CHAPTER II

METERIALS AND METHODS

2.1 Instruments and apparatus

1. Automatic autoclave (Ogawa model KT-30L,Japan)
2. Autoclave (Horizontal steam sterilize, Taiwan)
3. Laminar flow (Holten protective 1.2,Germany and BVT 123,Germany)
4. Bunsen lamp
5. Hemacetometer bright line improved nebular
6. Olympus microscope model CH30RF, Germany
7. Incubator (Graft, Germany)
8. Cool incubator (WTB Binder model KB53, Germany)
9. Hot air oven (Memmert ULE 500,Germany)
10. Shaker incubator (Room temperature, Thailand)
11. Shaker incubator (Lab-Line, Taiwan)
12. High performance liquid chromatograph (HPLC): pump (Waters 600E), auto sampler (Waters 917), and diode array detector (Waters 996)
13. HPLC reverse phase column (Asahipak NH2P-50, Shodex, Japan)
14. UV-Visible spectrophotometer (Jenway 600)
15. UV-Visible spectrophotometer (Spectronic 3000)
16. pH meter (pH 3 scan, Eutech Instruments)
17. Hot-plated magnetic stirrer (Coning)
18. Syringe filter (0.45 um PTFE, Alltech)
19. Pipette man (P20, P200, P1000 and P5000, Gilson)
20. Water membrane filters (0.45 um cellulose nitrate, Millipore)
21. Membrane filter (whatmann no.41)
22. Viscometer (Ubbelohde)

23. Freeze-dryer (Freezone 77520, Benchtop, Labconco)
24. Centrifuge (Centuar 2, Sanyo)
25. Centrifuge (Hettich Zentrijugen Rotofix 32)
26. Locking dialysis membrane clamps (Membrane Filtration Product Inc.)
27. Vial-capped 1.5 mL (MCT-150-C Axygen)

2.2 Chemicals

1. Sabouraud dextrose agar (SDA), Diffco
2. Lactose broth (LB), Diffco
3. Potassium dihydrogen phosphate Fluka, Switzerland
4. Magnesium sulphate haptahydrates(Merck, Germany)
5. Ammonium sulphate (Merck, Germany)
6. Urea (Merck, Germany)
7. Ammonium nitrate (Merck, Germany)
8. Ferrous sulphate (Merck, Germany)
9. Manganese sulphate haptahydrates (Merck, Germany)
10. Squid pen β-chitin (Ta-Ming Enterprises, Thailand)
11. *N*-acetyl-D-glucosamine (Fluka Chemicals,Ltd., Switzerland)
12. *N,N'*-diacetylchitobiose (Seikagaku Corporation Co.Ltd.,Japan)
13. *N,N',N''*-triacetylchitotriose (Seikagaku Corporation Co.Ltd.,Japan)
14. *N,N'N'',N'''*-tetraacetylchitotetraose (Seikagaku CorporationCo.Ltd.,Japan)
15. Sodium azide (Riesal-deltaen, Germany)
16. Glacial acetic acid, analar grade (Merck, Germany)
17. Sodium Hydroxide, analar grade (Merck, Germany)
18. Citric acid, analar grade (Merck, Germany)
19. Sodium hydrogen phosphate (Fluka Chemicals,Ltd., Switzerland)
20. Gel filtration packing material (HW-40S, TOSOH Corporation, Japan)
21. Hydrochloric acid, Analar grade (Merck, Germany)
22. Potassium hexaferrocyanate (Merck, Germany)
23. Sodium acetate, (Fluka Chemicals,Ltd., Switzerland)
24. Sodium carbonate, analar grade (Carlo Erba, Itaty)
25. Potassium hydroxide analar grade (Merck, Germany)
26. Acetronitrile, chromatography grade (Merck, Germany)

27. Sodium potassium tartrate (Merck, Germany)

28. Copper II sulphate (Merck, Germany)

2.3 Fungal Strains

The fungal strains used in this thesis were as listed below.

- *Aspergillus fumigatus* TISTR 3045 from leaves dry bonita for seasoning
- *Trichoderma viride* TISTR 3160 from University Philippine Culturing Collect 3515
- *Trichoderma aureoviride* TISTR 3330 from soil (S. Artjariyasripong).
- *Trichoderma reesei* from botany department, faculty of science, Chulalongkorn University
- *Mucor sp.* TISTR 3302 from Decayed leaves.

2.4 Medium preparation and cultivation of fungi

2.4.1 Sauborand dextrose agar (SDA) medium preparation

The SDA medium (6.5 g) was prepared in DI-water (100 mL). The medium was sterilized by autoclaving at 121 °C for 15 minutes before pouring into a plate (20 mL/ plate).

2.4.2 Preparation of Lactose broth (LB)

The LB medium (1.3 g) was prepared in DI-water (100 mL). The medium was sterilized by autoclaving at 121 °C for15 minutes.

2.4.3 The squid pen chitin 50 μm

The squid pen chitin (β-chitin) was purchased from Ta-ming enterprise Co., Ltd. The chitin was ground by ultracentrifugal mill (Recter 970) at the Metallurgy and Materials Science Research Institute, Chulalongkorn University using 25 μm. cylindrical sieving blade.

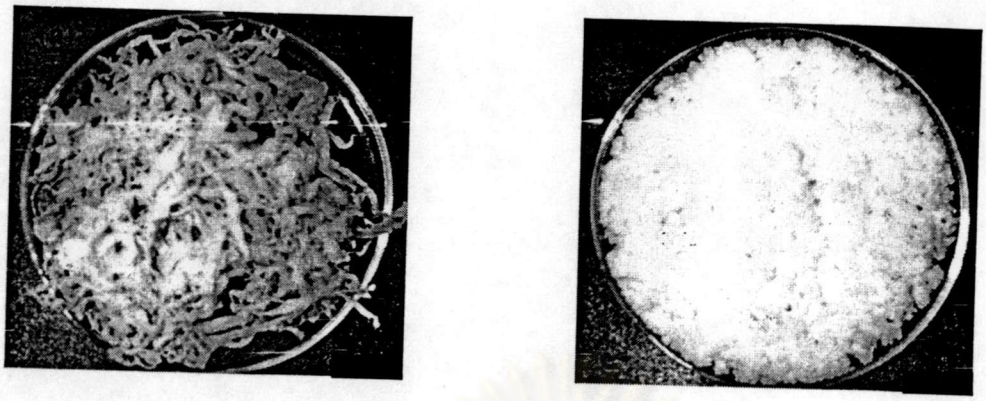

Figure 2.1 The squid pen chitin, (a) flak chitin, (b) fibrous β-chitin 25 × 50 μm

2.4.4 Swollen chitin

The squid pen chitin (20 g) from Ta-ming enterprise Co., Ltd. was cut into small 5 × 5 mm^2 and was ground in a food blender with DI-water (10 mL). The addition of DI-water (10 mL) was repeated when the chitin absorbed water and the grinding became inefficient. When the chitin was saturated with the absorbed water, the grinding could be continued efficiently for 20 more minutes.

2.4.5 Preparation of colloidal chitin

The concentrated hydrochloric acid was gradually added to α-chitin (crab chitin 50 g) in a beaker under vigrous stirring. The viscous mixture was stirred for 30 minutes. The solution was poured as a thin stream into vigorously stirred ice-water mixture (1 L) forming instantly a fine precipitate. The slurry was kept overnight in a refrigerator. The precipitate was washed with DI-water several times until the slurry was neutral and was kept in a refrigerator for further use.

2.4.6 Preparation of colloidal chitin minimum medium (CCMM)

Colloidal chitin minimum medium was used for the enzyme production from fungi. The ingredients were KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g) and urea (0.3 g) used as a nitrogen source, (0.5 g) colloidal chitin weight used as a carbon source. The ingredients were mixed in DI-water (1L). The mixture was adjusted to pH 4.5-5.0 by 1 N NaOH or 1 N CH_3COOH and sterilized by autoclave at 121 ° C for 15 minutes. One inoculum's loop of fungi from LB medium was transferred on to SDA medium (starter medium) and was incubated at 25 °C for 3 days.

2.4.7 Crude enzyme preparation.

Five strains of fungi were grown in CCMM. All strains were cultured at 30 °C on a rotary shaker at 150 rpm for 9-12 days when the maximum chitinolytic activity was obtained. Crude enzyme was separated from fungal cells and solid media by centrifugation at 6000 rpm for 15 minutes.

2.5 Protein assaying

2.5.1 Preparation of calibration curve

The stock solution A of standard protein was prepared by dissolving weighed bovine serum albumin (BSA, 100 mg) with DI water (10.0 mL), and stock solution B was prepared by dissolving weighed bovine serum albumin (BSA, 200 mg) with DI water (10.0 mL).

Table 2.1 Preparation of BSA standard solutions.

Standard No.	Volumn pipetted mL (stock)	DI-water (mL)	Conc. (mg/mL)
1	100 (A)	900	1.0
2	200 (A)	800	2.0
3	300 (A)	700	3.0
4	400 (A)	600	4.0
5	500 (A)	500	5.0
6	300 (B)	700	6.0
7	350 (B)	650	7.0
8	400 (B)	600	8.0
9	450 (B)	550	9.0
10	500 (B)	500	10.0

2.5.2 Biuret reagent

The Biuret reagent was prepared by mixing solution A: $CuSO_4.5H_2O$ (1.5 g) and $NaKC_4H_4O_6.4H_2O$ (6.0 g) in DI-water (500 mL) and solution B: 10 % NaOH (300 mL).

2.5.3 Preparation of standard calibration curve.

The designated amounts of standard solutions (Table 2.1) were pipetted into clean test tubes. The biuret reagent (4 mL) was added. The mixture was stirred and waited for 30 min. The amount of protein was determined by UV-Visible spectrophotometer at 550 nm, the standard curve was obtained by plotting the absorbance in y-axis against the concentration of protein (mg/mL) in x-axis.

2.5.4 Determination of the protein amount in the enzymes

The enzyme solution (1 mL) was pipetted into a clean test tube and biuret reagent (4 mL) was added. The mixture was stirred and waited for 30 min. The amount of protein was determined by UV-Visible spectrophotometer at 550 nm. The amount of protein was calculated by comparison with the standard curve of bovine serum albumin standard prepared in the previous section.

2.6 Reducing sugar

2.6.1 Preparation of standard curve for measuring the amount of sugar reducing end (GlcNAc) with K_3FeCN_6

The GlcNAc ($C_8H_{15}NO_6$ = 221.21) was purchased from Fluka Chemicals, Ltd. About 1 g of GlcNAc was dried under vacuum for 3 days. The stock solution A was prepared by dissolving GlcNAc (11.4 mg) in DI water (10.0 mL) and the stock solution B was prepared by dissolving GlcNAc (11.7 mg) in DI water (20.0 mL). The concentration of stock solution A was 5.1535 mM and stock solution B was 2.6445 mM. Dilution of the stock solutions A and B according to **Table 2.2** was prepared in the same fashion as the other standard solutions.

Table 2.2 Preparation of GlcNAc standard solution.

Standard No.	Volumn pipetted mL,(stock)	Amount of GlcNAc (μmole)
1	200 (A)	1.0307
2	180 (A)	0.9276
3	160 (A)	0.8246
4	140 (A)	0.7215
5	120 (A)	0.6184
6	100 (A)	0.5154
7	200 (B)	0.5289
8	160 (B)	0.4231
9	120 (B)	0.3173
10	80 (B)	0.2116
11	40 (B)	0.1058

2.6.2 Preparation of standard calibration curve.

Colloidal chitin (1.0 %, 0.1 mL) was added to the standards and made the volume to 1.5 mL with DI-water. The K_3FeCN_6 solution (0.5 g K_3FeCN_6/1L of 0.5 M Na_2CO_3) 2 mL was pipetted into each standard solution and the solution was boiled in hot water for 15 minutes. The absorption of each standard solution was measured with UV-vis spectrophotometer at 420 nm. The calibration line was obtained from a plot of ΔAbsorbance in y-axis and the amount of GlcNAc (μmole) in x- axis.

2.6.3 Determination of chitinolytic activity of the enzyme.

The chitinolytic activity of enzyme was assayed by measuring the amount of reducing sugars produced in a digestion of colloidal chitin with the enzyme according to Schales method. The colloidal chitin 0.10 mL (2.5 %), McIlvain buffer pH 3 (0.15 mL of 0.5 M) and (0.05 mL) of enzymatic were pipetted into a clean test tube and adjusted the volume to 1.5 mL with DI-water. The reaction was incubated at 37 °C for 30 minutes. After the incubation period, the K_3FeCN_6 (2.0 mL) was added.The mixture was boiled for 15 minutes. The solution was allowed to cool to room temperature. The small particles were removed by centrifugation at 2,500 rpm for 15 minutes. For the control experiment, the enzyme was boiled 15 for minutes before pipetted enzyme into the test tube and the same procedure described above was followed. The quantity of the reducing sugars was measured by UV-vis spectrophotometer at 420 nm using water as a blank. Both assayed

samples and controls were performed in three replicate and the average absorbance (A) was used in the activity calculation. The activity unit (U) per volume (mL) of the enzyme was calculated from the difference of the absorbance (ΔA) between the assay (A_1) and the control (A_0) according to the following equation.

$$\text{Activity (U)} = \frac{\mu\text{mole of reducing sugar}}{\text{Minute x mL of enzyme}}$$

$$= \frac{k\,\Delta A}{30 \text{ mins x } 0.05 \text{ mL}}$$

The factor (k) = 1/ 1.9662 was the slope of the calibration line used GlcNAc as a standard curve. One unit (U) of enzyme activity was defined as the amount of an enzyme able to produce reducing sugar equivalent to 1 µmole of GlcNAc per minute. Specific activity was defined as unit per mg of protein of enzyme sample.

2.7 Factors affecting the production of chitinolytic enzymes.

2.7.1 Carbon source.

Three strains of fungi were tested for the ability to produce chitinolytic enzyme in shaked flask. Each fungi was transferred from SDA (7 pieces with a diameter of 1.0 cm) into twelve flasks containing CCMM, LB, CMM and Buffer (100 mL). All flasks were incubated for 12 days at 30 ° C on a rotary shaker at 150 rpm. Samples were taken for analyses of chitinolytic activity by Schale's method.

2.7.2 Nitrogen source.

Five strains of fungi were tested for the ability to produce chitinolytic enzyme in shaked flask. Each fungi was transferred from SDA (7 pieces with a diameter of 1.0 cm) in to fifteen flasks containing of CCMM and added nitrogen source, NH_2CONH_2, NH_2NO_3 or NH_2SO_4 (100 mL). All flasks were incubated for 12 days at 30 °C on a rotary shaker at 150 rpm.

2.7.3 Incubation time.

The productions of enzyme at different time were determined. The fungi were sub cultivated in 0.5% CCMM and incubated at 30 ° C on a rotary shaker at 150 rpm. The samples of culture were taken on 3, 5, 7, 9, 12 and 15 days taken for analyses of chitinolytic activity by Schale's method

2.7.4 Inoculums of fungi.

Five strains of fungi were tested for the ability to produce chitinolytic enzyme in shaked flask. Each fungi was transferred from SDA (3, 5, 7, 10 or 14 pieces with a diameter of 1.0 cm) into six flasks containing CCMM (100 mL) were compared the quantity of fungi from SDA 3, 5, 7, 10 and 14 pieces in the CCMM. All flasks were incubated for 12 days at 30 ° C on a rotary shaker at 150 rpm. Samples were taken for analyses of chitinolytic activity by Schale's method.

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

The cultivation was performed by addition of $FeSO_4.7H_2O$ (0.5 mg/100 mL) and $MnSO_4.4H_2O$ (1 mg/100 mL) to the CCMM. The fungi were subcultivated in 0.5% CCMM with and without trace elements ($FeSO_4$ and $MnSO_4$) and incubated at 30 °C on a rotary shaker150 rpm. The samples of culture were taken at 5, 7, 9 and 12 days taken for analyses of chitinolytic activity by Schale's method.

2.7.6 Quantity of carbon source

The colloidal chitin concentrations were started at 0.5, 1.0, 2.0 and 3.0 % of dried chitin weight. Each cultivation was conducted at 100 mL in a shaked flask. The fungi was subcultivated in and incubated at 30 °C on a rotary shaker 150 rpm. Additional chitin was added after 5, 7 and 9 days of cultivation **(Table 2.3)**. The samples of culture were taken at 5, 7, 9 and 12 days for analyses of chitinolytic activity by Schale's method.

Table 2.3 Amount of colloidal chitin (g) added during incubation in shake flask.

Initial	5 days	7 days	9 days
0.5	-	-	-
0.5	0.5	0.5	0.5
1.0	1.0	1.0	1.0
2.0	2.0	2.0	2.0
3.0	3.0	3.0	3.0

2.7.7 Quantity of carbon source and inoculums of fungi.

The CCM medium was used and colloidal chitin concentrations were varied as 1.0, 2.0 and 3.0 % of dried chitin weight and varied inoculums fungi were transferred from SDA (7, 14 or 21 pieces with a diameter of 1.0 cm). The fungi was subcultivated in 0.5% CCMM and incubated at 30 °C on a rotary shaker 150 rpm. The samples of culture were taken at 5, 7, 9 and 12 days taken for analyses of chitinolytic activity by Schale's method.

2.7.8 Optimum temperature

Study the optimum temperature for produce chitinolytic enzyme at different time interval was determined. *Aspergillus fumigatus* was growth on SDA for 3 days at 30 °C. The fungi was subcultivated in 0.5% CCMM and incubated at 30, 40, 50 °C on a rotary shaker at 150 rpm. The samples were randomly taken at 5, 7, 9 and 12 days and analyses of chitinolytic activity by Schale's method.

2.8 Characterization of chitinolytic enzyme.

Modified Schale's method for colorimetric chitinolytic activity assaying.

2.8.1 Optimum pH for hydrolysis chitin

The optimum pH for chitinolytic activity was determined by incubation the enzyme (0.04 mL, 5.4 mU) in the McIlvaine buffers (0.15 mL) or phosphate buffer (0.15 mL) adjusted to various pH values and colloidal chitin (1.0 %) at 37 °C (**Table 2.4**). The reaction was monitored at 30 minutes.

Table 2.4 The amounts of reagents used in reaction in the study for optimum pH.

1.0 % of Colloidal Chitin (mL)	Enzyme (mL,mU)	Buffer			DI-water (mL)
		pH	Types (M)	Volume (mL)	
0.1	0.04, 5.4	6.5	None	0.15	1.21
0.1	0.04, 5.4	2.2	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	3.0	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	3.4	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	4.0	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	5.0	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	6.0	McIlvaine (0.5M)	0.15	1.21
0.1	0.04, 5.4	7.8	McIlvaine (0.5M)	0.15	1.21

2.8.2 Optimum temperature for hydrolysis chitin

In this experiment, the temperature of reaction was varied 30, 37, 45 and 55 °C The reaction mixture contained the enzyme (0.04 mL) in McIlvaine buffers (0.5 M, 0.15 mL) colloidal chitin (1.0 %). The reaction time was monitored at 30 minutes **(Table 2.5)**.

Table 2.5 The amounts of reagents used in reaction for optimum temperature.

Temperature (°C)	1.0 % of Colloidal Chitin (mL)	Enzyme (mL,mU)	McIlvaine 0.5 M (mL)	DI-water (mL)
30	0.1	0.04,8.16	0.15	1.21
37	0.1	0.04, 8.16	0.15	1.21
45	0.1	0.04, 8.16	0.15	1.21
55	0.1	0.04, 8.16	0.15	1.21

2.8.3 The chitinolytic enzyme on different substrates.

The enzyme was incubated with varied type of substrates (20 mg/mL) of colloidal chitin, β-chitin and α–chitin consisted of chitinolytic enzyme (1 mL), McIlvaine buffer pH 3 (0.5 M, 0.5 mL) and sodium azide (1%, 0.5 mL). The hydrolysis was incubated at 37 °C under continuous stirring of magnetic stirrer (**Table 2.6**). The reactions were monitored on the 1 day by colorimetric.

Table 2.6 The amounts of reagents used in reaction in the study for type of substrates.

Chitin Type	Enzyme (mL, mU)	McIlvaine (0.5 M)	1%NaN_3	DI-water
Colliodal chitin	1.0,126	0.5	0.5	3
β- chitin 25x50	1.0,126	0.5	0.5	3
α-chitin powder	1.0,126	0.5	0.5	3

2.8.4 Optimum enzyme/substrate ratio for hydrolysis chitin.

The enzyme/chitin ratio was varied in this investigation. The fibrous β-chitin (100 mg) was weighed into a screw-capped vial. NaN_3 (1%, 0.5 mL) and McIlvaine buffer pH 3.0 (0.5 M, 0.5 mL) was added. The appropriate amount of chitinolytic enzyme was added and made the reaction volume to 5.0 mL with DI-water **(Table 2.7)**. The hydrolysis was carried out at 45 °C under continuous stirring of a magnetic stirrer and monitored on 1 day by Schales' colorimetric method.

Table 2.7 The amounts of reagents used in the reaction for study enzyme /substrate ratio

Enzyme/substrate (mU/mg)	Enzyme (mL)	Chitin (mg)	McIlvaine (0.5 M, mL)	1%NaN_3 (mL)	DI-water (mL)
1.0	0.74	100	0.5	0.5	3.26
2.0	1.48	100	0.5	0.5	2.52
3.0	2.22	100	0.5	0.5	1.78
4.0	2.96	100	0.5	0.5	1.04
5.0	3.70	100	0.5	0.5	0.30
5.5	4.00	100	0.5	0.5	0.00

2.9 Characterization of chitinolytic enzyme by HPLC.

2.9.1 Optimum concentration of MacIlvaine buffer for chitin hydrolysis.

The concentration of buffer was examined in this experiment. The chitin mixture chitinolytic enzyme 176 mU/mL (1.5 mL), NaN_3 (1%, 0.5 mL), McIlvaine buffer pH 3 and made the volume to 5 mL by DI- water **(Table 2.8).** The reactions were incubated at 45 °C under continuous stirring and monitored after 5 days by HPLC.

Table 2.8 The amounts of reagents used in the reaction for finding the optimum concentration of McIlvaine buffer suitability.

McIlvaine (M)	Chitin (mg)	Enzyme (mL)	McIlvaine (mL)	1%NaN_3 (mL)	DI-water (mL)
0	100	1.5	0	0.5	3.0
0.05	100	1.5	0.5	0.5	2.5
0.1	100	1.5	1.0	0.5	2.0
0.2	100	1.5	2.0	0.5	1.0

2.9.2 Optimum substrate concentration for the hydrolysis of chitin

The concentration of chitin was increased in this investigation. The appropriate amount of fibrous β-chitin was added into a screw-capped vial (**Table 2.9**). NaN_3 (1%, 0.25 mL) and McIlvaine buffer pH 3.0 (0.5 M, 1.0 mL) were added. The chitinolytic enzyme (1.0 ml) was added and made the reaction volume to 5.0 mL with DI-water. The reactions were incubated at 45 °C under continuous stirring and monitored after 3 days of incubation by HPLC.

Table 2.9 The amounts of reagents used in reaction for substrate suitability.

Chitin (mg/mL)	Enzyme (mL, mU/mg)	McIlvaine (0.5 M, mL)	1%NaN_3 (mL)	DI-water (mL)
10	0.4, 1.8	1.0	0.25	3.35
20	0.8, 1.8	1.0	0.25	2.95
30	1.2, 1.8	1.0	0.25	2.55
40	1.6, 1.8	1.0	0.25	2.25
50	2.0,1.8	1.0	0.25	1.75
60	2.4, 1.8	1.0	0.25	1.35
70	2.4, 1.8	1.0	0.25	0.95
80	3.17,1.8	1.0	0.25	0.58

2.9.3 The fed-batch technique.

In this experiment two reactions were carried out at the same time. One with used a fed-batch technique using the initial amount of fibrous chitin at 2 g in the total reaction volume of 100 mL. Two more portions of fibrous chitin, 1.5 g and 0.73 g were added after 1 and 3 days of incubation. The substrate was incubated with the enzyme (8 U) in McIlvaine buffer pH 3 (0.1 M) at 45 °C for 5 days. The reaction was monitored after 1, 2, 3 and 5 days of incubation. For comparison, the reaction was also carried out with the initial amount of chitin at 4.3 g but no more addition of the substrate in a none fed-batch technique. The details of the amount of reagents used in this experiment are presented in **Table 2.10.**

Table 2.10 The amounts of reagents used in reaction for Fed-batch technique.

Type of reaction	Days of incubation	Chitin (g)	Enzyme (mL, U)	McIlvaine (mL)	NaN_3 (mL)	DI-water (mL)
None fed-batch	Initial	4.3	36,4	20	5	39
Fed-batch	1	2	40,8	20	5	35
	3	1.5	-	-	-	-
		0.73	-	-	-	-

2.9.4 Changing of preservatives from NaN_3 to toluene

The reactions were carried out using two types of preservatives, NaN_3 (1 %, 5 mL) and toluene (100 µL). The mixture of chitin (2 g/mL), chitinolytic enzyme (40 mL, 4 mU/mg), McIlvaine buffer pH 3 (0.1 M, 20 mL) was added with DI-water to make the volume to 100 mL by DI- water **(Table 2.11)**. The reactions were incubated at 45 °C under continuous stirring and monitored after 1, 2, 3 and 5 days of incubation by HPLC.

Table 2.11 Comparative preservation of NaN_3 and toluene

Type of reaction	Chitin (mg)	Enzyme (mL)	McIlvaine (mL)	DI-water (mL)
NaN_3	4200	40	20	35
Toluene	4200	40	20	39.9

2.9.5 Comparison of fibrous chitin with swollen chitin as substrates.

The hydrolysis chitin was performed by using fed-batch technique. The initial amount of fibrous chitin (2 g) was added into flask, toluene (100 μL) and McIlvaine buffer pH 3.0 (0.1 M, 20 mL) were added. The chitinolytic enzyme (40.0 mL, 4mU/mg) was added and made the reaction volume to 100.0 mL with DI-water **(Table 2.12)**. The reactions were incubated at 45 °C under continuous stirring and monitored after 1, 2, 3, 5, 7 days by HPLC. Two more portions of fibrous chitin, 1 g and 1.1 g were added after 1 and 3 days of incubation. Another reaction conducted by using added wollen chitin (10g) toluene (500 μL) and McIlvaine buffer pH 3.0 (0.1 M, 100 mL). The chitinolytic enzyme (150.0 ml, 4mU/mg) was added and made the reaction volume to 500.0 mL with DI-water. The reactions were incubated at 45 °C under continuous stirring and monitored after 1, 2, 3, 5, 7 days by HPLC. Two more portions of swollen chitin, 7 g and 3 g were added after 1 and 3 days of incubation.

Table 2.12 The amounts of reagents used in reaction.

Type of reaction	Days of incubation	Chitin (g)	Enzyme (mL)	McIlvaine (mL)	Toluene (mL)	DI-water (mL)
Fibrous chitin	Initial	2	40	20	0.1	39.9
	1	1	-	-	-	-
	3	1.1	-	-	-	-
Swollen chitin	Initial	10	180	150	0.5	249.5
	1	7	-	-	-	-
	3	3	-	-	-	-

2.10 Product analysis by HPLC

2.10.1 Preparation of calibration curve

The samples of exact weight of dried GlcNAc ($C_8H_{15}NO_6$=221.21) were dissolved with DI-water (5.00 mL) in two vials to make two stocked solutions A, and B **(Table 2.13)** The six standard solutions (C, D, E, F, G, H, I, and J) were prepared by dilution of A and B.

Table 2.13 Preparation of GlcNAc standard solutions.

Standard solution	Concentration (mg/mL)	Preparation Method
A	4.840	GlcNAc (24.2 mg.) + water 5 mL
B	2.480	GlcNAc (12.4 mg.) + water 5 mL
C	1.210	A (0.500 mL.) + water (1.50 mL)
D	0.484	A (0.200 mL.) + water (1.80 mL)
E	0.242	A (0.200 mL.) + water (3.80 mL)
F	0.121	A (0.100 mL.) + water (3.90 mL)
G	0.186	B (0.225 mL.) + water (2.775 mL)
H	0.062	B (0.100 mL.) + wate (3.90 mL)
I	0.0186	G (0.200 mL.) + water (1.80 mL)
J	0.0093	

Each standard (0.3 mL.) was mixed with acetonitrile (0.7 mL) and filtered through a 45 μm PTFE membrane filter. The standard solutions (20 μL) were injected into HPLC (pump Waters 600 E. Autosample Waters 917 and diode array detector waters 996) and detected at 210 nm. The mobile phase was acetonitrile: water (70:30) at a flow rate of 1.0 mL/min. An Asahipak NH_2P-50, Shodex (Japan) column was used as the stationary phase. The calibration curve was obtained by plotting the concentration of GlcNAc (mM) on the X-axis against the Peak area (mV*sec) on the Y-axis.

The calibration curve for $(GlcNAc)_2$ ($C_{16}H_{28}N_2O_{11}$=424.42) was obtained using the similar procedure described for that for GlcNAc **(Table 2.14).**

Table 2.14 Preparation of $(GlcNAc)_2$ standard solutions.

Standard solution	Concentration (mg/mL)	Preparation method
A	1.600	$(GlcNAc)_2$ (2.4 mg) + water (1.5 mL)
B	1.000	$(GlcNAc)_2$ (2.1 mg) + water (2.1 mL)
C	1.280	A (0.4 mL) + water (0.1 mL)
D	0.320	A (0.2 mL) + water (0.8 mL)
E	0.600	B (0.6 mL) + water (0.4 mL)
F	0.200	B 0.2 mL) +water (0.8 mL)
G	0.100	F (0.25 mL) + water (0.25 mL)
H	0.050	F (0.25 mL) + water (0.75 mL)

The calibration curve for $(GlcNAc)_3$ ($C_{24}H_{41}N_3O_{16}$ = 627.63) was obtained using the similar procedure described for that for GlcNAc **(Table 2.15).**

Table2.15 Preparation of $(GlcNAc)_3$ standard solutions.

Standard solution	Concentration (mg/mL)	Preparation method
A	2.000	$(GlcNAc)_3$(3.0) + water (1.5 mL)
B	1.000	$(GlcNAc)_3$ (2.1 mg) + water (2.1 mL)
C	1.600	A (0.4 mL) + water (0.1 mL)
D	1.200	A (0.3mL) + water (0.2mL)
E	0.800	B (0.4mL) + water (0.1mL)
F	0.600	B (0.3 mL) +water (0.2 mL)
G	0.400	B (0.4mL) + water (0.6 mL)
H	0.200	G (0.25 mL) +water (0.75 mL)
I	0.050	G (0.125 mL) +water (0.875 mL)

2.10.2 Analysis of products in the hydrolysates

After the designated time, each hydrolysate (100 μL) was pipetted into 2 mL plastic capped vial and diluted with DI-water (900 μL) (dilution factor = 10) The mixture was boiled for 15 min and centrifuged at 2,000 rpm for 20 minutes. The supernatant (0.3 mL) was pipetted out and mixed with acetronitrile (0.7 mL). The solution was filtered through a 0.45 μm PTFE filter before injecting into the HPLC. The same instrument system and condition as previously described in the preparation of calibration line was used in the analysis of the products. The GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ were detected at a retention time of 5.6, 6.5, and 7.9 min respectively. The peak areas were used to calculate the amount of the GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ according to following equation:

$$[\text{GlcNAc}]\ (\text{mM}) = \frac{\text{PeakArea} \times \text{dilution factor}}{384.65}$$

$$[(\text{GlcNAc})_2]\ (\text{mM}) = \frac{\text{PeakArea} \times \text{dilution factor}}{424.03}$$

$$[(\text{GlcNAc})_3]\ (\text{mM}) = \frac{\text{PeakArea} \times \text{dilution factor}}{834.32}$$

The factors of 384.65, 424.03, and 834.32 were obtained from the slope of the calibration lines of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ respectively.