Effect Of Degrees Of Deacetylation On The Antimicrobial Activities Of Chitosan

ENYERIBE C.C, KOGO A.A, YAKUBU M.K, OBADAHUN J, AGHO B.O, KADANGA, B

ABSTRACT: Chitosan, a natural polysaccharide polymer derived from chitin and was investigated for the effect of degree of deacetylation and its antimicrobial activity. The chitin was subjected to modification using 30%, 40% and 50% NaOH to obtain chitosan of various degree of Deacetylation and were subjected to physiochemical analysis such as: solubility, moisture content, ash content, viscosity and degree of deacetylation, Ultra-violet visible spectroscopy (UV-Visible), Fourier-Transform Infrared Spectroscopy Analysis (FT-IR) spectroscopy, Differential Scanning Calorimetry (DSC) and the antimicrobial study (zone of inhibition, minimum inhibitory concentration and minimum bacteriocidal/ fungicidal concentration) The degree of deacetylation was 80% when 50% w/v NaOH was used and 67% 30% w/v NaOH, which shows that as the concentration of NaOH increases the more acetyl groups are been removed.

Keywords. Chitin, Chitosan, Deacetylation and antimicrobial study

1.0 INTRODUCTION
Chitin, poly [β-(1-4)-N-acetyl-D- glucosamine] is a natural polysaccharide of major importance, first identified in 1884. It consist of 2-acetamide-2-deoxy-β-D-glucose through a β – (1,4) linkage (poly-N-acetyl-D-glucosamine). This kind of linear muco-polysaccharide is abundant in nature mainly present in animal support or tissue of lower evolutionary organisms such as crustaceans, insects and mushrooms, only less abundant in nature than cellulose[1]. Chitin, the second-most abundant biopolymer, and its deacetylated product, chitosan are high molecular- weight biopolymers and are recognized as versatile environmentally friendly raw materials in many applications [2]. Chitosan, poly [(1,4)-β-linked 2-amino-2-deoxy-D-glucose] [polyglucosamine], is the N-deacetylated form of chitin with amino groups along with the chitosan structure (polyglucosamine) also known as 2-amino-2-deoxy-(1,4)-β-D-glucopyranan[3][4]. This N-deacetylated is almost never complete. Chitosan is considered to be the most widely distributed biopolymer, it is a cationic, non-toxic, biodegradable and biocompatible polyelectrolyte with a pKa of approximately 6.5[5][6]. The solubility of chitosan in acidic solutions is the generally accepted criterion for identifying chitin and chitosan. Chitosan polymer is hydrophobic in nature although consisting of hydrophilic functional groups such amine groups and hydroxyl groups. The polycation chitosan is normally insoluble in water at near neutral pH and in most common organic solvents such as dimethylsulphoxide (DMSO) and organic alcohols, mainly due to its crystalline structure with extensive intra-molecular and intermolecular hydrogen bonding [7]. Chitosan is a semi-crystalline polymer in its solid state. The crystallinity of chitosan is lower than that of chitin, the free amino groups at the C2 position of the polymer backbone (D-glucosamine unit) are protonated and the polymer becomes fully soluble below pH 5[8]. Therefore, chitosan salt particles or flakes or other form-like polymers can be prepared from these aqueous solutions.

2.0 MATERIALS AND METHODS

2.1 Materials
Chitin, Hydrochloric acid, sodium hydroxide, acetic acid, Mueller Hinton agar, Mueller Hinton broth, potato dextrose agar and nutrient agar (oxido),Thermometer, beaker, crucible, desiccator, conical flask, cork borer, burette, Heating mantle, magnetic stirrer, brookfiedsynchr-lectic viscometer, Agilent pH meter, Agilent FT-IR spectrophotometer, Agilent UV-Visible
spectrophotometer, TA instrument differential scanning calorimeter, autoclave and incubator.

2.2 Methodology

2.2.1 Conversion of Chitin to Chitosan (Deacetylation)
The chitin was deacetylated in 30% (w/v) NaOH for 5 hours at 100°C using a heating mantle. After deacetylation, the chitosan was washed thoroughly with water followed by distilled water. The resulting chitosan was dried to constant weight at 65°C. Further deacetylation were carried out using 40% and 50% (w/v) NaOH, to obtain other samples with different degrees of deacetylation as shown in the reaction scheme below.

![Scheme 1: Extraction of chitosan from chitin](image)

2.2.2 Determination of Degree of Deacetylation
Dried chitosan (0.2 g) was dissolved in 20 cm³ 0.1 M hydrochloric acid and 25 cm³ deionized water. After 30 minutes continuous stirring, next portion of deionized water (25 cm³) was added and stirring continued for 30 minutes. When the chitosan completely dissolved, the solution was titrated with a 0.1 M sodium hydroxide solution using a pH meter to monitor the pH. Degree of deacetylation (DA) of chitosan will be calculated using formula:

\[ DA(\%) = \frac{V_2 - V_1}{M + 0.0042(V_2/V_1)} \times 100 \text{ Equation 1} \]

2.2.3 Solubility Analysis
Solubility analysis of the sample was performed in different selected solvents. For this purpose, the sample was added to the solvent at the concentration of 5 mg/mL at 25°C and their solubility was evaluated.

2.2.4 Determination of Percentage Moisture Content
The crucible was dried in an oven at 80°C for 20 minutes, cooled in a desiccator and weighed (W₁)g. 2g of the sample was then placed into the crucible and reweighed (W₂)g, the crucible with the sample was dried in the oven at 105°C until a constant weight was obtained after successive cooling in desiccator and weighing. It was finally transferred from the oven to the desiccator to cool and then quickly weighed (W₃)g. The percentage moisture content was calculated using formula:

\[ % \text{Moisture content} = \frac{(W_2 - W_3) \times 100}{W_2 - W_1} \text{ Equation 2} \]

2.2.5 Determination of Ash Content
The crucible was dried by holding in the Bunsen flame for about two minutes, then transferred into a desiccator to cool before weighing (W₁)g. 2g of the sample was weighed inside the crucible (W₂)g, the crucible with the sample was heated gently in a Bunsen burner in a fume cupboard till the smoke ceased, which was then transferred to the muffle furnace, preheated at 550°C. The heating was continued until all the carbon had been burnt away; the crucible was taken away with a pair of tong and immediately covered and placed in a desiccator to cool before weighing (W₃)g. The ash content was calculated using the formula:

\[ \% \text{Ash Content} = \frac{(W_3 - W_1) \times 100}{W_2 - W_1} \text{ Equation 3} \]

2.2.6 Viscosity
Viscosity of chitosan was determined with a Brookfield. Chitosan solution was prepared in 1% acetic acid at a 1% concentration on a dry basis at 25°C.

2.2.7 Fourier-Transform Infrared Spectroscopy Analysis (FT-1R)
The sample was thoroughly mixed with KBr, the dried mixture was pressed which resulted into a homogeneous sample disk. The measurement was carried out over the frequency range of 400-4,000 cm⁻¹ using the Agilent FTIR spectrophotometer [9].

2.2.8 UV-Visible Spectroscopy (UV-VIS)
The UV-Visible absorption spectra were measured using Agilent UV-visible spectrophotometer. 0.1M acetic acid solution of the sample was used while for the cationated samples distilled water was used and the spectra was recorded from 200-650nm wavelength frequency range [10].

2.2.9 Differential Scanning Calorimetry (DSC)
The differential scanning calorimeter (TA Instrument, Q200, USA) was employed to study the thermal property of the sample. 2.5mg of the sample was placed in an aluminum pan and sealed. The lid was perforated before sealing. Empty closed aluminum pan was used as the reference. Samples was scanned from a temperature range of 50°C to 450°C at a heating rate of 10°C/min under nitrogen atmosphere.

2.3 Antimicrobial Profile (Sensitivity Test) of the polymer using agar well diffusion method.
The standardized inocula of both the bacterial and fungus isolates were streaked on sterilized Mueller Hinton and potato dextrose agar plates respectively with the aid of a sterile swab sticks. Wells were pounced on each inoculated plates with a sterile cork borer with a diameter of 6mm. The wells were properly labeled according to different concentrations of the polymer which were 50, 25, 12.5 and 6.25mg/ml. each well was filled up with approximately 0.2ml of the polymer solution. The inoculated plates with the polymers were allowed to stay on the bench for about one hour, this was to enable the polymer solution to diffuse into the agar. The plates were then incubated at 37°C for 24 hours (plates Mueller Hinton agar) while the plates of potato dextrose agar were
incubated at room temperature for about 3-5 days. At the end of the incubation period, the plates were observed for any evidence of inhibition which appeared as a clear zone that was completely devoid of growth around the wells (zone of inhibition) the diameter of the zones were measured using a transparent ruler calibrated in millimeter.

2.3.1 Determination of Minimum Inhibitory Concentration (MIC)
The minimum inhibitory concentration of the sample was determined using the broth dilution method[11][12]. Mueller Hinton broth was used as the diluent. The lowest concentration of the polymer solution showing inhibition for each organism when the polymer solution was tested during sensitivity test was serially diluted in the test tubes containing Mueller Hinton broth. The organisms were inoculated into each tube containing the broth and the polymer solution. The inoculated tubes were then incubated at 37°C for 24 hours. At the end of the incubation period the tubes were examined for the presence or absence of growth using turbidity as a criterion, the lowest concentration in the series without visible sign of growth (turbidity) was considered to be the minimum inhibitory concentration (MIC).

2.3.2 Determination of Minimum Bactericidal/ fungicidal Concentration (MBC/MFC)
The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal concentration (MBC) of the polymers. A sterilized wire loop was dipped into the test tubes that did not show turbidity (clear) in the MIC test and a loopful was taken and streaked on a sterile nutrient agar plates. The plates were incubated at 37°C for 18-24 hours. At the end of incubation period, the plates were examined for the presence or absence of growth. This is to determine whether the effects of the polymers are bacteriostatic or bacteriocidal[11][12].

3.0 RESULTS AND DISCUSSION
Synthesis of chitosan was carried out by heating chitin from shrimp with various percent Concentrations of sodium hydroxide 30%, 40% and 50% so as to obtain chitosan of various degrees of deacetylation, the chitosan obtained was dissolved in 1% acetic acid for solubility which is an indication that chitosan has been synthesized,[13] stated that the solubility of chitosan also controlled by the distribution of the acetyl groups remaining along the chain. The deacetylated chitin (chitosan) obtained was tested with acetic acid to see its solubility in organic acid, they were all soluble in 1% acetic acid but with a variation in solubility, 50% w/v NaOH chitosan had the highest solubility, followed by that of 40% w/v NaOH chitosan and 30% w/v NaOH chitosan had the least solubility this can be attributed to the various degree of deacetylation of the chitin. Thus, the more deacetylated the chitin is, the better the solubility. This is a necessary condition to ascertain that chitosan have been obtained from chitin, since chitin is not soluble in acetic acid. The moisture content of the chitosan ranges from 8.07%-11.6%, that produced using 30% w/v NaOH has 8.07% moisture content, 40% w/v NaOH had 9.86% moisture content and 50% w/v NaOH had 11.60% moisture content this is because as the degree of Deacetylation increases the polymer becomes less crystalline and absorbs more water, the permitted level is below 20%. The moisture content may vary depending on season, relative humidity and intensity of sunlight [14]. The ash content of the 50% w/v NaOH chitosan is 6.02%, while that of 40% w/v NaOH is 5.97% and 30% w/v NaOH is 5.88%. The ash content is an indication of the effective removal of inorganic minerals from shrimp. The ash content is due to the presence of calcium carbonate which is found in large amount in shrimp shells[15]. Viscosity is an important factor in the conventional determination of molecular weight of chitosan[16]. Higher molecular weight chitosan often provides highly viscous solutions, which may not be desirable for industrial application. The chitosan extracted have a viscosity of 144.9 cp, 131.8cp and 108.3cp for 30% w/v NaOH, 40% w/v NaOH and 50% w/v NaOH respectively. From literature the viscosity of chitosan general ranges from 60 to 780cp[17]. The decrease in viscosity of the extracted chitosan as the concentration of NaOH increases may be attributed to the increase in the degree of deacetylation. The viscosity of chitosan is affected by some factors such as temperature, pH, ionic strength, concentration, molecular weight and degree of deacetylation [18].

Table 1: physicochemical characterization of chitosan

<table>
<thead>
<tr>
<th>Parameters</th>
<th>30% NaOH</th>
<th>40% NaOH</th>
<th>50% NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD%</td>
<td>67.02</td>
<td>74.64</td>
<td>80.39</td>
</tr>
<tr>
<td>Solubility (1% acetic acid)</td>
<td>soluble</td>
<td>soluble</td>
<td>soluble</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>8.07</td>
<td>9.86</td>
<td>11.6</td>
</tr>
<tr>
<td>Ash content (%)</td>
<td>5.88</td>
<td>5.97</td>
<td>6.02</td>
</tr>
<tr>
<td>Viscosity (cp)</td>
<td>144.9</td>
<td>131.8</td>
<td>108.3</td>
</tr>
</tbody>
</table>

The degree of deacetylation of the chitosan extracted was determined using potentiometric titration with a degree of deacetylation of 67%, 73% and 80% for 30% w/v NaOH, 40% w/v NaOH and 50% w/v NaOH respectively, which are higher than that reported by Isa et al., (2012), of 50% for shrimp shell lower than the reported degree of deacetylation of 98.38-98.79% achieved by [19] using shrimp shell. This may be attributed to the nature of the raw material used, its immediate environment and also the methods applied during the processes. There is an indication that chitosan has been extracted in the work, since the necessary condition as stated by some literature is that the degree of deacetylation should be above 50% and it should in acidic media [2]. The chitosan was found to dissolve completely in 1% acetic acid.
The UV spectra of the chitosan, was recorded in the range of 180nm-600nm as shown in Figure 2-4 the absorption band of chitosan were noticeably shown in the UV spectrum at 237nm, 227nm and 226nm for 30%, 40% and 50%NaOH respectively, these values are in agreement with the report of [20].

FT-IR spectra of chitosan synthesized using different concentrations of sodium hydroxide 30% w/v, 40% w/v and 50% w/v NaOH respectively. They all showed a broad peak at 3500-3300cm⁻¹ for OH and NH₂ stretching vibrations [21][22][23]. The amide I band or C=O and amide II band or NH deformation appeared at 1651cm⁻¹ and 1580cm⁻¹ respectively[21][24] They all showed absorption peaks at 1423-1416cm⁻¹ which are due to CH₂ bending[24][25]. They also showed peaks at the range of 1155-1148cm⁻¹ and 1077-1069cm⁻¹ ascribed to C-O and C-O-C stretching frequencies respectively [21][26][27]. The various chitosan synthesized using 30% w/v, 40% w/v and 50% w/v NaOH all showed the chitosan saccharide ring stretching observed at 895cm⁻¹.

The DSC curves as shown in figure 8-10 the extracted chitosans showed endothermic peaks at a range of 79.33°C-84.74°C. The endothermic peaks, often called dehydration temperature are due to the evaporation of water molecules. This values are in close agreement with the report of 79°C. The presence of the dehydration temperature suggests that some bound water was still not removed from the samples after drying. While it gives an exothermic transition at 345°C which likely corresponds
to chitosan decomposition since polysaccharides do not melt.

Table 2: zone of inhibition of chitosan

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>30% NaOH</th>
<th>40% NaOH</th>
<th>50% NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>19</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>17</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>16</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>16</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>18</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>20</td>
<td>23</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 3: Minimum Inhibitory Concentration of chitosan

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>30% NaOH</th>
<th>40% NaOH</th>
<th>50% NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>25</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>25</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>25</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>25</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>25</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>25</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

The antimicrobial studies, showed that the polymers have a strong activity against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumonia, Candida albicans and Aspergillus niger*. The zone of inhibition of the organisms that showed sensitivity ranges from 16mm-26mm with 30% w/v NaOH having the least activity while and the synthetic antimicrobial polymer having the highest activity as shown in Table 2-3 The MBC/MBF values of the polymers are 50mg/ml for 30% NaOH for all the organisms that showed sensitivity except it was unable to kill it at that concentration, 40% w/v NaOH, with MBC of 12.5mg/ml for *Escherichia coli*, 25mg/ml for *Staphylococcus aureus, Bacillus subtilis and Pseudomonas aeruginosa. Staphylococcus aureus* is known to play an important role in skin diseases including superficial.

Conclusion

The concentration of sodium hydroxide used in the deacetylation, as the concentration increases the degree of deacetylation of the chitosan increases, which further enhance the antimicrobial activity of the selected microbial organisms, while the thermal stability decreases as the degree of deacetylation of the chitosan decreases, because the chitosan became less crystalline.

REFERENCES


