Crab *(Brachyura)* shell Acid and Alkali Treatments: Influence on Thermal and Structural Properties of Isolated Acetamide-Rich Natural Polymer

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ABSTRACT: Exoskeleton of crab comprises a dominating mineral (calcium carbonate, $CaCO_3$), protein and a natural polymer (chitin). Chemical treatments have been employed at different instances to isolate pure chitin from different sources. It is thus necessary to investigate how this treatment will influence the features of chitin isolated from the same source (crab). In this study, 0.4, 0.8 and 1.2 M hydrochloric acid (HCl) were separately used to demineralize crab shell particles and this was followed by deproteinization with 0.4 and 1.2 M sodium hydroxide (NaOH) at 100 ^{0}C. Results showed that chitin properties were influenced by concentrations of reagents. Fibrils of different forms and surface appearance were observed via Scanning Electron Microscopy (SEM). The highest crystallinity index of 71% was possessed by chitin extracted using 0.4 M HCl and NaOH while 65.5% remained the least displayed by chitin extracted with 1.2 M HCl and NaOH. This trend was similar for chitin's thermal stability where Thermogravimetric analysis (TGA) results informed that using the highest concentrations of 1.2 M HCl and NaOH provided chitin with 80.12 kJ/mol activation energy. On the other hand, 112.54 kJ/mol was calculated for chitin isolated with the minimum demineralization and deproteinization reagents used in this study.

KEYWORDS: Activation energy, chitin, crab, demineralization, deproteinization

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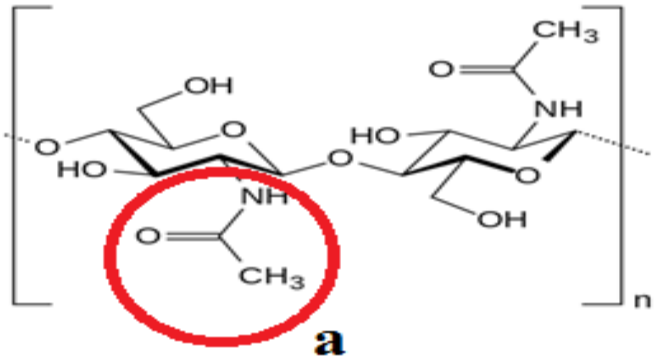
I. INTRODUCTION

Chitin is a structural polysaccharide with continual units of β-(1→4) - linked 2-acetamido-2-deoxy-β-D-glucopyranose. In terms of abundance, it is second to cellulose which is found in ubiquitous lignocellulosic materials (Gbenebor *et al*, 2022). These two natural polymers impart similar biological functions in the sense that while chitin beefs up the strength of fungi cell walls, cellulose fortifies the plants' cell walls (Malinovsky *et al*, 2014; Kittle, 2012). They both have hydroxyl (OH) groups in their molecules (Figure 1) but the absence of acetamide group (CH_3CONH_2) in cellulose makes the difference between the two in terms of structure; chitin can thus be regarded as cellulose derivative with CH_3CONH_2 groups.



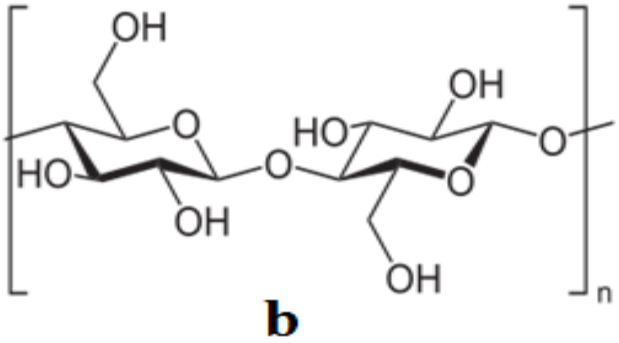


Figure 1: (a) Chitin (b) Cellulose molecular structures (Younes and Rinaudo, 2015; Wang and Uchiyama, 2013)

Exoskeletons of crustacean have been investigated by Aranaz *et al*, (2009) to comprise 30–50 % $CaCO_3$, 30% to 50% $CaCO_3$, 30% to 0 % protein and 20% to 30 % chitin. It was affirmed that there exists a protein-chitin matrix that is calcified to engender shells' hardness (Younes and Rinaudo, 2015). According to Zhao, *et al*, (2010), protein is covalently bonded with chitin via aspartyl or histidyl residues (or both) and thus produces a stable complex of glycoproteinsm. Chemical extraction technique is a conventional method of isolating chitin from crustacean shells where $CaCO_3$ is often removed using organic or mineral acids (demineralization)

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such as HCl, (Gbenebor *et al*, 2017a) nitric acid, HNO_3 (Amelia *et al*, 2021), sulphuric acid, H_2SO_4 (Ali *et al.*, 2019), acetic acid, CH_3COOH (Gbenebor *et al*, 2018), and formic acid, HCOOH (Charoenvuttitham *et al*, 2006). Deproteinization entails protein removal (mostly with NaOH), which is usually carried out after $CaCO_3$ is completely removed. Considering both stages, parameters such as reagent concentration, reaction time temperature and solute/solvent ratio, are often considered to yield desirable results (Abdulkarim *et al*, 2013; Yen *et al*, 2009). The crustacean to be considered in this study is crab. Crabs are decapods found in oceans, fresh water and some lands in designated regions of different countries of the world. In Nigeria, crab is one of the sea foods that are being consumed by some coastal dwellers in the part of the country. It is a source of protein (especially amino acids) and minerals, which is useful to the body Chitin chemically extracted from crab shells has found usefulness in the food industry (Harkin *et al*, 2019), water treatment (Rodriguez et al, 2009) and biomedical applications (Islam *et al*, 2017). Different parameters considered during the isolation process as stated earlier is responsible for chitin's diverse properties which gives rise to its different responses during applications.

Demineralization of 65 μm crab shell particles has been done with 1 M HCl at room temperature (Yen *et al*, 2009). Protein removal was followed for 3 h with the use of 1:10 (w/v) NaOH aqueous solution at 100 ^{0}C. Chitin extracted via this treatment was compared with that which was further treated with 1 % potassium permanganate ($KMnO_4$) solution followed by a similar concentration of oxalic acid ($C_2H_2O_4$). Each treatment lasted for 1 h for the purpose of pigments removal to yield pure chitin. Sharp peaks from Wide-angle X-ray diffraction (WAXD) were exhibited with the purified samples which justified their dense crystalline features. Unlike the untreated chitin, morphological characterizations revealed well-arranged micro fibrils in the purified variant. Yen *et al*, (2009) affirmed that acid and alkali treatments may not be sufficient for obtaining pure chitin; inclusion of depigmentation after treatments would achieve this. Different concentrations of 2.5% and 7.0% v/v HCl were used in isolating minerals (carbonates and phosphates) from 0.297 mm shells of crabs and shrimps (Andrade et al, 2012). Deproteinization and depigmentation were performed with the use of 5% w/v NaOH and 0.36% v/v solution of sodium hypochlorite (NaOCl) respectively to obtain pure chitin samples. More chitin (56%) was isolated from crab shells compared to 41 % realized from shrimp exoskeleton. Chitin from both sources exhibited similar particle geometry which was fine and irregular while 400 ^{o}C - to 500 ^{o}C measured the decomposition temperature range of CH_3CONH_2 groups in both chitin molecules. In contrast, the investigation by Isa *et al*, (2012) reported that 8.15% chitin was sourced from shrimp shells while crab shell yielded 7.8, 2%. They employed 1 M each of HCl (at room temperature) and NaOH (at 100 ^{0}C) for demineralization and deproteinization processes. Shrimp chitin was noticed to be more crystalline as sharp peaks were displayed in its XRD. Crab chitin displayed broad and less intense peaks. Comparing crab and shrimp exoskeletons, Gbenebor *et al*, (2016) reported that there lies a strong interaction between $CaCO_3$ and chitin, which resulted to more consumption (ml) of 0.4 M HCl used for demineralization of both shells. Their investigation showed that more chitin was yielded from the shell of shrimp than that of the crab. It may thus be said that the harder the shells, the more the volume of HCl (depending on the concentration) consumed. This was realized with chitin isolated from periwinkle and snail shells (Gbenebor *et al*, 2017b; Akpan *et al*, 2018). Diverse works have presented the use of various concentrations of demineralization and deproteinization reagents (in addition to depigmentation) to obtain pure chitin from crabs at different instances; it will be pertinent to investigate how these two processes will influence the features of chitin isolated from the same source. In this study, 0.4, 0.8 and 1.2 M HCl with 0.4 and 1.2 M NaOH were used for demineralization and deproteinization of aquatic crab shells.

II. MATERIALS AND METHODS

A. *Materials*

Crabs used for this study were sourced from fresh water environment located in Badagry, Lagos state. Analytical grade NaOH pellets (LOCA CHEMIE PVT. LTD) were used for deproteinization while 37.0% analytical grade HCl (CHEMSOLUTE) was used for demineralization. Depigmentation was carried out with the use of LabChem 30% hydrogen peroxide (H_2O_2).

B. *Extraction of chitin*

Crab shells were washed, dried and milled to *2*50 μm particle sizes. At room temperature, demineralization was carried out using 0.4, 0.8 and 1.2 M HCl after which particles were continuously washed with distilled water to neutral pH to leave a residue devoid of minerals and chlorine. Dried demineralized particles were refluxed with 0.4 and 1.2 M NaOH at 100 ^{0}C for 1 h to complete the deproteinination process. Particles were washed with distilled water to neutral pH, filtered and dried in an oven at 70 ^{0}C. To remove pigments that may be present, chitin particles were soaked in 1 M H_2O_2 overnight at room temperature. Chitin particles were dried after being washed and filtered as done earlier.

C. *Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray Analysis (EDS)*

Morphology of chitin particles was observed with the use of an ASPEX 3020 model variable pressure SEM equipment attached to Noran-Voyager energy dispersive spectroscope.

D. *X-Ray Diffraction (XRD)*

Crystallinity index and crystallite sizes from samples' diffractogram were recorded with the use of PAN analytical X' Pert PRO MPD X-ray diffraction system PW3040/60 equipment, where a monochromatic Cu Kα radiation (k = 1.5406 Á) at 40 kV and 40 mA were deployed.

The expression in Eqn. 1 was used in calculating crystallinity index. (Juarez de-la Rosa *et al*, 2015) while Eqn. 2 was used in calculating the crystallite size normal to the h k l plane (Wang et al, 2013).

$$Crl = \left(\frac{I_c}{I_c + I_a}\right) x\ 100 \quad (1)$$

$$D_{hkl} = \frac{k\lambda}{\beta cos\ \theta} \quad (2)$$

Where I_c and I_a represent the intensities of the crystalline and amorphous regions respectively.

K is a constant assumed to be 1 It is an indicative of crystallite perfection; λ(Å) is the wave length of incident radiation (1.5406 Å); β (rad) is the width of the crystalline peak at half height, and θ (deg) is the diffraction angle corresponding to the crystalline peak.

E. Thermogravimetric Analysis (TGA)

Two milligram of chitin particles was heated from 0 to 50 ^oC at the rate of 10 ^oC/min using TGA Q500 instrument. Temperatures for the commencement of thermal decomposition (Tonset), rapid decomposition (Tmax) and end of decomposition (Tfinish) were determined from the thermal curves.

Activation energy, Ea was calculated from the TGA curves by employing Broido approach (Broido, 1969) as shown in Eqn. (3):

$$Ln\ (-Ln\ (1-X)) = -\frac{E_a}{RT} + Const. \quad (3)$$

Where E_a is the activation energy of the degradation reaction (kJ/mol), R is the universal gas constant (8.314 J/mol·K) and T is the absolute temperature (K). The degree of decomposition X is given by Eqn. (4):

$$X = \frac{W_o - W_i}{W_o - W_f} \quad (4)$$

Where W_o is the initial weight of the sample, W_i is the instantaneous weight of the sample at time t and W_f the final weight of the sample. The plot of *Ln (- Ln (1- X))* against 1/T gives a straight line whose slope is –E/R.

III. RESULTS AND DISCUSSIONS

A. SEM/EDS

Presence of calcium (Ca) and oxygen (O) atoms with strong peak intensities in EDS of virgin crab shell particles shown in Figure 2a is an indication of $CaCO_3$ existence which is dominant in the exoskeleton. Treatment with 0.4 M HCl and NaOH yields 96% and 4.8% reduction in $CaCO_3$ and O, respectively with 4.2% and 68% increase in nitrogen (N) and carbon (C) (see Figure 2b) when compared with that of virgin crab shell particles. This implies that acid and alkali treatment play a significant role in removing $CaCO_3$ drastically and increase the formation of C-N prevalent in chitin. Increasing the acid concentration to 0.8 M (Figure 2c), elimination of $CaCO_3$ remains 96% while C increases by 2%. This shows that higher concentration of HCl also imparts changes on the shell's surface with more of C enrichment. Morphology of virgin crab particles (Figure 2a) is granular and fibrillar in structure while treatment with 0.4 and 0.8 M HCl (Figure 2b and c) show nodule-like fibrils without agglomerations. Using the highest concentration of 1.2 M HCl (Figure 2d) produces a surface morphology of agglomerated fibrils.

Morphologies of chitin samples extracted with the use of 0.4, 0.8 and 1.2 M HCl with a higher concentration of deproteinization reagent (1.2 M NaOH) are shown in Figure 3. When compared with the morphology of virgin crab shell (see Figure 2a), it is evident that these acid concentrations possess the potency of eliminating a much reasonable amount of $CaCO_3$ as reduction amounts of97%, 96% and 98% of the mineral are recorded for 0.4, 0.8 and 1.2 M HCl. With these order of concentrations, there exist 36%, 42% and 35% increase in N content when compared to virgin crab shell. This also justifies the formation of more N-acetyl groups (coupled with increase in C content) in isolated chitin than that embedded in the virgin shell. As observed with 1.2 M HCl/0.4 M NaOH (Figure 2d), demineralizing and deproteinizing at 1.2 M HCl and NaOH also show agglomerated fibrils with shiny surfaces (Figure 3c); this could be an indication of chitin deacetylation.

B. XRD

The XRD pattern of virgin crab shell in Figure 4 shows the dominant crystalline peaks of $CaCO_3$ whose highest intensity is diffracted on (104) at 2θ = 29.5°. Other $CaCO_3$ peaks are indexed on ($11\bar{3}$), ($11\bar{6}$) and (018), which represent diffractions on 2θ = 39.59°, 48.96°, and 47.59^o respectively. Chitin embedded in the exoskeleton are represented on 2θ = 26.84° (013), 23.33° (012), 36.27° (110), 44.2° (202) and 57.57° (122). Results agree with the findings of Rahman *et al*, (2013), Rahman and Halfar, (2014). Chitin in the embedded shell matrix cannot be fully maximized as the mineral plays a dominant role in the properties of the shell such as hardness and thermal stability. This could be the reason why it is often used as concrete aggregates in building constructions (Lim *et al*, 2021).

The XRD patterns of chitin sourced from crab shell after demineralization with 0.4, 0.8 and 1.2 M HCl and deproteinization at 0.4 M NaOH are presented in Figure 5. Calcium carbonate peaks are eliminated and diffractions on 2θ = 12.5°, 26.3°, 20.6° and 19.0° corresponding to (021), (013), (120) and (110), indicate the existence of α-chitin peaks Chitin in its α-form was investigated to maintain peaks at 2θ = 9.6°, 19.6°, 21.° and 23.7°; the β-form, at 2θ =9.1° and 20.3°, while 9.6° and 19.8°are characteristic diffraction angles for γ-chitin (Jang et al, 2004) .Crystallinity index for 0.4 M HCl/0.4 M NaOH, 0.8 M HCl/0.4 M NaOH and 1.2 M HCl/0.4 M NaOH are 71.0%, 68.8% and 67.6%. A comparable pattern is also displayed with the use of similar concentration range of HCl (as earlier employed) and 1.2 M NaOH (Figure 6). At this alkali concentration, the maximum crystallinity index of 68.9 % is calculated with the use of 0.4 M HCl. This is followed by 67.9 % (use of 0.8 M HCl) and the least 65.5% when 1.2 M HCl is used in the isolation process. It can be observed in this study that raising the concentrations of both acid and alkali solutions lowers the crystallinity index of chitin. Gbenebor et al, (2017a) reported that reduction in chitin's crystallinity implies a distortion in its structure which would give room for aqueous solution penetration that facilitates degradation. This is an indication of gradual transformation of CH_3CONH_2 to NH_2 (amino) groups as it happens in chitosan formation. Material such as this is important for biomedical applications - it possesses mechanical strength owing to the presence of CH_3CONH_2 in its chains (Cui *et al*, 2016) and degrades in aqueous media by virtue of gradual depletion of CH_3CO (acetyl) groups. Table 1 shows the crystallite sizes (a measure of the coherent volume for chitin in the diffraction peak) of

each sample. This study has shown that chitin with a reduced crystallite size is more structurally arranged (compact) than the large one; hence, as the crystallite size of chitin increases, its

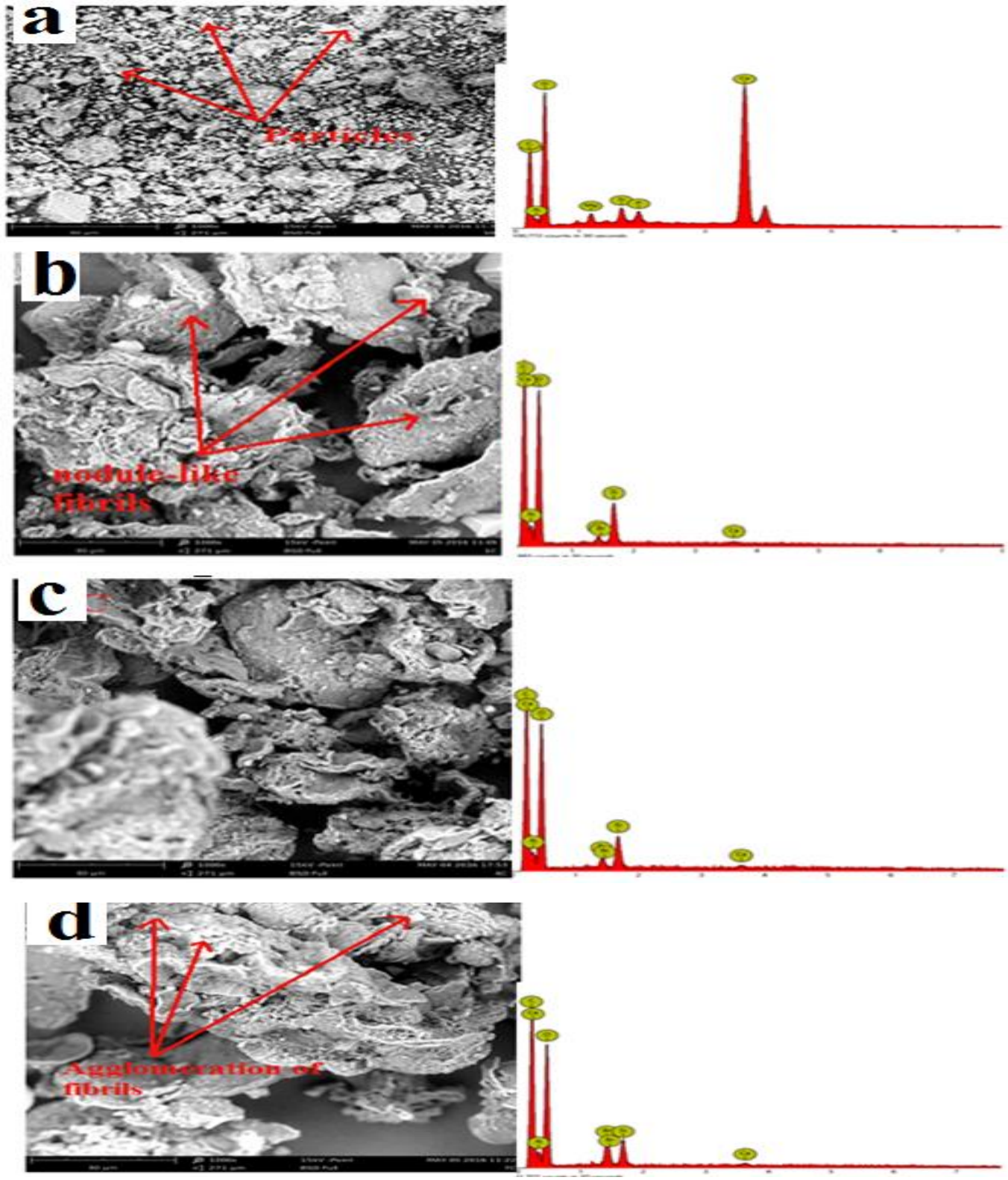


Figure 2: SEM/EDS of (a) Virgin crab shell (b) shell demineralized with 0.4 M (c) 0.8 M (d) 1.2 M HCl each deproteinized with constant 0.4 M NaOH (x 1000).

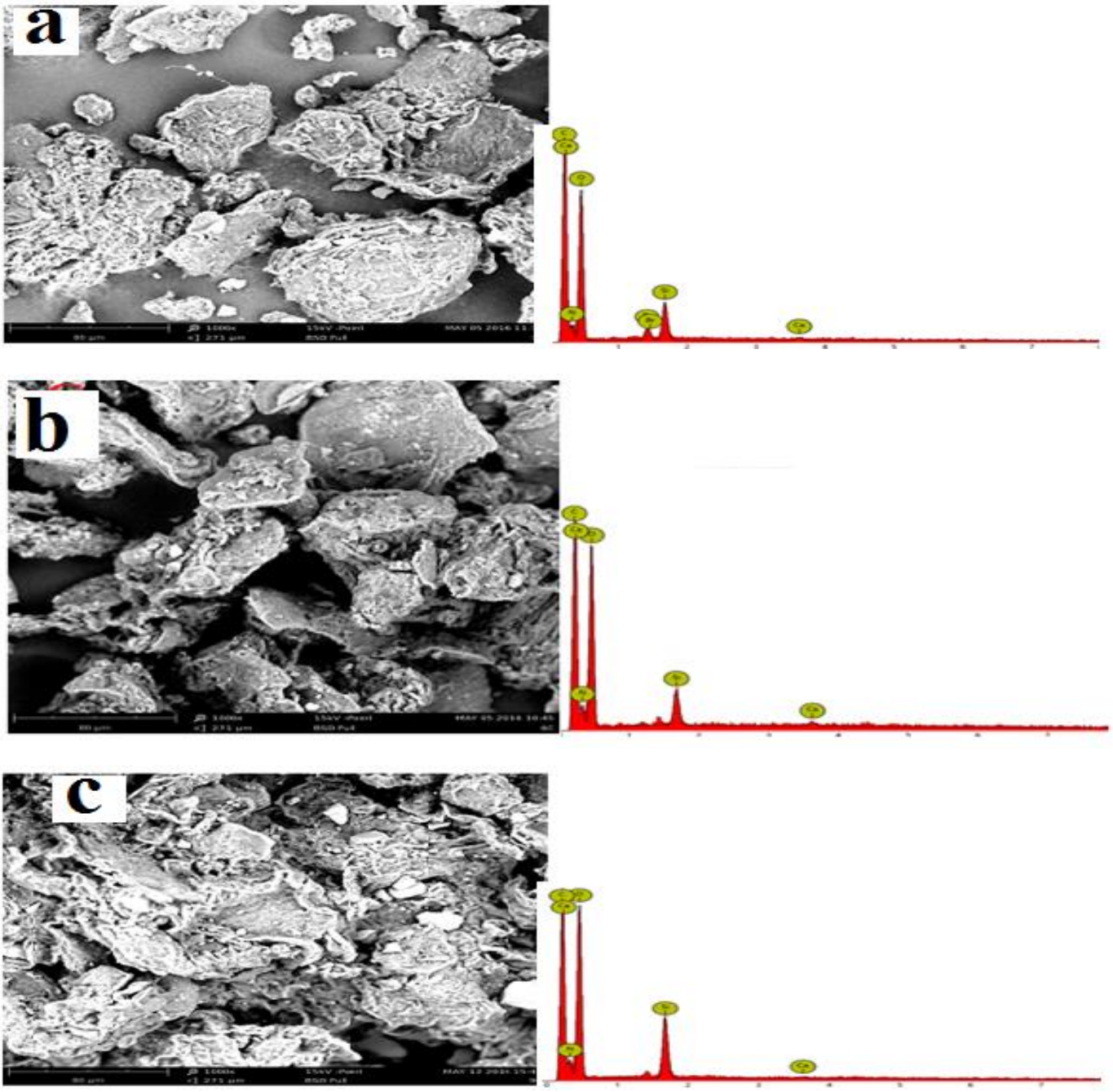


Figure 3: SEM/EDS of (a) Virgin crab shell (b) shell demineralized with 0.4 M (c) 0.8 M (d) 1.2 M HCl each deproteinized with constant 1.2 M NaOH (x 1000)

crystallinity index declines. As illustrated in the Table, increase in demineralization and deproteinization concentration is responsible for the gradual widening of crystallite size and depreciation of crystallinity index of chitin.

C. TGA-DTG

The TGA for virgin crab shell shown in Figure 7 shows a three-stage decomposition curve with the first representing 3.88 % weight loss between 60 °C to 100 °C. A temperature range of 316 °C to 397 °C is observed at the second stage with 10.38% weight loss. The maximum weight loss in virgin crab shell TGA curve (30.93%) occurs at the highest magnitude of temperature range between 682 °C and 729 °C. The first decomposition stage is attributed to the elimination of water; the second shows the presence of chitin embedded in the shell while presence of $CaCO_3$ is evident in the third stage. The maximum temperature at which the decomposition was rapid (T_{max}) is represented by the DTG curve. This occurs at 356.8 °C and 717.07 °C for embedded chitin and $CaCO_3$ respectively; residue content of 54.89% was yielded from the thermal process.

Thermal curves for chitin shown in Figure 8 a- f re characterized by two decomposition stages, where the first is

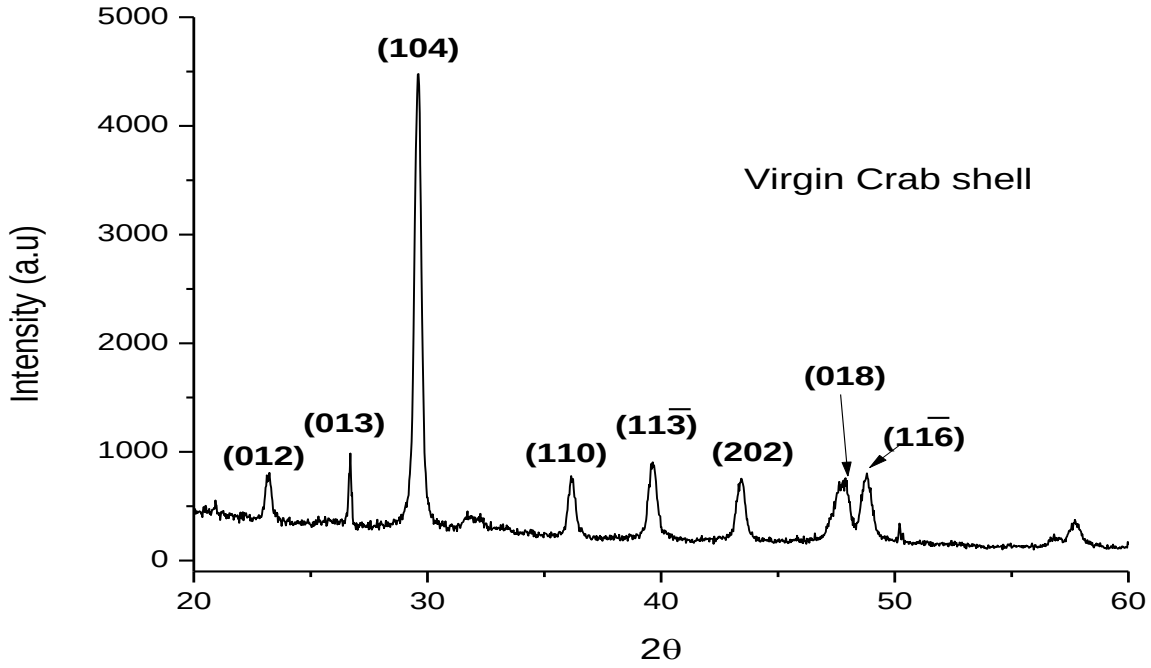


Figure 4: XRD of virgin crab shell

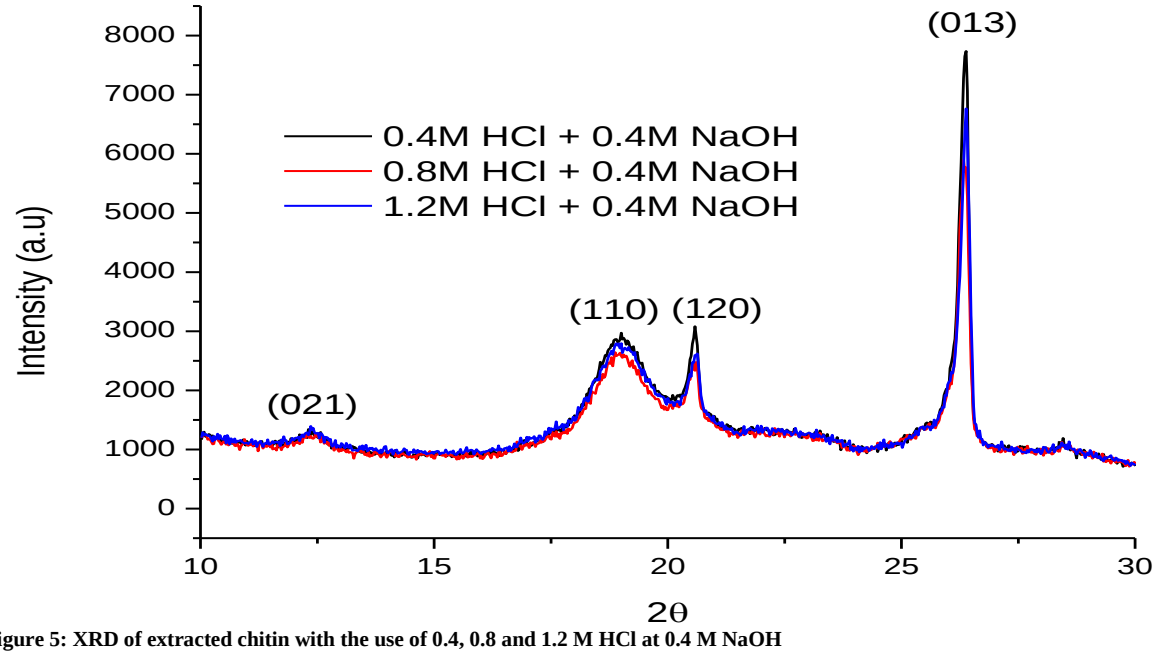


Figure 5: XRD of extracted chitin with the use of 0.4, 0.8 and 1.2 M HCl at 0.4 M NaOH

attributed to water loss and the second, the presence of chitin. Each chitin sample has no evidence of $CaCO_3$ presence as thermograms are devoid of a third decomposition stage. More chitin is yielded on removal of $CaCO_3$ showing that there exists a strong $CaCO_3$ – chitin bond which needs to be broken to achieve more pure chitin yield (Gbenebor *et al*, 2016). With the different acid and alkali concentrations, decomposition of chitin occurs between 340 and 416 ^oC and T_{mx}, between 367 ^oC and 398 ^oC. The activation energy, E_a (Figure 9) is used in this study as a measure of thermal stability of samples. This value

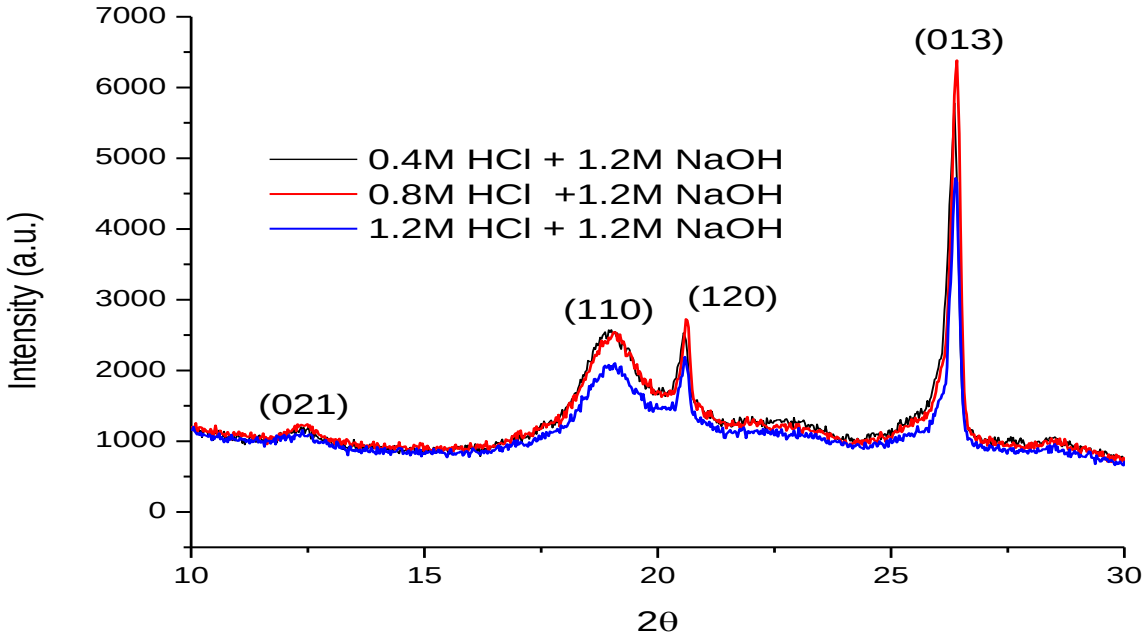


Figure 6: XRD of extracted chitin with the use of 0.4, 0.8 and 1.2 M HCl at 1.2 M NaOH

Table 1: Crystallite properties of chitin

S/N	Acid: Alkali (M)	D_{110} (Å)	D_{120} (Å)	D_{013} (Å)	CrI (%)
1	0.4:0.4	0.02730	0.0759	0.0296	71.0
2	0.8:0.4	0.02860	0.0878	0.0316	68.8
3	1.2:0.4	0.02957	0.0989	0.0334	67.6
4	0.4:1.2	0.02828	0.0847	0.0312	68.9
5	0.8:1.2	0.02930	0.0961	0.0330	67.9
6	1.2:1.2	0.03110	0.1216	0.0362	65.5

gradually decreases when HCl concentration is increased at constant NaOH concentration; same is witnessed when NaOH concentration is varied at constant HCl concentration. The maximum magnitude of 112.54 kJ/mol is recorded when 0.4 M HCl and 0.4 M NaOH are used in the isolation process while the least (80.12 kJ/mol) is calculated for chitin extracted with the use of 1.2 M HCl and 1.2 M NaOH. All isolated chitin samples are thermally stable than the one embedded in the shell (52 kJ/mol) and this implies that chitin will not be well maximized unless in its pure form. Presence of $CaCO_3$, protein and other pigments in the shell could be attributed to this.

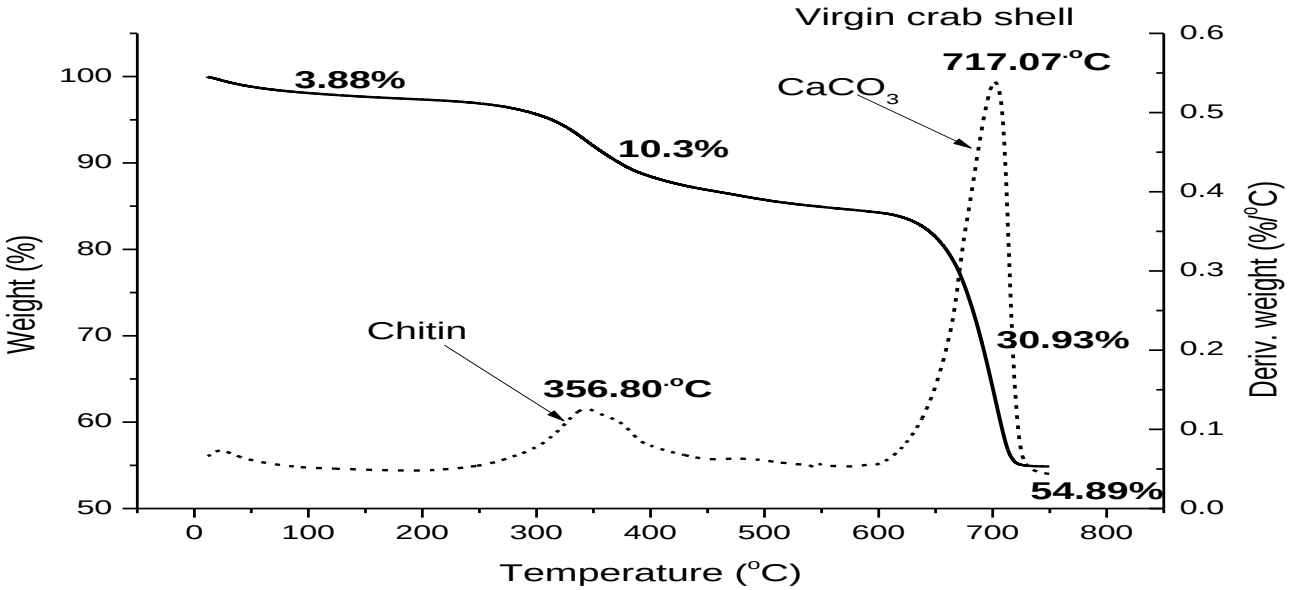
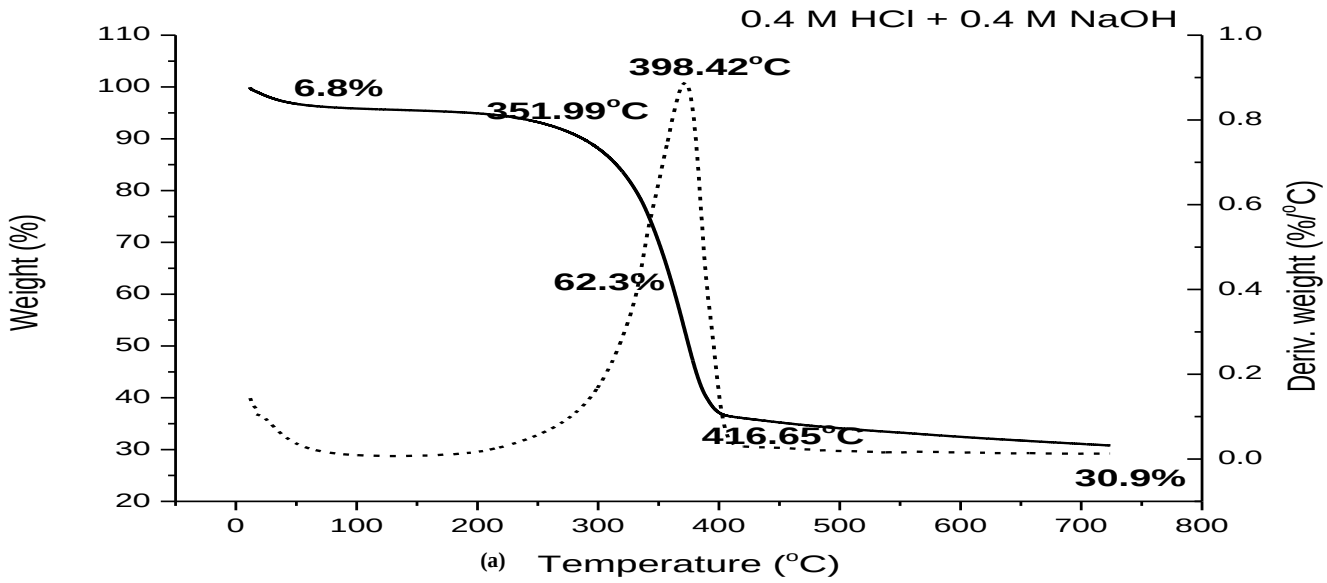
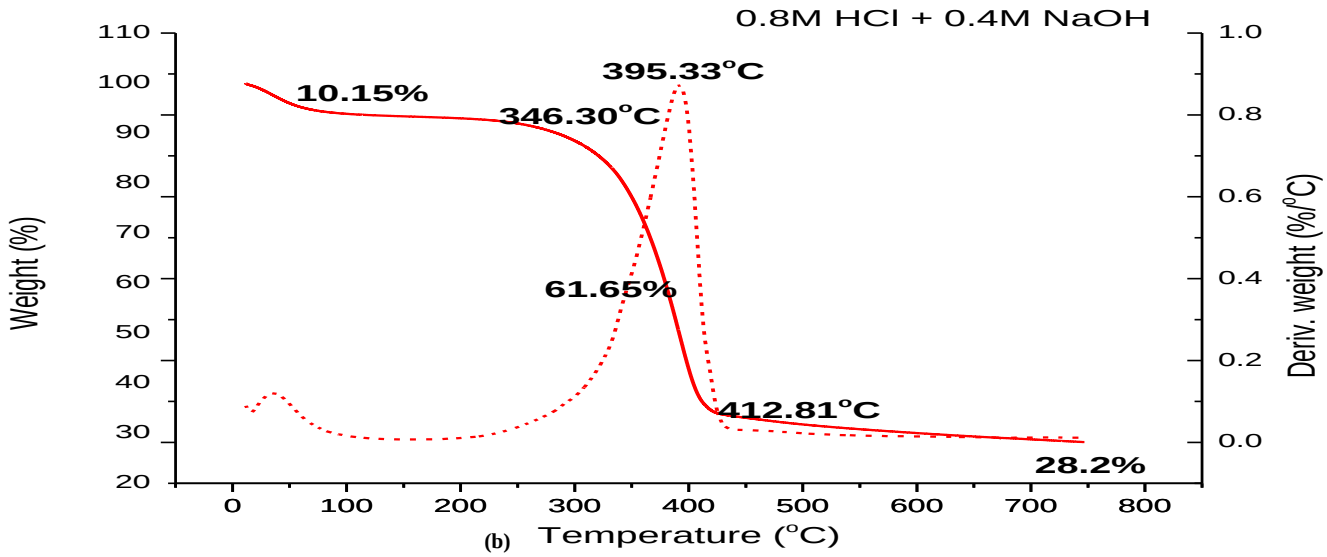


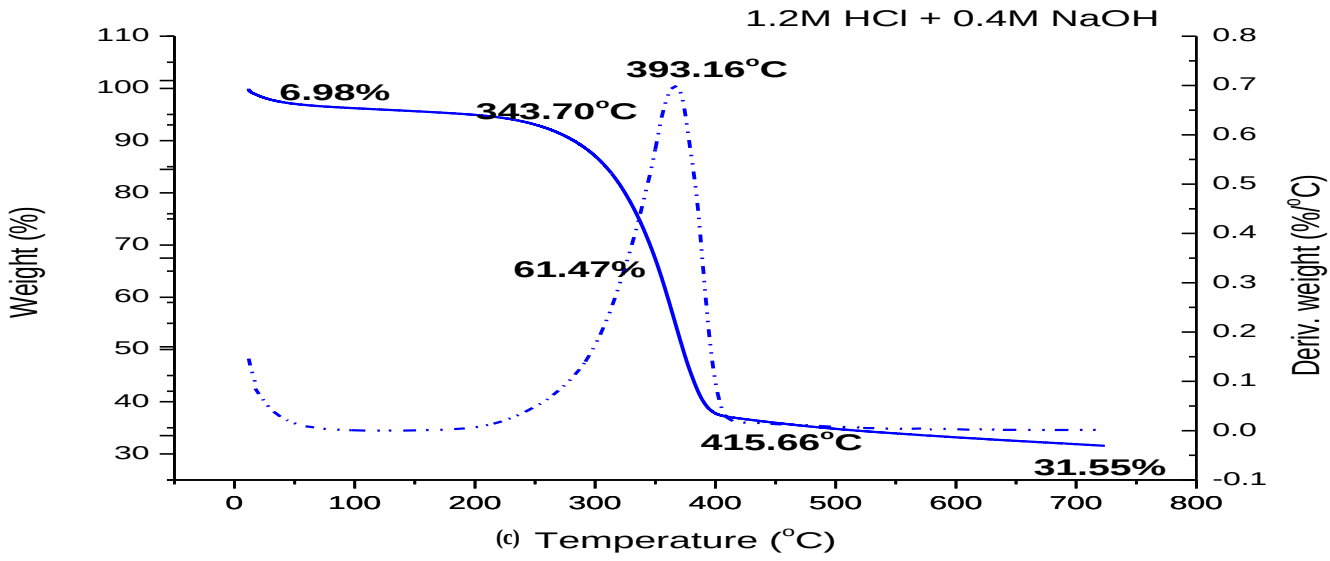
Figure 7: TGA-DTG curve for virgin crab shell


0.4 M HCl + 0.4 M NaOH
6.8%
351.99°C
398.42°C
62.3%
416.65°C
30.9%
Weight (%)
Deriv. weight (%/°C)
Temperature (°C)

(a)


0.8M HCl + 0.4M NaOH
10.15%
346.30°C
395.33°C
61.65%
412.81°C
28.2%
Weight (%)
Deriv. weight (%/°C)
Temperature (°C)

(b)


1.2M HCl + 0.4M NaOH
6.98%
343.70°C
393.16°C
61.47%
415.66°C
31.55%
Weight (%)
Deriv. weight (%/°C)
Temperature (°C)

(c)

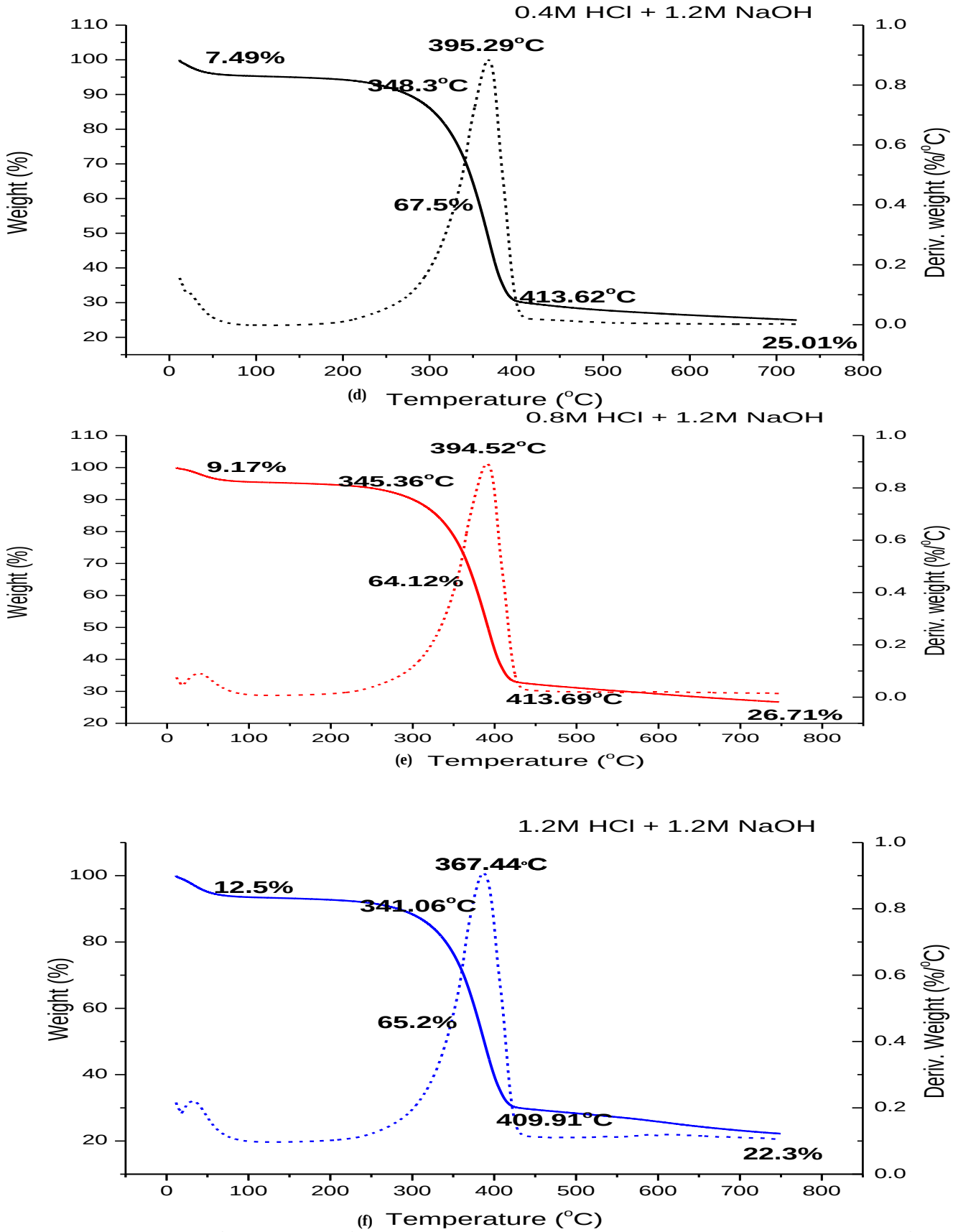


Figure 8: TGA-DTG curves for chitin extracted from crab shell with (a) 0.4 (b) 0.8 (c) 1.2 M HCl and 0.4 M NaOH (d) 0.4 (e) 0.8 and (f) 1.2 M HCl and 1.2 M NaOH

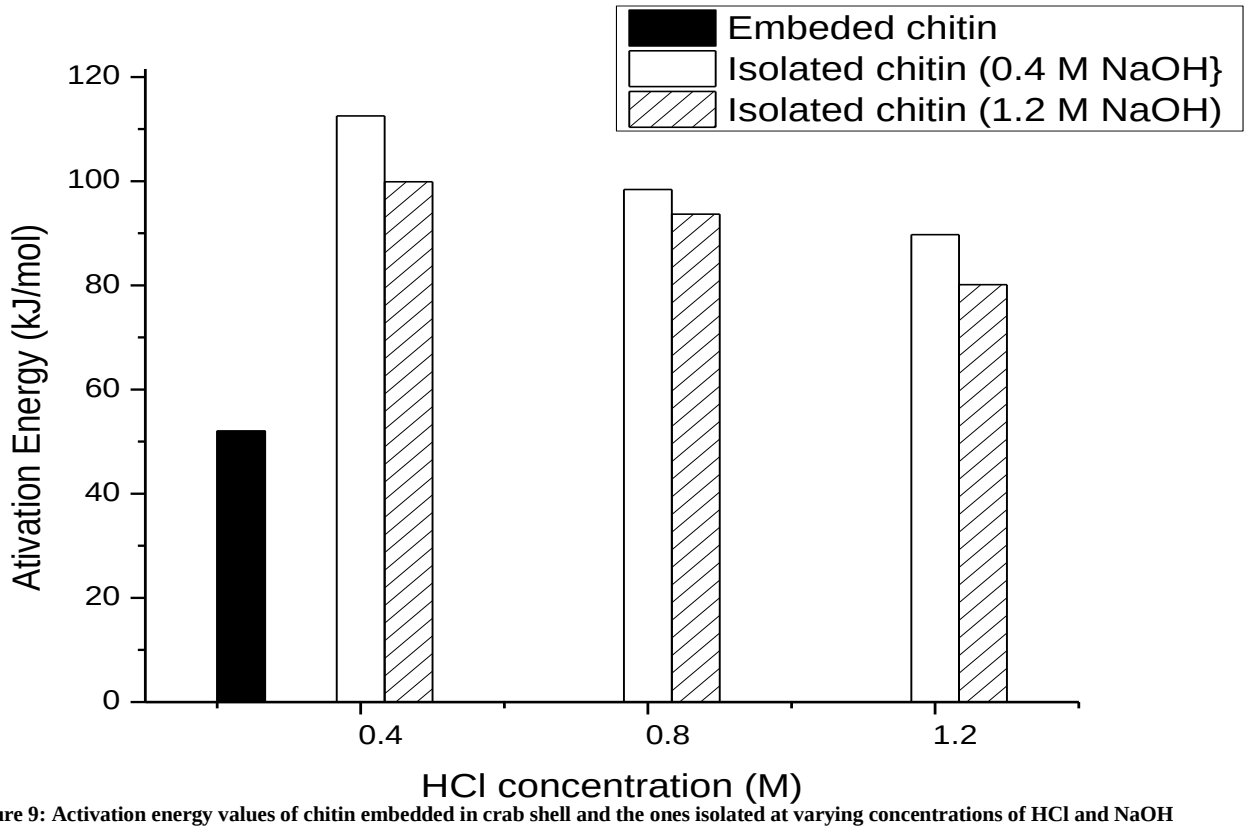


Figure 9: Activation energy values of chitin embedded in crab shell and the ones isolated at varying concentrations of HCl and NaOH

As observed with gradual declining of crystallinity index (Figures 5 and 6), reduction of *N*-acetyl (GlcNAc) units in chitin are engendered by high concentrations of reagents which in turn lower chitin's thermal stability. The high $T_{onset} - T_{finish}$ of all isolated chitin samples (341 ^oC to 416 ^oC) qualifies them as potential materials in the packaging of foods at low and elevated temperatures.

IV. CONCLUSION

This study focused on how thermal and structural properties of chitin can be affected via chemical treatment of crab shell particles. Chitin has been successfully isolated from crab exoskeleton with different concentrations of demineralization and deproteinization reagents using HCl and NaOH respectively. There is reduction I crystallinity index and thermal stability of chitin as concentration of HCl and NaOH increases; morphologies of chitin particles are also affected. This is an indication of gradual removal of C=O in CH_3CONH_2 to form NH_2. Variation in mechanical strength and hydrophylicity will be imparted. It can be concluded that synthesizing chitin of tailored features for different applications can be achieved by varying concentrations of HCl and NaOH as a chemical method of chitin isolation.

AUTHOR CONTRIBUTIONS

O.P. Gbenebor: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Writing - original draft; Writing - review & editing. **C.C. Odili**: Methodology. **G.I. Lawal:** Conceptualization; Methodology; Project administration; Resources; Supervision. **S.O. Adeosun:** Conceptualization; Investigation; Methodology; Project administration; Supervision, review & editing.

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