Extraction of chitosan from Fungal cell wall by Sulfuric acid
Studying the effect of Deacetylation degree and temperature on recovery chitosan

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Keywords:

*R.pulusilus, M.indicus*, Extraction, recovery, chitosan, Fungal cell wall, Sulfuric acid, Deacetylation degree, temperature
Abstract:

The goal of this project is extraction of chitosan optimally by surveys of temperature changes along with 1% Sulfuric acid utilization. Microbial chitosan is isolated as a bio-component from cell wall of two kinds of Zygomycetes by some extraction methods. This project compares ability of two type Fungi (R. pulusilus and M.indicus) from Zygomycets for production of chitosan.

To extract of chitosan is a combinational method with 40 %( w/w) Hydroxyl sodium for cell disruption and diluted Sulfuric acid (1% w/v) for chitosan extraction from cell wall as major chemical components. 40% NaOH is used to get different degrees of deacetylation (DD) from chitin for chitosan. In addition, it is examined 1% Sulfuric acid in a combination of temperature factor changes. It is needed dialysis for chitosan purification from bonded phosphate groups. Standard curves of acetic acid experiences for DD and water phosphate determination were accomplished.

It has resulted if degree of deacetylated chitin is about 50%; it has an average lost more than 50% in 1% (v/v) Sulfuric acid, hence less recovery as a no privilege that it can be relapsed by acetone in chitosan solution. Factor of temperate in same times shows important effect on extraction yield of chitosan by 1% Sulfuric acid. Extracted chitosan in 120°C has DD about 50%. Absolutely, its solubility will be more and it needs to an intricate solution for separation of chitosan from phosphate bonds as a major impurity by dialysis, but in 90°C, DD of chitosan is more with less solubility in water.

Between two Fungi, in experienced temperatures, hence, R. pulusilus has more recovery about 0.87/AIM (g/g) in 90°C, which have more much DD than 50%, and M.indicus has 0.79/AIM (g/g) in 120°C that it has DD about 50%.

Keywords: R.pulusilus, M.indicus, extraction, recovery, chitosan, Fungal cell wall, Sulfuric acid, Deacetylation degree, temperature
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**Nomenclature**

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<thead>
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<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid residues</td>
</tr>
<tr>
<td>CDA</td>
<td>chitin deacetylase</td>
</tr>
<tr>
<td>CS</td>
<td>chitin synthase</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>GlcN</td>
<td>2-amino-2-deoxy-d-glucopyranose, $\alpha$-(1-4)-linked in chitin/chitosan</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>2-acetamido-2-deoxy-d-glucopyranose, $\alpha$-(1-4)-linked in chitin/chitosan</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>Mw</td>
<td>mass average of molecular mass</td>
</tr>
<tr>
<td>RMP(R)</td>
<td>Rhizomucor pulusilus</td>
</tr>
<tr>
<td>Mocur(M)</td>
<td>M. indicus</td>
</tr>
<tr>
<td>AIM</td>
<td>alkali insoluble material</td>
</tr>
<tr>
<td>HAIM</td>
<td>hot acid insoluble material</td>
</tr>
<tr>
<td>DD</td>
<td>deacetylation degree</td>
</tr>
<tr>
<td>AAIM</td>
<td>alkali- and acid-insoluble material</td>
</tr>
</tbody>
</table>
Chapter 1. 

Introduction

In 1811, Braconnot isolated a material from a Fungus and named it "Fungin". In 1823, Odier found a similar component in constituents of the exoskeleton of insects and called it "chitin", which means envelope in Greek [1]. Rouget and Hoppe-Seiler, in 1859 and 1894 respectively, treated chitin with concentrated alkaline solution and prepared chitosan [2]. Both chitin and chitosan are naturally occurring biopolymers that are obtained from crab and shrimp shells, as waste materials of marine and food processing industries. These materials are biodegradable, biocompatible and can be used in some technologies.
Chapter 2.

**Overview on chitin and chitosan**

2.1. Molecular Structure and Conformation

A linear (1, 4)-linked 2-acetamido-2-deoxy-β-d-glucopyranan (N-acetyl-β-d-glucosaminan) is chitin, and chitosan is the deacetylated derivative of chitin (see Fig.1). Unlike chitin, chitosan is soluble in aqueous solution of some acids e.g., acetic acid and hydrochloric acid. The physical properties of chitosan depend on to the degree of deacetylation (DD), the molecular mass and the distribution of free amino groups in the chain [3]. Degree the deacetylation is a relative number of amino groups to the total groups (acetamido groups and amino groups) in the chitin and chitosan chain. When the DD is high (e.g. DD>50%) or amino groups are dominant, the biopolymer is named chitosan [4].

![Fig 1: Chitin and chitosan structure](image)

2.1.1. Chitin

X-ray diffraction patterns show two polymorphs of chitin (α- and β-chitin). In α-chitin the chains have an antiparallel and in β-chitin they have a parallel orientation [5]. Among different conformations of chitin, α-chitin is more stable. The β-chitin can be converted to α-chitin by acid treatment but the reverse reaction does not occur [7].

2.1.2. Chitosan
Two and eight-fold helix make up two molecular forms of chitosan respectively type I (an extended two-fold) and type II (a relaxed two-fold helix) (Fig. (2)). High humidity is caused transformation of the eight-fold helix to the two-fold helix. chitosan does not have any of these conformations in aqueous acidic solutions. Increasing the degree of N-deacetylation, the ionic strength in the solutions and the temperature lead to more molecular flexibility of chitosan solution [6].

![Two and eight-fold helix making two molecular forms of chitosan](image)

Fig 2: Molecular conformations of chitosan at the solid state, Type I is shown on the right and Type II is shown on the left.

### 2.2. Raw Materials and Production

Annual Production of chitin is about $100 \times 10^9$ t/y. chitin is supplied from the exoskeleton of crustaceans (crab, shrimp etc.), the cartilages of mollusks (krill, squid etc.), the cuticles of insects (cockroach, beetle etc.), and the cell walls of micro-organisms (Fungi). chitosan is produced through a chemical deacetylatation from chitin. chitosan also presents in the cell wall of a group Fungi called Zygomycetes. At present, the major industrial source of chitin and chitosan are the shell wastes of crabs and shrimps. Generally, the shellfish is made of 20-30%chitin [8,9].

### 2.3. Fungal cell wall as a source for Chitin and chitosan

#### 2.3.1. The Fungal kingdom

A Fungus is any member of order of eukaryotic organisms, Unikonts, Opisthokont, which includes microorganisms such as yeasts, molds and mushrooms. The Fungi are classified as a kingdom that is separate from plants, animals and bacteria. One major difference is that
Fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. Fungi perform an essential role in the decomposition of organic materials and have fundamental roles in nutrient cycling and exchange [10].

The Fungal cell wall occupies about 40 per cent of the cell volume and it has a uniform thickness around the protoplast [13]. Fungal cell wall has an array of polysaccharides and glycoproteins that is a unique structure in eukaryotes, and is essential for Fungal growth and viability. Actually, Electron microscopy shows that the Fungal cell wall is composed of two or more layers. Usually, cell walls of mycelia contain about 29 percent glucan, 31 percent mannann, and 25-30 percent chitin [11].

Chitin is widely distributed in Fungi, occurring in Basidiomycetes, Ascomycetes, Zygomycetes and Phycymycetes, where it is a component of the cell walls of mycelia, stalks, and spores. Variations in the amounts of chitin in the cell wall may depend on physiological parameters in natural environments as well as on the fermentation conditions in biotechnological processing or in cultures of Fungi. Cell walls of mycelia contain mostly chitin in the form of fibrillar polymer and may constitute 25-30 percent of the dry weight of the cell wall [12].

![Diagram of Fungal cell wall and presence of Glycophosphatidylinositol](image)

**Fig 3:** Schematic of Fungal cell wall and presence of Glycophosphatidylinositol

### 2.3.2. Fungal Taxonomy

[4]
Fig 4 generally shows the taxonomy of Zygomycets. On basis of scientific classification they are in Kingdom: Fungi, Division: Zygomycota, Class: mold, and their Orders are

1) Mucoromycotina: Endogonales, Mucorales, Mortierellales
2) Kickxellomycotina: Asellariales, Kickxellales, Dimargaritales, Harpellales
3) Entomophathoromycotina: Entomophthorales
4) Zoopagomycotina: Zoopagales

Zygomycota, like all other Fungi, produce cell walls containing chitin. They grow primarily as mycelia, or filaments of long cells called hyphae. Among different Fungal orders, only Zygomycetes are useful for chitosan production [15].

Fig 4: Taxonomy of Zygomycetes

---

Zygomycetes are comprised of 4 orders, 32 families, 124 genera, and 870 species with only about 1% of the known species of Fungi. They are fast-growing and primary colonizers of substrates containing carbon sources like sugar or starch. Zygomycetes can grow by the formation of sexual spores (Zygospores), vegetative mycelium asexual reproduction (Mucorales and Zoopagales). In addition, Arthrospores, Chlamydomosporas, and yeast cells can be formed by some species [14]. Some well-known examples of this family are black bread mold (Rhizopus stolonifer), and Pilobolus species which are capable of ejecting spores several meters through the air. Medically relevant genera include Mucor, Rhizomucor, and Rhizopus [15].

---

2.4. Isolation of Chitin from Crab and Shrimp Shells sources

Chitin is isolated from crab and shrimp shells in a process containing: (1) demineralization: dissolution of Ca₂CO₃ in dilute HCl, (2) decolorization: extraction of Astaxanthin pigments and lipids by organic solvents, e.g., acetone and ethanol, (3) deproteinization: extraction of proteins, by dilute NaOH or digestion by proteases. Flakes are original form of chitin in the process. The pigments are consisting of conjugated double bond which is broken down by sunlight in the air-drying step, because they are very sensitive to ultraviolet light. For a white-colored product, treatment with H₂O₂ or NaOCl is used as an oxidative bleaching [16].

2.4.1. Preparation of chitosan from Chitin

Produced chitin from shellfish can be converted to chitosan by three methods:

1) Heterogeneous deacetylation: chitin flakes are treated in suspension with 30-60% aqueous solution of NaOH at 80-120 °C for 4-6 h. This method can give highly N-deacetylated products during long treatment time. Generally, depolymerization can happen in period of repeated treatments [17].

2) Homogenous deacetylation: Treatment of alkaline chitin in the form of solution of the sodium salt of chitin in 1.4% NaOH, at 25 °C. The process can produce partially N-deacetylated derivatives of chitosan, which are soluble in water. However, this method is not very efficient and a random distribution of N-acetyl groups is found in these products. [18]

3) Enzymatic deacetylation: In this method, the powdered chitin is treated with the chitin deacetylase (EC3.5.1.41). This method has some benefits such as low degree of depolymerization [19]. chitin deacetylases have glycoproteins structure and they have two kinds of secretion in periplasmic area and extra-cellular. Both of them have stability at their optimum temperature (50°C) with different molecular weight. Extra-cellular kinds have a range of optimum pH more than 7, and a specific characteristic that acetate does not inhibit
deacetylation reaction, but acetate has inhibiting effect on activity of intra-cellular enzymes and these kinds of enzymes have an optimum pH less than 7. Zygomycetes such as Mucor and Rhizopus produce intra-cellular chitin deacetylases [20].

\[
\text{chitin (- GlcNAc)} + \text{H}_2\text{O} \xrightarrow{\text{chitin deacetylase}} \text{chitosan (- GlcN)} + \text{acetate}
\]

2.5. Fungal chitosan

Beside of industrial production of chitosan by chemical deacetylation of chitin, alternatively chitosan is produced in the cell wall of Zygomycets Fungi. As it is shown in Table 1, chitosan is one of the major ingredients of cell wall in these Fungi while it is not found in any other groups of Fungi. Of course, the amount of chitosan between species is different. Table two compares the amount of chitosan between different strains. Among different strains in the table *Rhizopus oryzae TISTR3189* produces the highest amounts of chitosan [21]. The culture media is important to grow Fungi and chitosan production in cell wall e.g. *R. oryzae TISTR3189* in Soybean residue grows better and produces more chitosan rather than mungbean residue (respectively 4.3 and 1.6 g chitosan/kg substrate) [22].

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>Fibrillar polymers</th>
<th>Matrix polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oomycets</td>
<td>β(1,3), β(1,6)- Glucan; cellulose</td>
<td>Glucan</td>
</tr>
<tr>
<td>Chytridomycetes</td>
<td>Chitin; glucan</td>
<td>Glucan</td>
</tr>
<tr>
<td>Zygomycetes</td>
<td>Chitin; chitosan</td>
<td>Polyglucuronic acid; glucuronomannoproteins</td>
</tr>
<tr>
<td>Basidiomycetes</td>
<td>Chitin; β (1,3)- β (1,6) glucans</td>
<td>α(1,3)-glucan; xylomannoproteins</td>
</tr>
<tr>
<td>Ascomycetes/ deuteromycetes</td>
<td>Chitin; β (1,3)- β (1,6) glucans</td>
<td>α(1,3)-glucan; glucuronomannoproteins</td>
</tr>
</tbody>
</table>

Table 2: Amount of produced chitosan by different Fungi [22]

<table>
<thead>
<tr>
<th>Species</th>
<th>chitosan produced (mg g(^{-1}))^(a)</th>
<th>chitosan content (%)^(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizopus oryzae TISTR3189</em></td>
<td>138</td>
<td>14</td>
</tr>
<tr>
<td><em>Lentinus edodes no. 1</em></td>
<td>33</td>
<td>3.3</td>
</tr>
<tr>
<td><em>Pleurotus sajo-caju no. 2</em></td>
<td>12</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Zygosaccharomycetes rouxii TISTR5058</em></td>
<td>36</td>
<td>3.6</td>
</tr>
<tr>
<td><em>Candida albicans TISTR5239</em></td>
<td>44</td>
<td>4.4</td>
</tr>
</tbody>
</table>

^a^ cell dry weight
2.5.1. Isolation of chitosan from Fungal cell wall

For extraction of chitosan from cell wall, first the cell wall is isolated from the Fungal biomass through an alkali treatment (with dilute NaOH solution) at elevated temperature (e.g. 90-120%). NaOH solution, in this condition, dissolves proteins, lipids, and alkali-soluble carbohydrates and the cell wall is remaining as alkali insoluble material (AIM). In the next step traditionally, chitosan is separated from AIM by dissolution in an acid solution (e.g. 2–10% acetic acid at 25–95 °C for 1–24 h). In this step, the other components of cell wall are remaining as alkali- and acid-insoluble material (AAIM). At the end, precipitation of Fungal chitosan is accomplished by increasing the pH to 9-10 and chitosan is recovered by centrifugation [25]. When acetic acid or HCl is used for extraction of the chitosan, a product is obtained with a low yield and high phosphate impurity [23]. However recently, treatment of cell wall with hot dilute Sulfuric acid solution has resulted in a high yield and more purity of chitosan [25]. Unlike acetic, citric, lactic and hydrochloric acids, chitosan is not soluble in dilute Sulfuric acid solutions in room temperature. However, it is soluble in hot boiling solution of Sulfuric acid. The temperature dependent Solubility of chitosan in Sulfuric acid solutions is not shared with the other components of cell wall such as chitin and polyphosphates. Therefore, by treating the cell wall with hot dilute Sulfuric acid, chitosan become soluble in hot acid and it can be separated from other components of cell wall by filtration. In the next, step chitosan can be recovered from Sulfuric acid by cooling. Zamani et al. reported that extraction of chitosan from cell wall of Zygomycetes Fungi by Sulfuric acid results in a product with higher purity and yield compared to traditional extraction method by e.g. acetic acid.

2.6. Application of chitosan

chitosan is a cationic biopolymer that has a wide variety of applications e.g. in waste water treatment, food industry, medical industry, biotechnology, agriculture, cosmetics, pulp and
paper industry and membrane technology. A detailed description of chitosan application in each field is presented in Table 3.

Table 3: chitosan Application

| Wastewater treatment | -Removal of metal ions  
|                      | -Flocculent /Coagulant: Protein, Dye, Amino acids |
| Food industry        | -Removal of Dye, Suspended solids etc.  
|                      | -Preservative  
|                      | -Color Stabilization  
|                      | -Animal feed additive |
| Medical industry     | -Bandages  
|                      | -Blood Cholesterol Control  
|                      | -Skin Burn  
|                      | -Contact Lens  
|                      | etc |
| Biotechnology        | -Enzyme Immobilization  
|                      | -Protein Separation  
|                      | -Cell Recovery  
|                      | -Chromatography  
|                      | -Cell Immobilization |
| Agriculture          | -Seed Coating  
|                      | -Fertilizer  
|                      | -Controlled Agrochemical Release |
| Cosmetics            | -Moisturizer  
|                      | -Face, Hand and Body Creams |
| Pulp and paper industry | -Surface Treatment  
|                      | -Photographic Paper |
| Membrane industry    | -Permeability  
|                      | -Reverse Osmosis |

2.7. The project goal

It is tried to survey methods of high yield of chitosan from biomass by using of dilute Sulfuric acid [24]. First, it must be understood how chitosan is produced from cell wall and the methodology in which section will effect and second, it should be searched the effect of different temperature at step of 1% Sulfuric acid adding. Actually, the project aim is basis of 1% Sulfuric acid usage that was followed in a general view of determination of De-acetylation Degree in under construction. This structure can help to better following in methodology:

A) In commercial chitin

1) NaOH on chitin → **De-acetylation** → chitosan
2) 72% Sulfuric acid on chitosan for determination of De-acetylation degree

B) In microbial chitosan (30 min test)

1) NaOH on cell wall → destruction of cell wall

2) 1% Sulfuric acid → dissolution of chitosan from cell wall

3) 72% Sulfuric acid on chitosan for determination of De-acetylation degree
Chapter 3.

Methodology

3.1. Production of chitosan with different degree of deacetylation

40 ml sodium hydroxide 40% (w/w) was mixed with 2 gr chitin (Sigma, crabcells), and it was purged with N₂. Then, it was placed in an oven at 100°C for (20min- 8h). After that, it was filtrated and washed with water. After the desired time, the samples were cooled on ice in order to stop the reaction. Then, the product was dried in on oven it is dried in oven after washing.

\[
\text{chitin} + \text{NaOH (40\%)} \xrightarrow{20 \text{ min-8h}} \text{chitosan}
\]

Fig 5: the steps of Chitin deacetylation

3.2. Production of water-soluble chitosan

In some references, it was referred that chitosan is solved in H₂O in certain deacetylation degree (DD≅ 50%) [25], so this problem causes to less recovery in progressing of experiments, if it is faced to that. For discovery of required time that it causes to produce chitosan with this DD, the samples were prepared to DD determination with 72% Sulfuric acid. The result of HPLC and related calculations show critical point of time is 30min that had DD about 50%. For verify, distillated water was added to about 0.25g treated chitin with NaOH (30min, 100°C), then it was prepared to dialysis, and after that for freeze-drying.

3.3. Solubility of chitosan in H₂O

0.2g of the deacetylated samples of chitin by NaOH from “20min, 30 min, 1h, and 2h” were put it in a beaker, and 50 ml water was added and mixed well for 1h. Then the pH of the
mixture was measured. After that, the mixture was centrifuged and the samples were collected, dried, and weighted.

3.4. Sulfuric acid treatment on chitosan

After treatment of chitin with NaOH, and preparation of chitosan, this was treated with 1% Sulfuric acid at 120°C for 20min in autoclave [29]. After the treatment, the mixture was filtered and the filtrate (called liquid A in this text) was cooled down on ice to precipitate chitosan. The precipitated chitosan was washed with water and dried. Actually, this stage was planned for comparison in real test with M.O. (AIM analogue).

3.4.1. chitosan Recovery tests

In order to recover the chitosan from Sulfuric acid solution, in addition to cooling, four different methods were applied.

A) Acetone test: 1ml of liquid A was mixed with 4ml acetone in a glass tube and the mixture was left for 12h.

B) Ethanol test: 1ml of liquid A was mixed with 4 ml of ethanol 70% and mixture was left for 12h.

C) Sodium hydroxide test: 4% (w/w) sodium hydroxide was added to liquid A until pH around 8-10 and mixture was left for 12h.

The same experiments as mentioned in A and B were repeated on potassium phosphate solution (instead of liquid A) to check the precipitation of phosphate, because these components are dissolved along with chitosan from cell wall after treatment by NaOH.

I) Acetone was added to 0.1gr of KH₂PO₄ in 50ml tube.

II) Ethanol was added to 0.02 gr of K₂PO₄ in 12ml tube.

D) Dialysis and freeze-drying method: In this method, the liquid A was dialyzed against running water for 1-2 days. The dialysis was stopped if the pH of the liquid around 7. Then the liquid was frozen and freeze-dried and the solid product was collected and weighted.
3.4.2. Glucosamine Test

Glucosamine is an amino derivative of glucose (C₆H₁₃NO₅) which is a component of many polysaccharides and is the basic structural unit of chitin.

After providing of each sample, it is taken two tubes for measurements(samples and blank). As a sample test, 0.5 ml of that is mixed with NaNO₂ (69g per 100 ml). As a blank test, 0.5 ml of that is mixed with distillated water.

It is closed all of the samples tubes tightly, and they are mixed, then they leave for 6 hours under hood, because the samples can release NO₂, which is poison gas. After this time, the samples tubes are opened and leaved for overnight under hood.

It is added 0.5 ml of ammonium sulfate (12% w/v) to each sample, it must be mixed, then wait for 4 min. Again, it is added but 0.5 ml of MBTH (0.5% w/v). Samples are leaved 1h without mixing. 0.5 ml of FeCl₃ (0.5%w/v) is added to all of them along with mixing, and it should be kept for 1 hour in dark place, then whole samples are diluted 100 times (1ml...
sample + 99ml water). At the end, they are measured the absorbance of them against blank at 650 nm.

3.5. Cultivation of Fungi

3.5.1. Cultivation of Fungi

Two kind of M.O. were used in these experiments, *Mucor indicus* and *Rhizomucor pulusilus*, so they should be cultured for more amounts. Preparation of culture media was done on basis of under structure.

A- Make the following solutions:

**Solution1**: containing \{15g (NH$_4$)$_2$SO$_4$; 7g KH$_2$PO$_4$; 1.5g MgSO$_4$·7H$_2$O; 10g yeast extract\} in 1600 ml water.

**Solution 2**: containing \{100g glucose; 2g CaCl$_2$·2H$_2$O\} in 400 ml water. Dissolve these materials in the same way as you did for solution A.

Take 100 ml glass bottles (8) and 500 ml flasks (8) for ready solution. To each bottle, put 50 ml of solution1. In addition, to each flask add 200 ml of solution 2.

- Close the bottles with blue caps and the flasks with cotton.
- Take one more bottle, mark it by H$_2$O, add 50 ml water in to it, and close it.
- Fill the Ependorf tip box with blue tips and close it with autoclave tape.
- Sterilize all of flasks, bottles and box (at 120℃ for 20 min).

B- Inoculation: it was done in bath shaker for 3 days with continuous surveillance.

3.5.2. Cultivation of strains on agar plate

Agar plate was prepared with \{glucose 4g, agar 2g, peptone 2g\} for slant preparation of Fungi.

These components were mixed in 100 ml water. It was autoclaved in 120℃ for 20 min, then by spinning movement, 0.5 ml of culture method was spread some samples on media, and it was placed into oven in 32℃ for 3 days for incubation. (Notice: the dishes were backed,
because of evaporation drops after autoclave). Cultivation was done in aseptic conditions for prohibition from impurities.

3.6. Preparation of cell wall for getting AIM

Harvesting of Fungi was included preparation of cell wall and extraction of chitosan.

i) Harvest the biomass on a screen, wash it with water, and freeze it.

ii) Dry the biomass with freeze dryer.

iii) Make the biomass as a powder with coffee grinder that have in the lab.

iv) Weigh the biomass and divide it into 2g portions. Put each portion in a 100 ml bottle, add sodium hydroxide solution (2%, 60 ml) in to it, and put in the autoclave at 120°C for 2 min.

v) Centrifuge the mixture, get the solid, and wash it several times with water to get natural pH (this solid was named AIM, which was our cell wall).

vi) Freeze the AIM and freeze dry it.

vii) Take 0.25g of the AIM for Sulfuric acid treatment.

3.7. Extraction of chitosan from cell wall by Sulfuric acid

3.7.1. Extraction Liquid A and HAIM from AIM:

For extraction of AIM, it should be followed these steps:

1) Work with a four decimal number scale for 0.25g AIM.
2) Put the samples of 50, 70 and 90°C in bath shaker.

3) After incubator (120 °C, 20 min) or autoclave (50, 70, 90 °C) stage, tubes solutions were transferred on filter rapidly for keeping the temperature in about 90°C. It could be used from a water bath for keeping. Then the liquid A was separated for one hour on ice (it was better about 20 ml) from HAIM (it can have chitin, N-acetylene components, which we did not have any information about that) which was washed repeatedly to natural pH. Put filter papers in Oven (50°C) for drying.

4) Please notice, maybe some solids are on funnel under filter paper, so it should be added to liquid A with water washing.

5) Centrifuge the Liquid A, keep the Aqueous phase of liquid A after that (for some measurement such as phosphate test), wash the Solid phase of Liquid A to natural pH, wash it with Acetone for extraction improvement as a pure polysaccharide, and then dry the solid (it must be chitosan).

In continue other experiences were implemented, so it was decided to be determined DD by UV on basis of presence of Acetic acid.

\[
\text{NAc components (sample: Chitin, Chitosan, other)} \rightarrow \text{GLcN + Acetic Acid}
\]

### 3.7.2. AIM treatment

This section was included AIM treatment in four steps on two kinds of cultivated Fungi, _Mucor_ (M) and _R. pulusilus_ (P). In continue, it was used easy-phrase, which was called the AIM-M (alkali-insoluble material derived from _Mucor indicus_) and AIM-P (alkali-insoluble material derived from _Rhizomucor pulusilus_).

Some notes before following of four steps:

1) Experiments should be done in duplicate (A & B) so there would be four samples in total: AIM-M-A, AIM-M-B, AIM-P-A and AIM-P-B).
2) Because of precipitation of chitosan in cold liquid, it was better to be prepared some Bain-maries for needed temperature.

3) Centrifugation should be done in 5 min and 10000 rpm.

4) Washing and freeze-drying were done for probable extracted chitosan.

5) Phosphate test needed 0.3 ml from liquids, so it was put away 0.5 ml.

6) After Extraction I, it should be performed the Extraction II, hence they were used AIM2, AIM3 and AIM4 respectively in steps of 2, 3 and 4.(HAIM: hot acid insoluble material), (AIM: alkali-insoluble material)

Now, it should be followed on basis of under steps.

Step 1:

![Diagram of AIM Treatment Test](Fig 9: Step 1 of AIM Treatment Test)
Step 2:

Fig 10: Step 2 of AIM Treatment Test

Step 3:

Fig 11: Step 3 of AIM Treatment Test
Step 4:

**Fig 12: Step 4 of AIM Treatment Test**

### 3.8. Measurement of the degree of deacetylation (DD)

DD was determined by mixing of H$_2$SO$_4$ (0.3 ml, 72%) with 0.01g of sample and they were blended with glass bar. The time of effect was 90 min in room temperature. After that, it is added 8.4 ml of water to each sample, and they were mixed with spatula. At the end, samples were autoclaved at 121°C for 20 minutes. Preparations of standard and purified NAc do not forget. After Autoclave, they were put in refrigerator for 10 min for cooling, then 1 ml of them was mixed with 3 ml of distilled water in tubes (10 ml), with syringe and HPLC filter was poured 1.5 ml of them in ependorfs, at the end 1ml of them were used for measurement of HPLC in special tube of that. Of course, the standards (st1.st2, st3) were diluted 4times (1:3) for measurement.

### 3.9. Phosphate measurement and HPLC analysis

The extracted chitosan from biomass was along with phosphate components, so it must be designed a test for phosphate determination after extraction and dialysis:

Standard solution: some 100 ml flasks were ready for mixture of phosphate standard in 0.5, 1, 1.5, 2, 2.5 ml with 40 ml of H$_2$O. It was added ascorbic acid (2 ml, 1%V/V) with 4 ml of acid mobyldate, also from each 0.3 ml of Liquid A, two times (as A& B samples), was taken and
mixed with 40 ml of H₂O in flask with same components for standard tests. At the end, they were filled with water up to 100 ml. Between 10 to 30 min; whole of samples should be transferred for UV assay in 880 nm.

Notice: 1) with distillated H₂O, UV device was fixed on zero.

2) Cleaning of flasks were done with HCl (1%v/v) and a detergent like Rica.
Chapter 4.

Result and Discussion

4.1. DD determination

The first experiments show non-suitable degree of deacetylation (DD) for 1 to 8 hours time for deacetylation. It is necessary to obtain DD ≅ 50% for future optimization, in mentioned method for chitosan production from biomass, some cellular chitosan is disappear that it has DD ≅ 50% (the survey of acetic acid and GLcN show the amount of 50% for both of them from cellular wall, hence chitosan DD can be 50%).

Table 4: the first experience about time and DD

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.80 ± 0.55</td>
</tr>
<tr>
<td>2</td>
<td>77.83 ± 0.52</td>
</tr>
<tr>
<td>3</td>
<td>82.5 ± 0.86</td>
</tr>
<tr>
<td>4</td>
<td>83.81 ± 1.33</td>
</tr>
<tr>
<td>5</td>
<td>83.01 ± 0.136</td>
</tr>
<tr>
<td>6</td>
<td>85.23 ± 0.11</td>
</tr>
<tr>
<td>7</td>
<td>85.51 ± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>86.31 ± 0.53</td>
</tr>
</tbody>
</table>

It is considerable to calculate DD it’s needed some data which is gained from making solution of ST1, ST2, ST3 respectively standard solution 1, standar solution 2 and standard solution 3. In addition, it is needed monomer chitin (N-acetyl-D-glucosamine) for future calculations. By HPLC and standard solutions, it can be drawn standard concentration (vertical axis) against of HPLC curve under area (horizontal axis) with y=7822976.87x& R$^2$=1.00. This is for first-degree deacetylation (DD). These DD’s are calculated in basis of this order:

1-Standard solution Concentration 1=0.1*1.05/60*5(mol/l)
2-Standard solution Concentration 2=(0.1*1.05/60*5)/2(mol/l)
3-Standard solution Concentration 3=(0.1*1.05/60*5)/4(mol/l)

4-N-acetyl theoretical concentration = amount of monomer chitin/8.7*1000(mol/l)

5-Practical concentration by HPLC from monomer chitin =(curve under area in HPLC)/7822976.87*221(mol/l)

6-Ratio average of N-acetyl practical to theoretical concentration (Z)

7-chitosan concentration=amount of chitosan/8.7*1000(mol/l)

8-N-acetyl content= (curve under area in HPLC)/7822976.87/Z*204/ (chitosan concentration) *100

9-DD= 100- (N-acetyl content)

In the next exercises, it is was decided to be worked below of 1 hour for degree of deacetylation, on basis of this idea that shorter time of reaction between NaOH and chitin will give less degree deacetylation and closer to DD≈50%.

Table 5: the second experience about time and DD

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>18.29 ± 9.1</td>
</tr>
<tr>
<td>30</td>
<td>49.25 ± 6.7</td>
</tr>
<tr>
<td>45</td>
<td>60.70 ± 1.39</td>
</tr>
<tr>
<td>60</td>
<td>66.69 ± 0.45</td>
</tr>
</tbody>
</table>

In second experienced standardization, By HPLC and standard solutions, it can be drawn standard concentration (vertical axis) against of HPLC curve under area (horizontal axis) with y= 7822976.87x & R²=1.00. Degree of deacetylation is calculated on basis of above same way.

**4.2. Production of water-soluble chitosan**

After determination of deacetylation degree, it is needed to be sure about the chitosan solubility in water, because maybe deacetylated chitin from cell wall in a certain DD will be
solved, so it should be recovered. Exactly, the data of Table 6 shows deacetylated chitin in 30min solves in water about half of the amount of chitosan. Even, acetone cannot take back it.

<table>
<thead>
<tr>
<th>Table 6: solubility of chitosan in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>chitosan Weight</td>
</tr>
<tr>
<td>0.2506±0.0003</td>
</tr>
</tbody>
</table>

4.3. 30 min Test

After some experience on commercial chitin, it is understood that 30 min samples have degree of deacetylation about 50%. That is why, it is focused on a series of experiments on deacetylated chitin and treatment of them with 72% Sulfuric acid for determination of deacetylation degree before autoclave stage (120°C and 1h), because it is guessed the time of treatment will have high effect on DD, but after treatment, this consumption was wrong. This experience is only for conformation. Table 7 shows this fact. Absolutely, these data have its equation standard with y=7838149.66x with R²=0.95.

<table>
<thead>
<tr>
<th>Table 7: the effect of treatment of time Sulfuric acid on AIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min samples</td>
</tr>
<tr>
<td>DD</td>
</tr>
</tbody>
</table>

4.4. Solubility of chitosan in H₂O

In some references, it is referred that chitosan is solved in H₂O in certain DD, so this problem causes to less recovery. It is decided before solving of deacetylated chitin in 72% H₂SO₄ and degree deacetylation determination, it is solved by H₂O. Table 8 shows the solubility of chitosan in H₂O; hence, it can be expected in some certain DD, more chitosan will be disappear, and from results, it can be gotten amount dissolved chitosan of 30 min in H₂O will be more.
Table 8: solubility of chitosan in H₂O

<table>
<thead>
<tr>
<th>time</th>
<th>20min</th>
<th>30min</th>
<th>1h</th>
<th>2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>0.1871±7</td>
<td>0.208±0.001</td>
<td>0.2005±0.0003</td>
<td>0.2014±0.0003</td>
</tr>
<tr>
<td>PH</td>
<td>7.56±0.294</td>
<td>7.555±0.095</td>
<td>7.624±0.076</td>
<td>8.057±0.068</td>
</tr>
<tr>
<td>After drying</td>
<td>0.1802±0.0013</td>
<td>0.2028±0.0009</td>
<td>0.1924±0.001</td>
<td>0.1932±0.0014</td>
</tr>
</tbody>
</table>

4.5. Sulfuric acid treatment on chitosan

Table 9: amount of deacetylated chitin recovery after treatment with Sulfuric acid 1%

<table>
<thead>
<tr>
<th>time</th>
<th>initial sample</th>
<th>insoluble</th>
<th>soluble</th>
<th>Inso./so.=x</th>
<th>x/ini.samp.</th>
<th>average-recovered</th>
<th>average-lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>20min</td>
<td>0.251±0.0014</td>
<td>0.0138±0.0048</td>
<td>0.0254±0.0084</td>
<td>0.0392±0.0036</td>
<td>0.1557±0.0134</td>
<td>0.1558</td>
<td>0.8442</td>
</tr>
<tr>
<td>30min</td>
<td>0.254±0.002</td>
<td>0.0037±0.0002</td>
<td>0.075±0.0051</td>
<td>0.0573±0.0268</td>
<td>0.3104±0.0233</td>
<td>0.3103</td>
<td>0.6896</td>
</tr>
<tr>
<td>1 h</td>
<td>0.250±0.003</td>
<td>0.0075±0.0012</td>
<td>0.1262±0.0015</td>
<td>0.1336±0.0026</td>
<td>0.5336±0.0096</td>
<td>0.5335</td>
<td>0.4665</td>
</tr>
<tr>
<td>2h</td>
<td>0.255±0.002</td>
<td>0.01±0.0043</td>
<td>0.1727±0.0024</td>
<td>0.1826±0.0067</td>
<td>0.7161±0.021</td>
<td>0.7160</td>
<td>0.2839</td>
</tr>
</tbody>
</table>

This experience is planned to determine whether 1% Sulfuric acid has effect on solubility of chitosan or not, and if it has, how much the chitosan will be disappeared. Unfortunately, the experiments data in Table 9 show that chitosan with less percentage of deacetylation degree give less recovery, hence it must be thought about a profitable recovery method.

4.6. Solubility of chitosan in ethanol and acetone

Some experiences are set to determine whether obtained chitosan from chitin will be settled as sediment by acetone or 70% ethanol. Ethanol does not have any effect on obtained chitosan form chitin for sedimentation, but acetone shows to have more effect on this phase in more long time, but on basis of quantity view is not enough. Acetone changes aqueous phase color and to increase turbidity visibly in 1 hour, but it does not make happen to sedimentation. Below pictures show change amount of obtained chitosan form chitin in acetone in 20 min, 45 min and one hour. Absolutely the progress of time is suitable for recovery.
After these experiments, again, it has been repeated, because the result from first stage shows solution chitosan is extracted unstable by acetone, in this stage the amount of obtained chitosan form chitin is 5 ml and acetone is 20 ml. Results as same as first stage are obtained.

Table 10: recovery percentage in different samples

<table>
<thead>
<tr>
<th>TIME</th>
<th>DD</th>
<th>INITIAL SAMPLE</th>
<th>SOLUBLE (precipitation)</th>
<th>INSOLUBLE (HHIM)</th>
<th>ACETON</th>
<th>SUM</th>
<th>RECOVERY%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20min</td>
<td>18.29 ± 9.1</td>
<td>0.2537 ± 0.003</td>
<td>0.0018 ± 0.001</td>
<td>0.1762 ± 0.0068</td>
<td>0.0275 ± 0.0017</td>
<td>0.2055</td>
<td>81</td>
</tr>
<tr>
<td>30min</td>
<td>49.25 ± 6.7</td>
<td>0.2506 ± 0.0003</td>
<td>0.0197 ± 0.0082</td>
<td>0.0214 ± 0.0002</td>
<td>0.1781 ± 0.029</td>
<td>0.2192</td>
<td>87.47</td>
</tr>
<tr>
<td>45min</td>
<td>60.70 ± 1.39</td>
<td>0.2078 ± 0.0003</td>
<td>0.0581 ± 0.002</td>
<td>0.0037 ± 0.0003</td>
<td>0.1166 ± 0.0051</td>
<td>0.1784</td>
<td>85.85</td>
</tr>
<tr>
<td>1h</td>
<td>64.80 ± 0.55</td>
<td>0.2522 ± 0.0045</td>
<td>0.1443 ± 0.0073</td>
<td>0.0038 ± 0.0014</td>
<td>0.094 ± 0.01</td>
<td>0.2421</td>
<td>95.99</td>
</tr>
</tbody>
</table>

Table 10 shows a proportional recovery about 87.47% from 30 min sample that it is deacetylated chitin as chitosan. Absolutely, the recovery will be more in more time of contraction between acetone and aqueous phase, but these experiments focused on DD≈50%. It can be amazing for future works.

It is important for us to know after the precipitation by Acetone, whether it is soluble in water or other solvents or not, so from fist Acetone experiment, it is taken one sample, and all of the acetone is removed by pipette then it is added 4 ml of water in it. It is mixed manually, and leaves it overnight, it is seen which is non-soluble. In Phosphate test, it is cleared to sediment KH$_2$PO$_4$ in Acetone, K$_2$PO$_4$ is not solved in Ethanol (4 ml, 70%), chitosan in water is solved along with phosphate and it is not solved along with Phosphate in ethanol for
overnight. Phosphoric acid (2ml, 85%) has effected on chitosan in 50, 60 and 75 °C, for 2h, 1h and 45min respectively, but in room temperature, it does not back. 0.01gr of chitosan is not soluble in 8 ml of water completely, because after some minutes, it returns in extent, but it will be not obvious.

These experiences are designed for Fungal cell wall and its extracted chitosan from the cell wall, because it is considered the effect of other components, which are in the cell wall. That is why; it is dissolved 0.1gr of potassium di-hydrogen phosphate (KH₂PO₄) in Sulfuric acid (5ml, 1%), KH₂PO₄ will be solved, but when it is added 20ml acetone into this solution, again, KH₂PO₄ will be precipitated.

Actually, it can be understood that phosphate components along with Fungal chitosan cannot be separated by acetone, because it is settled with chitosan after treatment.

In continue as reminding, fig 21 shows to use the Sulfuric acid 1%, and hot treatment on chitin for resulted chitosan. [29], this diagram is extraction method of chitosan from cell wall of R. pulusilus (Rhizomucor puillus CCUG 11292).

After extraction, an aqueous phase will be remained which is named Liquid A. this Liquid has solved chitosan with degree deacetylation about 50%. Chemical methods doesn’t have any response for settling and separating of phosphate components from that, so it is tested in a dialysis method as a physical way (fig 7). The result of that was great, because phosphate
components can be separated by water flow in 2-3 days with a low water velocity. After that, it is transferred to freeze-drying that its result is the chitosan like wool (fig 15).

Fig 15: after hydrolysis, dialysis and freeze-drying

Note: any experiences in microbial environments need to keep away from contamination. For example, if the culture media for these Fungi is contaminated, it will have Fungi with incomplete grow, because the other M.O. use from media as feed. These Fungi grow in media for 3 days, after that, it should have a clear view from aqueous phase in culture media as health culture for Fungi in 3 days. Fig 16 shows clear media after Fungi growth.

Figure 16: Rhizomucor pulusilus and Mucor indicus (from left to right)

4.7. Phosphate test

This test is designed for determination of phosphate components along with chitosan after dialysis solution. 30 min samples are used as practical sample. Preparation of phosphate standard solutions is done in 0.5, 1, 1.5, 2, 2.5 ml volume, and color change is measured by UV spectrometry in Blue domain, along with phosphate measurement in sample. In continue, this experience helps to measure real amount of chitosan in samples with curve equation standard of $y=0.304x-0.017$ and $R^2=0.982$ ($x$: sample volume, $y$: absorbance).
Table 11: Phosphate test after dialysis

<table>
<thead>
<tr>
<th>St₁(0.5ml)</th>
<th>St₂(1ml)</th>
<th>St₃(1.5ml)</th>
<th>St₄(2ml)</th>
<th>St₅(2.5ml)</th>
<th>Sample₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.145</td>
<td>0.247</td>
<td>0.461</td>
<td>0.627</td>
<td>0.716</td>
<td>0.0165±0.01</td>
</tr>
</tbody>
</table>

This test is necessary, because chitosan is attached to phosphate salt, so it must be distinguished after washing with water in dialysis whether this salt is removed from chitosan or not.

4.8. chitosan extraction I

Table 12: Crude data from Extraction I

<table>
<thead>
<tr>
<th>Step 1(50°C)</th>
<th>AIM-P</th>
<th>DD</th>
<th>AIM-M</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-1</td>
<td>0.192±0.008</td>
<td>0.313±0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate-2</td>
<td>0.225±0.005</td>
<td>0.3685±0.0025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-1</td>
<td>0.0022±0.0012</td>
<td>0.005±0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-2</td>
<td>0.0091±0.0026</td>
<td>81.1±1.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-3</td>
<td>0.0023±0.0003</td>
<td>0.0131±0.0008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-4</td>
<td>0.0011±0.0006</td>
<td>79.71±3.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.0146±0.0022</td>
<td>0.0234±0.0043</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2(70°C)</th>
<th>AIM-P</th>
<th>DD</th>
<th>AIM-M</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-1</td>
<td>0.0465±0.0015</td>
<td>0.0575±0.0095</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate-2</td>
<td>0.0825±0.0135</td>
<td>0.081±0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-1</td>
<td>0.0113±0.0049</td>
<td>90.3±1.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-2</td>
<td>0.0142±0.0035</td>
<td>78.2±3.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-3</td>
<td>0.0021±0.0005</td>
<td>0.013±0.0003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-4</td>
<td>0.0001±0.0000</td>
<td>85.9±13.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.0277±0.0079</td>
<td>0.0294±0.0087</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3(90°C)</th>
<th>AIM-P</th>
<th>DD</th>
<th>AIM-M</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-1</td>
<td>0.049±0.001</td>
<td>0.0475±0.0035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate-2</td>
<td>0.054±0.006</td>
<td>0.037±0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-1</td>
<td>0.0273±0.0019</td>
<td>93.82±0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-2</td>
<td>0.0147±0.0008</td>
<td>71.86±4.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-3</td>
<td>0.0006±0.0001</td>
<td>0.0012±0.0004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-4</td>
<td>0.0001±0.0001</td>
<td>0.0007±0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.0435±0.0027</td>
<td>0.0638±0.0057</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 4(120°C)</th>
<th>AIM-P</th>
<th>DD</th>
<th>AIM-M</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-1</td>
<td>0.028±0.011</td>
<td>0.014±0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-1</td>
<td>0.0068±0.0004</td>
<td>0.0067±0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitosan-2</td>
<td>0.0016±0.0003</td>
<td>0.0052±0.0043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.1297±0.0025</td>
<td>0.1176±0.0004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This step is named to extraction I, because after experiment on basis of drawn diagram (p.23, 24 & 25), it is understood that experience face to more mistakes, because from each step e.g. step of 50°C which is made HAIM-1, this hot acid insoluble material (HAIM) is transferred to
step of 70°C as raw material (biomass). If the experience face to some wrongs in first step, it will be transferred to other steps in continue, and it can be understood the real reason of mistake in progress.

For calculation of Degree of deacetylation, in Extraction I, a standard equation is prepared with \( y=8.521,447.62x \) and \( R^2=1 \) (X= concentration (mol/l) and \( y= \) curve under area in HPLC).

After each dialysis, amount of phosphate will be decreased and when the higher temperatures is used amount of chitosan extraction will be increased, so in 90°C, more chitosan is received, but this method has more uncertainty, because samples is ready from last step to next step, and it can be along with mistakes.

N.B. determination of deacetylation degree (DD) needs at least 0.01g of chitosan as sample.

| Table 13: Phosphate analysis of distilled water in Extraction I |
|------------------|-----------------|-----------------|
| \( \text{KH}_2\text{PO}_4 \) | \= 136.09 g | \text{PO}_4^{3-} | \= 95 g |
| g | mol/l | PO_4 (g/l) | A (abso.) | A-r |
| \text{KH}_2\text{PO}_4 | 0.2196 | 0.001614 | 0.153296 |
| r(ml) | PO_4 (g/l) | A (abso.) | A-r |
| water | 0 | 0.013 | 0 |
| ST1 | 0.5 | 0.000766 | 0.195 | 0.182 |
| ST2 | 1 | 0.001533 | 0.413 | 0.4 |
| ST3 | 1.5 | 0.002299 | 0.564 | 0.551 |
| ST4 | 2 | 0.003066 | 0.723 | 0.71 |
| ST5 | 2.5 | 0.003832 | 0.898 | 0.885 |

Phosphate analysis in Extraction (I) need to know about the phosphate of distilled water, because it must be corrected by amount of that in water. Absolutely, it should be prepared phosphate calibration curve which is made standard equation of \( y=234.8x \) and \( R^2=0.996 \) (x=PO_4 (g/l), y=A-r). This standard equation of phosphate as calibration curve is useful to calculate amount of phosphate in *Rhizomucor pulusilus* (*R.pulusilus*) and *Mucor indicus* (*M.indicus*) and recovery of that.

After experiments in phase I, it is decides to correct amount of phosphate (g) in 50ml. Table 13 shows real amount of these component after correction, because the distillated water had
Table 14: phosphate of first stage in Experiment I

<table>
<thead>
<tr>
<th>P</th>
<th>A (Phosphate 1) ±</th>
<th>A-r (Phosphate 1) ±</th>
<th>Calibration PO₄(g/l) ±</th>
<th>Phosphate 1(g) ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.192±0.008</td>
<td>0.179±0.008</td>
<td>7.62<em>10⁻⁴±3.41</em>10⁻⁵</td>
<td>0.0127±0.0006</td>
</tr>
<tr>
<td>70°C</td>
<td>0.046±0.001</td>
<td>0.034±0.001</td>
<td>1.45<em>10⁻⁴±5</em>10⁻₆</td>
<td>0.0024±0.0001</td>
</tr>
<tr>
<td>90°C</td>
<td>0.049±0.001</td>
<td>0.036±0.001</td>
<td>1.55<em>10⁻⁴±5</em>10⁻₆</td>
<td>0.0026±0.0001</td>
</tr>
<tr>
<td>120°C</td>
<td>0.028±0.011</td>
<td>0.015±0.011</td>
<td>6.5<em>10⁻⁵±4.5</em>10⁻⁵</td>
<td>0.0011±0.0008</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>0.313±0.015</td>
<td>0.3±0.015</td>
<td>1.25<em>10⁻³±5</em>10⁻₅</td>
<td>0.0213±0.0011</td>
</tr>
<tr>
<td>70°C</td>
<td>0.058±0.009</td>
<td>0.045±0.009</td>
<td>1.8<em>10⁻³±4</em>10⁻₅</td>
<td>0.0032±0.0007</td>
</tr>
<tr>
<td>90°C</td>
<td>0.048±0.004</td>
<td>0.035±0.003</td>
<td>1.45<em>10⁻³±1.5</em>10⁻⁵</td>
<td>0.0025±0.0019</td>
</tr>
<tr>
<td>120°C</td>
<td>0.014±0.003</td>
<td>0.002±0.000</td>
<td>1<em>10⁻⁵±0.0</em>10⁻⁵</td>
<td>0.0002±0.0000</td>
</tr>
</tbody>
</table>

phosphate which has effect on measurements for optimization. Calculation is done on basis of below steps:

1- A, Phosphate 1: from table 12
2- A-r, Phosphate 1: A, Phosphate 1- 0.013(from table 13, asorbance of water)
3- Calibration PO₄ (g/l) : A-r, Phosphate 1/234.8
4- Initial PO4 (g/l) : Calibration PO₄ (g/l)*100/0.3
5- Initial PO4 (g/50ml) : Initial PO4 (g/l)/20
6- Phosphate 1(g) : Initial PO4 (g) in 50 ml

Calculation steps in table 15 are similar to table 12, only it has two more steps.

7- Phosphate 2-1: Phosphate 2(g) - Phosphate 1(g)
8- Δ/AIM: Phosphate (2-1)/amount of biomass

Table 15: biomass amount

<table>
<thead>
<tr>
<th>Sample kind</th>
<th>Biomass(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM-P</td>
<td>0.5120±0.0076</td>
</tr>
<tr>
<td>AIM-M</td>
<td>0.4641±0.0028</td>
</tr>
</tbody>
</table>
Table 16: phosphate of second stage in Experiment I, and amount of biomass

<table>
<thead>
<tr>
<th>P</th>
<th>A-Phosphate</th>
<th>Phosphate 2(g)</th>
<th>Phosphate 2-1</th>
<th>∆/AIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.225± 0.005</td>
<td>0.0151± 0.0004</td>
<td>0.0024± 0.0003</td>
<td>0.0045± 0.0004</td>
</tr>
<tr>
<td>70°C</td>
<td>0.0825± 0.013</td>
<td>0.0048± 0.0011</td>
<td>0.0026± 0.0009</td>
<td>0.0049± 0.0002</td>
</tr>
<tr>
<td>90°C</td>
<td>0.054± 0.006</td>
<td>0.0029± 0.0004</td>
<td>0.0004± 0.0001</td>
<td>0.0007± 0.0001</td>
</tr>
<tr>
<td>120°C</td>
<td>0.028± 0.011</td>
<td>0.0011± 0.0007</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 17: Total recovery in Extraction I

<table>
<thead>
<tr>
<th>P</th>
<th>chitosan1/AIM</th>
<th>chitosan2/AIM</th>
<th>Phosphate1/AIM</th>
<th>Final Recovery: Total/AIM(g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.0043± 0.0024</td>
<td>0.044± 0.0118</td>
<td>0.0237± 0.0004</td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>0.0222± 0.0099</td>
<td>0.0693± 0.0179</td>
<td>0.0049± 0.0001</td>
<td></td>
</tr>
<tr>
<td>90°C</td>
<td>0.0533± 0.0029</td>
<td>0.0115± 0.0026</td>
<td>0.0049± 0.0001</td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>0.0132± 0.0011</td>
<td>0.008± 0.0016</td>
<td>0.0005± 0.0001</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.0931± 0.0104</td>
<td>0.1929± 0.0019</td>
<td>0.0339± 0.0005</td>
<td>0.8787± 0.0335</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>chitosan1/AIM</th>
<th>chitosan2/AIM</th>
<th>Phosphate1/AIM</th>
<th>Final Recovery: Total/AIM(g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.0108± 0.0087</td>
<td>0.0706± 0.0047</td>
<td>0.0459± 0.0021</td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>0.0265± 0.0109</td>
<td>0.0809± 0.0268</td>
<td>0.0068± 0.0015</td>
<td></td>
</tr>
<tr>
<td>90°C</td>
<td>0.0739± 0.0035</td>
<td>0.1490± 0.033</td>
<td>0.0052± 0.0006</td>
<td></td>
</tr>
<tr>
<td>120°C</td>
<td>0.0144± 0.0015</td>
<td>0.0059± 0.001</td>
<td>0.0003± 0.0001</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.1258± 0.0216</td>
<td>0.3465± 0.0376</td>
<td>0.0581± 0.0005</td>
<td>1.3101± 0.0496</td>
</tr>
</tbody>
</table>

After the measurement of phosphate, it must be calculated the ratio of chitosan to biomass in first and second steps, because only in these steps, degree of deacetylation (DD) is calculated.

For total recovery, it is needed to consider Phosphate of first step, which is related to chitosan
in first and second steps, because total recovery should be calculated only in some steps that DD is obtained, the other data is not effective in experiments. For DD calculation needs at least 0.01 gr of chitosan from biomass.

The recovery from two steps can show acceptable amount from 4 steps total, but it can be along with some faults, for example incomplete recovery from each step can be effect on next steps which is important, and so it is decided to change the method of measurement which is suitable theoretically.

In table 12, DD of some steps are measured, because it would be like to know whether DD is about 50% or not. The aim of this project is optimization chitosan, and it must be noticed to 50% DD for more recovery, because about of this DD, it can be disappeared the cell wall chitosan, so the measurement of DD is a necessary factor for certainty. That is why; the results of DD give definiteness for progressing of work.

**4.9. chitosan Extraction II**

<table>
<thead>
<tr>
<th>kind</th>
<th>M,50H</th>
<th>M,70H</th>
<th>M,90H</th>
<th>M,120H</th>
<th>M,50D,C₂</th>
<th>M,70D,C₂</th>
<th>M,90D,C₂</th>
<th>M,120D,C₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>60.77±0.42</td>
<td>50.69±4.55</td>
<td>47.3±7.9</td>
<td>49.29±1.73</td>
<td>81.1±5.2</td>
<td>86.21±5.6</td>
<td>62.38±4.11</td>
<td>59.87±4.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>25.37±2.65</td>
<td>27.9±0.8</td>
<td>23.8±0.83</td>
<td>20.29±0.3</td>
<td>70.72±6.75</td>
<td>77.05±4.5</td>
<td>61.3±1.36</td>
<td>51.6±5.19</td>
</tr>
</tbody>
</table>

H: HAIM  D: Degree deacetylation  
C₁: chitosan1, C₂: chitosan2  
M: *Mucor indicus* and P: *Rhizomucor pulusilus*

II phase of chitosan excartion is performed, because of some errors which is transferred potencially by HAIM (hot acid insoluble material) as biomass and phosphate components amount from last to next steps in I phase, which is effected on the result. In this phase, It is
decided which is performed each step (50, 70, 90, 120°C) separately, and compared the result of their DD (degree of deacetylation). Standard equation of DD is $y=7935085.71x$ and $R^2=1$ ($x=\text{concentration (molar)}, y=\text{curve under area}$). Table 15 has some results such as:

1- In M, (90°C and 120°C) H and P, (90°C and 120°C) H amount of chitosan acetylation degree is less other temperatures about 50-70. It shows the most extraction is must be done in 90 and 120°C. Absolutely, this explanation kind cannot be exact. In period of phase II of project, unfortunately, it is understood that water is mixed with NaOH. This happening was strange!

By the way, the other results are:

2- With increasing of temperature to 120°C, degree of deacetylation is closer to 50%, thus chitosan has more solubility in high temperatures about 120°C.

3- Solubility of *R. pulusilus* chitosan maybe is more than *M.indicus* in 120°C.

4- These cells show kind of Fungi effects on degree of deacetylation, thus DD of *M.indicus* is more than *R. pulusilus*.

5- Degree deacetylation in HAIM shows this material has chitosan, because the acetic acid is released from chitosan after treatment with 72% Sulfuric acid.

![Fig 17: comparison of chitosan DD in different temperature in *R. pulusilus*](image-url)
Table 19: Crud data from Extraction II

<table>
<thead>
<tr>
<th>P</th>
<th>AIM</th>
<th>Final HAIM</th>
<th>chitosan1</th>
<th>chitosan2</th>
<th>Phosphate1</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.2724±0.0011</td>
<td>0.2673±0.0015</td>
<td>0.0002±0.0001</td>
<td>0.0123±0.0009</td>
<td>0.008±0.0003</td>
</tr>
<tr>
<td>70°C</td>
<td>0.2711±0.0002</td>
<td>0.2641±0.0036</td>
<td>0.0003±0.0002</td>
<td>0.019±0.0003</td>
<td>0.0103±0.0003</td>
</tr>
<tr>
<td>90°C</td>
<td>0.2728±0.0003</td>
<td>0.2232±0.0001</td>
<td>0.0035±0.0003</td>
<td>0.0475±0.0027</td>
<td>0.0136±0.0006</td>
</tr>
<tr>
<td>120°C</td>
<td>0.2725±0.0022</td>
<td>0.1968±0.0058</td>
<td>0.0129±0.0006</td>
<td>0.0634±0.0012</td>
<td>0.0176±0.0004</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>0.4048±0.0001</td>
<td>0.3572±0.0018</td>
<td>0.0007±0.0002</td>
<td>0.0102±0.0002</td>
<td>0.0113±0.0007</td>
</tr>
<tr>
<td>70°C</td>
<td>0.4054±0.0022</td>
<td>0.3358±0.0008</td>
<td>0.0001±0.0000</td>
<td>0.0205±0.0038</td>
<td>0.0124±0.0015</td>
</tr>
<tr>
<td>90°C</td>
<td>0.4132±0.0008</td>
<td>0.3056±0.0195</td>
<td>0.0012±0.0007</td>
<td>0.0565±0.0085</td>
<td>0.0185±0.0002</td>
</tr>
<tr>
<td>120°C</td>
<td>0.4049±0.0044</td>
<td>0.1691±0.0298</td>
<td>0.0007±0.0001</td>
<td>0.1006±0.0198</td>
<td>0.0286±0.0037</td>
</tr>
</tbody>
</table>

Table 19 shows amount of experimental data after extraction II, actually these data is crude. These data are used for recovery calculation like extraction I, but with one different, in this extraction period, from each biomass separate its HAIM e.g. HAIM 2 is for AIM 2.

Phosphate Test II:
Table 20: Phosphate analysis II

<table>
<thead>
<tr>
<th>KH₂PO₄</th>
<th>=136.09</th>
<th>PO₄³⁻</th>
<th>=95</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/l</td>
<td>mol/l</td>
<td>g/l</td>
<td></td>
</tr>
<tr>
<td>main</td>
<td>0.2196</td>
<td>0.001614</td>
<td>0.153295613</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>r(ml)</th>
<th>PO₄³⁻ (g/l)</th>
<th>A (abso.)</th>
<th>A-r</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>0</td>
<td>0.013</td>
<td>0</td>
</tr>
<tr>
<td>ST1</td>
<td>0.5</td>
<td>0.000766</td>
<td>0.169 0.156</td>
</tr>
<tr>
<td>ST2</td>
<td>1</td>
<td>0.001533</td>
<td>0.357 0.344</td>
</tr>
<tr>
<td>ST3</td>
<td>1.5</td>
<td>0.002299</td>
<td>0.538 0.525</td>
</tr>
<tr>
<td>ST4</td>
<td>2</td>
<td>0.003066</td>
<td>0.697 0.684</td>
</tr>
<tr>
<td>ST5</td>
<td>2.5</td>
<td>0.003832</td>
<td>0.864 0.851</td>
</tr>
</tbody>
</table>

Absolutely, for recovery calculation of Fungi chitosan, it should be considered amount of phosphate components in distellet water aslo. Its method is exactly similar to phosphate analysis I. The equation standar of phosphate test is $y=223.2x$ with $R^2=0.999$ ($x=\text{PO}_4(\text{g/l}), y=\text{A}-\text{r}(\text{corrected A})$).

In both Fungi, higher temperate will increase amount of phosphate, because temperature causes to broke down binds between chitosan and phosphate.

Table 21: Total recovery in different temperature for $R. pulusilus$ and $M.indicus$

<table>
<thead>
<tr>
<th>P</th>
<th>chitosan 1/AIM</th>
<th>chitosan2/AIM</th>
<th>Phosphate1/AIM</th>
<th>Final Recovery: Total/AIM(g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.0008±0.0004</td>
<td>0.0452±0.0032</td>
<td>0.0279±0.0002</td>
<td>0.271±0.0086</td>
</tr>
<tr>
<td>70°C</td>
<td>0.0011±0.0007</td>
<td>0.0701±0.0011</td>
<td>0.0390±0.0001</td>
<td>0.4068±0.0019</td>
</tr>
<tr>
<td>90°C</td>
<td>0.0126±0.0013</td>
<td>0.1741±0.0101</td>
<td>0.052±0.0001</td>
<td>0.8778±0.0403</td>
</tr>
<tr>
<td>120°C</td>
<td>0.0475±0.0019</td>
<td>0.2326±0.0025</td>
<td>0.063±0.0005</td>
<td>1.2598±0.0037</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>0.0016±0.0001</td>
<td>0.0252±0.0005</td>
<td>0.0281±0.0018</td>
<td>0.1355±0.0025</td>
</tr>
<tr>
<td>70°C</td>
<td>0.0002±0.0000</td>
<td>0.0506±0.0097</td>
<td>0.0307±0.0041</td>
<td>0.2015±0.0348</td>
</tr>
<tr>
<td>90°C</td>
<td>0.003±0.0016</td>
<td>0.1678±0.0292</td>
<td>0.0449±0.0005</td>
<td>0.4471±0.2069</td>
</tr>
<tr>
<td>120°C</td>
<td>0.0017±0.0002</td>
<td>0.2487±0.0516</td>
<td>0.0706±0.0099</td>
<td>0.7943±0.1597</td>
</tr>
</tbody>
</table>

Some results take from this table. They are:

1) With dialysis, amount of chitosan in second steps is more than first steps usually.
2) Final Recovery: Total amount of chitosan in two steps plus phosphate (g) / AIM(g) gives the suitable result in 90 and 120°C in *R. pulusilus*, but *M.indicus* doesn’t have nice recovery which can related to their DD, because it is near to 50%,and chitosan is solved in this area, and their recovery will be difficult even with dialysis.

3) Unfortunately, final recovery shows some errors that it does not any reason for that.

4) Generally, discontinue method has less errors for extraction of chitosan and phosphate components from AIM.

Table 22: average of phosphate from *R. pulusilus* and *R. pulusilus* and *M.indicus* in double experiences

<table>
<thead>
<tr>
<th>Phosphate Average in P</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.008±0.0003</td>
</tr>
<tr>
<td>70°C</td>
<td>0.0103±0.0003</td>
</tr>
<tr>
<td>90°C</td>
<td>0.0136±0.0006</td>
</tr>
<tr>
<td>120°C</td>
<td>0.0176±0.0004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phosphate Average in M</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>0.0113±0.0007</td>
</tr>
<tr>
<td>70°C</td>
<td>0.0124±0.0015</td>
</tr>
<tr>
<td>90°C</td>
<td>0.0185±0.0002</td>
</tr>
<tr>
<td>120°C</td>
<td>0.0286±0.0037</td>
</tr>
</tbody>
</table>

Fig 19: comparison of phosphate deliverance in different temperatures of extraxtionII for *R. pulusilus* and *M.indicus*

Fig 19 approves that more amount of Phosphate is released from cell wall with more temperatures, hence in 90 and 120°C is released more chitosan respectively.

This fig shows to remain less chitosan in high temperatures from 50 to 120°C, of course it can be along with unaccuracy and errors which is explained before.
To forbidden from errors, it is decided to do each temperature separately with AIM (alkali insoluble material) as biomass. This decision is along with nice result for separation of phosphate and chitosan.

Also, this fig shows to remain less chitosan in high temperatures from 50 to 120°C, of course it can be along with unaccuracy and errors which is explained before.

Accurate results have been received in extraction II, more temperature that causes more separation of chitosan and phosphate.
In extraction of I phase, much inaccuracy happened between experimental method, so it cannot be trusted to show its data, only it is focused on extraction II for more discussion.

This project was done on Fungal chitosan, so it must be compared to acquired data from same experience in past. First, it is not considered the species of *M.indicus*. It can be understood this new method has more efficiency, because J. Synowiecki and N. Al-Khateeb in 1997 took about 42% chitosan on basis of AIM of *M.rouxii* [30], but now it is resulted about 68% chitosan on basis of AIM, of course they didn’t consider the phosphate components, this effects on efficiency also, and maybe it will be less. In other example, *Gongronella butleri* USDB 0201 gives yield with 4–6 g chitosan/100 g mycelia [31].

Fig 22: amount of chitosan in 2 steps in *R. pulusilus*

Fig 22 shows amount of chitosan in 120°C in second step is more than 50, 70 and 90°C, because this temperature gives more energy for distruption of phosphate and chitosan bond. One thing is important, 120°C is not suitable, because degree of deacetylation in this area is about 50%, and hence probably solubility of chitosan will be more. This happening needs dalysis to separate chitosan from phosphat.
If compare the fig 23 it could be understood the chitosan extraction characteristics is same in *R. pulusilus* and *M.indicus*, hence amount of chitosan is more in 120 in second step. tow amazing point, both of *R. pulusilus* and *M.indicus* give more chitosan in second step, also *M.indicus* has less chitosan rather than to *R. pulusilus* in first step.

Comparison of chitosan and phosphate in *R. pulusilus* shows in more high temperature, it can be separated more phosphate and chitosan from AIM (alkali insoluble material). Amount of chitosan will increase with more slant, but phosphate has more slop.
In *M. indicus*, high temperature has more efficiency on chitosan extraction and unrestricted phosphate. This fig shows a behavior similar to chitosan and phosphate components of *R. pulusilus*.

Recovery is total of chitosan in first and second step plus phosphate in first step to AIM (alkali insoluble material). In comparison between two Fungi it is resulted in 50°C, *M. indicus* has more recovery, but *R. pulusilus* has more efficiency in 70, 90 and 120°C totally, but it must be noticed that chitosan has the degree of deacetylation about 50%. It means more trouble for extraction of that.
Fig 27: comparison of DD and chitosan in *R. pulusilus* and *M.indicus* in Linear format

Fig 28: comparison of DD and chitosan in *R. pulusilus* and *M.indicus*

Generally, the functional properties of chitosan depend on intrinsic factors such as degree of deacetylation (DD) distribution of acetyl group, molecular weight (MW). Different MWs chitosan is prepared by hydrolysis methods. Apparently, DD has effect on degradation rate and rate constant, because hydrolysis of alkali insoluble material (AIM) by 1% Sulfuric acid decreased with increasing DD of chitosan, hence more chitosan is exist in less DD. This confusing point be due to the bond energy of acetamido is higher than β(1-4) glucoside linkage or hydrogen bond. Breakage of β(1-4) glucoside linkage will result in lower
molecular weight and increasing reaction rate and the rate constant. In other word, in the higher DD has the easier molecular degradation of chitosan during of 1% Sulfuric acid. Actually, for the red area, it does not have any idea, unless exist of experimental errors. The fig. 27 and 28 also show R. pulusilus has more chitosan in different steps in corresponding to related DD rather than M.indicus.

![Deacetylation degree of Chitosan 2](image)

Fig 29: deacetylation degree in different temperature

**Comparison of DD and recovery in R.pulusilus and M.indicus**

![Comparison of DD and recovery in R.pulusilus and M.indicus](image)

Fig 30: Comparison of DD and Recovery in M.indicus and R.pulusilus
If the above discussion will be correct, so it can be understood in the less degree of deacetylation will be more chitosan, because in high temperature will be more the total chitosan of first and second steps. High temperature give more energy to disrupt the phosphate bond, thus the amount of phosphate will be more also, actually the recovery, which is chitosan in first and second step plus phosphate, will be high.
Chapter 5.

Conclusion

It is resulted that more time of alkalytions of chitin with NaOH (40% w/w) will give chitosan with high Degree of deacetylation. After dissolving of chitin in 1%H$_2$SO$_4$ and chitosan in H$_2$O, it is resulted less recovery and more solubility respectively. This experiences show that temperature as a factor needs for optimization of chitosan extraction, hence it should be considered for extraction of Fungal chitosan from bonded salt phosphate in alkali insoluble material and this factor has relationship on different amounts of phosphate bond. Actually, Try and error method was done in this project for the effect of temperature on optimization as a factor. A problem gets appearance on high temperature (120°C) that is deacetylated chitosan degree is about 50%, which has high solubility in water, so it needs to more researches, because it has effect on being disappear chitosan and usage of dialysis as an extraction method. Dialysis method with continue water flow is effective to separate chitosan from phosphate bond, but certainly, it is a time consuming solution. In high temperature (120°C), amount of total chitosan in before and after first dialysis is more than the other temperature (90, 70 and 50°C) in same way. In other word, the total phosphate deliverance before first dialysis in 120°C is more than the other temperatures respectively.

These experiences show that two Fungi of *R. pulusilus* and *M.indicus* have same diagram about amount of chitosan and phosphate deliverance in high temperature (120°C) rather than to 90, 70 and 50°C in before and after first dialysis as well recovery will be more in *R. pulusilus* rather than to *M.indicus* in 90°C.
Chapter 6.

*Future work*

To complete of this project is better to experience the usage of distilled water to chitosan solution, and then to add the acetone for separation in some steps, at the end it take apart by centrifuge. Utilization of electricity can be proposed to make an electric field for increasing of electro-negativity difference between phosphate and chitosan, and they are separated between two electrodes. It can be saved time rather than to dialysis. After taking the results, Response Surface Methodology is the best solution to find the effect of temperature and time on extraction of chitosan from Fungi.
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