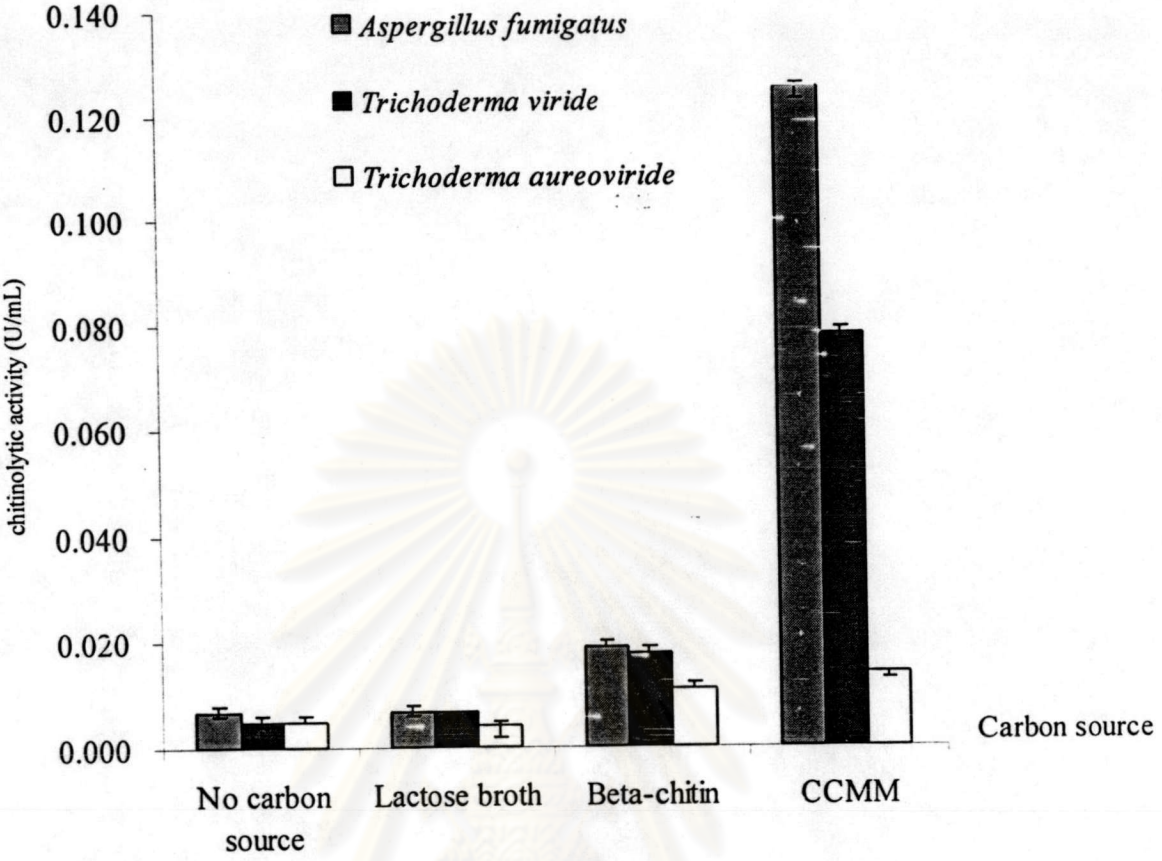
CHAPTER III

RESULTS AND DISCUSSION

3.1 Factors affecting the production of chitinolytic enzyme.

3.1.1 Carbon source

The activities of chitinolytic enzymes produced in various carbon sources were determined by the modified Schale's colorimetric method using colloidal chitin as a substrate. CCMM containing of colloidal chitin gave the highest activity for all species of fungi tested **(Figure 3.1)**. In lactose broth produced only low chitinolytic activity. In phosphate buffer the fungi produced very slow and produced only low chitinolytic activity. The fungi growth in a culture medium using β-chitin fiber as a carbon source was faster than that in lactose broth but the fungi produced slightly higher chitinolytic activity. When the carbon source was changed to the colloidal chitin, the fungi produced considerably higher chitinolytic activity indicating that the use of colloidal chitin as a carbon source provides an appropriate stress for these fungi to produce chitinolytic enzymes. Under the experimental conditions using CCMM as a carbon source, the chitinolytic activities were 125 mU/mL for *Aspergillus fumigatus*, 78 mU/mL for *Trichoderma viride* and 14 mU/mL for *Trichoderma aureoviride*. These results indicated that *Aspergillus fumigatus* was the most active fungus that can produce high chitinolytic enzymes.

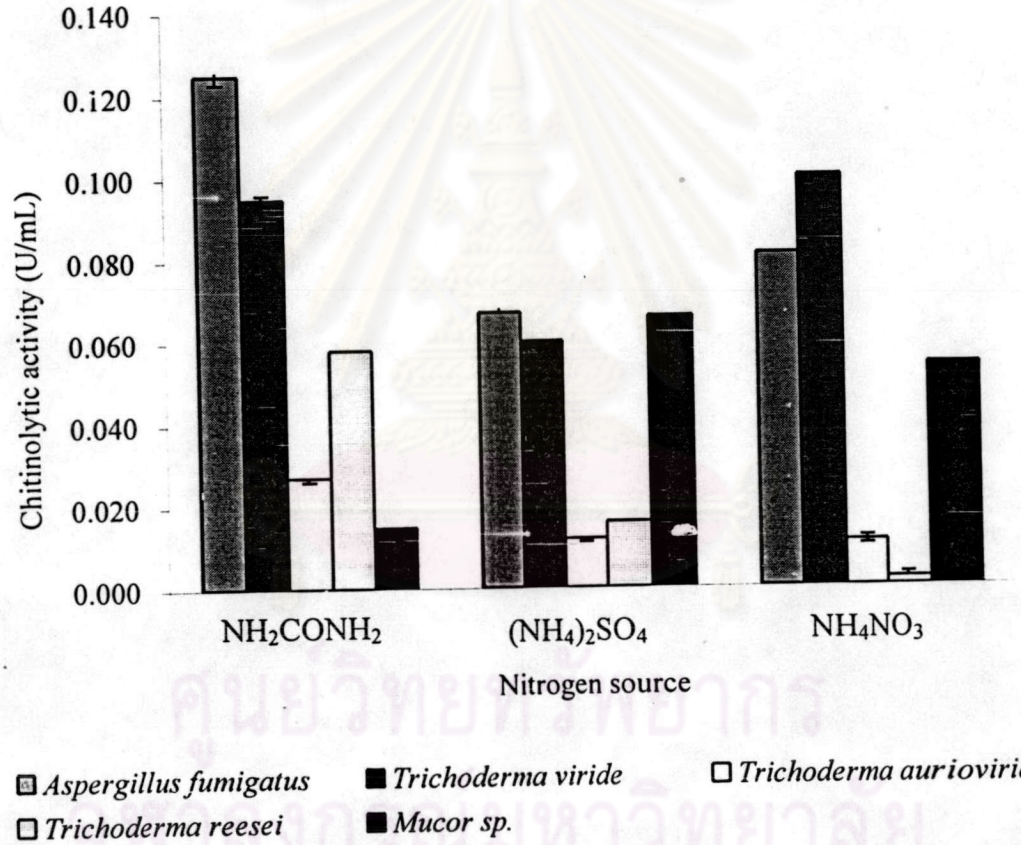


Incubation conditions: pH = 4.5, temperature = 30 °C, time = 12 days.

Figure 3.1 The effect of carbon source on chitinolytic enzyme production from *Aspergillus fumigatus, Trichoderma viride* and *Trichoderma aureoviride*.

3.1.2 Nitrogen source

CCMM medium with three different nitrogen sources: urea, ammonium sulfate and ammonium nitrate were used in the production of chitinolytic enzymes from five species of fungi. For most fungi, except for *Mucor sp.*, urea was the best nitrogen source that allows the fungi to produce the highest chitinolytic activity. Among the five fungi species, *Aspergillus fumigatus* gave the highest chitinolytic activity per milliliter of enzyme preparation (125 mU/mL) **(Figure 3.2)** and further optimization was performed on this fungi using urea as the nitrogen source. These results were different from the literature reporting that ammonium sulfate was the most suitable nitrogen source for *Aspergillus fumigatus*.[23]

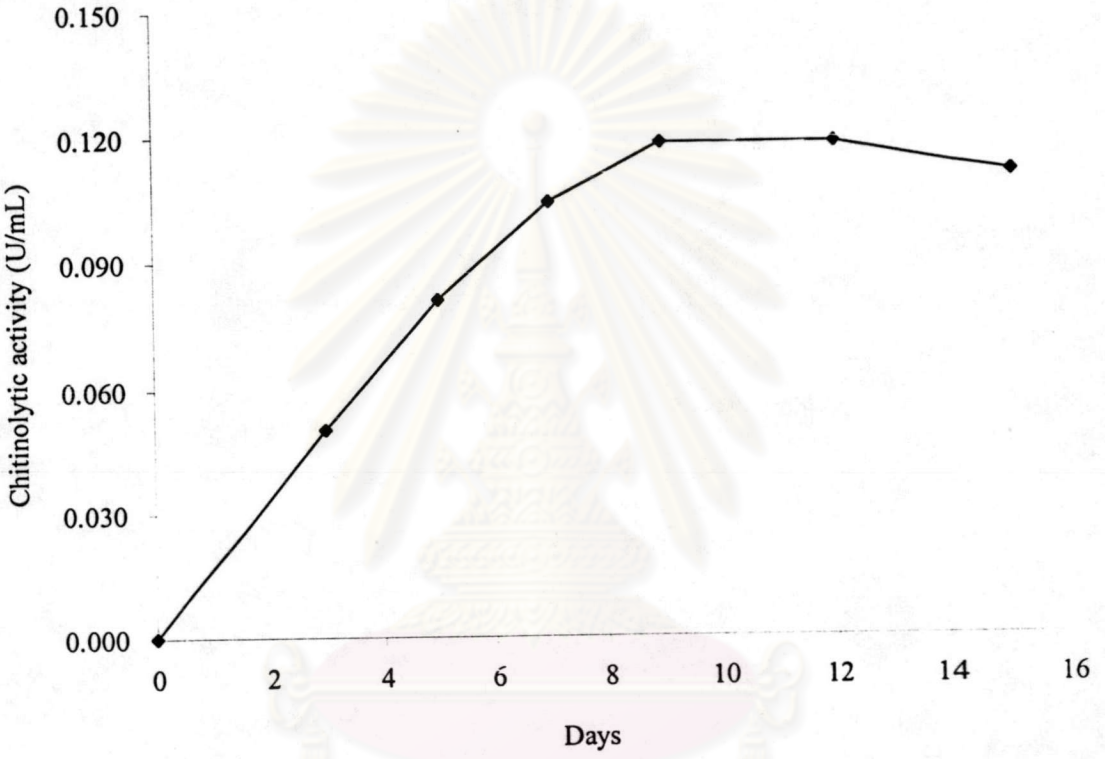


Incubation conditions: pH = 4.5, temperature = 30 °C, time = 12 days; Medium = 0.5 % colloidal chitin in CCMM.

Figure 3.2 The effect of the nitrogen sources on chitinolytic enzyme production from *Aspergillus fumigatus*, *Trichoderma viride*, *Trichoderma aureoviride*, *Trichoderma reesei* and *Mucor sp.*

3.1.3 Incubation time

The chitinolytic activity of the enzymes produced during cultivation of *Aspergillus fumigatus* in 0.5 % colloidal chitin as a carbon source in CCMM and urea as a nitrogen source was increased with the cultivation period to reach the maximum activity after 9 days **(Figure 3.3)**. The cultivation time of 9 days was thus used throughout this thesis.

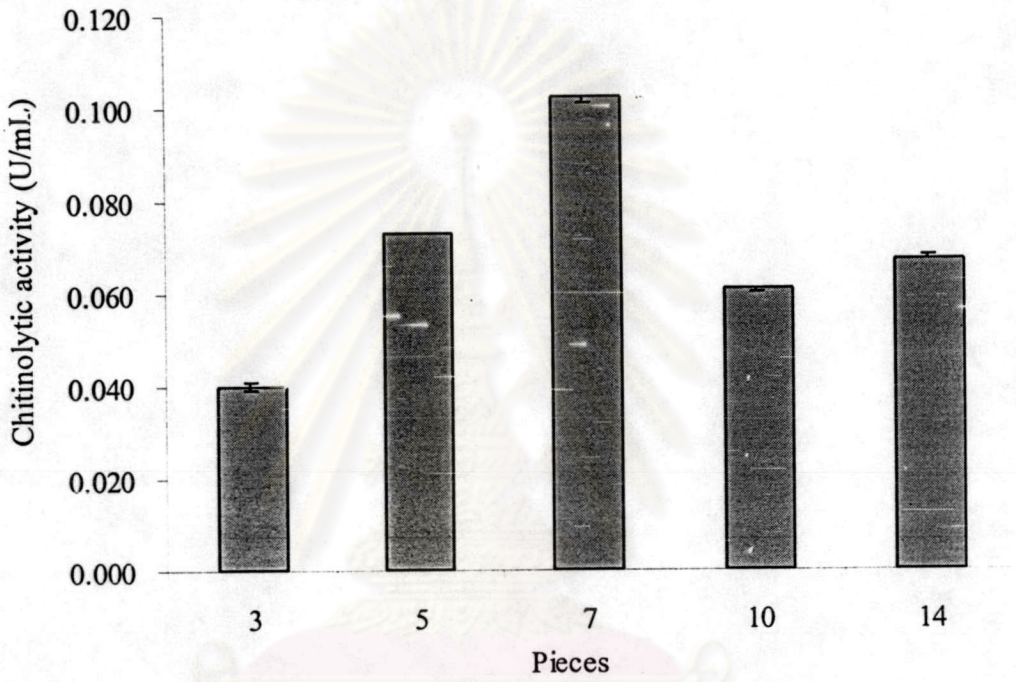


Incubation conditions: pH = 4.5, temperature = 30 °C

Figure 3.3 The time course of the chitinolytic activity obtained from the cultivation of *Aspergillus fumigatus*.

3.1.4. Inoculum amounts of *Aspergillus fumigatus*

The chitinolytic activity produced from various inoculum amounts of *Aspergillus fumigatus* was determined by the colorimetric method using colloidal chitin as substrate. The maximum enzyme activity was obtained with 7 inoculum pieces of fungi **(Figure 3.4)**, indicating the optimum initial amount of the fungus to be used for the chitinolytic enzyme production.

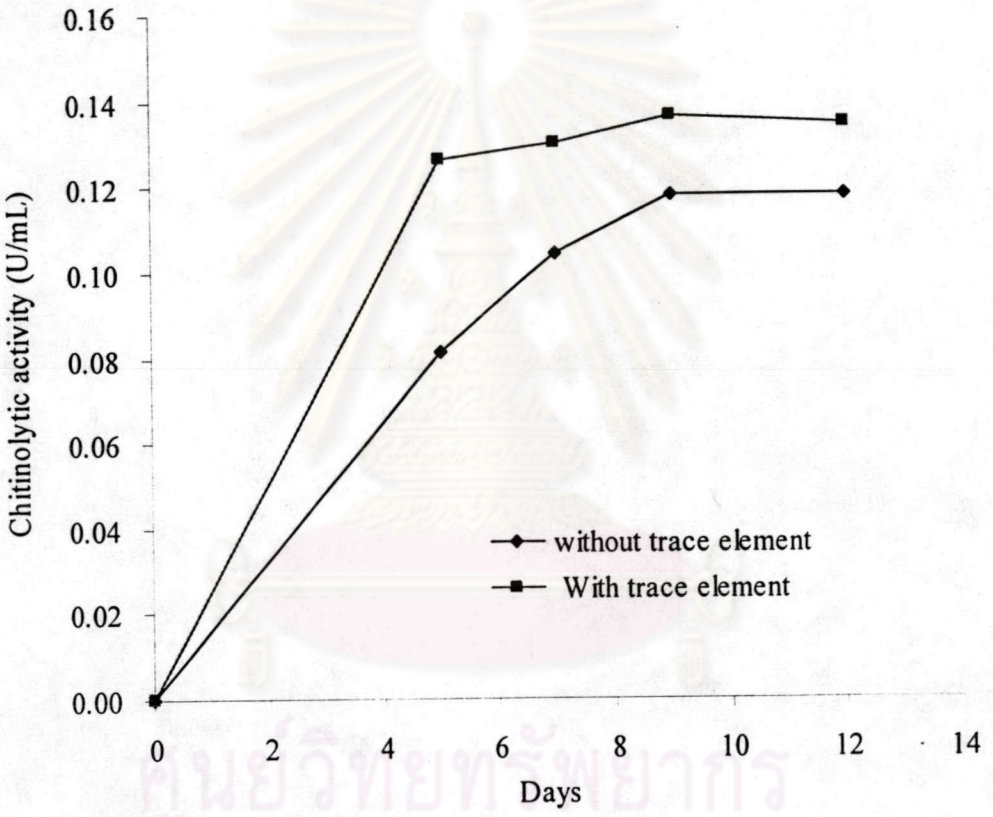


Incubation conditions: pH = 4.5, temperature = 30 °C Medium = 0.5 % colloidal chitin and 0.03 % urea as nitrogen in CCMM.

Figure 3.4 The effect of inoculums size of *Aspergillus fumigatus* on chitinolytic enzyme production.

3.1.5 Trace elements ($FeSO_4$ and $MnSO_4$)

The inclusion of some trace elements, such as Fe^{2+} and Mn^{2+} in the cultivation media (CCMM), increased the rate of production of chitinolytic enzymes from *Aspergillus fumigatus* **(Figure 3.5)**. The maximum activity was obtained after 5 days of the cultivation with trace elements. Trace elements such as manganese and iron have been reported to be important for enzyme functioning in living system including fungi.[40-41]

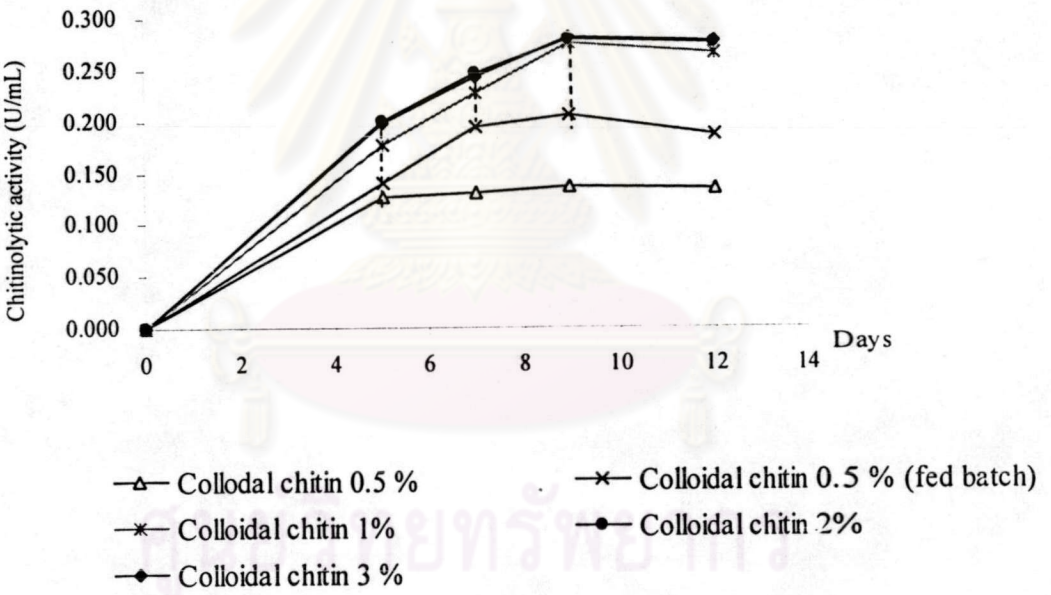


Incubation conditions: pH = 4.5, temperature = 30 °C

Figure 3.5 The effect of trace elements ($FeSO_4$ and $MnSO_4$) on the chitinolytic enzyme production from *Aspergillus fumigatus*.

3.1.6 Amounts of colloidal chitin

From the previous experiment, the production of chitinolytic enzymes did not increased after 5 days. The colloidal chitin in CCMM medium was also completely consumed in 5 days. In this experiment, more colloidal chitin (0.5 g) was added after 5, 7 and 9 days. With additional chitin in this fed batch experiment, the production of chitinolytic enzyme increased further **(Figure 3.6)**. The maximum activity was obtained on 9 days and the enzyme production rate increased with the concentration of colloidal chitin up to 1 % of colloidal chitin, where the fungi produced 281 mU/mL of enzyme activity. Further increment of chitin concentration did not provide higher activity of enzymes produced from the fungi. The saturation of enzyme activity might be the result of the limiting cell density **(Figure 3.6)**.[35]

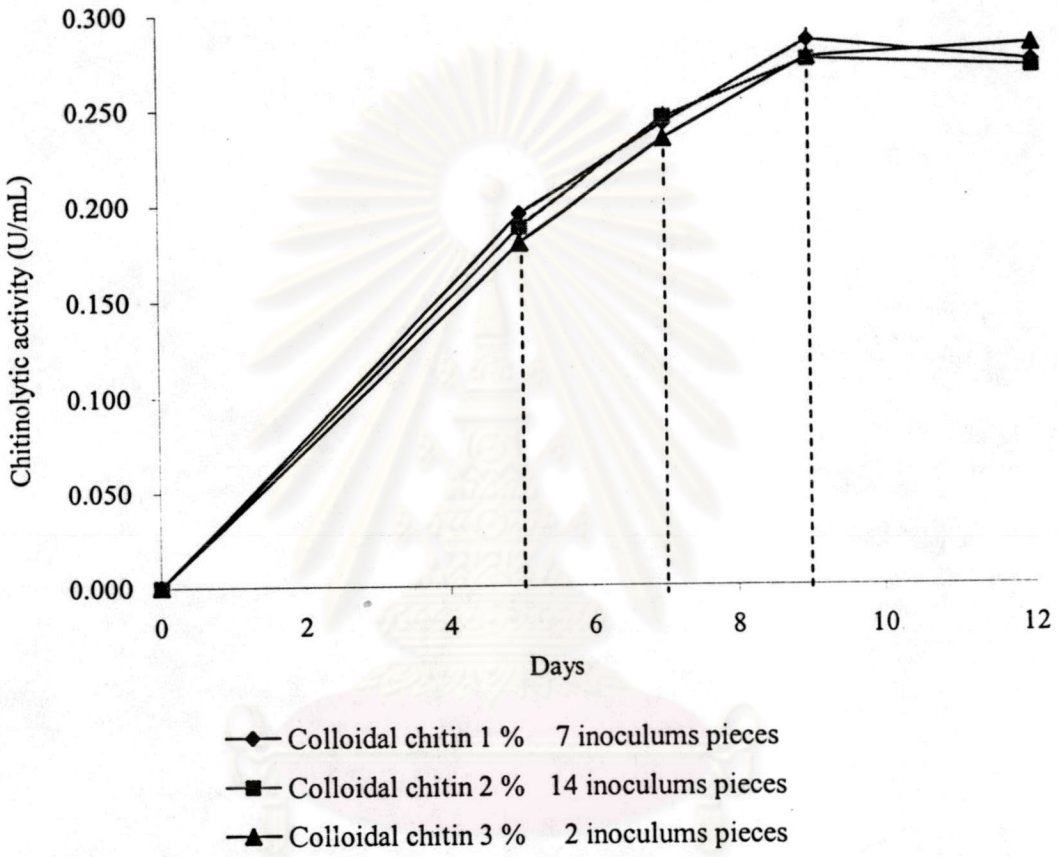


Incubation conditions: pH = 4.5, temperature = 30 °C

Figure 3.6 Amount of colloidal chitin on chitinolytic enzyme production from *Aspergillus fumigatus*. Dashed lines indicate the addition of chitin in the fed batch cultivation.

3.1.7 Initial amounts of colloidal chitin and fungal inoculums

In this experiment, the initial amounts of colloidal chitin were varied as 1%, 2% and 3% and the initial amount of *Aspergillus fumigatus* were varied proportionally 7, 14 and 21 inoculum pieces. The enzyme production profiles were virtually identical for all three conditions **(Figure 3.7)**. These results substantiated the hypothesis on cell density limiting growth and enzyme production.

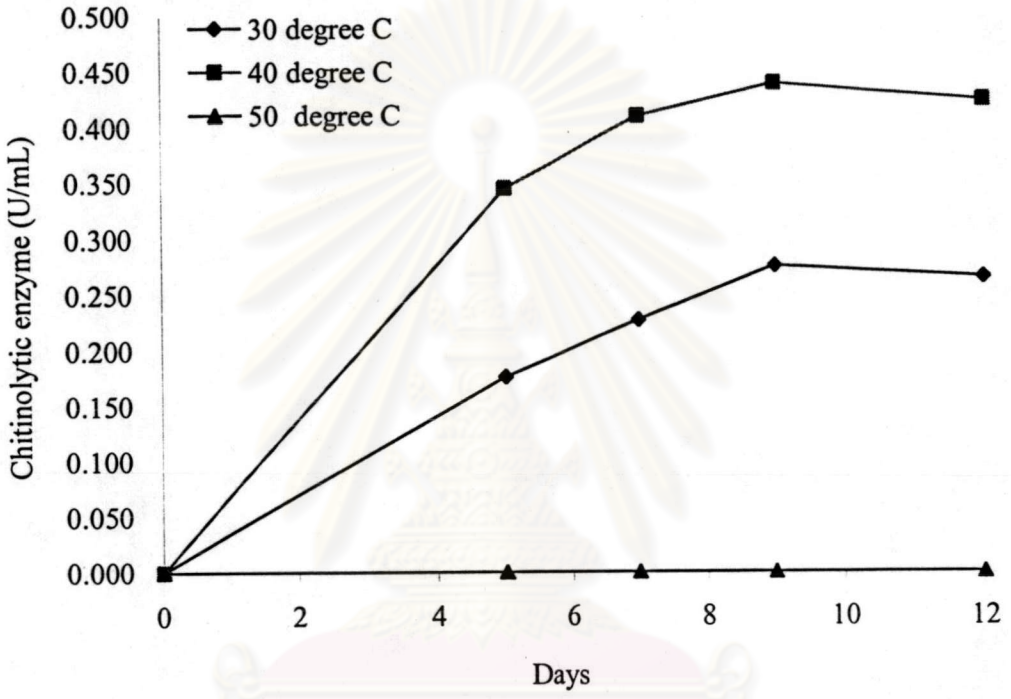


Incubation conditions: pH = 4.5, temperature = 30 °C

Figure 3.7 Amount of colloidal chitin and amount of *Aspergillus fumigatus* on chitinolytic enzyme production. Dashed lines indicate the addition of chitin in the fed batch cultivation.

3.1.8 Temperature

The cultivation of *Aspergillus fumigatus* was performed at three different temperatures: 30, 40 and 50 °C. The cultivation at 40 °C provided the highest chitinolytic activity of 438 mU/mL **(Figure 3.8)**. It is also interesting to note that the fungus could not grow at 50 °C.



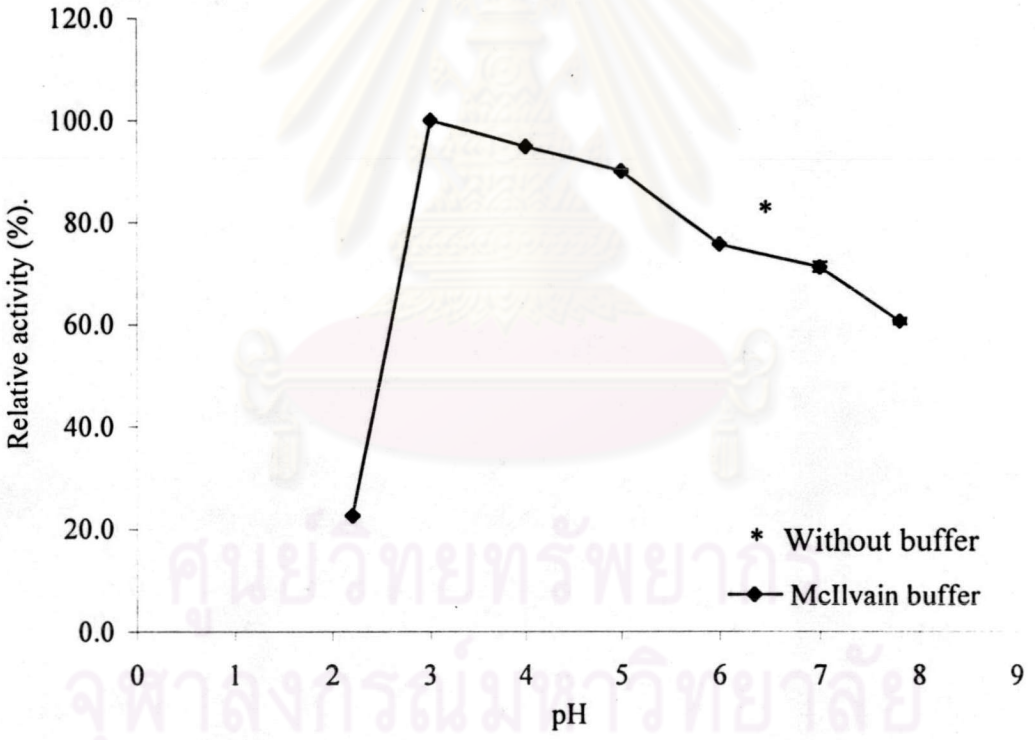
Incubation conditions: pH = 4.5.

Figure 3.8 Temperature for culturing *Aspergillus fumigatus* on chitinolytic enzyme production. Dashed lines indicate the addition of chitin in the fed batch cultivation.

3.2 Study hydrolytic activity of the ensemble of chitinolytic enzymes from *Aspergillus fumigatus* by enzyme assaying with modified Schale's method.

3.2.1 pH dependence

The pH dependence of the chitinolytic activity of the enzymes obtained from *Aspergillus fumigatus* was investigated in the pH range of 2.2-7.8 using modified Schale's method. The assaying was also conducted without buffer solution pH 6.5. Crude enzymes showed a broad optimum pH range, pH 3-5 **(Figure 3.9)**. This optimum pH range is common for chitinolytic enzymes from many fungi.[27, 32] Also, note that the hydrolysis of chitin with enzyme from *Aspergillus fumigatus* in a solution without a buffer showed about 85% of the maximum activity.

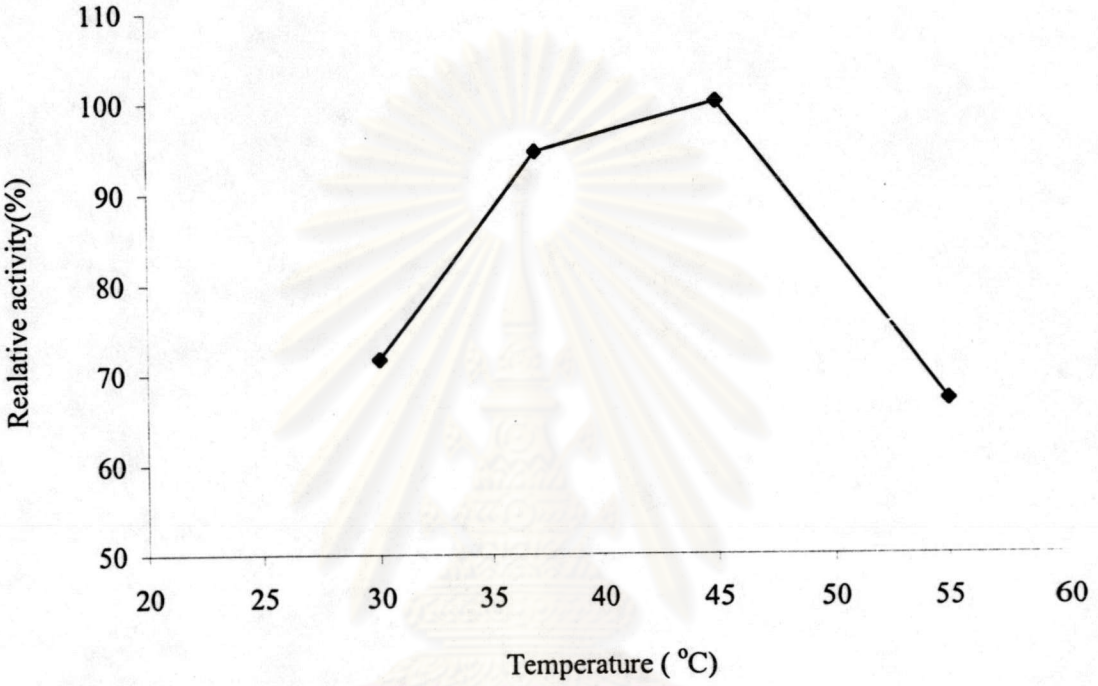


Conditions: Incubation at 37 °C for 30 minutes.

Figure 3.9 pH dependence of the activity of the enzymes from *Aspergillus fumigatus*.

3.2.2 Temperature dependence

The hydrolysis of chitin with the ensemble of chitinolytic enzymes was carried out at 30, 37, 45 and 55 °C. The chitinolytic activity was assayed by modified Schale's method. The chitinolytic enzymes showed relatively broad optimum temperature range, 37- 45 °C. The highest activity appeared at 45 °C **(Figure 3.10)**.

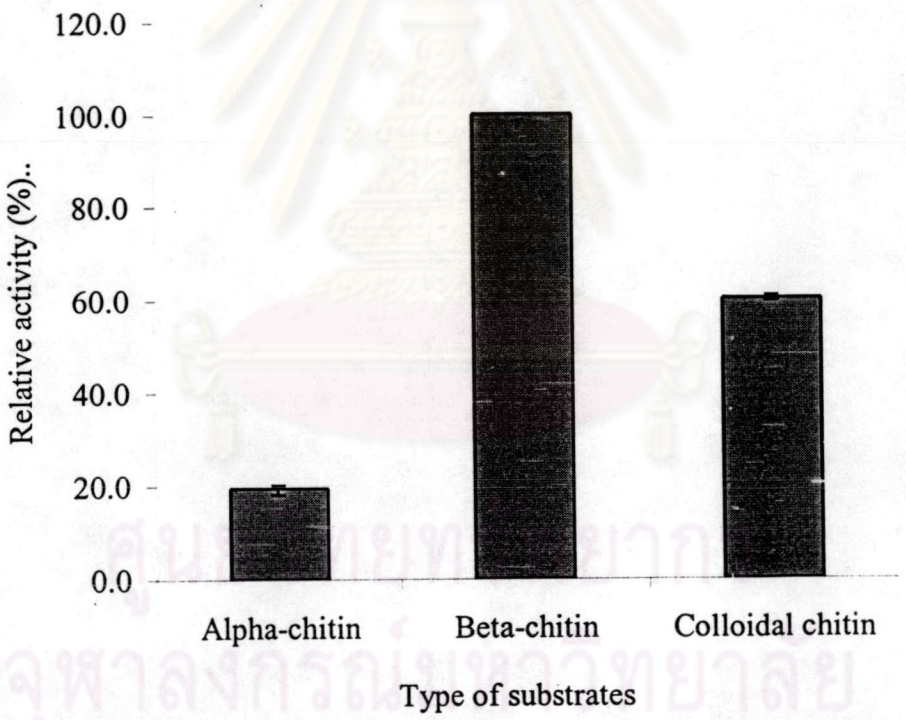


Conditions: McIlvaine buffer pH 3 (0.05 M, 0.15 mL) and incubation time = 30 minutes

Figure 3.10 Temperature dependence of the activity of the enzymes from *Aspergillus fumigatus*.

3.2.3 The chitinolytic susceptibility of different substrates

Three different substrates, colloidal chitin, β-chitin and α–chitin were hydrolyzed with the enzymes from *Aspergillus fumigatus*. The results revealed that β-chitin possessed the highest chitinolytic susceptibility toward the ensemble of chitinolytic enzymes from *Aspergillus fumigatus*. It is common for β-chitin to have greater chitinolytic susceptibity than α–chitin as the β-chitin has looser packing of chitin chains in the crystalline domains comparing to the α–chitin. It is however quite unusual for β-chitin to have higher chitinolytic susceptibity than the amorphous colloidal chitin. The results thus indicated that the ensemble of chitinolytic enzymes from *Aspergillus fumigatus* had some specificity toward the crystalline structure of chitin **(Figure 3.11)**.

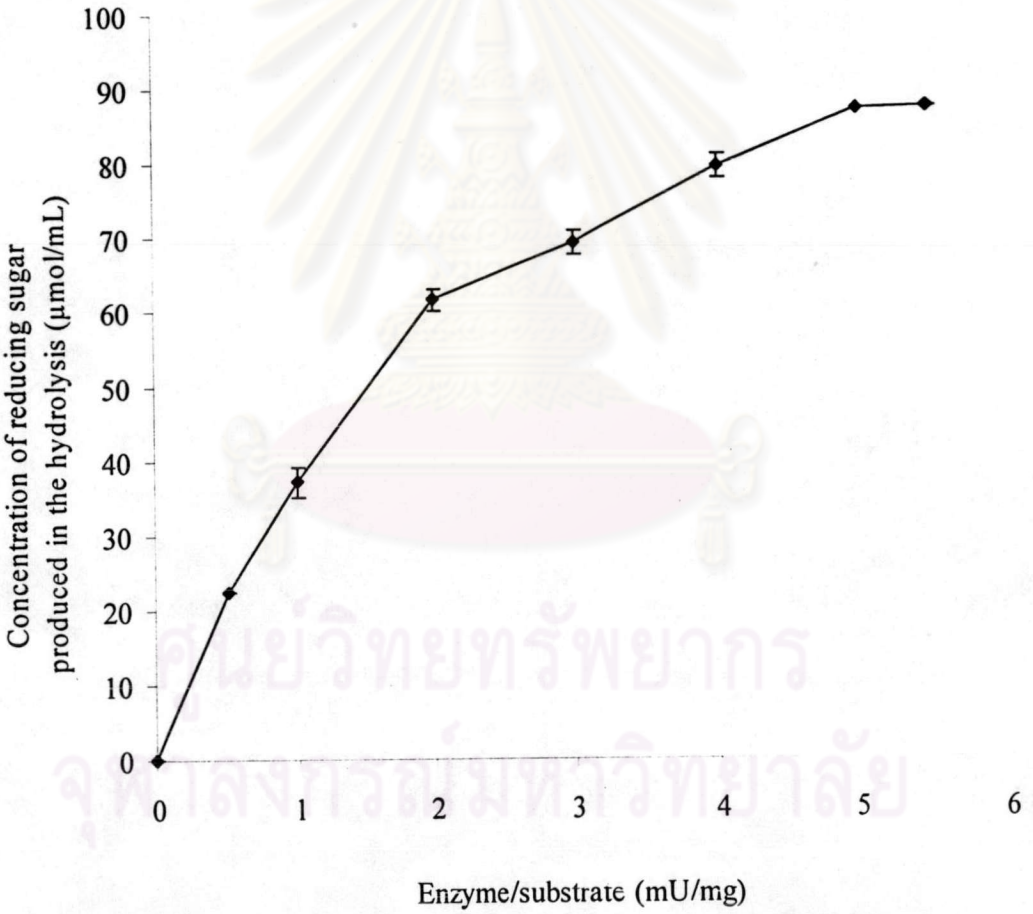


Conditions: Enzyme /β-chitin 1.26 mU/mg, McIlvaine buffer pH 3 (0.05M, 0.5 mL) and incubation at 45 °C for 1 day.

Figure 3.11 The substrate dependence of the hydrolytic activity of the enzymes from *Aspergillus fumigatus*.

3.2.4 Enzyme/substrate ratio

The ratio of the enzyme/substrate was varied as 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 5.5 mU of enzymes per milligram of β-chitin by fixing the amount of chitin substrate at 20 mg/mL. The results showed that the concentration of products increased with the augmentation of enzyme/chitin ratio **(Figure 3.12),** more pronouncedly up to 4.0mU/mg. On that account, the enzyme/chitin ratio of 1.0-4.0 mU/mg was chosen in further study as these ratios also provided appropriate concentration of the products to be effectively analyzed by the colorimetric method over the course of the hydrolysis.



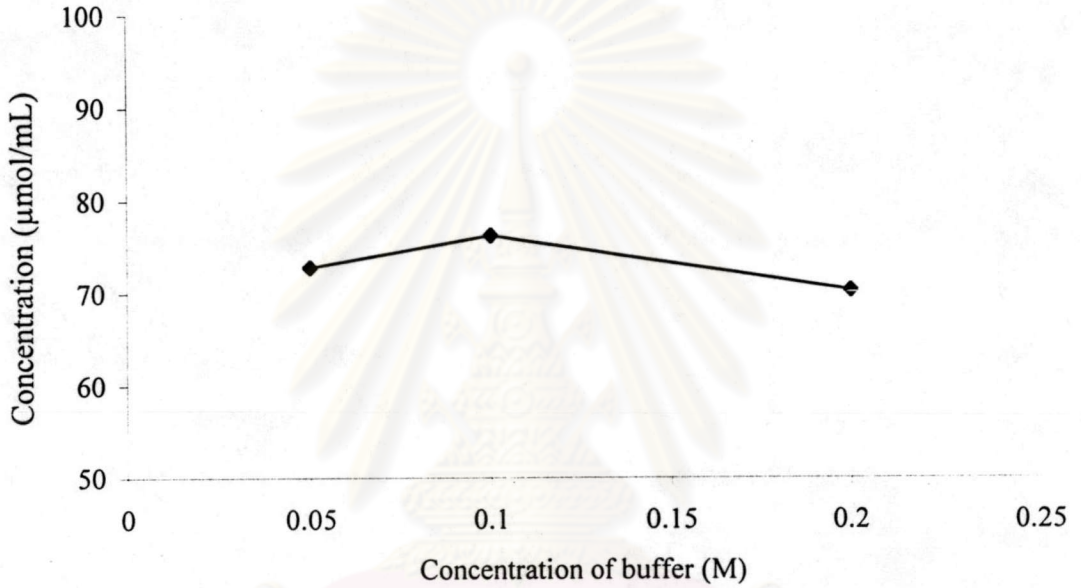
Condition: [β-chitin] = 20 mg/mL, McIlvaine buffer pH 3.0 (0.05M, 0.5 mL) and incubation at 45 °C for 1 day

Figure 3.12 The effect of enzyme/chitin ratio on the chitinolysis.

3.3 Study for optimum conditions toward a preparative scale of GlcNAc using HPLC

3.3.1 Effect of buffer concentration.

The concentration of the buffer was varied from 0.05 to 0.2 M at pH 3. The yield of GlcNAc was only slightly affected by the buffer concentration **(Figure 3.13)**. The results suggested that the ensemble of enzymes from *Aspergillus fumigatus* was not sensitive to the ionic strength and pH variation, which might occur during the course of the hydrolysis.

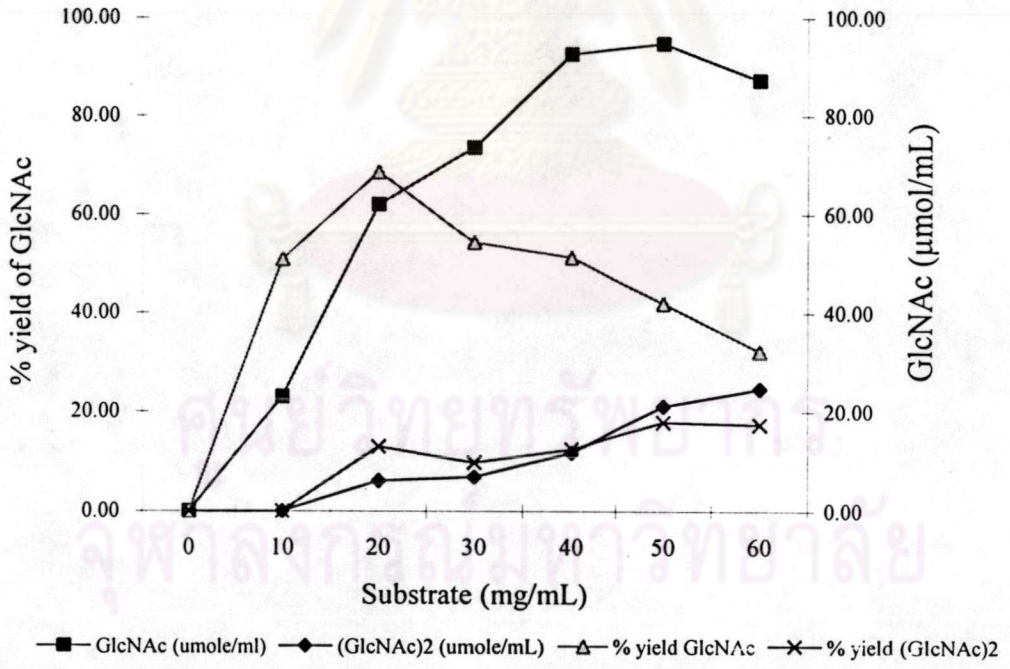


Conditions: enzyme/chitin ratio = 4 mU/mg and incubation at 45 °C for 5 days.

Figure 3.13 The effect of concentration of buffer on the chitinolysis activity of the enzymes from *Aspergillus fumigatus*.

3.3.2 Effect of substrate concentration.

The most obvious means to increase the product yield and concentration is to raise the concentration of the chitin substrate while maintaining the enzyme/chitin ratio at (1.8 mU/mg). The yields and % yield of GlcNAc went up with the increasing concentration of chitin from 10 mg/mL to 20 mg/mL **(Figure 3.14)**. As the concentration of chitin increased to 30 mg/mL and 40 mg/mL, the yield of GlcNAc increased but the % yield of GlcNAc gradually dropped. When the concentration of chitin increased to 50 mg/mL the yield of GlcNAc became relatively unchanged indicating the saturation of the hydrolysis efficiency that may be due to the difficulty in stirring the sticky slurry. The concentration of chitin at 20 mg/mL was thus chosen for further study in the production of GlcNAc. It is worth noting here that the yield of $(GlcNAc)_2$ increased gradually with the increasing concentration of chitin. The results suggested that at higher concentration of chitin, the ensemble of enzymes displayed relatively lower *β-N*-acetylhexosaminidase activity in comparison to chitinase activity.

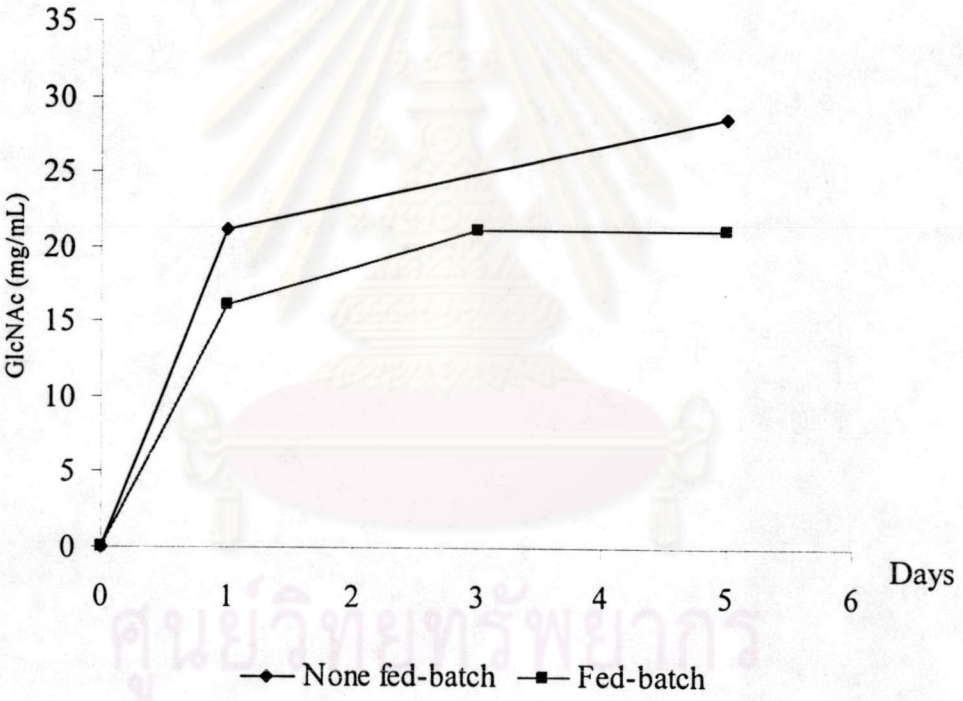


Conditions: enzyme/chitin ratio = 1.8 mU/mg, McIlvaine buffer pH 3.0 (0.1M, 1mL) and incubate at 45 °C for 5 days

Figure 3.14 The effect of chitin concentration on the chitinolytic activity of enzymes from *Aspergillus fumigatus*.

3.3.3 Fed batch technique of fibrous chitin.

In a fed-batch technique, the reaction started with fibrous chitin (2 g), the ensemble of chitinolytic enzymes (4 mU/mL) in McIlvaine buffer (0.1 M) with total reaction volume of 100 mL. The mixture was incubated at 45 °C and two more portions of chitin (1.5 g and 0.73 g) were added after 1 and 3 days of incubation to bring the final concentration of chitin used in the hydrolysis to about 42 mg/mL. This fed-batch technique did not increase the yield and concentration of GlcNAc product **(Figure 3.15)** comparing to the reaction staring with 42 mg/mL of chitin. The results indicated that the fed-batch technique was not necessary for the hydrolysis of fibrous chitin.

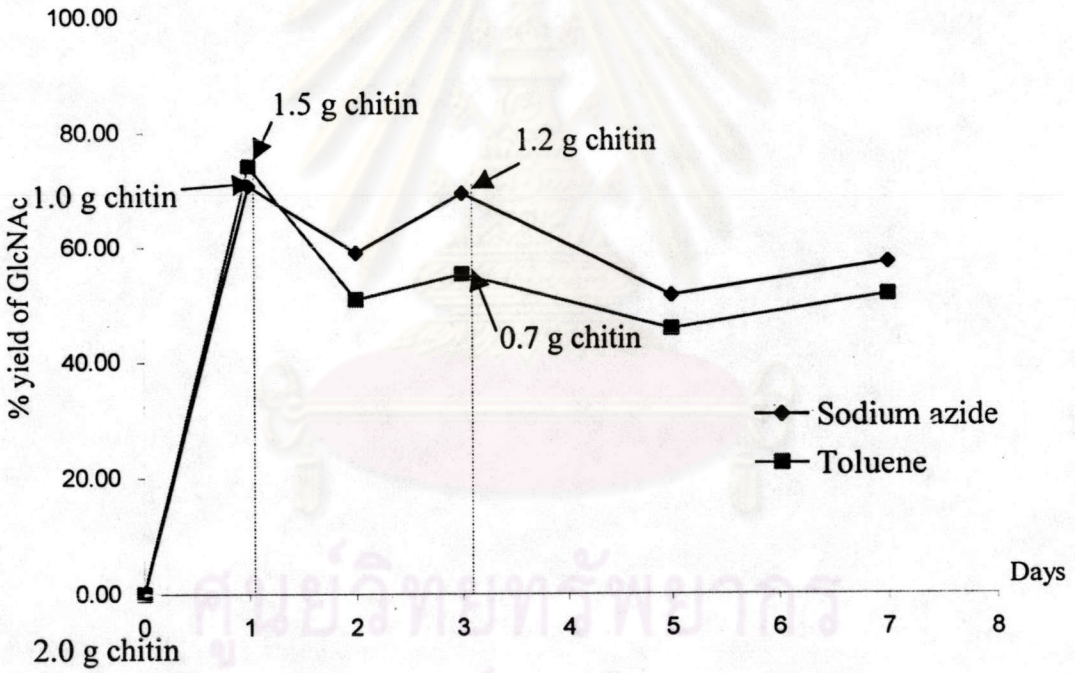


Conditions: chitinolytic enzymes (8 U) in McIlvaine buffer pH 3 (0.1 M, 20 mL). The mixture was incubated at 45 °C and chitin was added after the 1 and 3 days of incubation.

Figure 3.15 Yield of GlcNAc from fed-batch experiment compared to a single batch experiment.

3.3.4 Preservatives.

All of the previous experiments, NaN_3 was added as a preservative to prevent growth of microorganism which may consume the desired product during the reactions. In this experiment toluene was utilized in comparison to NaN_3. The results showed virtually identical hydrolytic profiles for both preservatives **(Figure 3.16)**. Toluene is a better candidate for preservative in an industrial production as it could be easily removed from the reaction product by evaporation. Note that the two experiments were fed batch experiments with different reaction scales (see section 2.9.4). The percentage yields were thus used for comparison to cancel this different. The percentage yields were also dropped after each addition of chitin (1st and 3rd dates) due to higher denominator in the % yield calculation.

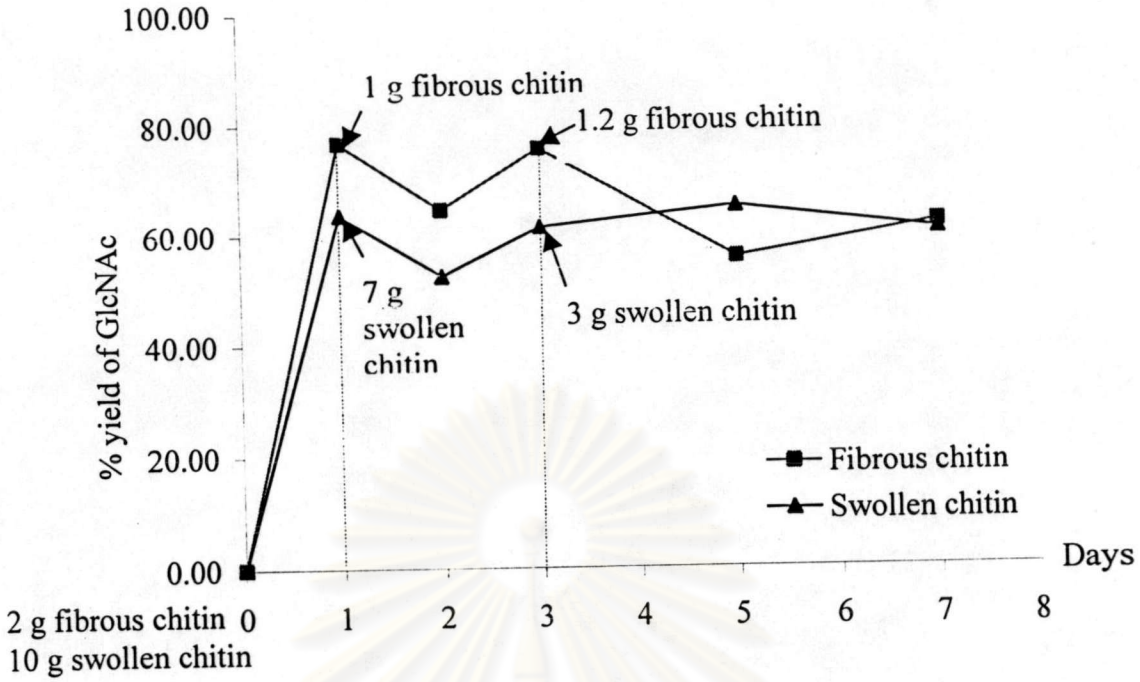


Conditions: chitinolytic enzymes (8 U) in McIlvaine buffer pH 3 (0.1 M, 20 mL). The mixture was incubated at 45 °C and chitin was added after 1 and 3 days of incubation. Dashed lines indicate the addition of chitin in fed batch technique.

Figure 3.16 Hydrolytic profiles for different preservative.

3.3.5 Comparison of fibrous chitin versus swollen chitin.

As the preparation of fine fibrous chitin, used in all previous experiments, required an expensive equipment, centrifugal milling machine, an alternative substrate preparation was sought. Instead of using centrifugal milling machine, a simple food blender was used in grinding β-chitin in the presence of water to produce swollen chitin. This substrate preparation technique was convenient and over 20 g of swollen chitin could be produced in a short time in common laboratory. The study on the hydrolysis of this swollen chitin comparing to the hydrolysis of fibrous chitin using fed-batch technique was conducted. The fed-batch technique was necessary for swollen chitin as the concentration of swollen chitin at 40 mg/mL prevented an efficient stirring and hydrolysis. The reaction was thus started with 10 g of swollen chitin in 500 mL (20 mg/mL of chitin) reaction mixture. Two more portions, 7 g and 3 g of swollen chitin, were added after 1 and 3 days of incubation. The hydrolysis of fibrous chitin was conducted using the same fed-batch technique but smaller amount of chitin was used. The hydrolysis of fibrous chitin started with (2 g) of chitin in 100 mL of reaction mixture. Two more portions, 1 g and 1.2 g of fibrous chitin were added after 1 and 3 days of incubation. The results showed that both substrates gave very similar hydrolytic profiles especially for the final yields after 7 days of around 60% or the final concentration of GlcNAc around 26.50 mg/mL **(Figure 3.17)**. These experiments also presented in terms of percentage yields which decreased after an addition of more chitin substrate.



Conditions: chitinolytic enzymes (8 U for hydrolysis fibrous chitin and 40 U for hydrolysis swollen chitin) in McIlvaine buffer pH 3. The mixture was incubated at 45 °C and chitin was added after the 1 and 3 days of incubation.

Figure 3.17 Hydrolytic profiles for swollen and fibrous chitin.