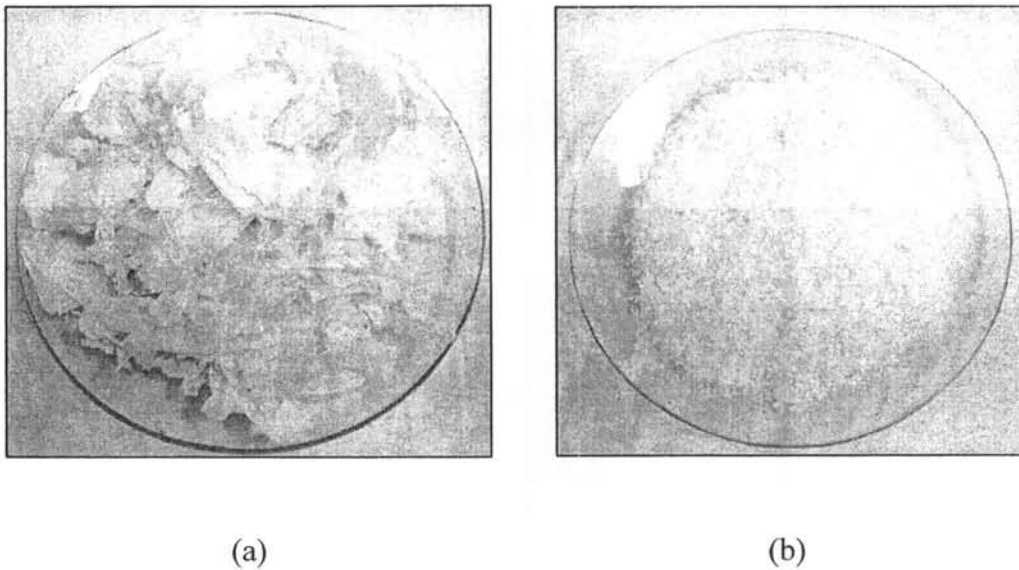


## CHAPTER III

### Results and discussion

#### 3.1 Shrimp chitin

Commercial shrimp chitin purchased from industry was in a form of thick fibrous sheets (Figure 3.1 a). The chitin sheets were ground by a 500 watt food blender to provide 10-60 mesh chitin powder (Figure 3.1 b, Table 3.1).



**Figure 3.1** Photographs of shrimp chitin (a) as purchased and (b) after grinding

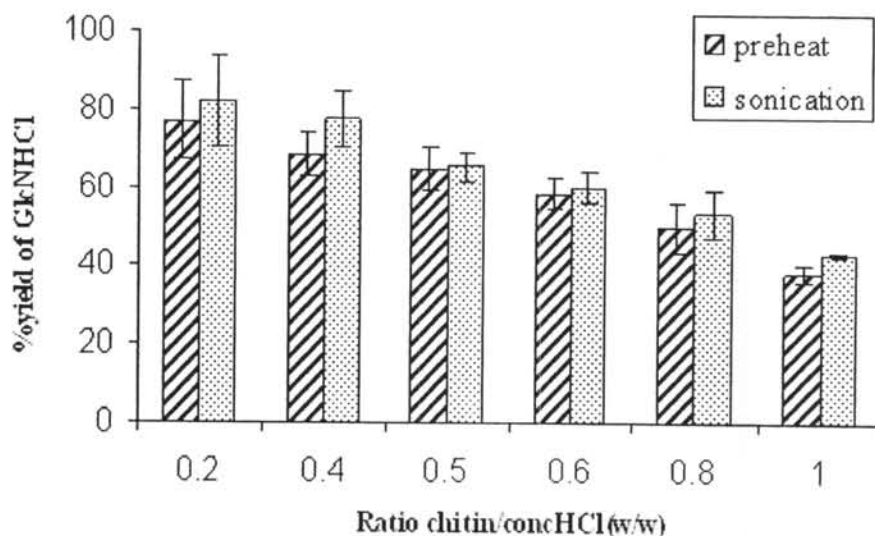
**Table 3.1** Size of chitin measured by a sieving shaker

Chitin powder	Weight(g)	%Weight
<10 mesh	0	0
10-40 mesh	28.3	57
40-60 mesh	9.15	18
> 60 mesh	12.18	25
Total	50.01	100

### 3.2 Preparation of glucosamine hydrochloride (GlcNHCl)

#### 3.2.1 Effect of sonication and chitin/conc. HCl weight ratio

At room temperature, natural chitin dissolves sluggishly in concentrated HCl. The hydrolysis of chitin without allowing the complete dissolution of chitin thus give irreproducible results and usually low yield of GlcNHCl. Gandhi, N. *et al* suggested that preheating conc. HCl to 60 °C before the addition of chitin brought about the complete dissolution of chitin and thus increased the yield of GlcNHCl and reproducibility of the reaction. In this thesis work sonication techniques was studied in comparison to the preheating method. Chitin (10 g) was dissolved in various amount of either preheated conc. HCl or conc. HCl in sonicating bath. After about 20-30 minutes the complete dissolution of chitin was observed for both treatments. The chitin solution was heated to 95 °C and allowed for the hydrolysis to take place for 75 minutes. GlcNHCl was isolated by precipitation, activated charcoal decolorization and ethanol washing. The percent isolated yields of GlcNHCl obtained from the hydrolysis at various weight ratios of chitin/conc. HCl were compared. The sonication treatment seemed to give only slightly higher yield of GlcNHCl than the pre-heating method (**Figure 3.2**) indicating that both methods are effective for dissolution of chitin. The results also showed that the GlcNHCl yield dropped significantly when the ratio of chitin/conc. HCl increased. The lower percent yield observed at higher chitin/conc. HCl ratio is probably because, at high concentration of chitin, the solution is so highly viscous that the hydrolysis rate becomes slow.

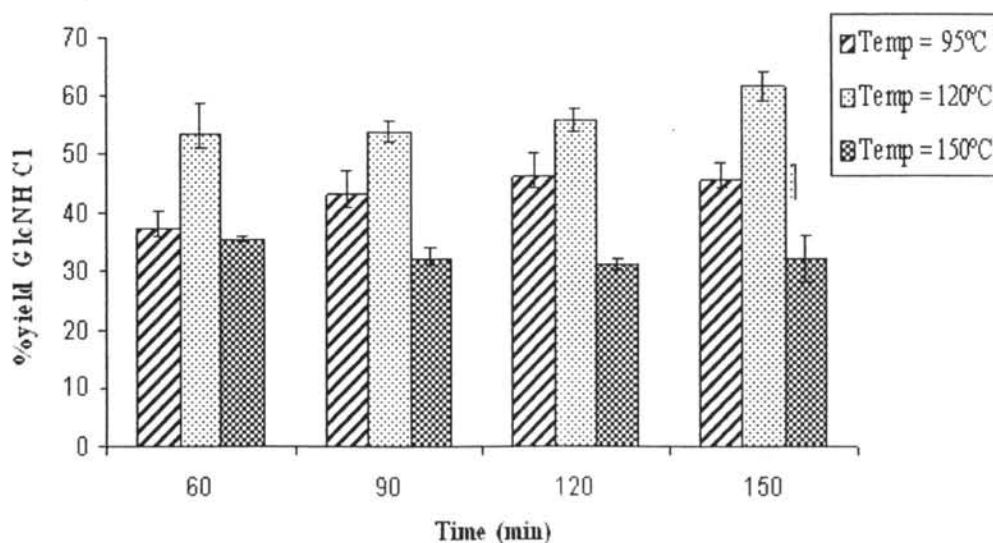


**Figure 3.2** GlcNHCl yields obtained from the hydrolysis of chitin ratio of chitin/conc HCl (w/w). The averages from duplicated experiments are used in the plot.

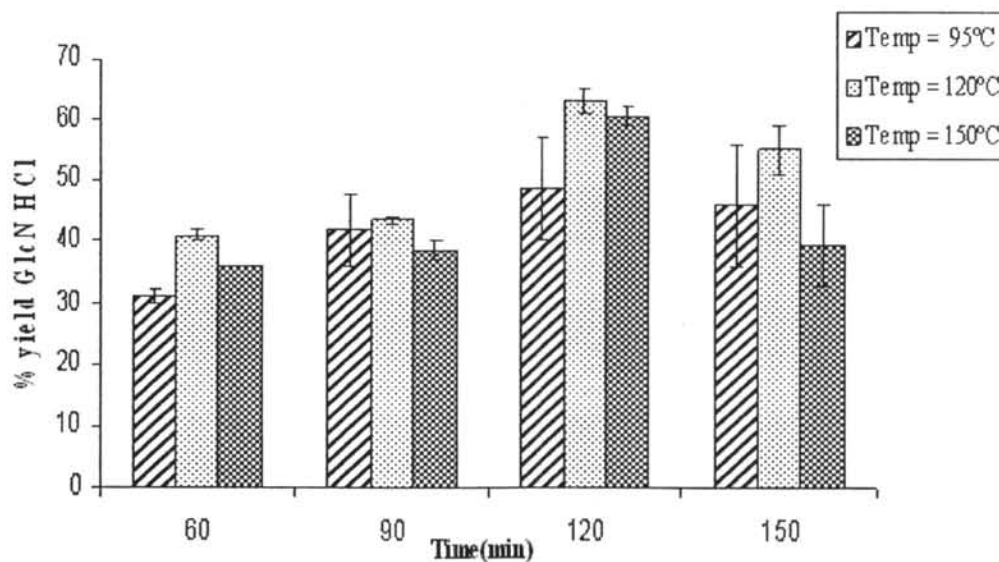
*Condition:* pretreatment period time = 30 min, pretreatment temp = 30-60 °C, hydrolysis time = 75 min, hydrolysis temp = 95 °C

### 3.2.2 Effect of hydrolysis temperature and time

The production of GlcNHCl must be effectively achieved by using the lowest amount of the acid without having to compromise its percent yield. To improve the yield of GlcNHCl at chitin/conc. HCl ratio of 1:1(w/w), the effects of hydrolysis temperature and time were studied. Chitin (10 g) was hydrolyzed in concentrated hydrochloric acid (10 g) at various hydrolysis temperatures (95-150 °C) and times (60-150 minutes) after the dissolution of chitin by either pre-heating or sonication. Overall, the yields of GlcNHCl are dependent on the hydrolysis temperature and time and showing its optimum condition. In general, low temperature and short reaction time lead to incomplete hydrolysis while high temperature and prolonged reaction time result in charring and polymerization. As for the optimum condition, high GlcNHCl yield was obtained at the hydrolysis temperature and time of 120°C and 120-150 minutes, respectively (**Figure 3.3**). Since several factors contribute to the isolated yield of GlcNHCl, it is impossible to unambiguously rationalize for all the detail differences in the yields between each condition.



(a)



(b)

**Figure 3.3** GlcNHCl yields obtained from the hydrolysis of chitin using various hydrolysis times and temperatures after (a) preheating and (b) sonication treatments. The averages from duplicated experiments are used in the plot.

*Condition:* ratio of chitin:conc HCl = 1:1(w/w), pretreatment period time = 30 min, pretreatment period temp = 30-60 °C

### 3.3 Sonication assisted hydrolysis of chitin for preparation of GlcNAc

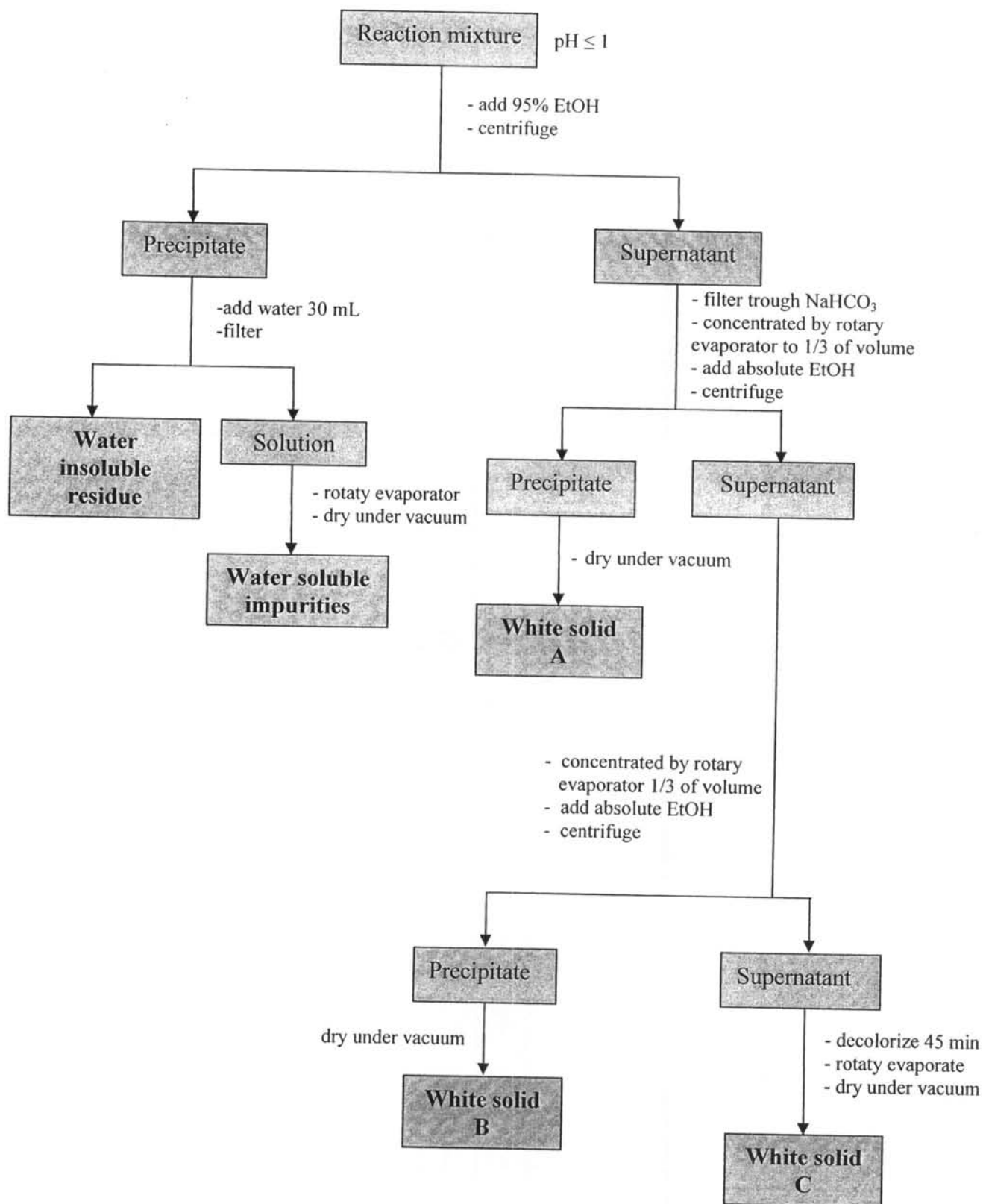
As described in the previous section, the hydrolysis of chitin at temperature, higher than 90 °C, gives GlcNHCl as the major product and no significant difference in the GlcNHCl yields between two different dissolution methods, pre-heating and sonication. The results indicate that sonication does not affect the hydrolysis rate but serves as the dissolution facilitator. It was noted during the experiments that the sonication brought about the dissolution of chitin even at room temperature. It is thus possible to perform the hydrolysis of chitin in homogeneous state at lower temperature that may allow the glycosidic bond to be hydrolyzed selectively to give GlcNAc.

Chitin (10 g) was hydrolyzed by concentrated hydrochloric acid (25 g). The hydrolyses of chitin with and without sonication pretreatment were studied at three temperatures *i.e.* 20, 40 and 60 °C. The pretreatment of chitin/conc. HCl mixture with sonication resulted in complete dissolution of chitin and less water insoluble residue (**Scheme 3.1**) obtained, this therefore indicated high degree of hydrolysis when pre-sonication was performed (**Table 3.2**).

**Table 3.2** Hydrolysis of chitin with concentrated HCl with and without sonication assisted solubilization

Temperature	w/o sonication		sonication	
	residue (g)	% hydrolysis	residue (g)	% hydrolysis
20°C	1.6246	3	8.9468	12
40°C	4.2924	57	2.0277	80
60°C	0.9220	91	0.2898	97

*Condition:* chitin/conc HCl = 1:3 (w/w), sonication period = 30 min, hydrolysis time = 120 min.

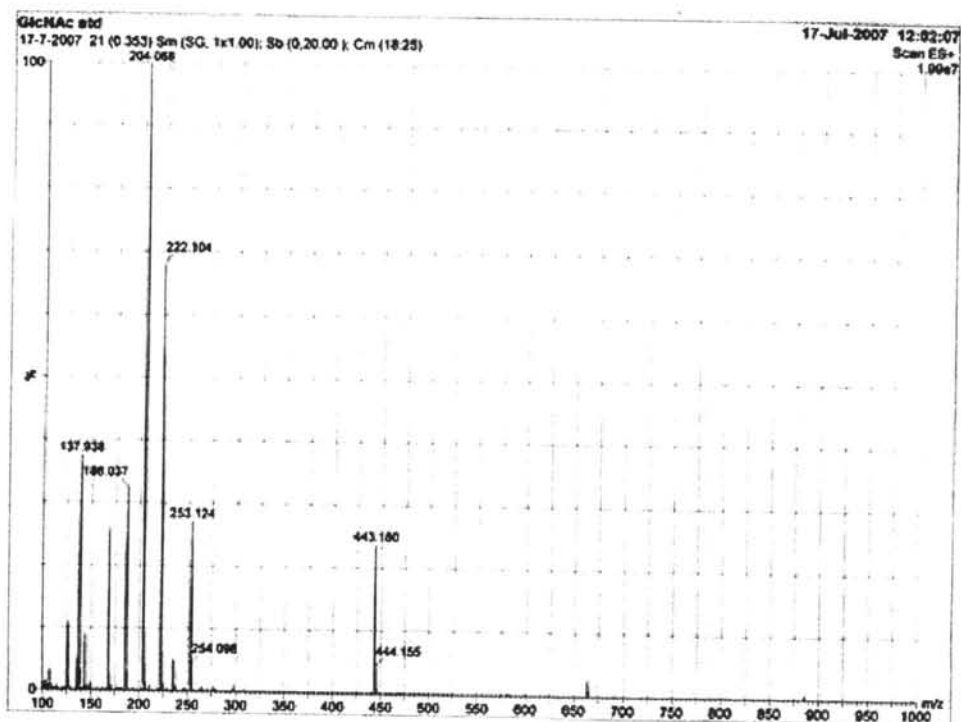


**Scheme 3.1** Isolation of GlcNAc

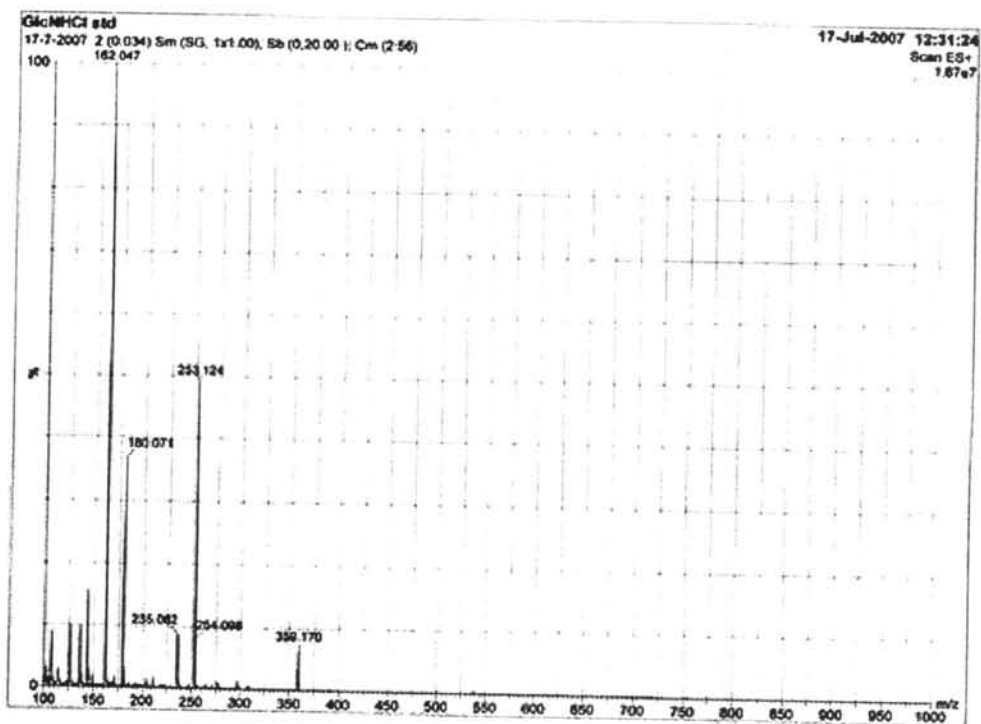
As expected, the degree of hydrolysis (% hydrolysis =  $100 \times (\text{initial chitin weight} - \text{weight of residue}) / \text{initial chitin}$ ) is higher with the sonication pretreatment and increase with the temperature. At 20 °C, the hydrolysis of glycosidic bond was slow that considerable amount of water insoluble white residue, representing the unhydrolyzed chitin and long chain oligosaccharides, remained after the reaction. At higher temperature *viz.* 60 °C a high degree of hydrolysis and only little of dark residue were obtained. The dark residue is probably charred chitin occurred at high temperature reaction.

### 3.4 Monitoring of the hydrolysis of chitin by ESI-MS

The GlcNAc product produced in the hydrolysis of chitin with sonication assisted solubilization was evaluated by electro spray mass spectrometer (ESI-MS). Chitin (10 g) was hydrolyzed in conc. HCl (50 g) at various hydrolysis time and temperature. The sonication temperature was controlled to be the same as each hydrolysis temperature. The reaction was monitored by ESI-MS with MS scan mode at designated time intervals. Using the optimum instrumental conditions, standard GlcNAc were observed as the signals of  $[\text{GlcNAc} + \text{H}_3\text{O}]^+$  at  $m/z = 204$ ,  $[\text{GlcNAc} + \text{H}]^+$  at  $m/z = 222$  (**Figure 3.4a**) and standard GlcNHCl were observed as the signal of  $[\text{GlcN} + \text{H}_3\text{O}]^+$  at  $m/z = 162$ ,  $[\text{GlcN} + \text{H}]^+$  at  $m/z = 180$  (**Figure 3.4b**).



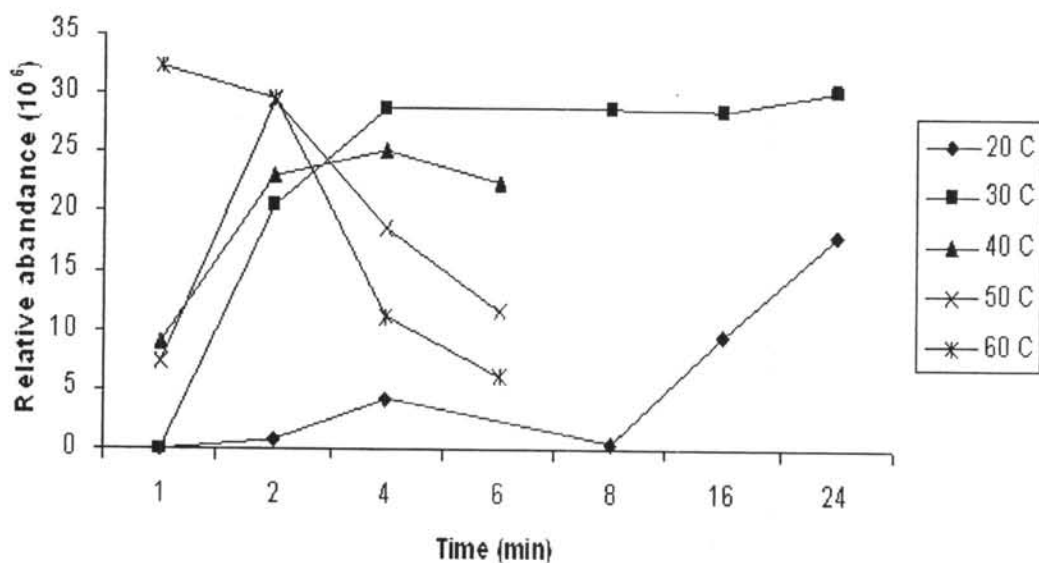
(a)



(b)

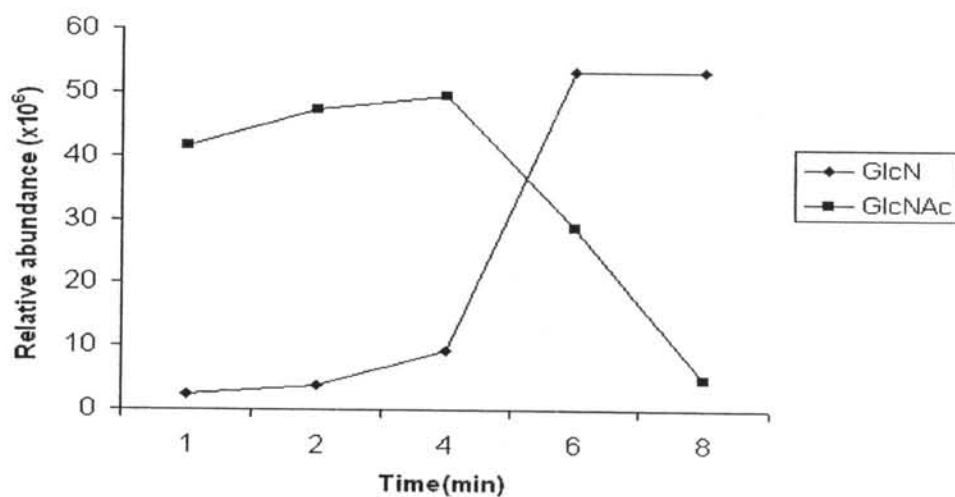
Figure 3.4 Mass spectra of GlcNAc (a) and GlcNHCl (b)





**Figure 3.5** Relative abundance of GlcNAc detected by ESI-MS at various hydrolysis temperature and time.

*Reaction condition:* chitin/conc. HCl = 1:5 (w/w), sonication period = 30 min.



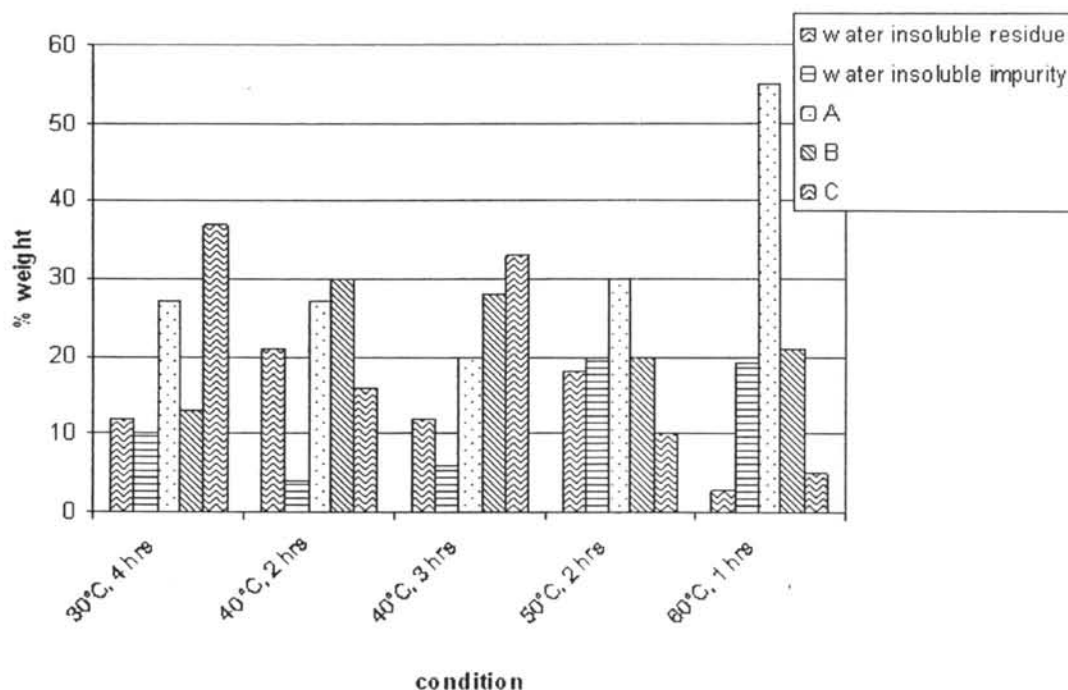
**Figure 3.6** Relative abundance, MS signal intensity of GlcNAc and GlcN during hydrolysis of chitin.

*Reaction condition:* chitin/conc. HCl = 1:3(w/w), sonication period = 30 min, hydrolysis temp = 40 °C

At hydrolysis temperature of 50 and 60 °C, the yield of GlcNAc dropped sharply as the hydrolysis time extended beyond 2 hours. When using the hydrolysis temperature of 30 °C and 40 °C, the yield of GlcNAc reached its maximum after 3-4 hours. At low hydrolysis temperature of 20 °C, GlcNAc started to appear after 8 hours of hydrolysis and increased very slowly afterward(**Figure 3.5**). The results showed the glycosidic bond (depolymerization) and the amide bond (deacetylation) were rapidly hydrolyzed at high temperature (50-60°C). At hydrolysis temperature of 40 °C, the deacetylation occurred after 4 hours, evidently seen in Figure 3.6 as an increase of GlcN content(**Figure 3.6**). At low temperature of 20°C, the depolymerization slowly occurred after 8 hours.

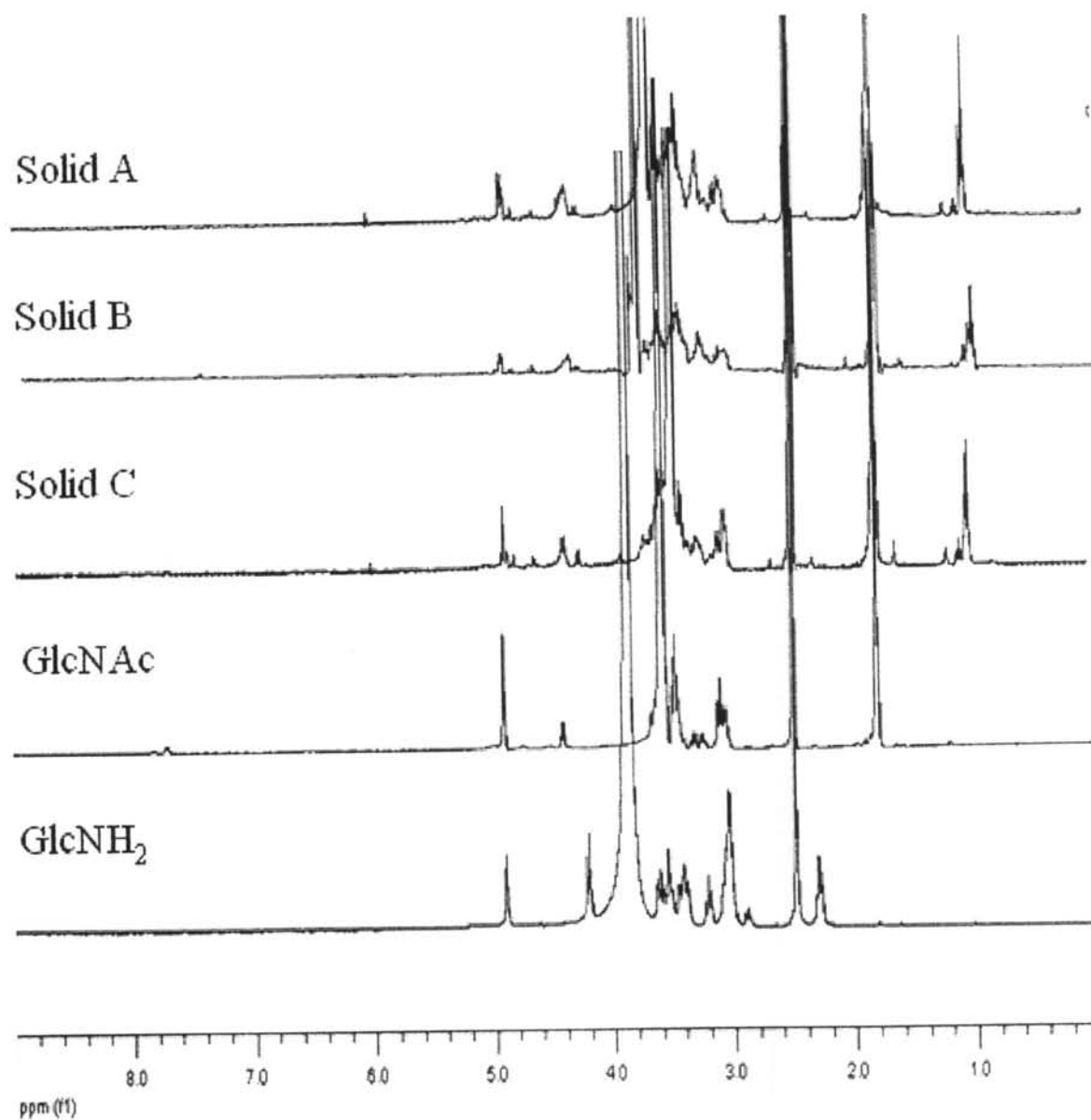
### 3.5 Preparation of GlcNAc

$\alpha$ -Chitin (10 g) was hydrolyzed in concentrated HCl (30 g) at the optimal hydrolysis temperature and time obtained from the ESI-MS results. The GlcNAc product was isolated according to **Scheme 3.1**. The weight of solid precipitates obtained from each precipitation step were determined and calculated as %weight of the initial chitin (**Table 3.1**). The total solid recovery well accounts for almost 100% of the initial chitin. The water insoluble residue is presumably the unhydrolyzed chitin or long chain oligosaccharides that decrease with the increase of the hydrolysis temperature and time. On the other hand, the water soluble impurity precipitated from the addition of 95% ethanol increases with the hydrolysis temperature and time. The main component of this impurity is GlcNHCl which is well soluble in water but not in 95% ethanol. Solid A, B and C (**Scheme 3.1**) are not precipitated by the addition of 95% ethanol but can be fractionally precipitated by the addition of absolute ethanol. These solid precipitates were analyzed by <sup>1</sup>H NMR and ESI-MS to determine their identity and purity.



**Figure 3.7** Weight of solid precipitate obtained from fractional precipitation of the hydrolysate from the hydrolysis of chitin using sonication assisted solubilization.

Comparing the  $^1\text{H}$  NMR spectra of solid A, B and C with those of the standard  $\text{GlcNH}_2$  and  $\text{GlcNAc}$  (**Figure 3.8**), the spectra of solid A, B and C are closely resemble to  $\text{GlcNAc}$  without the characteristic signals of  $\text{GlcNH}_2(\text{GlcNHCl})$  at 2.3 and 4.2 ppm. The spectra of solid A, B and C also show some extra minor peaks indicating the existing of certain impurities at 4.3 ppm. To obtain the  $\text{GlcNAc}$  purity, each solid was analyzed quantitatively against  $\text{GlcNAc}$  standard by ESI-MS.



**Figure 3.8**  $^1\text{H}$  NMR spectra of standard GlcNHCl, GlcNAc and solid precipitates (A, B and C) in  $(\text{CD}_3)_2\text{SO}$  in the presence of an aliquot of  $\text{D}_2\text{O}$ .

### 3.6 Determination of GlcNAc purity by using ESI-MS

The exact weight of solid sample (W) was dissolved in an exact amount of DI-water. The solution was filtered through a 0.45  $\mu\text{m}$  PTFE syringe filter and injected into an ESI mass spectrometer. Under MS scan mode, all injections and ionization parameters were adjusted to give the highest signals corresponding to GlcNAc. The purity of GlcNAc in the sample was determined from the abundance (A) of  $[\text{GlcNAcH}^+ - \text{H}_2\text{O}]$  signal at 204 against two solutions of GlcNAc standard with similar weight ( $W_1$  and  $W_2$  where  $W_1 < W < W_2$ ) in the same amount of DI-water according to the following equation.

$$\% \text{ purity} = \frac{W_1 + [(A - A_1) \times (W_2 - W_1) / (A_2 - A_1)]}{W} \times 100$$

**Table 3.3** % Weight and % purified of solid A, solid B and solid C from the purified products

Hydrolysis condition	Solid A		Solid B		Solid C		Total GlcNAc yield (%)
	%purity	%weight	%purity	%weight	%purity	%weight	
30°C, 4 hrs	75	27	77	13	95	37	65
40°C, 2 hrs	65	27	73	30	97	16	55
40°C, 3 hrs	69	20	67	28	95	33	64
50°C, 2 hrs	67	30	75	20	92	10	43
60°C, 1 hrs	71	55	81	21	92	5	60

Condition : ratio of chitin/concHCl = 1:3(w/w), sonication period = 30 min, sonication temp = 30°C

At the hydrolysis temperature of 30°C using ultrasonic wave for assisting the dissolution, the highest total yield of GlcNAc was 65% yield at the optimum hydrolysis temperature and time were 30°C and 4 hours, respectively. The hydrolysis produced *N*-acetyl-*D*-glucosamine as a major product in 37% isolated yield. The optimum temperature is the same temperature that *Varum et al.* was found. The purity of solid C was higher than solid B and solid A, respectively because the impurity was precipitated in absolute ethanol.