GlcNAc)_2$ **(Tables 3.2 and 3.3)** suggesting that cellulase *Ac* had β-N-acetylhexosaminidase activity corresponding to the hypothesis.

**Table 3.2** The hydrolysis of $(GlcNAc)_2$ with cellulase *Ac*.

| Time (hour) | [GlcNAc] (mM) | $[(GlcNAc)_2]$ (mM) |
|---|---|---|
| 1 | 0.459 | 3.451 |
| 5 | 0.542 | 3.357 |
| 24 | 0.583 | 3.148 |

Conditions: $[(GlcNAc)_2]$ = 3.65 mM; [cellulase *Ac*] = 10 mU; McIlvaine buffer pH 3.0 (0.1 M); 37 °C.

**Table 3.3** The hydrolysis of $(GlcNAc)_2$ with Chi 60.

| Time (hour) | [GlcNAc] (mM) | $[(GlcNAc)_2]$ (mM) |
|---|---|---|
| 1 | N.D. | 3.830 |
| 5 | N.D. | 3.950 |
| 24 | N.D. | 3.859 |

Conditions; $[(GlcNAc)_2]$ = 3.90 mM; [Chi 60] = 10 mU; McIlvaine buffer pH 6.0 (0.1 M); 37 °C.