STANDARDIZATION OF OPTIMUM CONDITIONS FOR THE PRODUCTION OF GLUCOSAMINE HYDROCHLORIDE FROM CHITIN

Thesis submitted to
Cochin University of Science and Technology
in partial fulfillment of the requirements
for the degree
of
Doctor of Philosophy
By

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June-2006

DECLARATION

I, Martin Xavier K.A. do hereby declare that the thesis entitled **"STANDARDIZATION OF OPTIMUM CONDITIONS FOR THE PRODUCTION OF GLUCOSAMINE HYDROCHLORIDE FROM CHITIN"** is a genuine record of research work done by me under the guidance of Dr. K. G. Ramachandran Nair, Joint Director and Head of Fish Processing Division, Central Institute of Fisheries Technology, Cochin and that no part of this work has previously formed the basis for the award of any degree, diploma, associate-ship, fellowship or other similar title of any University or Institution.

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CERTIFICATE

This is to certify that this thesis entitled **"STANDARDIZATION OF OPTIMUM CONDITIONS FOR THE PRODUCTION OF GLUCOSAMINE HYDROCHLORIDE FROM CHITIN"** is an authentic record of the research work carried by Mr. Martin Xavier K.A, under my supervision and guidance in the Central Institute of Fisheries Technology, Cochin in partial fulfillment of the requirements for the degree of Doctor of Philosophy and that no part of this work thereof has been submitted for any other degree, diploma, associate ship, fellowship or any other similar title.

(Dr. K. G. Ramachandran Nair)

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ACKNOWLEDGEMENT

I wish to express my heartfelt gratitude and deepest sense of indebtedness to my guide Dr. K. G. Ramachandran Nair, Joint Director and Head of Fish Processing Division of Central Institute of Fisheries Technology, Cochin for his encouragement, inspiration and enthusiastic guidance throughout my research work.

I express my humble gratitude to Dr. K. Devadasan Director, CIFT, Cochin for giving permission, providing facilities and encouragement to carry out this work.

My heart felt thanks go to Dr. V. Geethalekshmi, Senior Scientist, EIS division of CIFT for helping the statistical analysis. I also express my gratitude to Dr. P.T. Mathew, Principal Scientist, Fish processing Division, Dr. Jose Joseph, Principal Scientist, Fish processing Division, Dr. T.K. Thankappan, Principal Scientist, Fish processing Division, Dr. Ashok Kumar, Senior Scientist, QAM division, Dr. T.V. Shankar, Senior Scientist, Biochemistry division, Dr. Suseela Mathew, Senior Scientist, Biochemistry division for providing critical remarks and valuable suggestions for preparing this thesis work.

I feel great pleasure in expressing my regards and profound indebtedness to Shri. M, Asharaf, Senior scientist of Fishing technology division for his help. I extend my gratitude to All Scientists and Staff of Fish Processing Division for giving me all co-operations during the study.

I am very much thankful to Dr. Nikita Gopal, Senior Scientist of EIS division, Dr. Ashaletha Senior Scientist of EIS division and Dr. G. R. Unnithan, Principal Scientist of EIS division for providing all the help in compiling the work.

The assistance rendered by the library staff Mr. Devasya, Smt. Shylaja and Mr. Baskaran for the collection of necessary literature are gratefully acknowledged. I am thankful to Mr. R. D. Goswami personal assistant for his assistance through out my work.

I wish to share my deepest feeling of gratitude to my colleagues, Anoop Krishna, Shine Kumar C.S. Sini T.K., Santhosh S., Aneesh kumar, Jijoy T., Jones varkey, Gipson and Arun Mallick for their constant support and help rendered throughout my academic period.

I take this moment to remember all my teachers and well-wishers for their blessings and support. I owe everything achieved to my loving parents, grandmother, brother and sister for their affection, inspiration and above all keeping me always beside the divine power.

(Martin Xavier K.A)

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1. INTRODUCTION

Shellfish processing industries around the world turn out a significant quantity of head and shell as industrial waste. This waste must be removed immediately from the premises with out allowing to spoil to prevent the contamination to the processing environment, and should be disposed of in a manner that is not detrimental to the receiving environment. The magnitude of the problem of waste management in the fish industry depends on the volume of waste, its polluting charge, rate of discharge and the assimilatory capacity of the receiving medium. The disposal of shellfish waste generated is a serious problem as it poses environmental problems. The techniques that are available for their disposal include ocean dumping, incineration or disposal of landfill sites (Rehav-moiseev and Carroad, 1981).

Annual world wide crustacean shell production has been estimated to the 1000 million metric tones. In India availability of shrimp processing waste is estimated to be nearly 1.25 lakh tons and it is the single largest fishery waste of the country. Crab shell is yet another waste thrown out in large quantities from seafood processing. A part from these squilla, another species of crustacean which has no use as such also statutes a considerable portion of the trawler catch in certain seasons. The species of squilla available in Indian waters *Oratosquilla nepa* is not generally used for human consumption as it does not contain much meat and hence almost the entire catch is thrown back to the sea. In the absence of a commercial fishing for squilla, its abundance cannot be correctly estimated. However, it is believed to be almost equal to that of prawns.

On an average the head and shell constitute not less than 50% weight of the whole prawn. At present only a small portion of this finds use as ingredient in shrimp/poultry feed mix. The shellfish waste contains mainly protein, minerals and chitin. Over the years, there has been considerable effort to develop techniques for the recovery and utilization of this biopolymer from these wastes. Serious environmental problems caused by prawn shell waste can be avoided by using it as raw material for production of chitin and its derivatives. In addition to controlling environmental pollution, it saves the

expenditure incurred by the industry to disposing the waste and generates more employment and the economy of the industrial processing of crustaceans can be improved by the full utilization of chitin and protein present in the waste.

Chitin

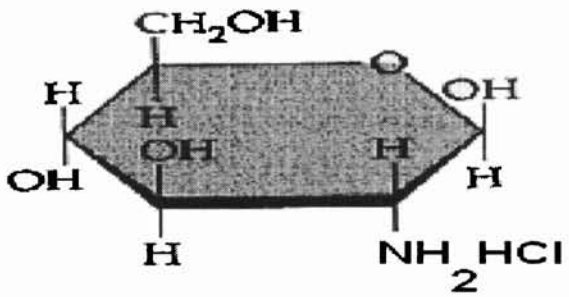
Chitin is a polymer formed primarily of repeating of β (1-4) 2-acetamido 2-deoxy-D-glucose or N-acetyl glucosamine. Chitin is a nitrogenous polysaccharide, which is white, hard, inelastic, found in the outer skeleton of insects, crab, shrimp and lobsters and in the internal structures of other invertebrates. Its structure is similar to the structure of cellulose, except that acetyl amino groups have replaced the hydroxyl groups in position-2. Native chitin has a limited application potential, but the deacetylated chitin has a wide spectrum of applications ranging from large scale technical applications, in functional membranes, food technology and protein precipitation to very sophisticated applications in medicine and cosmetics (Knorr, 1991; Tsugita, 1990). Despite its early discovery and being the second most abundant polysaccharide after cellulose with an estimated annual production of at least 1000 million metric tons per year in the biosphere, chitin has remained an almost unused biomass resource until the last quarter of 20^{th} century. The traditional and commercial source of chitin is by chemical or microbiological processes and which can be extracted by some Fungi like *Aspergillus niger, Mucor rouxii, Penecillium notatum* (Tan et al., 1996, Knorr, 1984) On treatment with concentrated acid chitin gives a relatively pure amino sugar called Glucosamine and it is used as a supplementary food for various purposes.

In the present world, after IT revolution, we are passing through a phase of nutraceutical revolution. Demand for these products increased recently. Developed countries spend millions of dollars for Nutraceutical research and product development. The term Nutraceutical coined in 1989 refers to substances that may be considered food or part of a food and provide medical or health benefits, including prevention and treatment of diseases. The movement to nutraceuticals is one that could represent the

most significant trend affecting the food industry during the next decades. Glucosamine can be considered as a nutraceutical product by virtue of its properties.

Glucosamine hydrochloride

Glucosamine and its derivatives like N-acetylglucosamine (GlcNAc) and D-glucosamine hydrochloride (GlcNHCl) have attracted much attention owing to their therapeutic activity in arthritis and been approved as a food supplement by FDA. Glucosamine is an amino sugar synthesized in the body from glucose through hexosamine path way and it is naturally occurring in the connective tissues contributing to maintenance of strength, flexibility and elasticity of these tissues.



Structure of Glucosamine hydrochloride

Glucosamine is also important for healthy skin. Adequate amounts of it in the blood are necessary for the production of hyaluronic acid, one of the substances essential to heal skin injuries. Therefore, glucosamine plays a major role in the healing of surgical incisions and skin wounds. In a few studies, participants who began taking supplemental glucosamine before surgery and continued taking it until their incisions were completely healed showed generally faster healing with less scarring than other participants who did not take glucosamine. Wound dressings that contain a modified type of glucosamine known as poly-N-acetyl glucosamine are being studied to stop major bleeding. In other research, a few studies evaluating whether supplemental glucosamine helps to prevent or reverse wrinkling have had

mixed results. Because natural production of hyaluronic acid decreases as individual's age, decreases in it may contribute to wrinkling of the skin. It is thought that increasing glucosamine may help the skin stay more resilient.

Glucosamine is brought to the attention of the chemical selection-working group as a widely used dietary supplement that would be consumed over a period of many years to maximize potential beneficial effects. No chronic toxicity studies to support the safety of such long-term use were found in the available literature. Used alone or with chondroitin sulphate glucosamine salts alleviate pain and inflammation from Osteo-arthritis and reportedly have beneficial effects on degenerated joints. Thus dietary supplements containing glucosamine and chondroitin sulphate have a potential market of hundreds of millions of people who suffer from osteoarthiritis, athletes and dancers who may have joint overuse, and aging body boomers interested in maintaining their joints.

Glucosamine is a poly-hydroxylated primary amine, used in the body as a molecular element for special macromolecules, the proteoglycans, and important constituents of the articular cartilage. Virtually no information on the potential toxicity of orally administered glucosamine was found in the available literature. Adverse events reports from limited clinical trials have not mentioned significant toxicity in humans at the doses consumed. Glucosamine hydrochloride, Glucosamine sulphate and N-acetylglucosamine are the commonly used glucosamine derivatives.

Enzymatic and chemical hydrolysis of chitin are widely employed for the production of glucosamine. During hydrolysis, cleavage occurs in the glycosidic bonds and subsequent deacetylation occurs. The resulting products contain glucosamine along with intermediate compounds. Considering the simplicity, speed and cost of production acid hydrolysis is extensively used for the production of glucosamine hydrochloride from chitin.

The market for dietary supplements containing glucosamine and chondroitin sulphate is large. Between July 1998 and May 1999, the retail sales in the United States were estimated to be more than 500 million dollars (Adebowale, 2000). These dietary supplements are marketed primarily for

pain and inflammation relief in osteoarthiritis and related autoimmune diseases that affect the joints. 40 million Americans have been reported to suffer from osteoarthiritis. In Europe, glucosamine has been approved as prescription drug for over a decade (Adebowale et al., 2000; Hippocrates, 2000). Large food manufacturers have begun to add glucosamine compounds to fruit juices. For example, Pepsi' s widely available *SoBe Sport System* ® line, which contains glucosamine, is promoted for use among young athletes. Elation ®, another fruit-flavored beverage containing glucosamine, is produced by Coca-Cola and Procter & Gamble (Barnes and Winter, 2001).

Glucosamine hydrochloride can be considered as a nutraceutical by virtue of its properties. As a pharmaceutical product its preparation has only begun now in India. A number of products have been launched in the Indian market with glucosamine hydrochloride and glucosamine sulphate as the major ingredient. Glucosamine is a highly valued commercial product and hence the details of its production technology and its chemical properties are not widely available in literature. In this context it is felt that the technologies for the production of glucosamine hydrochloride in the purest form with maximum yield and least investment will help the industry significantly. The objectives of the proposed work are,

- To develop a feasible method for the production of glucosamine hydrochloride of high purity from crustacean processing waste.
- To study the effect of demineralization of prawn shell during chitin production and its effect on hydrolysis of chitin.
- To study the effect of deproteinisation of chitin on the yield of glucosamine hydrochloride.
- To study the effect of acid concentration on chitin hydrolysis.
- To study the effect of volume of hydrochloric acid on chitin hydrolysis.
- To study the effect of duration of treatment of chitin with

hydrochloric acid on glucosamine hydrochloride conversion.

- To study the effect of temperature on hydrolysis of chitin.
- Standardization of conditions for the purification of glucosamine hydrochloride.
- Eco-friendly approach for utilization of spent acid.
- Storage studies of glucosamine hydrochloride.

2. REVIEW OF LITERATURE

2.1. Origin of Chitin research

The first explicit account of chitin was in 1811, attributed to Prof. Henri Braconnot, Director of the botanical garden at Nancy, France. He described and named the alkali resistant fraction from isolates of certain type of mushroom "Fungine". A decade later, a similar substance was found to be present in certain insects in 1823 by Odier who named it "chitin" after the Greek word "Khiton" that denotes "envelop" in reference to the cuticle. Subsequently, the chemical character of chitin began to be elucidated. The presence of nitrogen in chitin is attributed to Payen in 1843. In 1859, Prof. C. Rouget subjected chitin to alkali treatment and observed that unlike chitin, the substance resulting after alkali treatment dissolved in acids; which was named as "Chitosan" by Hoppe-seiler in 1894 .

During 1930's and 1940's these biopolymers of glucosamine gained much interest within the oriental world, mainly in applications in the field of medicine and water purification. During 1970's the interest in these bio-macromolecules renewed at a brisk pace resulting in the first ever Chitin-Chitosan conference held in the United States in1977. It has taken more than hundred years to arrive at the chemical identity of chitin and the revelation of its polymeric properties.

2.2. Source of Chitin

Chitin was found in animals and plant sources. The major sources of chitin are shrimps, crabs, squilla, lobsters, krill, clams, oysters, squid, insects and fungi. Allan et al. (1978) estimated the chitin content of selected crustacean, insects, molluscan organs and fungi. Though the main source of chitin is animals, it is frequently present as a cell wall material in plants, replacing cellulose or some times occurring together with cellulose. This polymer in the deacetylated form ie. chitosan is present in various fungi eg. Zygomycetes contain both chitin and Chitosan. (Austine et al.,1981, Rudall, 1969). Chitin is extensively produced from fungi varieties such as

Aspergillus niger, Mucor rouxii, Penecillium notatum (Tan et al., 1996, Knorr, 1984). Chitin is present in marine diatoms, protozoa and the cell walls of several fungal species. Chitin from the diatom spines such as *Cyclotella cryptica* and *Thalassiosira fluviatilis* are the only form reported to be 100% poly N-acetylglucosamine that is not associated with proteins and is termed Chitan (McLachlan et al., 1965). Fungal chitin is covalently tented to other molecules such as glucans, a requirement of their biological role. Apart from these sources byproducts from citric acid and antibiotic manufacturing plants may also become an important source for chitin.

2.3. Chitin structure

Chitin is a biopolymer comprising of N-acetyl glucosamine polymers with β (1-4) glycosidic bonds between each monomeric unit (Figure-1). The term chitin currently refers to a polymer of β (1-4) N-acetyl-D-glucosamine, where a minority of the acetyl groups has been lost.

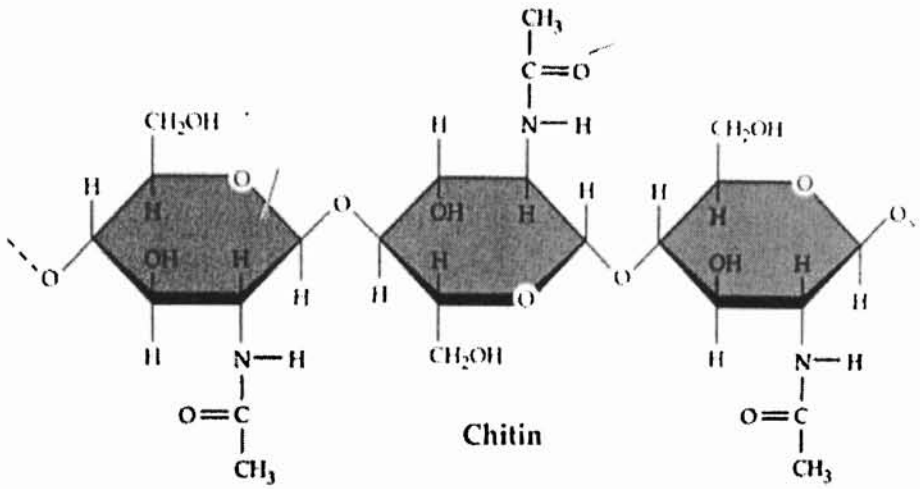


Figure-1 Structure of Chitin

Chitin occurs in three polymorphic solid-state forms designated as α, β, and γ chitin, which differ in the arrangement of molecular chains with in the crystal cell. α chitin is the tightly compacted, most crystalline polymorphic form where the chains are arranged in an anti-parallel fashion, β chitin is the form where the chains are parallel and γ chitin is the form where two chains

are 'up' to everyone 'down" (Muzzarelli, 1977).

α chitin is the most abundant form, found in shellfish and also in fungal cell wall. α chitin is the more stable. Isolation methods for α chitin involve harsher treatments than extraction methods for β chitin. β chitin is found in the diatom spines and squid pens. It should be noted that because of the greater mobility of β chitin chains, they are readily transformed irreversibly by steam annealing into α chitin. β chitin is more readily soluble in a number of solvents and highly reactive than α chitin as the inter-molecular forces are weak. Therefore chitin derived from squid pen is expected to have a higher molecular weight. γ chitin was found in stomach lining of squid and cuttle fish. α, β and γ chitins were found in the different organs of Squid

2.4. Extraction of Chitin

Chitin is a natural polymer mainly composed of β (1-4)-linked N-acetyl-D-glucosamine (NAG) units and it occurs generally as a chitin-protein complex, the protein content of which is in the range of 30-70%. The complex it self may also be calcified, and the mineral content were as high as 75% in the crustacean shells. Purification of chitin involves deproteinization with hot alkali followed by demineralization with cold dilute acid. Such specimen of chitin generally contains some de-acetylated residues and there has been some speculation that these may be the points for covalent linkage to the protein (Blackwell, 1982). It has been reported that native chitin contains 82.5% of acetyl glucosamine, 12.5% of glucosamine and 5% water (Muzzarelli, 1977). For the β-chitin in loligo pen, it appears that the frequency of free amino group is about one in every five or six residues, a structure slightly less acetylated than the one found by Giles et al., (1958) for the α-chitin of lobster shells (Muzzarelli, 1977). Complete removal of protein from shellfish sources, is especially important for biomedical applications, as a percentage of human population is allergic to the shellfish, the primary culprit being the protein content (Knor, 2001). Wide ranges of chemicals have been tried for deproteinisation with variation in the temperature and duration of treatment. The use of NaOH invariably results in partial deacetylation of chitin and hydrolysis of the biopolymer that lowers the molecular weight of

chitin (Brine, 1981). Burtos and Healy (1994) have found that chitin obtained by bacterial deproteinisation had a higher molecular weight compared to chemically prepared chitin. The use of proteolytic enzymes such as pepsin, papain or trypsin has been shown to minimize deacetylation and depolymerization in the chitin isolate. Other proteolytic enzymes such as tuna trypsin, Rhozyme- 62, Cod trypsin and bacterial proteinase have also seen demonstrated to remove proteins from crustacean shells (Hall, 1994). The enzymatic digestion and separation of the shell waste proteins should allow the recovery of the protein hydrolysate with a well-balanced amino acid composition (Jozef and Nadia, 2000). The production of chitin on an industrial scale was started recently and it was concentrated in maritime countries of the world. Global production of chitin is about 2000 tonnes with Japan and Poland as the leading producers. Significant quantity is manufactured now in India also.

2.5. Effect of alkali concentration on chitin quality

In deproteinisation, covalent chemical bonds have to be destroyed between the chitin-protein complex. This is achieved with some difficulty especially if performed heterogeneously utilizing chemicals that will also depolymerize the chitin. A wide range of chemicals have been tried for deproteinisation which include reagents like NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $Ca(OH)_2$, Na_2SO_3, $NaHSO_3$, $CaHSO_3$, Na_2PO_4 and Na_2S. In deproteinisation alkali strength, temperature of reaction etc. are significant. Holan et al., (1971) reported that chitin in 3% sodium hydroxide, after 3-hour results in 3.4% higher deacetylation at 60°C and 98% after 2 hour at 100°C. The effect of alkali treatment on the macromolecule length during the production of chitosan from chitin is less pronounced than that of the acid treatment. With increasing cooking time in strong alkali, the degree of deacetylation increases with increasing deproteinisation resulting the production of low molecular weight chitin.

2.6. Effect of acid concentration on chitin quality

Harsh chemical treatments are usually required to remove carbonate

and other minerals and protein from raw chitinous material. A mineral free chitin i.e. with very low ash content would be required for applications that have very low impurity tolerance. In demineralization, type of acid, concentration, temperature and extent of reaction time are the important factors, which determines chitin quality. Because of its low cost and ready availability commercial Hydrochloric acid is commonly used for demineralization. Muzzarelli (1977) reported that any acid treatment carried out on chitin or chitinous raw materials, leads to a partial or extended depolymerization and deacetylation. Holan et al., (1971) determined that in 2% HCl at 98°C during 14.5 hr, the deacetylation is 29.5% and the yield of glucosamine is 4.4% of chitin. Chitin in 40% nitric acid and 30% hydrochloric acid showed that extensive degradation occurred even at 0°C.

2.7. Effect of temperature on chitin quality

Chitin and Chitosan in air at high temperature undergo degradation. Thermal analysis showed that these polymers couldn't with stand temperature higher than 100 - 120°C (Bihari *et.al.,* 1975). Thermal decomposition of N - acetyl -D- glucosamine takes place in maximum rate at about 200°C followed by a second process at 400 to 450°C (Muzzarelli; 1977). The thermal stabilities of the three polymers such as chitin, chitosan, and chitosan hydrochloride are significantly different, with chitin being the most stable. Chitosan hydrochloride is the least stable, presumably because the hydrogen chloride liberated in the probe at elevated temperatures attacks the β- glycosidic links between the monomer units, causing rapid depolymerization. This observation confirms that chitosan hydrochloride darkens rapidly when heated in an oven at 125°C. Water loss from chitin polymer takes place at 60° C and the main thermal degradation process takes place at 300°C and 270°C for chitin and chitosan respectively. Higher temperature and prolonged time increase the percentage of deacetylation and reduce the molecular size. Rao *et.al.* (1987) reported that deacetylation of chitin is always accompanied by partial degradation and reduction in molecular weight. Reduction of the reaction temperature from 100°C to 60°C offers not only energy saving but also quality improvement presumably as a

result of milder treatment.

2.8. Biodegradation of chitin

The term biodegradable normally refers to a material being susceptible to enzymatic degradation and other bio-based reactions when exposed to natural environment. Implicit in the concept of a biodegradable biomaterial is the ability for the biomaterial to perform its required function for a predetermined time period with the gradual dissipation of the biomaterial until ultimately, it is totally assimilated by nature. The estimated amount of chitin synthesized by all known biological systems that produce these biopolymers is nearly one billion tons per year (Hirano, 1994).

2.9. Enzymatic hydrolysis

Enzymatic hydrolysis of chitin to acetyl glucosamine is performed by a system consisting of two hydrolases: Chitinase (chitin glycan hydrolase, E.C. 3.2.1.14) and Chitabiase (Chitabiase acetyl-amino deoxygluco hydrolase, E.C. 3.2.1.29). Chitinases are widely distributed enzymes synthesized by bacteria, fungi and digestive glands of animals whose diet includes chitin. Reviews had been made on this subject by Jeuniaux (1963, 1966 and 1971). The occurrence of chitinase in sugar maple and oaks is explained by Wargo (1975) in terms of protection against invasion by microorganisms.

2.10. Chitosanases (E.C 3.2.1.99)

Chitosanases are found to be active in hydrolyzing chitosan (Monaghan et al., 1973). The molecular weight of the chitosanase was estimated to be about 31,000 by gel filtration on Sephadex G-100. Chitosanases are stable at temperatures below 50°. If the enzyme is incubated at 70°C for 15 minutes about 70% of the original activity remains. Chitosanase is most active at pH 5.6 and it is inactivated rapidly at alkaline pH. Minoru (1988) isolated chitosanase-producing bacteria from soil and the enzyme was purified to homogeneity. The enzyme has a molecular weight of 27000 and shows a pH of 9.2 with high substrate specificity to chitosan.

2.11. Chitin hydrolyzing bacteria

Chitin degrading microorganisms representing a variety of genera have been isolated from a wide range of habitats. Warnes and Rux (1983) isolated chitinolytic bacteria from fresh water lakes which belong to the genera *Myxobacteria spp, Pseudomonas spp* and *Serratia spp.* Okutani (1978) reported that at the most 10% of the heterotropics bacteria were chitin decomposers in the bottom mud, while N-acetylglucosamine decomposers reached a height of 100% i.e. the population of chitin decomposers was scarce as compared to that of starch decomposers. Zobell and Rittenberg (1937) reported that most chitin is probably utilized by biological agents, since little of it accumulates in marine sediments, its decomposition is probably largely due to microbial action. The microbiology of chitin decomposition in soil has been studied by a number of workers (Gray et al., 1963; Gray, 1968; Okafor, 1966; Veldkamp, 1955). They isolated the following strains

1. Bacillus spp (2 strains)
2. Streptomyces spp (4 strains)
3. Arthrobacter sp
4. Trichoderma sp
5. Penicillium sp
6. Aspergillus sp.

During chitin decomposition, CO_2 is released from chitin. Observations of the rate of CO_2 release from the chitin-amended soil indicate that most of the purified chitin added to the soil disappeared in about 3 weeks.

2.12. Chitinolytic activities in the digestive tract of fish

Since many crustaceans are consumed as bait by fish, it can be assumed that chitinolytic enzymes must be present in fish digestive organs. Okutani (1978) isolated chitin-decomposing bacteria from fish gut at 10^9 cells

per gm of the digestive contents. These bacteria showed the characteristics of genes Aeromonas, Vibrio or Alginomonas. Chitinolytic bacteria have been isolated from the gastrointestinal tracts of whales (Seki and Taga 1965). Chitinolytic enzyme was widely distributed in the fish digestive organs. It was found that the chitinolytic enzyme of non-bacterial origin was highly active in the stomach of fish, but absent or relatively inactive in the pyloric caeca and intestine. The main substances formed by the action of the enzyme are N-acetyl D glucosamine and its oligosaccharides when precipitated chitin is used as the enzyme substrate. Jeuniaux and Cornelius (1978) extracted and purified chitinolytic enzymes devoid of any lysozymic activity, taken from the gastric mucosa of mammals, indicated that these enzymes are true chitinases, rather than lysozymes with chitinolytic activity.

2.13. Chitin hydrolysis in human beings

Chitin is not present in human body, but its constituents N-acetyl glucosamine and D-glucosamine are present in human body. There are several enzyme systems in human beings that can break down chitin. First, macrophages in the body do contain Chitinases and lysozymes as part of their phagocytic arsenal (Boot *et al.,* 1995). In addition the terminal non-reducing N-acetyl glucosamine residue of chitin is also exposed to hydrolysis by N-acetyl-D-glucosaminidase.

2.14. Oligosaccharides

Oligosaccharides are bioactive compounds, manufactured by the hydrolysis of chitin or chitosan by chemical or enzymatic method. Usually oligosaccharides are composed of 2-10 units of D-glucosamine. Chitin and its oligomers have been of great help in the past for the progress of medical and biochemical knowledge. Chitin oligomers were used to study the behavior and the chemical characteristics of lysozymes. Deacetylated oligosaccharides can be obtained from the degradation of chitosan while controlled acidic treatment lead to chitin degradation to produce N-acetylated oligosaccharides. Capron and Foster (1970) examined two methods for the preparation of de-o-acetylation and hydrolysis in 2 M hydrochloric acid

followed by neutralization and desalting on sephadex G-25: the later method was found to be more convenient. Muraki (1993) prepared D-glucosamine oligosaccharides of degree of polymerization 5-9 from chitosan by enzymatic hydrolysis with the cellulose of *Trichoderma viridae.* Rupley (1964) studied the hydrolysis of chitin over a range of acid concentrations and temperatures for the preparation of low molecular weight substances for lysozyme (N-acetyl muramide-glycanohydrolase). Harowitz (1957) dissolved chitosan hydrochloride in water and hydrolysed with concentrated acid up to 24 hours at 53 °C, glucosamine was obtained together with oligomers while after 72 hours the conversion was to monosaccharide. Lenk *et al.,* (1961) produced further results by chromatography on Dowex 50. Barker *et al.,* (1958) also studied the acidic hydrolysis of chitosan at 100°C for 34 hours. They obtained deacetylated oligosaccharides in the range mono to hexa units and mentioned the difficulty of the chitosan degradation in acids. The stability of chitosan to depolymerization was due to the stabilizing effect of free amino group (Muzzarelli, 1977).

Yaku and Koshijima (1976) prepared glucosamine oligosaccharide by partial degradation of chitosan with 0.1 N HCl with $NaNO_2$ at room temperature for 15 hrs and the resulting degraded fraction contain 17 glucosamine units. As 2 amino-2-deoxy-D-glucosyl linkage in chitosan is very resistant to acid hydrolysis, the preparation of glucosamine oligosaccharide by partial hydrolysis with acid is difficult. Chitosan in 0.1 N hydrochloric acid was therefore treated with a specific amount of sodium nitrite, causing a partial deamination and cleavage of the chain on the chitosan molecule. N-Acetyl glucosamine has been produced by Pope and Zilliken (1959) by a fermentation process where a mold of the *Aspergillus genus* is propagated in a medium containing chitin. They found that if the aerobic fermentation conditions necessary during the propagation of the mold are changed to anaerobic after the fermentation has proceeded for a period of time, the N-acetylglucosamine, which is held by the mycelium in a form in which it is not easily recovered from the medium, is converted to a suitable form for recovery.

2.15. Chitin hydrolysis

When chitin is dissolved in concentrated hydrochloric acid, hydrolysis of the glycosidic linkages commences almost immediately, followed by a much slower removal of the acetyl groups. In hydrochloric acid solution, chitin initially has $[\alpha]_D^{20}$ -14° which slowly changes to + 56° due to hydrolysis. This optical rotation change is indicative of β -D linkages (Foster and Webber 1961). The similarity of the glycosidic linkages in chitin and in cellulose is reflected in the values of approximately 29 k.cal. obtained for the hydrolytic heats of activation of both polysaccharides.

Meyer and Wehrli (1937) hydrolysed chitin in 50% HNO_3, in order to compare it with cellulose, which it resembled. Hackman (1962) investigated the effect of HCl and other strong mineral acids on the size of the chitin chain, with particular regard to changes occurring under the mild conditions used to prepare colloidal chitin extensive acid hydrolysis of chitin has been employed in the preparation of glucosamine and N-acetyl glucosamine.

2.16. Glucosamine

The origin of glucosamine research was initiated by Ledderhose, a premedical student at Gottingen University in 1876 by mineral acid hydrolysis of the lobster shell and was named "glycosamin". Although this amino sugar was believed to have the D-glucose configuration, and was synthesized by Fisher and Leuchs in 1903. It was not until 1939 that the configuration was unequivocally proved by synthesis. Ledderhose (1878) observed that acetic acid was also produced simultaneously during the hydrolysis and it was subsequently shown by Brach (1912) that the two products formed in equimolar proportions. Frankel and Kelly (1902) isolated 2-acetamido-2-deoxy-D-glucose, the true structural component of chitin during milder hydrolysis.

Ikan (1969) accomplished the degradation of chitin by heating with concentrated hydrochloric acid. Crude glucosamine hydrochloride was purified by filtration through celite and activated carbon. Final purification of crude glucosamine was done by dissolving the product in hot water and

adding ethanol, where upon the α-anomer crystallizes, while the ß-anomer remains in solution and recovered by precipitation with ether. Purchase and Braun (1946) described the preparation of glucosamine-based on the use of filter aids, concentration under diminished pressure and crystallization. Their method was slightly simplified by Navratil *et al.*(1975) who used 12M hydrochloric acid at 95° for 30-60 min. Oeriu *et al.*, (1962) suggested the use of 30% hydrochloric acid at 90-95°C for best results during 5th hour. Matsushima (1948) boiled chitin with concentrated hydrochloric acid for 2 hrs; glucosamine hydrochloride crystallizes out on cooling and can be purified by recrystallisation and decolouration. Ramachandran (1967) determined absolute configuration of α-D-glucosamine hydrochloride by X-ray diffraction method and the results reveal that the molecule exists in the chain form.

Ingle (1973) prepared glucosamine hydrochloride from shrimp canning waste by hydrolysis of 1 part of shell with 3 parts of 10N hydrochloric acid. Total hydrolysis was done for 2 hrs at 100°C and the crude glucosamine obtained was dissolved in hot water and precipitated in absolute alcohol and vacuum dried. Fisher and Nebel (1955) hydrolysed 100 mg chitin in 5.6ml concentrated hydrochloric acid at 50°C followed by dilution with distilled water to yield a 7N acid. The hydrolysis was carried out at 100°C during 4 hours. They observed optimum glucosamine yield under this condition.

2.17. Environmental occurrence

Glucosamine is the principal component of O-linked and N-linked glycosaminoglycans (Sialic acid, Hyaluronan, Keratin sulphate, Heparin sulphate, Dermatan sulphate, Chondroitin sulphate). Glycosamine glycans form the matrix of all connective tissue. Glucosamine is also the precursor for the biosynthesis of all the hexosamines that will form Sialic acids and proteoglycans (Deal and Moskowitz, 1999; Kelly, 1998)

2.18. Acute toxicity studies of glucosamine

The LD_{50} values of glucosamine hydrochloride are given in table-1

Animal	Mode of administration	LD_{50} values
Mouse	Oral administration	15gm/kg body wt.
Mouse	Intravenously	1100mg/kg body wt.
Mouse	Subcutaneous	6200mg/kg body wt.

The oral LD_{50} value of glucosamine sulphate in the mouse was reported to be > 500 mg/ kg, a dose that did not produce mortality (Senin *et al.*, 1987)

2.19. Uses of glucosamine hydrochloride

Glucosamine derivatives are important intermediates in the synthesis of oligosaccharides and glycoconjugates (Banoub *et al.,* 1992). Both natural and synthetic glucosamine containing compounds have demonstrated potent anti-coagulation and immuno-modulatory activity and are used clinically to treat heart disease, arthritis and kidney disorders (Petitou, 1997; Hyers, 1998; da Camara, 1998). Bekesi and Winzler (1970) reported that glucosamine hydrochloride could inhibit tumor growth in animals under some circumstances.

Glucosamine hydrochloride is technically classified as a rare sugar and is required in the pharmaceutical industry and for biochemical research. Nikolaeva (1968) reported that glucosamine could be administered along with tetracycline and other similar antibiotics, since its addition promotes the absorption of antibiotics in the blood system. Its incorporation in the treatment of rheumatic diseases (Dennis and Rainbaud, 1967) as well as in drugs prescribed for anti-pepsin and anti-ulcer activities (Namekata, 1967). Sorkin (1956) reported that glucosamine inhibit the growth of cancer cells. Masquelier (1968) suggested glucosamine as a valuable ingredient in the preparation of diet meal for infants. Glucosamine can be utilized as a starting material for the preparation of valuable intermediates such as D-arabinose

and D-arabonic acid recommended in the manufacture of riboflavine and of L-alanine required for the production of vitamin B_6 (Matsushima, 1957; Wolfrom, 1949). Fieser (1956) reported that emulsifiers can be prepared from glucosamine and Shimizu (1953) prepared clarifying agents by using glucosamine as the basic raw material.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Shrimp head and shell waste

Shrimp head and shell waste (Metapenaeus dobsoni) was collected from a peeling shed near Kochi, Kerala, India. This was brought to the laboratory in iced condition in insulated boxes.

3.1.2. Chemicals

Analytical grade reagents supplied by B.D.H, E.Merck, SRL and Sigma were used for the experiments.

3.2. EXPERIMENTAL METHODS

3.2.1. Preparation of chitin

Shrimp head and shell waste was washed thoroughly in water to remove adhering sand, dirt etc. and drained.

Deproteinization: Washed and drained shell was transferred to steam jacketed kettle, to which sufficient quantity of 3% NaOH was added and boiled for about 30 minutes. It was allowed to cool and drained off the solution containing the dissolved protein from the prawn shell. The residue was washed with water to remove the alkali completely.

Demineralization: The residue was transferred to a tank. Sufficient quantity of 1.25 N Hydrochloric acid was added and kept for 1hour with occasional stirring. The excess acid containing minerals, drained off and the residue was washed with water repeatedly with stirring to make it free from acid. The acid free residue was collected and pressed under screw press to remove water. This residue was then sun dried to a minimum moisture level. (<10)

3.2.2. Preparation of chitin to study the effect of deproteinization on chitin hydrolysis

Shrimp head and shell wastes of single species prawn (Metapenaeus dobsoni) collected from the processing plant at Kochi, and brought to the laboratory in iced condition in insulated boxes. The raw material was washed thoroughly in water to remove adhering sand, dirt etc and drained the water. These shells were demineralized in 1.25 N Hydrochloric acid solution for 1hour with occasional stirring. The excess acid containing minerals, drained off and the residue was washed with water repeatedly with stirring to free it from any adhering acid. The acid free residue was collected and pressed under screw press to remove water. These demineralized shells were dried to moisture content less than 10. From these dry shells deproteinization was done by boiling for about 30 minutes with different concentration of NaOH ranging from 2 - 7%(w/v). It was allowed to cool and drained off the solution containing the dissolved protein from the shell and head. The residue is washed with water to remove the alkali completely.

3.2.3. Preparation of chitin to study the extent of demineralization on chitin hydrolysis

Shrimp head and shell wastes of single species prawn (Metapenaeus dobsoni) collected from the processing plant at Kochi, and brought to the brought to the laboratory. The raw material washed thoroughly in water to remove adhering sand, dirt etc and drained the water. These shells were deproteinised with sufficient quantity of 3% NaOH and boiled for about 30 minutes. When the residue is settled, drained off the solution containing the dissolved protein from the raw material. The residue was washed with water to remove the alkali completely and pressed under screw press to remove water. The deproteinised shells were divided into six batches and each batch was separately demineralized with 1 N and 1.5 N Hydrochloric acids for different periods (30min, 60min, 90min) with occasional stirring. The resulting chitin samples are used for the study.

3.2.4. Glucosamine hydrochloride preparation

Dry Chitin powder was hydrolyzed with concentrated Hydrochloric acid in a glass lined reactor equipped with reflux condenser in a thermostatically controlled digital water bath with occasional stirring. The temperature of the reaction mixture was slowly raised to 95^0C and maintained at that level for the completion of reaction until the solution no longer gives opalescence on dilution with water. During the process the liberated HCl gas was absorbed in water. The excess acid can be distilled off under vaccum after completion of the reaction. The reaction mixture still contained undissolved residue, was filtered after adding equal quantity of water. To this mixture 10% activated charcoal was added and the solution was warmed to 60^o C for 30 minutes and filtered. Resulting pale yellow solution was evaporated to dryness under reduced pressure in a flash evaporator (IKA-ERKE RV 06 ML). After Complete crystallization the mixture was washed with alcohol. The alcohol washed glucosamine hydrochloride samples were dried at 50^oC in a vacuum Oven (Heraeus Vacutherm). Yield of glucosamine was calculated on the basis of raw chitin using the following equation.

$$\text{Yield of GHC (\%)} = \frac{\text{Weight of dry Glucosamine hydrochloride}}{\text{Weight of dry chitin}} \text{X100}$$

3.2.5. Solubles from crude glucosamine hydrochloride on washing with different solvents

Hydrolyzed chitin sample was decolorized, filtered and crystallized under reduced pressure. These crystals were washed with solvents such as acetone, isopropanol, ethanol and methanol and filtered through sintered disc by vacuum suction. This filtrate contains soluble fraction of crude glucosamine.

3.2.6. Optimum volume of hydrochloric acid for chitin hydrolysis

Dry Chitin samples were hydrolyzed with 1 part, 2 part, 3 parts, and 4 parts of concentrated Hydrochloric acids at optimum level and each hydrolyzed samples were decolorized, crystallized under reduced pressure, washed with alcohol and the yield was calculated.

3.2.7. Optimum concentration of hydrochloric acid for chitin hydrolysis

Chitin powder was hydrolyzed in a number of round bottom flasks with 32%(w/v) and 38% (w/v) Hydrochloric acids up to 150 minutes. Samples were drawn at regular intervals of 15 minutes. Total 10 samples were taken. Each hydrolyzed samples were decolorized, crystallized under reduced pressure and yield was calculated.

3.2.8. Effect of time and temperature on chitin hydrolysis

Chitin powder was hydrolyzed with concentrated Hydrochloric acid at different temperatures ranging from 80^0 C - 100^0 C in a digitally controlled water bath. Samples were drawn at 15 minutes intervals up to 150 minutes. A total of 10 samples were taken. Each hydrolyzed samples were decolorized, crystallized under reduced pressure, washed with alcohol and the yield was calculated.

3.2.9. Effect of solvents on glucosamine purification

Chitin was treated with concentrated hydrochloric acid in a glass-lined reactor equipped with reflux condenser in a thermostatically controlled digital water bath with occasional stirring. The temperature of the reaction mixture was slowly raised to 95°C and maintained at that level for the completion of reaction until the solution no longer gives opalescence on dilution with water. The reaction mixture, which still contained undissolved residue, was filtered after adding equal quantity of water. To this mixture charcoal was added and kept at 60^0 C for decolourisation. After filtering the clear filtrate was divided into four equal portions and each portion was evaporated at reduced pressure (25mmHg) using flash Evaporator (IKA-ERKE RV 06 ML) to recover the glucosamine hydrochloride. After complete crystallization of each portion the dry glucosamine hydrochloride obtained was washed with respective solvents such as acetone, isopropanol, ethanol and methanol. Glucosamine hydrochloride thus purified was dried at 50°C in a vacuum oven (Heraeus

Vacutherm). Yield of glucosamine was calculated on the basis of raw chitin using the equation.

$$\text{Yield of GHC (\%)} = \frac{\text{Weight of dry Glucosamine hydrochloride}}{\text{Weight of dry chitin}} \times 100$$

3.3. ANALYTICAL METHODS

3. 3. 1. Determination of moisture: (AOAC, 2000)

A known weight of sample (10 gm) was weighed in a preweighed clean petridish in an electronic balance. The samples were dried to constant weight by placing in a hot air oven at 100°C for 12-16 hrs. Then cooled in a desiccator and weighed. The moisture content was calculated and expressed as percentage.

3. 3. 2. Determination of ash content: (AOAC, 2000)

About 1-2 gm of the sample was transferred into a pre-weighed silica crucible. The sample is carbonized by burning at low red heat and then placing the crucible in a muffle furnace at 550°C for 4 hrs until a white ash was obtained. Crucibles were weighed after cooling in a desiccator and percentage of ash was calculated.

3. 3. 3. Estimation of crude fat: (AOAC, 2000)

About 2-3 gm of accurately weighed moisture free sample was taken in a thimble plugged with cotton and extracted with petroleum ether (40-60°C boiling point) in a Soxhlet apparatus for about 10 hrs at a condensation rate of 5-6 drops per second. Excess solvent was evaporated and the fat was dried at 100°C to constant weight. The crude fat was calculated and expressed as percentage.

3. 3. 4. Determination of nitrogen content in glucosamine hydrochloride: (AOAC, 2000)

About 0.5- 1 gm of the glucosamine was transferred into a Kjeldhal

flask of 100 ml capacity. A few glass beads and a pinch of digestion mixture and 10 ml of concentrated sulphuric acid were also added. It was digested over a burner until the solution turned colorless. To the digested and cooled solution distilled water was added in small quantities with intermittent shaking and cooling until the addition of water generated heat. It was transferred quantitatively into a100 ml standard flask and made up to the volume. With a 2 ml pipette made up solution was transferred to the reaction chamber of the Micro-Kjeldhal distillation apparatus. Two drops of phenolphthalein indicator and 40% sodium hydroxide were added to the chamber. Distillation was done for 5 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro's indicator. The amount of ammonia liberated was determined by titration with 0.02 N standard sulphuricacid.

3. 3. 5. Protein in prawn shell and other crustacean wastes (A.O.A.C. 1975)

Principle:

As prawn shell and other crustacean wastes contain chitin protein complex, protein cannot be estimated as in the case of other proteinaceous materials. The total nitrogen content is a mixed contribution from chitin and protein in the exoskeletons. So the method comprises of separate estimations of total nitrogen and chitin nitrogen. The difference between the total nitrogen and the chitin nitrogen will give the protein nitrogen. Total nitrogen is estimated by the Kjeldhals method. For the estimation of chitin nitrogen, the protein in the sample is removed by hydrolyzing with hot dilute alkali and the nitrogen in the residual material, that is, chitin, is estimated.

Procedure:

Total Nitrogen (TN):

1 Transfer 0.5 to 1.0g of the dry sample to a 100 ml Kjeldhal digestion flasks, add 15 ml conc. H_2SO_4 and digestion mixture and keep for digestion.

2 After digestion make up to 100 ml and estimate the nitrogen by Kjeldhal

method.

Chitin Nitrogen (CN):

1 Transfer quantitatively 0.5 to 1 g of the sample (preferable in flake form) to a 250 ml beaker.

2 Add 25 ml Sodium hydroxide solution and keep on a boiling water bath for 15 minutes.

3 Decant the supernatant through a What-man No.1 filter paper and discard the filtrate.

4 Repeat the treatment once again and wash the residue with hot distilled water. Transfer the residue to the filter paper and the residue along with the filter paper is digested as mentioned above using H_2SO_4 and digestion mixture.

5 Make up the digested solution to 100-ml. and estimate nitrogen. (Calculate the chitin content by multiplying the chitin nitrogen by 14.5).

Protein Nitrogen (PN) = TN - CN

Protein content of shell = PN x 6.25

3. 3.6. Determination of pH:

pH of the samples was determined according to the APHA (1998). About 1 gm of the sample was dissolved in 100 ml distilled water and the pH was recorded using a digital pH meter (Cyber-scan 510 model, UK)

3.3.7. Determination of hexosamine (Elson Morgan method, 1933)

Reagents

a. Acetyl acetone test solution: Distilled and purified colorless acetyl acetone (B.P. 138 - 140°C) 1.5 ml. + 1.2 N Sodium carbonate, making up to 50 ml using sodium carbonate.

b. p-dimethyl amino benzaldehyde test solution (Ehrlichs reagent); p-

dimethyl amino benzaldehyde 1.6 g. + hydrochloric acid 30 ml. Dissolve in 30 ml. 96% alcohol and mix.

Procedure:

1. To measure the absorbance of test solution of glucosamine hydrochloride

   a. Glucosamine hydrochloride 0.02 g + glass distilled water and make up to 100ml

   b. Pour 1 ml out of 100 ml in the test tube + acetyl acetone test solution 2 ml. Mix well and apply heat at 90°C for one hour. Cool down in running water and add 20 ml of 96% alcohol + p-dimethyl amino benzaldeyde test solution 2 ml and mix well.

   c. Leave them at room temperature for one hour. Measure the absorbance at 535 nm.

2. To measure the absorbance of standard solution of glucosamine hydrochloride

   d. D-Glucosamine hydrochloride+ glass distilled water > 100 -150 microgram/ml.

   e. Same as above method

   f. Same as above method

Calculating formula of the contents of Glucosamine hydrochloride is as follows

$$\text{Glucosamine hydrochloride}(\%)=\frac{A \times 1000}{\text{Amount of extract of inspected body (g.)}} \times \frac{a}{b}$$

Where;

A: Concentration of standard solution of glucosamine hydrochloride(as micro gram/ml of glucosamine hydrochloride)

a: Absorbance of test solution of Glucosamine hydrochloride

b: absorbance of standard solution of Glucosamine hydrochloride

3.3.8. Degree of N-acetylation (Baxter method- 1992)

Vacuum dried Chitin sample was made into KBr discs and the FTIR spectra obtained with a NICOLET AVATAR-360 FTIR- spectrophotometer. The average number of scans taken per sample was 50 in the spectral region between 400 - 4000 cm^{-1}. The degree of N-acetylation of the samples was calculated from the absorbencies at wavelengths 1655 and 3450 based on the following equation.

N-acetylation% = (A_{1655}/A_{3450}) x115

3.3.9. Estimation of chloride (AOAC 1990)

About 0.5-1gm of moisture free glucosamine hydrochloride was transferred into a conical flask and add 50ml distilled water and dissolve the sample. To this solution 1ml potassium chromate solution was added and titrates against standard silver nitrate solution with constant stirring until there is apparent perceptible reddish brown color was developed.

3.3.10. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared spectroscopic analysis was conducted on a NICOLET AVATAR-360 FTIR- spectrophotometer. Potassium bromide (KBr) discs containing samples to be analysed were prepared by mixing 01 - 2 mg of vacuum dried Glucosamine hydrochloride with approximately 100 mg of powdered KBr. The mixture was ground before being compressed in a special metal KBr die under pressure of 15 - 30 tonnes to produce transparent KBr discs. The average number of scans taken per sample was 50 in the spectral region between 400 - 4000 cm^{-1}

3.3.11. Residual Solvent (AOAC, 1975- Method No: 973, 69)

A. Reagents and Apparatus

(a) *Ethanol Standard Stock solution-* (I) 2% (v/v) - Dilute 5.0 ml absolute alcohol to 250 ml with H_2O (2) 0.2% (v/v) - Dilute 10.0 ml solution (I) to 100 ml with H_2O.

(b) *Isopropanol standard stock solution* -2% (v/v). Dilute 5.0 ml isopropanol to 250 ml with H_2O.

(c) *Acetone standard stock solution*-2% (v/v) Dilute 5.0 ml acetone to 250 ml with H_2O.

(d) *Methanol standard stock solution*-2% (v/v) Dilute 5.0 ml methanol to 250 ml with H_2O.

(e) *Acetonitrile internal standard stock solution* - 2% (v/v). Dilute 5.0 ml CH_3CN to 250 ml with H_2O.

(f) *Gas chromatograph*- with 1.8 m(6') x 4 mm id glass column. Packed with 80-100 mesh Porapak Q (Water Associates, Inc) and H_2 flame ionization detector, Approximate operating conditions temperatures (°C)- column 135, detector 155, injection port 165, N_2 carrier gas flow rate 120 ml/min. $CH_{3+}CN$ peak should elute in 5 min. Adjust H_2 and air flow rates and electrometer sensitivity so that 5 µL 0.2% ethanol standard solution gives 50-70% scale deflection.

B Preparation of GC Column

Carefully plug column exit with small pad of glass wool. Apply vacuum to exit and slowly add packing material through inlet, tapping very gently to pack firmly. Pack to within 1 cm of area heated by injection port. Plug with glass wool and condition overnight at 235°C with slow N_2 stream. Check column for voids and add more packing, if necessary.

C Preparation of Test Sample

(a) *Ethanol* - Prepare test solution containing ca 2% (v/v) ethanol by stepwise dilution with H_2O. Proceed as in D.

(b) *Isopropanol*-Prepare test solution containing 2% (v/v) isopropanol by stepwise dilution with H_2O. Proceed as in D

(c) *Acetone*- Prepare test solution containing 2% (v/v) acetone by stepwise dilution with H_2O. Proceed as in D.

(d) *Methanol*- Prepare test solution containing 2% (v/v) methanol by stepwise dilution with H_2O. Proceed as in D.

If methanol concentration is unknown, prepare 50% dilution of product with H_2O prepare methanol standard solution and inject test sample and standard as in D. To determine amount methanol, adjust product and standard dilutions to give comparable peak heights; % internal standard added to the 2 solutions should be equal to % methanol present in standard solution.

D. Determination

Pipet 10 ml test sample solution into 100ml volumetic flask. Pipet 10 ml each standard stock solution needed into separate 100 ml volumetric flask. Pipet 10 ml internal standard stock solution into each flask and dilute to volume with H_2O.

Inject 5 µl test sample and standard solutions, each in duplicate, using 10 µl syringe. Approximate retention times of peaks relative to CH_3CN internal standard peak are as follows: methanol, 0.72; ethanol, 0.76; acetone, 1.32; isopropanol, 1.40.

Calculate % methanol, ethanol, acetone, or isopropanol in test sample as:

$$C, \% = C' \times (H/H') \times (I'/I) \times \int$$

Where C and C' = % component in test sample and standard, respectively, H = average test sample peak height or area in test sample chromatogram, H' = average standard peak height or area in standard chromatogram, I and I' = respective values for internal standard and $\int$ = test sample dilution factor.

3.3.12. Transmittance of glucosamine hydrochloride solution

Transmittance of glucosamine hydrochloride solution was measured in spectrophotometer (Spectronic genesys-05) at 530nm wavelength using 1% glucosamine hydrochloride in distilled water.

3.3.13. Specific rotation of glucosamine hydrochloride

Specific rotations of glucosamine hydrochloride samples were measured using a Polarimeter (ADP 220 Bellingham Stanly Ltd). A cylindrical

glass cell of two-decimeter path having 10 ml capacity was used. Glucosamine samples were dissolved in distilled water at a concentration of 100 mg/ml and after standing overnight to establish mutarotational equilibrium, the readings were taken. The reading obtained was Angular rotation. Specific rotation of samples were calculated using the following equation

$$\text{Specific rotation} = \frac{\text{Angular rotation}}{\text{Concentration}} \times \text{length of tube in decimeter}$$

Several readings were taken and the mean values were expressed as result.

3.4. STATISTICAL ANALYSIS

All the experiments were done in triplicates. Statistical analysis was carried out using SPSS (10.0) package. Duncan's Multiple Range Test was also done to determine the significance of variance.

4. RESULTS AND DISCUSSION

4.1. Proximate Composition of Prawn Shell

Chitin, minerals and protein content present in prawn shell waste vary depending on the species. Shahidi and Synowiecki (1991) reported that crustacean shell waste contains 14-32% chitin and 18-42% protein on dry weight basis. Generally prawn shell waste contains 30-45% crude protein on dry basis. The raw material used for the study contains 41.2% protein, 22.8% chitin, 24.4% minerals and 9.8% lipids on dry basis (Table-2).

4.2. Effect of Deproteinisation on Chitin Hydrolysis

Chitin in prawn shell waste is associated intimately with proteins and minerals; therefore, deproteinisation of prawn shell for the chitin preparation is important. Enzymatic and chemical methods are usually employed for deproteinization. During deproteinization the protein present in the shell is hydrolyzed to peptones and amino acids. In chemical deproteinization the amount of alkali, temperature, shell to alkali ratio, pH, extent of reaction time, initial protein content etc. are the important factors which determine chitin quality. These parameters also cause variation in the levels of deproteinization (Gagne and Simpson, 1993).

4.2.1. Protein content in chitin prepared using different concentration of NaOH

The protein content in chitin obtained from prawn shell using different concentration of NaOH is given in Table-3. Total Nitrogen content of chitin prepared from prawn shell waste by different alkali treatments varies from 6.78 to 7.08%. The difference in total nitrogen and chitin nitrogen is regarded as protein nitrogen and it was found that the protein content in prawn shell decreases with increasing use of alkali concentration Chitin nitrogen content in chitin shows an increasing trend with increasing alkali concentrations used for deproteinisation.

Table-2. Proximate composition of shell waste (*Metapenaeus dobsoni*)

Constituent	Percentage (dry basis)
Protein	41.2
Ash	24.4
Chitin	22.8
Lipid	09.8

Table-3. Protein content of chitin prepared from prawn shell using different alkali concentrations

Alkali concentration (%)	Total Nitrogen (%)	Chitin Nitrogen (%)	Protein Nitrogen (%)	Protein content (%)
2	6.92	6.54	0.38	2.38
3	6.78	6.59	0.19	1.19
5	6.97	6.92	0.05	0.43
7	7.08	7.06	0.02	0.13

This is due to partial deacetylation of chitin during deproteinization. Madhavan and Nair (1974) reported that 3% NaOH at 100°C for 30 minutes is sufficient to get a good quality chitin from prawn shell waste. 2% NaOH is not sufficient for the deproteinisation of *Metapenaeus dobsoni* shell with the resulting chitin containing more protein than acceptable limit. When the alkali level increases chitin undergoes partial deacetylation. Alkali concentration of more than 3% may produce more 2-amino D-glucopyranose rather than 2-acetamido D-glucopyranose. Besides the formation of monomers (D-glucosamine units) increases the degree of deacetylation and as a result chitin nitrogen value increases.

4.2.2. Yield of Glucosamine hydrochloride

The rate of hydrolysis and yield of glucosamine hydrochloride increases with increasing degree of acetylation of chitin. Fig. 2 represents the yield of glucosamine hydrochloride obtained by the hydrolysis of prawn shell deproteinised with different alkali concentrations. Acid hydrolysis of the glycosidic linkage involves both protonation of the glycosidic oxygen and addition of water to yield the reducing sugar and group (BeMiller, 1967). Shells deproteinised with 3% NaOH on hydrolysis generates 71.46% glucosamine hydrochloride. However on the basis of pure chitin maximum yield of glucosamine hydrochloride was obtained by hydrolyzing deproteinised shell with 2% NaOH though the yield was 71.08%. Chitin prepared by deproteinised shell with 5% and 7% alkali on hydrolysis gave 69.61% and 66.59% of glucosamine hydrochloride respectively. These yields are 3 to 7 % less compared to the yield of glucosamine hydrochloride from shell deproteinised with 3% NaOH. The yield fluctuation between 2% NaOH and 3% NaOH is not significant ($P<0.05$). When alkali concentration for deproteinization increases, the yield of glucosamine hydrochloride decreases with a clear trend ($P<0.01$).

Non-glucosamine hydrochloride fraction, removed from hydrolysed crystallized matter as methanol soluble, was represented in Fig. 3. The non-glucosamine hydrochloride fraction increases from 23.42 to 29.03% with increase in the alkali concentration used for deproteinization. This trend is very clear and highly significant ($P<0.05$). Duncan's test reveals that these solubles are belonged to different subsets.

Increasing solubles may be an indication of deacetylation of chitin where there is increase in methanol soluble fraction there is relatively reduction in the yield of glucosamine hydrochloride. Thus the use of higher concentrations of alkalis should be avoided for deproteinization to get maximum yield of glucosamine hydrochloride.

4.2.3. Quality of glucosamine hydrochloride

Characteristics of glucosamine hydrochloride prepared from prawn shells deproteinized with various NaOH concentrations are given in Table-4. These glucosamine hydrochloride samples were validated against sigma grade glucosamine hydrochloride. Specific rotation values of prepared glucosamine hydrochloride range from 72.15 to 72.45. Among the different samples of glucosamine hydrochloride, chitin prepared from prawn shell with 2% alkali on hydrolysis gave relatively lower rotation value than others. This may be due to the residual protein in Chitin. All the samples show 100% transmittance. Chloride content and total nitrogen contents of the experimental samples were more or less same for all samples and there was no significant difference.

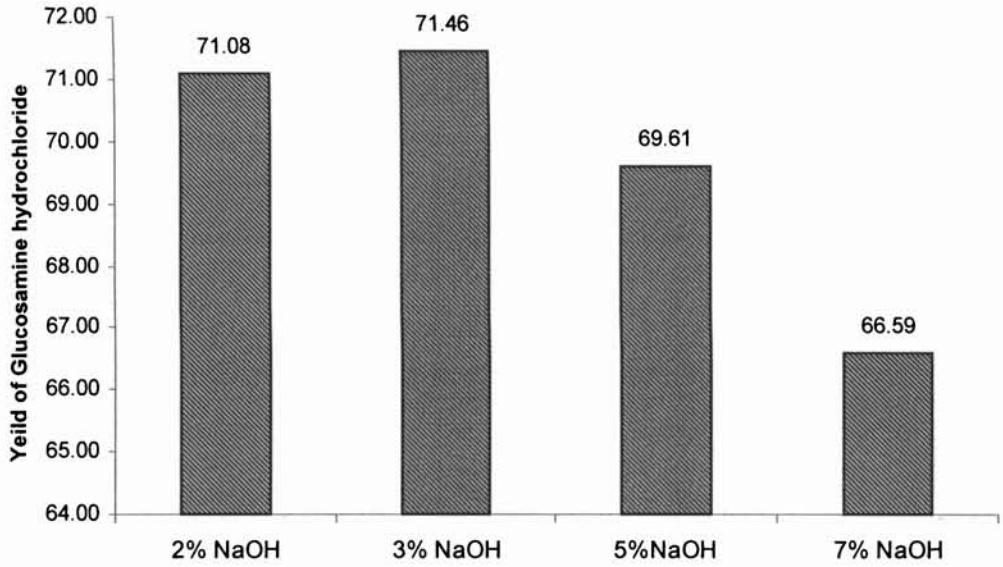


Fig. 2 Yield of glucosamine hydrochloride during deproteinisation of prawn shell with different alkali concentrations

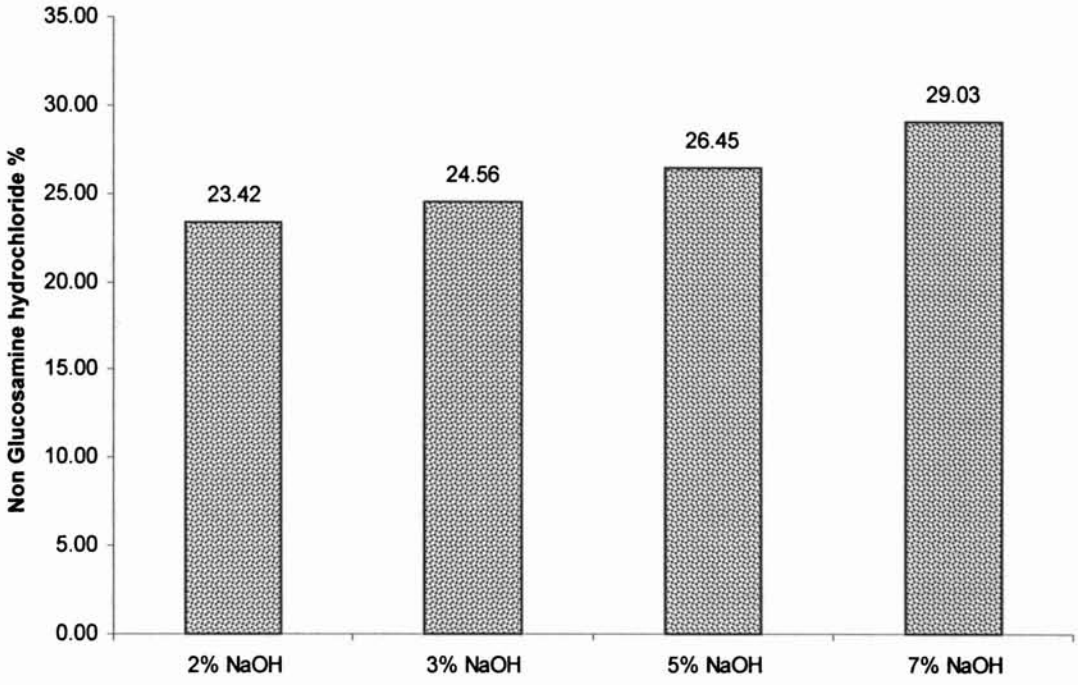


Fig. 3 Yield of non glucosamine hydrochloride fraction obtained during deproteinisation of prawn shell

Table-4 Characteristics of glucosamine hydrochloride prepared from prawn shell deproteinised with different alkali concentrations

Parameters	2%	3%	5%	7%
Glucosamine assay %	99.6 ± 0.02	99.9 ± 0.01	99.8 ± 0.01	99.8 ± 0.01
Specific rotation (C=10,water)	72.15 ± 0.05	72.45 ± 0.03	72.41 ± 0.03	72.43 ± 0.04
Transmittance %	100.5 ± 0.04	100.7 ± 0.05	100.7 ± 0.04	100.7 ± 0.04
Chloride content %	16.49 ± 0.01	16.49 ± 0.01	16.48 ± 0.01	16.49 ± 0.01
Total Nitrogen %	6.50 ± 0.01	6.49 ± 0.02	6.49 ± 0.01	6.50 ± 0.01

4.3. Effect of Conditions of Demineralization on Chitin Hydrolysis

Mineral content in deproteinised shell waste at 30 min, 60 min and 90 min intervals of demineralization are given in the Table 5. The strength of acid and duration of demineralization have significant effect the quality of chitin. 30 minutes of demineralization of prawn shell in 1N hydrochloric acid removed 77.5% of minerals present in the raw material. When the treatment duration was increased to 60 min and 90 minutes the residual mineral contents changed to 1.24 % to 1.06% respectively and the removal was 95% and 96% of initial value. The minerals present in chitin after demineralization with 1N hydrochloric acid belonged to different subsets ($P<0.05$). Based on the Duncan's multiple range test demineralizing the shells for 60 to 90 minutes with 1 N hydrochloric acid gives a chitin of acceptable quality. Similar trend was observed in the case of demineralization with 1.5N hydrochloric acid. Compared to demineralization with 1 N hydrochloric acid 90% minerals were removed at 30 minutes of treatment with 1.5N hydrochloric acid. ANOVA on demineralization with 1.5N acid reveals that the values are highly significant ($P<0.05\%$). When the strength of acid increased the removal of minerals from chitin became faster.

4.3.1. Chitin hydrolysis and Glucosamine yield

Extent of demineralization of chitin on hydrolysis to glucosamine hydrochloride is presented in Fig. 4. During hydrolysis amidic and glycosidic bonds of chitin were broken followed by deacetylation and depolymerization. Novikov (2003) reported that the yield of N-acetyl glucosamine during acid hydrolysis decreased with increased degree of deacetylation.

Prawn shell demineralized with 1N hydrochloric acid solution gives 61.96%, 71.06% and 69.27% glucosamine hydrochloride yield respectively for 30 min, 60 min and 90 minutes of demineralization while that with 1.5 N acid solution gives 68.03, 69.47 and 67.48% glucosamine hydrochloride respectively for 30, 60 and 90 minutes of demineralization time. This indicates that higher acid strength and longer duration of demineralization significantly influence the chitin hydrolysis and glucosamine yield at 5% level ($P<0.05$). When taking the yield of glucosamine hydrochloride on the basis of pure chitin (excluding the minerals) 30 minutes of demineralization time produces more yield than 60 and 90 minutes. Longer duration and higher acid concentration result in more deacetylated residues, thus conversion to glucosamine hydrochloride from N-acetyl glucosamine monomeric units decreases.

4.3.2. Effect of conditions of demineralization of chitin on non-glucosamine hydrochloride fraction (methanol solubles)

Fractions other than glucosamine hydrochloride during hydrolysis of different chitin samples were measured as methanol soluble. Fig. 5 represents the effects of demineralization of chitin on methanol soluble fraction, that is the non-glucosamine hydrochloride fraction obtained during chitin hydrolysis. Higher mineral content in chitin results in more methanol soluble fraction. Excluding the minerals present in non glucosamine hydrochloride fraction, it was found that methanol solubles increased with increasing acid concentration and duration of treatment. Demineralization of prawn shell for 60 minutes with 1 N and 1.5 N hydrochloric acid solutions results 24.63% and 26.5% non-glucosamine hydrochloride fractions while 90 minutes of demineralization increased this value to 26.06% and 27.93% respectively. 30 minutes of demineralization with 1N hydrochloric acid results 26.5% methanol soluble, out of which 22.51% was non-glucosamine hydrochloride fraction and the remaining was minerals. Demineralization with 1.5 N hydrochloric acid solution resulted in 28.44% methanol soluble, out of which 26.14% was non-glucosamine hydrochloride fraction.

Table-5 Effect of demineralization time and concentration of hydrochloric acid on mineral content of chitin

Demineralization Time in minutes	Mineral content in Chitin (gm%)	
	1 N Hydrochloric acid	1.5 N Hydrochloric acid
30	5.49 ± 0.01*	2.34 ± 0.02*
60	1.24 ± 0.03*	1.03 ± 0.02*
90	1.06 ± 0.02*	0.98 ± 0.01*

* Significant at 5% level

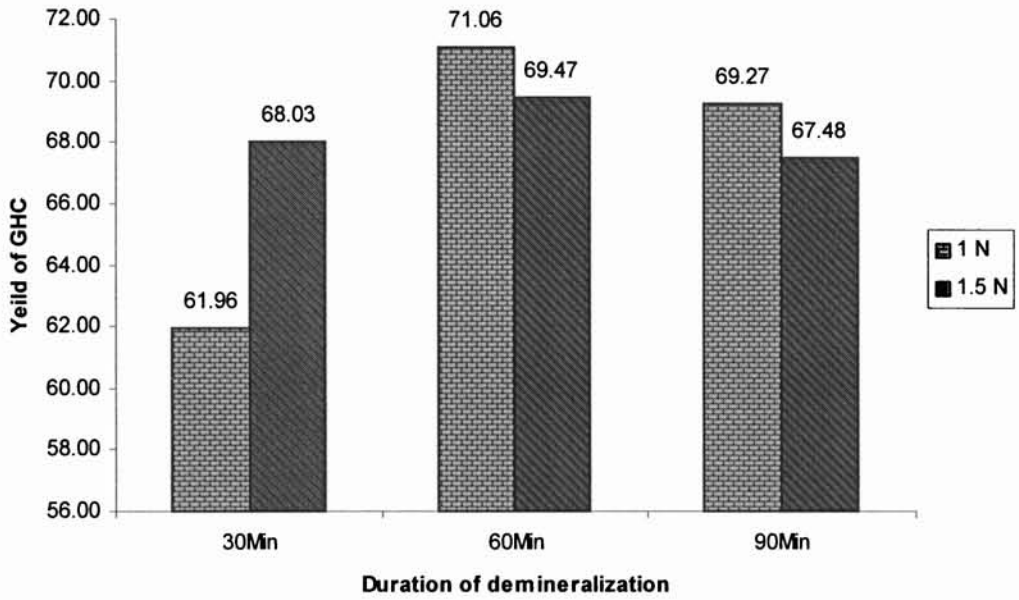


Fig. 4 Effect of concentration of hydrochloric acid used for demineralization of prawn shell on the yield of glucosamine hydrochloride

4.3.3. Total Nitrogen content of methanol solubles

Chitin is a polysaccharide containing Nitrogen. Table-6 gives the total nitrogen content of methanol solubles. Nitrogen content in Methanol solubles varies from 4.84% to 6.76% in case of shell demineralized with 1N hydrochloric acid while it varies 6.43% to 6.77% in the case of shells demineralized with 1.5N hydrochloric acid. This indicates that the nitrogen values of methanol solubles are higher than that of chitin and it indicates that is the deacetylated residues The ANOVA reveals that the nitrogen content in methanol soluble fraction is significant at 5% level ($P<0.05$). The Duncan's test also indicates that they belong to different subsets.

Table-7 represents the mineral content of hydrolysis products. Higher mineral content in chitin results in glucosamine hydrochloride with high ash content and the solubles are also rich in minerals. When the demineralization time increases the amount of minerals in glucosamine hydrochloride decreases

4.3.4. Glucosamine hydrochloride purity

Quality of Glucosamine hydrochloride prepared from chitin of various demineralization levels are given in Table 8. Among the important parameters specific rotation and glucosamine assay are significant. Specific rotation values ranges from 72.13% to 72.41% while glucosamine assay ranges from 99.52 to 99.99%. All the samples of glucosamine hydrochloride prepared from chitin with different mineral content showed 100% transmittance. Chloride content and Total nitrogen content did not show much difference. The results indicate that minerals present in chitin influences the quality and quantity of glucosamine hydrochloride. More reaction time results in lesser yield and more non-glucosamine hydrochloride fractions during chitin hydrolysis

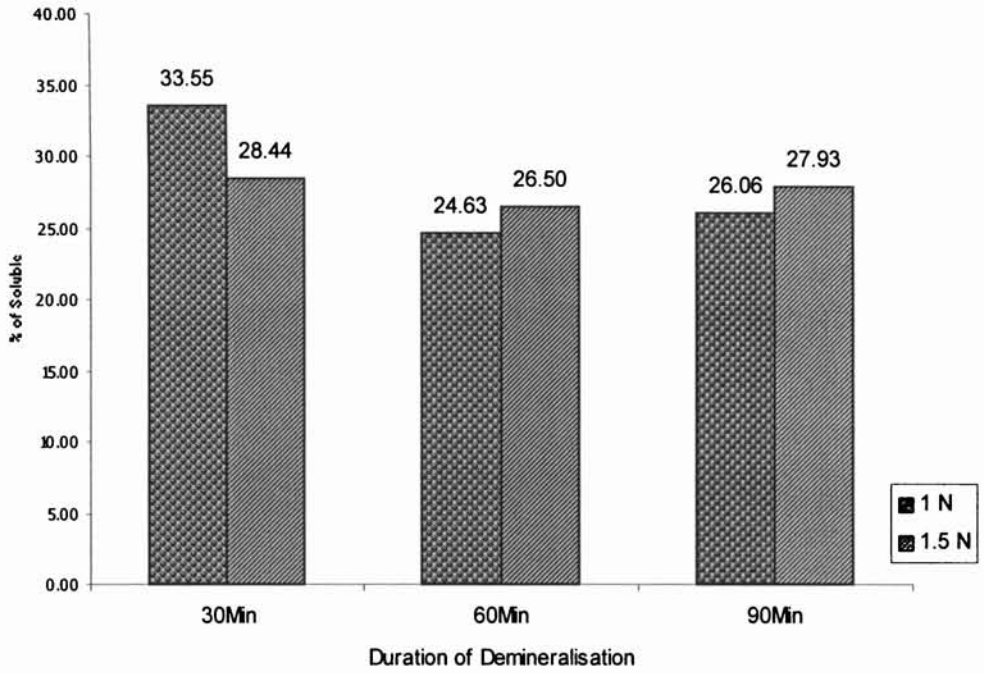


Fig. 5 Percentage of methanol soluble fraction obtained during glucosamine hydrochloride production from chitin after demineralizing the shell with different concentrations of hydrochloric acid

Table-6 Total Nitrogen content of methanol solubles (%)

Demineralization Time in minutes	Shell demineralized with1N hydrochloric acid	Shell demineralized with 1.5N hydrochloric acid
30	4.84 ± 0.01 *	6.43 ± 0.01 *
60	6.62 ± 0.02*	6.65 ± 0.02 *
90	6.76 ± 0.02 *	6.77 ± 0.02 *

* Significant at 5% level

Table-7 Effect of demineralization time on mineral contents in glucosamine hydrochloride and methanol solubles

Duration of demineralization	Glucosamine hydrochloride (%)		Methanol solubles (%)	
	1 N	1.5 N	1 N	1.5 N
30 Minutes	0.08* ± 0.00	0.03 ± 0.00	15.15 ± 0.06 *	8.07 ± 0.02 *
60 Minutes	0.07* ± 0.00	0.02 ± 0.00	4.55 ± 0.03 *	3.88 ± 0.05 *
90 Minutes	0.03* ± 0.00	0.02 ± 0.00	3.93 ± 0.01 *	3.43 ± 0.03 *

* Significant at 5% level

Table-8 Effect of concentration of hydrochloric acid and demineralization time of chitin on the characteristics of glucosamine hydrochloride

Parameters	30Minutes		60Minutes		90Minutes	
	1N	1.5N	1N	1.5N	1N	1.5N
Glucosamine assay%	99.52±0.22	99.60±0.15	99.62±0.21	99.82±0.12	99.84±0.10	99.90±0.08
Specific rotation	72.13±0.11*	72.32±0.10*	72.30±0.03	72.4±0.04	72.4±0.06	72.41±0.02
Transmittance%	100.63±0.15	100.66±0.12*	100.7±0.10	100.7±0.10	100.7±0.08	100.7±0.09
Chloride content%	16.49±0.02	16.50±0.02	16.49±0.01	16.50±0.01	16.49±0.01	16.49±0.01
Total Nitrogen%	6.47±0.01	6.47±0.01	6.48±0.01	6.48±0.01	6.48±0.01	6.48±0.01

* Significant at 5% level

Table-9 Characteristics of Chitin used for Experiment

Parameters	**Values** (dry basis)
Total Nitrogen	6.59 %
Chitin Nitrogen	6.38 %
Protein content	1.31 %
Ash content	1.27 %
Degree of deacetylation	14.2 %

4.4. Effect of Acid Concentration on Chitin Hydrolysis

4.4.1. Effect of concentration of acid on chitin hydrolysis

•Hydrolysis of chitin into glucosamine hydrochloride was measured as water-soluble fraction and it is indicated in Fig. 6. The acid used for the hydrolysis was having 38%(w/v) and 32%(w/v) strength. Chitin hydrolysis with 38% hydrochloric acid results in 74.47% conversion in 15 minutes while 32% acid results in only 61.27%. Reduction of 15% strength in concentration reduces the hydrolysis by 21.5%. Chitin hydrolysis shows three distinct stages. Initial accelerating phase, lag phase and a final stationary phase. During hydrolysis 50-70% of chitin was hydrolysed into glucosamine hydrochloride with in 15 minutes. Hydrolysis of chitin with 38% hydrochloric acid increases the conversion to 90.8% in 30 minutes of reaction, where as hydrolysis with 32% hydrochloric acid strength requires 2 hrs for 90% conversion. Hydrolysis of chitin with 38%(w/v) hydrochloric acid results a maximum value of 92.86% conversion with in 45 minutes and it remains in equilibrium. The results suggest that the values of hydrolysis are in the range of 91.08 to 92.81% after 30 minutes. These results suggest that hydrolysis with 38% HCl after 45 minutes are unworthful While the hydrolysis of chitin with 32% (w/v) Hydrochloric acid is highly significant ($P>0.05$) with respect to the yield. Chitin hydrolysis with 32% hydrochloric acid results 75.2% conversion after 30 minutes. This value increased to 77.13% after 45 minutes and then 80.69 within 60 minutes. For the 90% of chitin hydrolysis 120 minutes may require and the value increased to 93% after 150 minutes. Reduction in concentration significantly affect the hydrolysis of chitin Hydrolysis from 61.27% to 93.82% takes 135 minutes with 32%(w/v) hydrochloric acid where as 92% hydrolysis was reached within 45 minutes in concentrated acid of 38% (w/v).

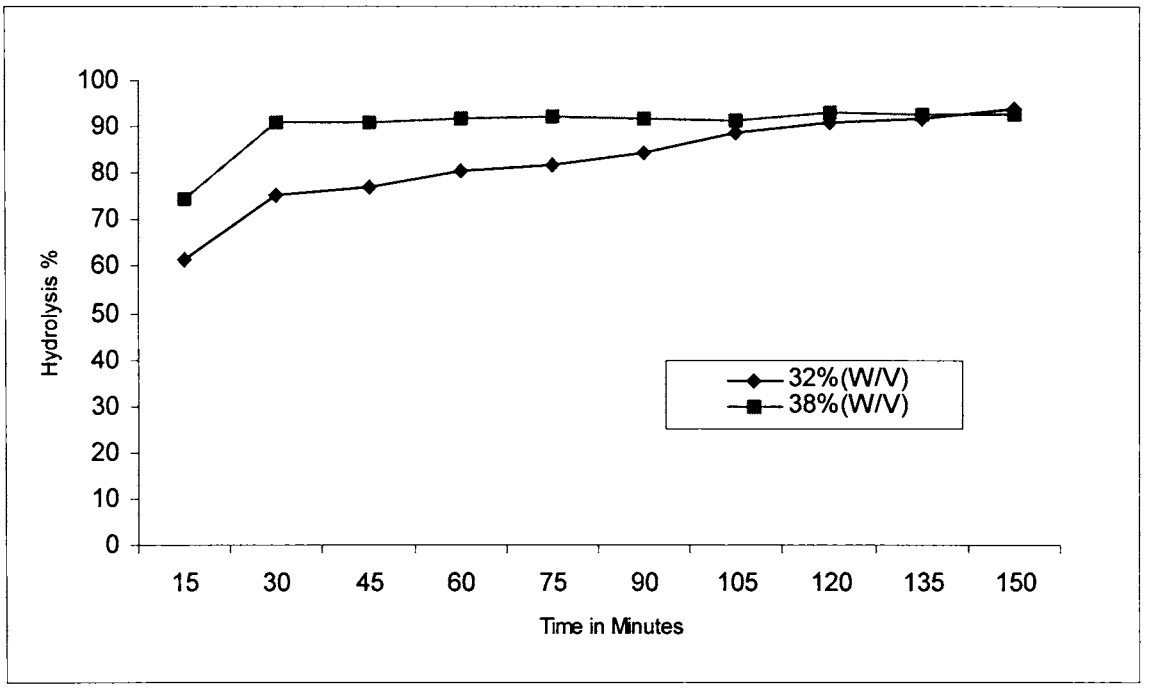


Fig. 6 Effect of acid concentration on the rate of hydrolysis of chitin at 95^0C

4.4.2. Effect of acid concentration on glucosamine hydrochloride yield

Effect of acid strength on glucosamine yield is represented in Fig.7. Initially the glucosamine hydrochloride was not present in reaction mixture. Similar to hydrolysis the glucosamine hydrochloride yield also shows the same trend. 38%(w/v) hydrochloride acid converts 54.58% pure glucosamine hydrochloride in 15 minutes. But this value increased to 69.36% after 30 minutes and remains more or less same for subsequent increase in duration. Glucosamine hydrochloride yield obtained during hydrolysis of chitin with 32% hydrochloric acid shows increasing trend up to 2.30 hrs and there after it remains the same. 32% acid strength results in 45.4% glucosamine hydrochloride after 15 minutes and it increased to 60.89% in 45 minutes. 70% glucosamine hydrochloride was obtained in 120 minutes and up to 75% glucosamine hydrochloride was produced after 150 minutes without much increase for the subsequent period. The results of acid concentration on hydrolysis indicates that glucosamine hydrochloride conversion is highly influenced by acid concentration and faster conversion of chitin to glucosamine hydrochloride took place with 38% hydrochloric acid than with 32% HCl. However more deacetylation takes place at higher acid concentrations and more yield was obtained in 32% strength acid than 38%.

4.4.3. Effect of concentration of acid on methanol soluble components

Methanol soluble components obtained during the purification of glucosamine hydrochloride are more, when the strength of hydrochloric acid was increased to 38% from 32% (Table-10). Chitin hydrolysis with 32% (w/v) hydrochloric acid resulted in 15.73% solubles at 15 minutes and it was increased to 16.62% after 30 minutes. There was no significant increase in the solubles after 30 minutes of hydrolysis of chitin and it is in the range 17.24 to 17.64% when hydrolyzed up to 150 minutes which indicates the solubles formed after 30 minutes are not significant.

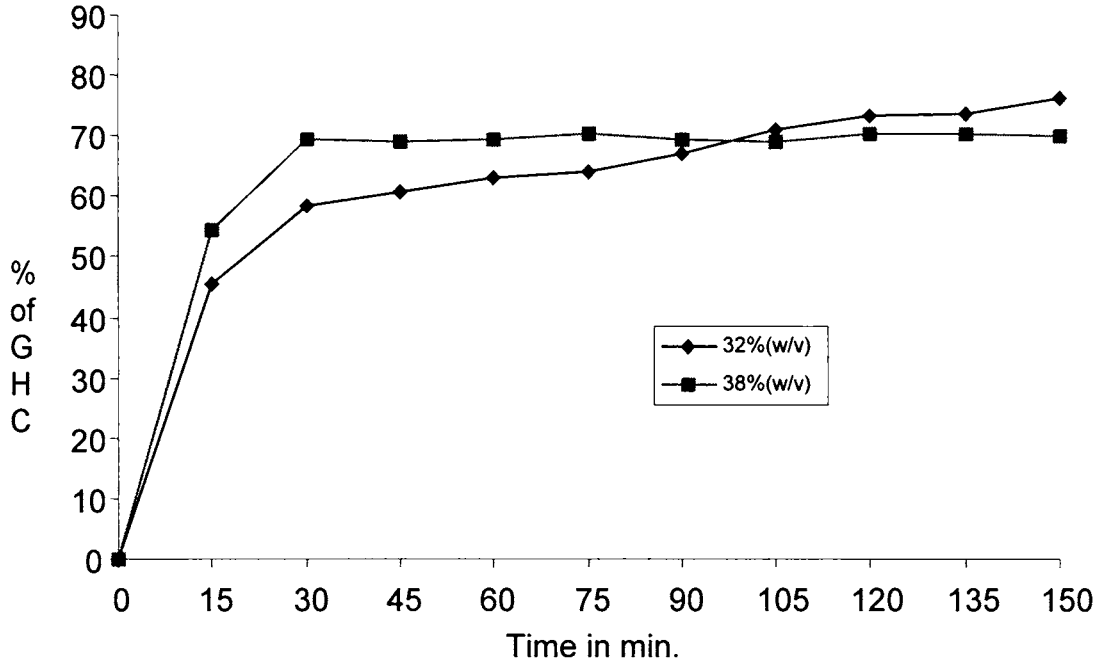


Fig. 7 Effect of concentration of acid on the conversion of chitin into glucosamine hydrochloride

Hydrolysis of chitin with 38% (w/v) hydrochloric acid produced 19.89% soluble components after 15 minutes and it was to 21.44% after 30 minutes and after 45 minutes it was 22.03 to 22.70% up to 150 minutes. When strength of hydrochloric acid was reduced by 15% the percentage solubles formed also reduced by 5%. This indicates that at higher concentration of hydrochloric acid the chitin undergoes deacetylation.

4.4.4. Specific rotation of Glucosamine hydrochloride

Specific rotation of glucosamine hydrochloride samples prepared at different acid concentrations with various levels of hydrolysis are represented in Table 11. Specific rotation value of glucosamine hydrochloride in water was initially having a value of 100 and it reduced and reached an equilibrium value of 72.5 after 4 hrs. Chitin hydrolysed with 32% hydrochloric acid generated glucosamine hydrochloride with specific rotation of 68.55 at 15 minutes of hydrolysis. Whereas glucosamine hydrochloride prepared with 38% hydrochloric acid resulted in specific rotation of 70.38 which is 2.6% higher than glucosamine hydrochloride prepared with 32% hydrochloric acid. Chitin hydrolyzed for 30 minutes with different acid strength resulted in glucosamine hydrochloride with 68.58 and 70.79 specific rotation value for 32% hydrochloric acid and 38% hydrochloric acid respectively. These values increased to 69.35 and 72.13 for 32% and 38% hydrochloric acids respectively after 45 minutes. Specific rotation values of glucosamine hydrochloride prepared with 32% hydrochloric acid with a hydrolysis time of 60 minutes resulted in the value of 69.98 where as for the same duration with 38% hydrochloric acid hydrolysis generates glucosamine hydrochloride with a specific rotation of 72.30.

Table-10 Effect of concentration of acid on the methanol soluble fractions

Time in Minutes	%Soluble components in hydrolysis with 32%(w/v) hydrochloric acid	Soluble components in hydrolysis with 38%(w/v) hydrochloric acid
15	15.73 ± 0.24	19.89 ± 0.33
30	16.62 ± 0.39	21.44 ± 0.54
45	17.24 ± 0.56	22.03 ± 0.47
60	17.47 ± 0.43	22.17 ± 0.60
75	17.64 ± 0.53	21.48 ± 0.44
90	17.44 ± 0.48	22.34 ± 0.35
105	17.53 ± 0.40	22.30 ± 0.51
120	17.53 ± 0.46	22.45 ± 0.22
135	17.86 ± 0.32	22.54 ± 0.28
150	17.60 ± 0.41	22.70 ± 0.36

Table- 11 Specific rotation of Glucosamine hydrochloride prepared at different concentrations of acid

Treatment durations	Specific rotation(C=10,Water)	
	32% hydrochloric acid	38% hydrochloric acid
15Min	68.55 ± 0.10	70.38 ± 0.13
30Min	68.58 ± 0.68	70.97 ± 0.16
45Min	69.35 ± 0.22	72.13 ± 0.10
60Min	69.98 ± 0.21	72.30 ± 0.18
75Min	70.83 ± 0.13	72.42 ± 0.08
90Min	71.28 ± 0.23	72.43 ± 0.08
105Min	71.60 ± 0.13	72.45 ± 0.05
120Min	71.42 ± 0.51	72.47 ± 0.06
135Min	71.75 ± 023	72.48 ± 0.03
150Min	71.72 ± 0.23	72.48 ± 0.02

4.5. Effect of Chitin-Hydrochloric Acid Ratio on Hydrolysis of Chitin

Volume of hydrochloric acid is an important factor for the hydrolysis of chitin. The effect of hydrochloric acid volume on chitin hydrolysis is presented in Fig. 8. Equal quantity of chitin and hydrochloric acid resulted in 68.9% hydrolysis of chitin. This increased to 92.33% when 2 parts of hydrochloric acid was used for chitin hydrolysis. Hydrolysis of chitin with 3 parts of hydrochloric acid produced 93.41% water-soluble fraction and when chitin to acid ratio was increased to (1:4) hydrolysis it improved to 93.95%. ANOVA on chitin-acid ratio on hydrolysis indicated that the acid volume is significant at 5% level ($P<0.05$). Duncan's multiple range test shows chitin hydrochloric acid ratio with 1:1 and 1:2 are highly significant and 1:3 & 1:4 belong to some category. Rate of hydrolysis improved with increasing volume of hydrochloric acid. But the rate of hydrolysis does not increase proportionally and the 1:2 ratio is sufficient for hydrolysis.

4.5.1. Effect of chitin acid ratio on the yield of glucosamine hydrochloride

The rate of chitin hydrolysis affects glucosamine yield. Though the extent of hydrolysis was different yield of glucosamine hydrochloride shows variation. Fig. 9 represents the effect of chitin hydrochloric acid ratio on the conversion of chitin into glucosamine hydrochloride. Chitin treated with equal quantity of hydrochloric acid produced 53.82% glucosamine hydrochloride. Chitin hydrolysed with double quantity of hydrochloric acid gives 69.98% glucosamine yield. With 3 parts of acid to chitin yield was slightly reduced to 69.78% and with 4 parts of acid produced 69.43% glucosamine hydrochloride. It is clear that chitin hydrolysis with 2 part of acid yield maximum glucosamine hydrochloride which was also significant at 1% level statistically. Duncan's multiple range test indicate that the chitin acid ratio of 1:2, 1:3 and 1:4 belong to same subsets.

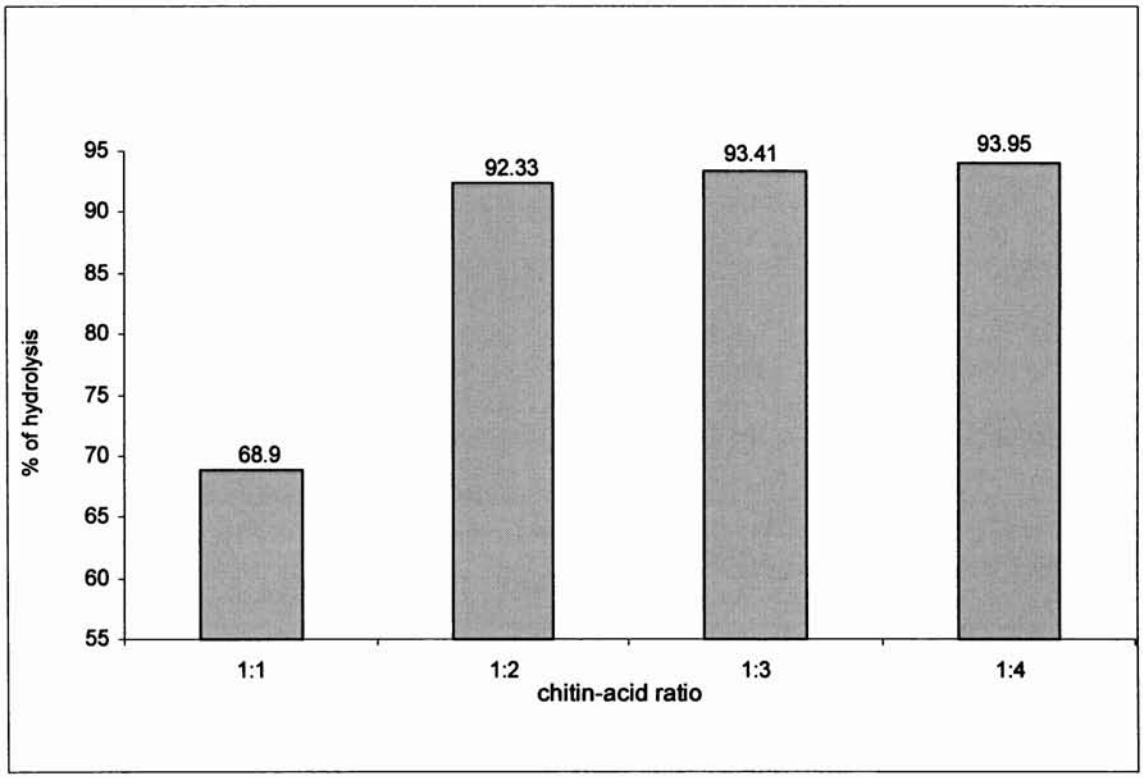


Fig. 8 Effect of Chitin-Acid ratio (w/v) on the extent of hydrolysis

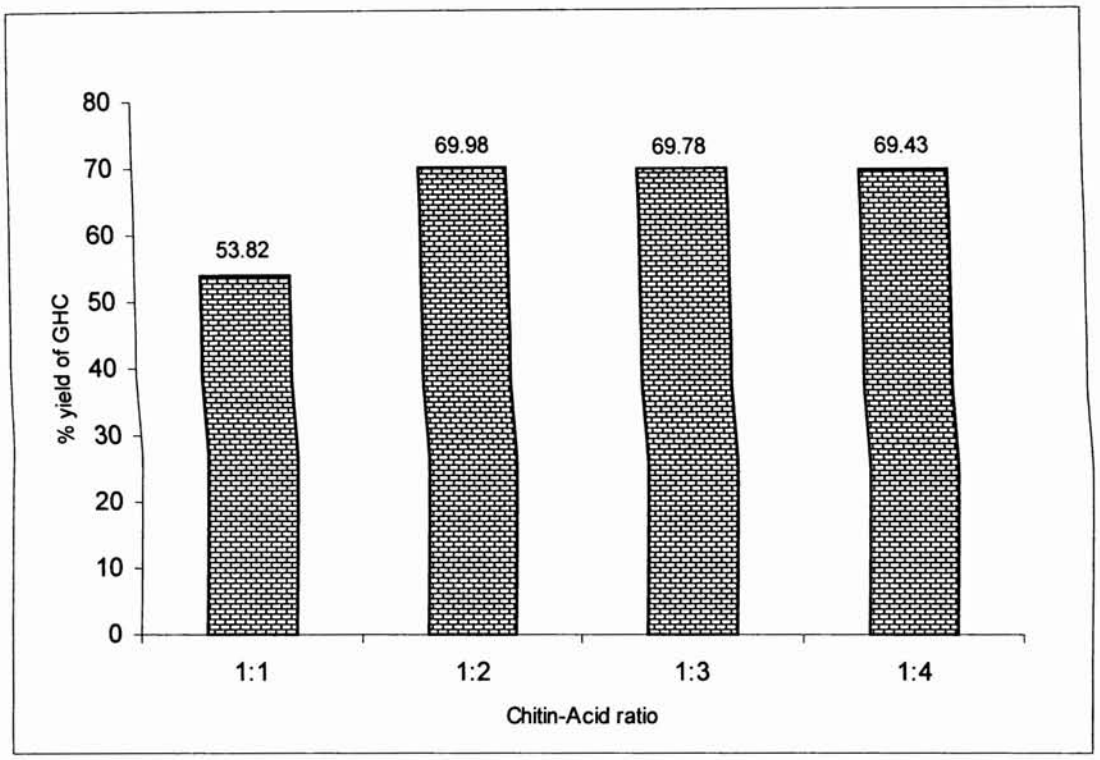


Fig. 9 Effect of chitin-acid ratio on the conversion of chitin into glucosamine hydrochloride

4.5.2. Effect of chitin acid ratio on methanol solubles

When the chitin-acid ratio used for the hydrolysis increased, the soluble fraction of chitin hydrolysis other than glucosamine hydrochloride also increased. Fig. 10 show the trend of solubles in solvent. Chitin with 2 part of concentrated hydrochloric acid resulted in 22.35% solubles while glucosamine prepared with 3 part of hydrochloric acid and 1 part of chitin possesses 23.63% solubles. Hydrolysis with 4 parts of concentrated hydrochloric acid and 1 part of chitin gives 24.52% soluble after glucosamine hydrochloride extraction. These results indicate that as acid volume increases the methanol soluble fraction formed also increases. ANOVA reveal that chitin acid ratio on soluble fraction are highly significant at 1% Level ($P<0.01$). Duncans test also proved that they were belonging to different subsets.

4.5.3. Characteristics of glucosamine hydrochloride prepared at different chitin-acid ratios

Glucosamine assay indicates that glucosamine hydrochloride prepared with 1 part of chitin and 1 part of hydrochloric acid gives relatively inferior value. Chitin hydrolysed with 2 parts of acid shows 99.68% purity whereas that prepared with 1:3 ratio gives 99.65% Assay. Hydrolysis of chitin with its 4 parts of concentrated hydrochloric acid shows 99.70% purity on the basis of glucosamine. ANOVA on glucosamine assay indicated that they were significant at 5% level. DMRT reveals 1:1 ratio significantly differs from others. Similarly specific rotation of glucosamine hydrochloride obtained at 1:1 ratio is having value of 71.87 and which is lesser than the other values. Other quality parameters did not have any significant difference among samples.

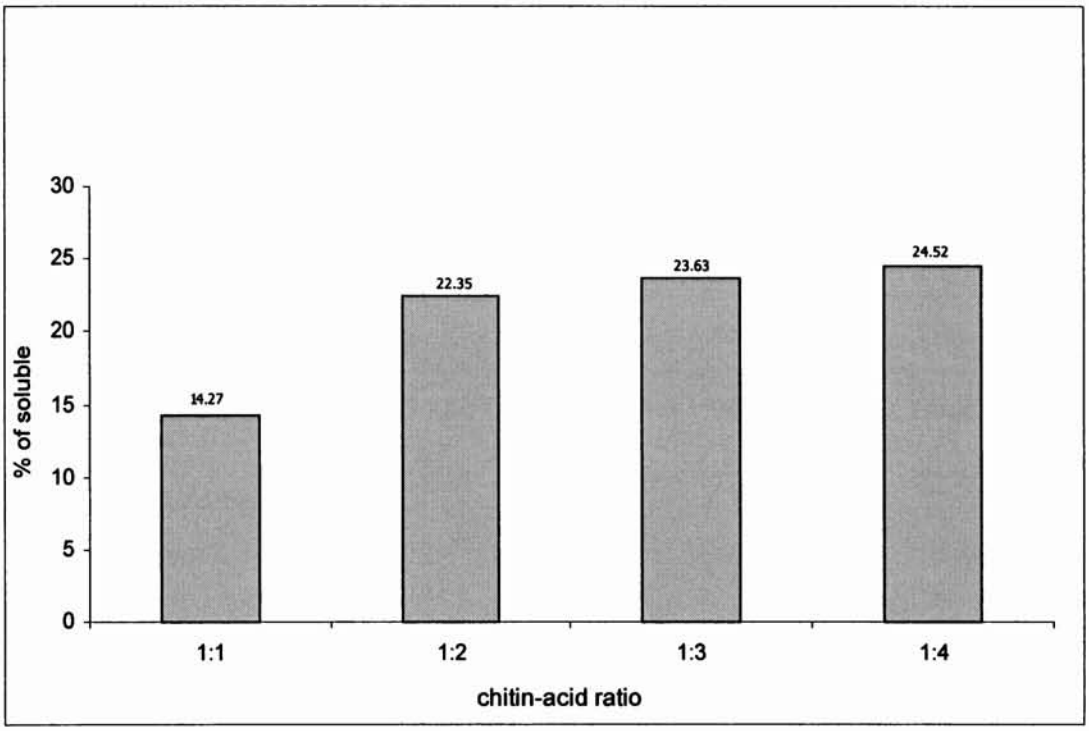


Fig. 10 Effect of chitin acid ratio on methanol solubles

Table 12 Characteristics of glucosamine hydrochloride prepared by hydrolyzing chitin using different chitin-acid ratios

Parameters	Chitin: Hydrochloric acid ratio			
	1:1	1:2	1:3	1:4
Assay%	98.02 ±0.18*	99.68±0.21	99.65 ±0.24	99.70 ±0.18
Specific rotation(C=10;Water)	71.87 ±0.03*	72.38±0.18	72.45 ±0.05	72.44 ±0.02
Chloride content%	16.48 ±0.01	16.49 ±0.01	16.49 ±0.01	16.49 ±0.01
Transmittance%	100.6 ±0.14	100.7 ±0.16	100.7 ±0.10	100.7 ±0.08
Ash%	0.02 ±0.00	0.02 ±0.00	0.02 ±0.00	0.02 ±0.00
Total Nitrogen%	6.50 ±0.01	6.50 ±0.01	6.50 ±0.01	6.51 ±0.01

* Significant at 5% level

4.6. Effect of Temperature and Duration of Treatment on Chitin Hydrolysis

4.6.1. Effect of temperature and duration of hydrolysis on the yield of Glucosamine hydrochloride

Yield of glucosamine hydrochloride at different temperatures is presented in Fig. 11. The glucosamine hydrochloride produced by hydrolysis of chitin was estimated for a range of temperatures 80°C to 100°C. Among these the lowest conversion of chitin to glucosamine hydrochloride was at 80°C. In the present study, the hydrolysis performed at 80, 85, 90 and 95 °C posted an increase in the yield in the initial 15 minutes. The calculated yields were 37.26%, 43.09%, 48.23% and 54.85% respectively. Interestingly, at 100^0C the rate of conversion was completed in 15 minutes and the rate of conversion was faster than the other temperatures, which resulted in only 67.43% of glucosamine hydrochloride yield.

Maximum yield possible for glucosamine hydrochloride by hydrolysis with 38%hydrochloric acid (w/v) was observed as 70% when this reaction proceeds at 80°C for 90 minutes. At higher temperatures at 85, 90, 95 and 100°C, the process time required for this yield was 60, 45, 30 and 15 minutes respectively. In general, 50 to 100% hydrolysis to glucosamine hydrochloride was completed in 15 minutes. It is also noted that rate of hydrolysis increases with increasing temperature. Even though the reaction was faster at 100°C, maximum yield was observed at 90-95°C which shows that deacetylation of chitin is caused at higher temperatures.

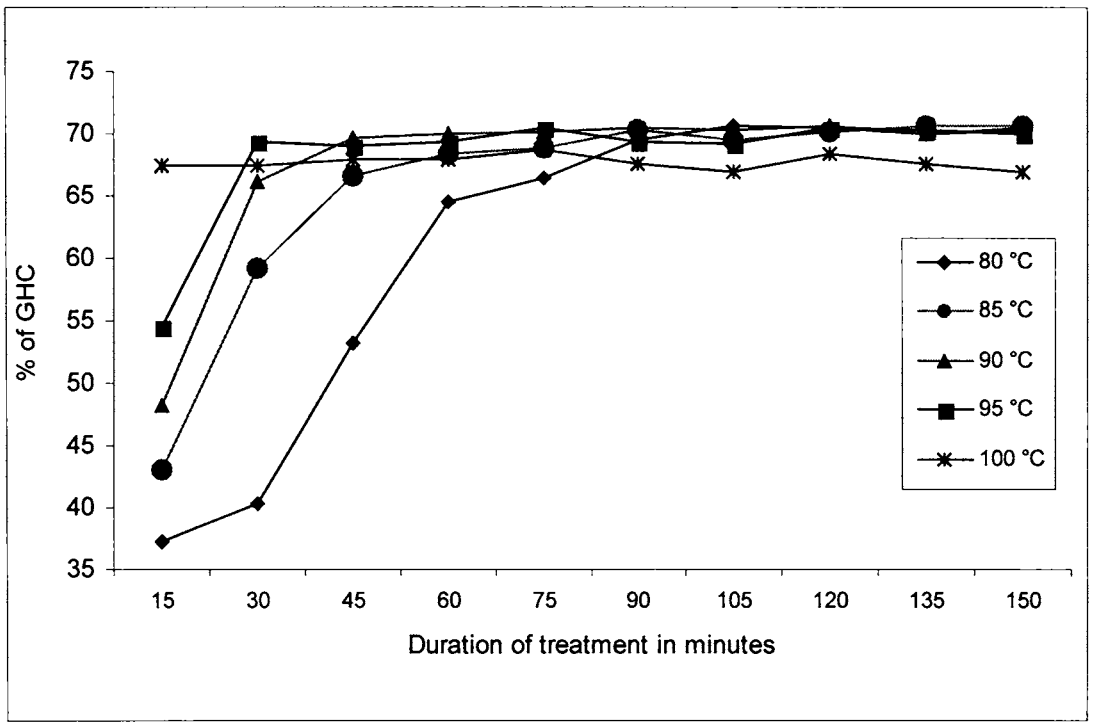


Fig. 11 Yield of Glucosamine hydrochloride at different temperatures

ANOVA was performed on glucosamine yield data for various temperatures. It was found that the yield varied significantly at different temperatures at 5% level. Duncans multiple range test was also performed on the yield data to identify the significance of the difference in yield at various temperature levels. It was found that when temperature was increased from 80^0C to 85^0C the increase in yield was significant. Raising the temperature further to 90°C, 95°C and 100°C did not improve significant increase in the yield. It can be concluded that optimum yield is obtained at 90°C to 95°C.

The yield data pertaining to varying process time at 80°C was analyzed to find out the effect of duration of hydrolysis on yield. The duration of hydrolysis had significant effect on the yield and DMRT revealed that optimum yield was obtained at 90 minutes of hydrolysis. Processing further did not give significant increase in yield. ANOVA was also performed for yield data varying at different process time and it was found that at 5% level maximum yield was at 60 minutes. ANOVA on duration of hydrolysis at 90°C, 95°C and 100°C reveals that optimum yield at 5% level was obtained at 45 minutes, 30 minutes, 15 minutes respectively.

4.6.2. Effect of temperature on specific rotation

Specific rotation values of different glucosamine hydrochloride samples obtained at different temperature ranging from 80°C to 100°C are represented in Fig.12. Specific rotation value of glucosamine hydrochloride obtained at 80°C was ranged from 68.55 to 71.60. Samples of glucosamine hydrochloride obtained under this temperature were inferior in quality. Glucosamine hydrochloride samples obtained at 85°C temperature having specific rotation value 68.7 to 71.86 Specific Rotation Values of Glucosamine hydrochloride obtained at 90, 95 and 100°C were 69.43 to 72.33 and 70.38 to 72.48 and 70.85 to 72.5 respectively. In all samples glucosamine hydrochloride obtained with in 15 minutes of hydrolysis and slightly inferior in quality. Glucosamine hydrochloride obtained at 95°C and 100°C are pure compared to that obtained at other temperatures. ANOVA on specific rotation at different temperatures indicated that the values are highly

significant ($P<0.01$).

4.6.3. Effect of temperature and duration of hydrolysis on transmittance

Transmittances of glucosamine hydrochloride sample obtained during chitin hydrolysis at different temperatures were given in Fig.13. Commercial grade glucosamine hydrochloride having above 98% and sigma grade poses 100% transmittance. Reduction in transmission was due to the impurities. At 80°C the glucosamine hydrochloride obtained by hydrolysis had 97% transmittance and it reaches 100% at 105 minutes. Hydrolysis of chitin at 85°C gives glucosamine hydrochloride with 98.57% transmittance and 100% transmittance was obtained at 60 minutes of hydrolysis. Glucosamine hydrochloride obtained at 90°C possessed 98.67% and reaches 100% at 45 minutes where as glucosamine hydrochloride obtained at 95 and 100°C had 100% transmittance from 15 minutes onwards. ANOVA on transmittance reveals that temperatures of hydrolysis are highly significant. Hydrolysis of chitin at 95 & 100°C gives better quality than that obtained at temperatures.

4.6.4. Effect of temperature and duration of hydrolysis on chloride content and pH

Chloride content of glucosamine hydrochloride samples obtained during chitin hydrolysis at different temperature were represented in Table-13. Chloride content in pure glucosamine hydrochloride was 16.47% and all the values obtained are with in range. The pH value for pure glucosamine hydrochloride lies between 4 to 5. pH values of glucosamine obtained during chitin hydrolysis at different temperatures were with in the specified range (Table-14). The effect of different temperatures on the values of chloride and pH obtained does not have any significance.

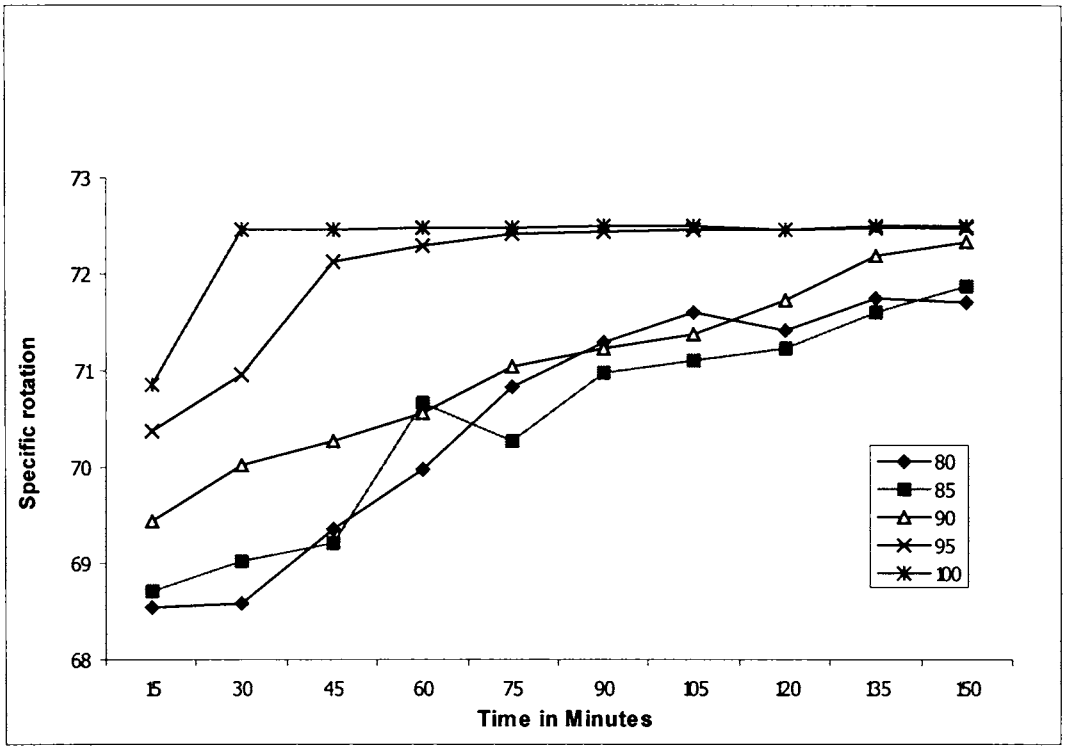


Fig. 12 Specific rotation of glucosamine hydrochloride prepared at different time-temperature combinations

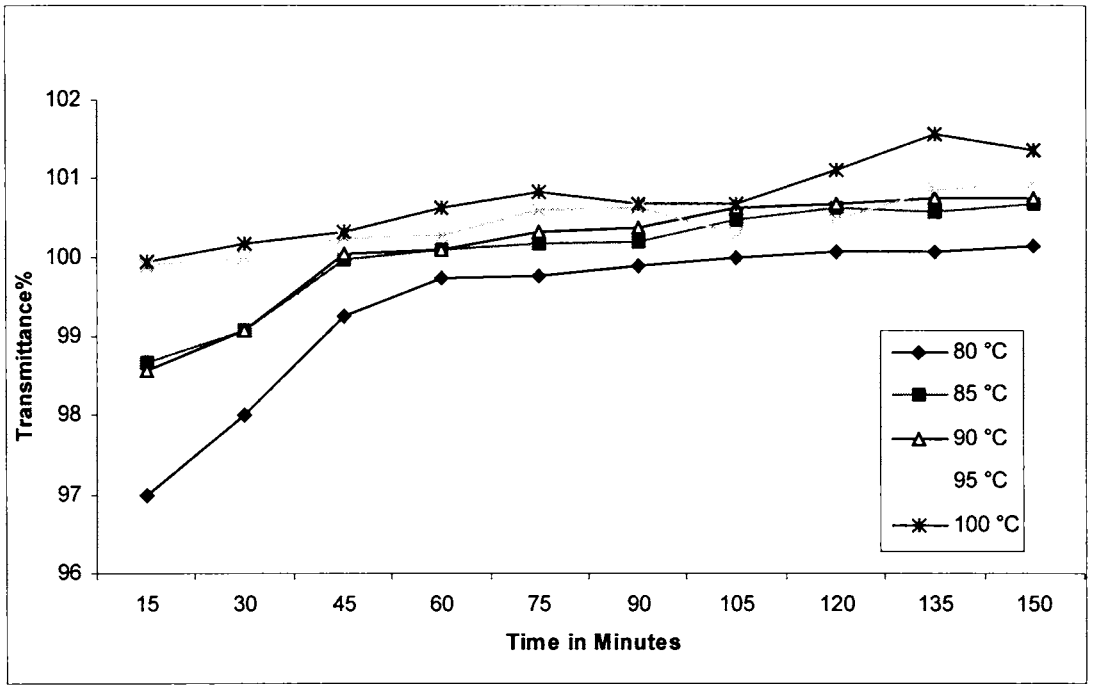


Fig. 13 Effect of temperature and duration of hydrolysis on transmittance of glucosamine hydrochloride

Table-13 Effect of temperature and duration of hydrolysis on chloride content (%)

Time in minutes	Temperature				
	80°C	85°C	90°C	95°C	100°C
15	16.46 ± 0.04	16.48 ± 0.01	16.48 ± 0.01	16.48 ± 0.01	16.49 ± 0.01
30	16.50 ± 0.02	16.49 ± 0.01	16.50 ± 0.01	16.50 ± 0.02	16.51 ± 0.02
45	16.48 ± 0.01	16.48 ± 0.01	16.49 ± 0.02	16.50 ± 0.01	16.50 ± 0.02
60	16.47 ± 0.01	16.49 ± 0.01	16.49 ± 0.01	16.50 ± 0.01	16.50 ± 0.02
75	16.48 ± 0.03	16.49 ± 0.02	16.51 ± 0.01	16.50 ± 0.01	16.51 ± 0.02
90	16.48 ± 0.02	16.50 ± 0.01	16.51 ± 0.01	16.50 ± 0.01	16.50 ± 0.01
105	16.50 ± 0.01	16.51 ± 0.01	16.51 ± 0.01	16.51 ± 0.01	16.50 ± 0.01
120	16.48 ± 0.04	16.52 ± 0.02	16.51 ± 0.01	16.52 ± 0.01	16.51 ± 0.02
135	16.48 ± 0.02	16.50 ± 0.01	16.50 ± 0.01	16.50 ± 0.01	16.50 ± 0.01
150	16.50 ± 0.02	16.51 ± 0.01	16.52 ± 0.01	16.51 ± 0.01	16.51 ± 0.01

Table-14 Effect temperature and duration of hydrolysis on pH of glucosamine hydrochloride

Time in minutes	Temperature				
	80^{0}C	85^{0}C	90^{0}C	95^{0}C	100^{0}C
15	4.20 ± 0.15	4.67 ± 0.13	4.45 ± 0.05	4.57 ± 0.06	4.59 ± 0.08
30	4.43 ± 0.12	4.59 ± 0.08	4.62 ± 0.08	4.63 ± 0.08	4.60 ± 0.11
45	4.40 ± 0.23	4.74 ± 0.06	4.65 ± 0.05	4.57 ± 0.07	4.69 ± 0.04
60	4.51 ± 0.06	4.73 ± 0.12	4.68 ± 0.03	4.72 ± 0.07	4.72 ± 0.07
75	4.51 ± 0.26	4.73 ± 0.10	4.68 ± 0.07	4.72 ± 0.05	4.72 ± 0.07
90	4.48 ± 0.02	4.55 ± 0.11	4.66 ± 0.05	4.72 ± 0.03	4.73 ± 0.09
105	4.45 ± 0.06	4.59 ± 0.11	4.77 ± 0.04	4.81 ± 0.01	4.77 ± 0.10
120	4.47 ± 0.14	4.70 ± 0.05	4.76 ± 0.07	4.77 ± 0.11	4.68 ± 0.16
135	4.47 ± 0.07	4.71 ± 0.16	4.83 ± 0.04	4.78 ± 0.05	4.76 ± 0.09
150	4.46 ± 0.10	4.71 ± 0.17	4.83 ± 0.04	4.77 ± 0.06	4.74 ± 0.09

4.7. Effect of Different Solvents on Purification of Glucosamine Hydrochloride

The yields of glucosamine hydrochloride when solvents such as acetone, isopropanol, ethanol and methanol are used for purification of the crude product after hydrolysis are shown in Fig.14. Glucosamine obtained from hydrolysed chitin after acetone washing was 90.27%. Isopropanol washed glucosamine hydrochloride yield was 87.56%. Ethanol washed dry chitin hydrolysate yields 81.90% glucosamine hydrochloride and methanol washing of crude glucosamine crystals gives 69.37% yield. Among the different solvents used for the purification of crude glucosamine maximum yield was obtained for acetone followed by isopropanol, ethanol and methanol.

In general hydrolyzed chitin on vacuum evaporation gives 85-95% solids, which contains glucosamine hydrochloride, deacetylated glucosamine units, oligomers, minerals, hydrolyzed protein fraction etc. During washing with different solvents some of these fractions were removed based on their solubility in the solvents used for the purification. The more yield means some of the impurities such as calcium chloride, oligomers, hydrolysed protein are retained along with glucosamine hydrochloride depending on the solvent.

Solubles in different solvents when used for glucosamine purification were given in Fig.15. Total residue in solvents varied from 2.063% to 21.947%. Crude glucosamine hydrochloride purified with acetone gives 2.063% residue in solvent. While glucosamine hydrochloride washed with isopropanol contains 5.067% solubles. Glucosamine hydrochloride purified with ethanol on evaporation contains 10.183% residue while methanol contains 21.947% residue. Among the different solvents used for purification of crude glucosamine highest solubles were obtained for methanol followed by ethanol and least from acetone.

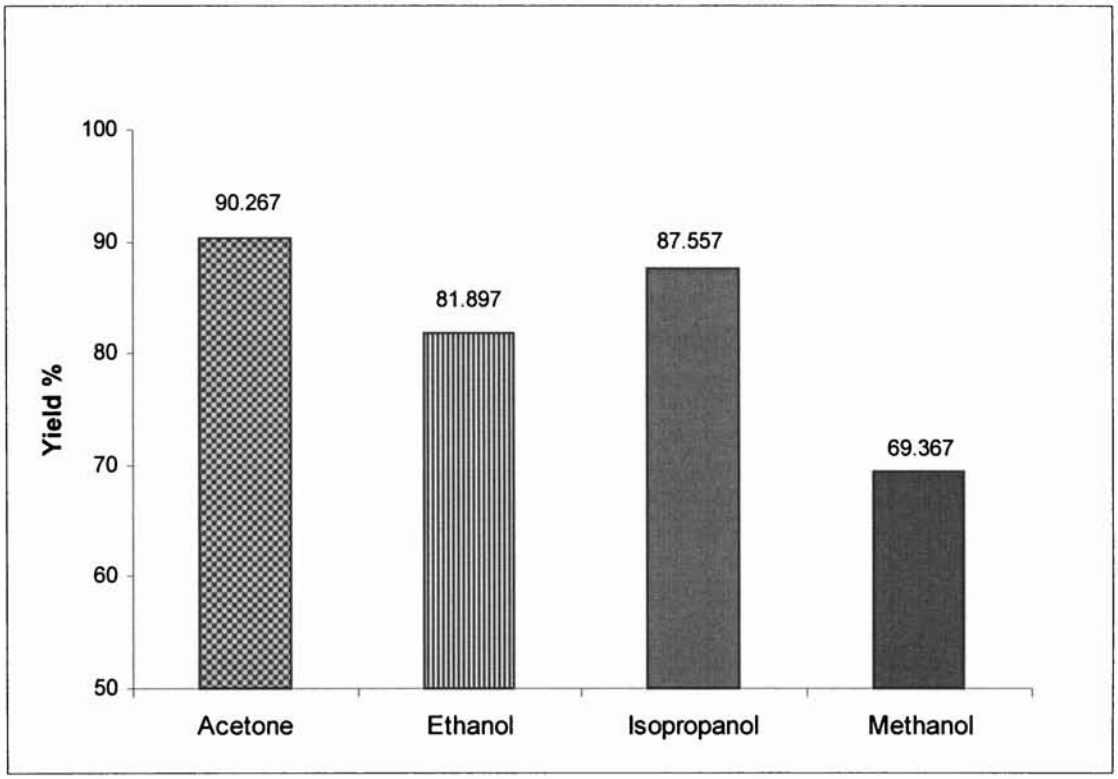


Fig. 14 Yield of glucosamine hydrochloride on purification with different solvents

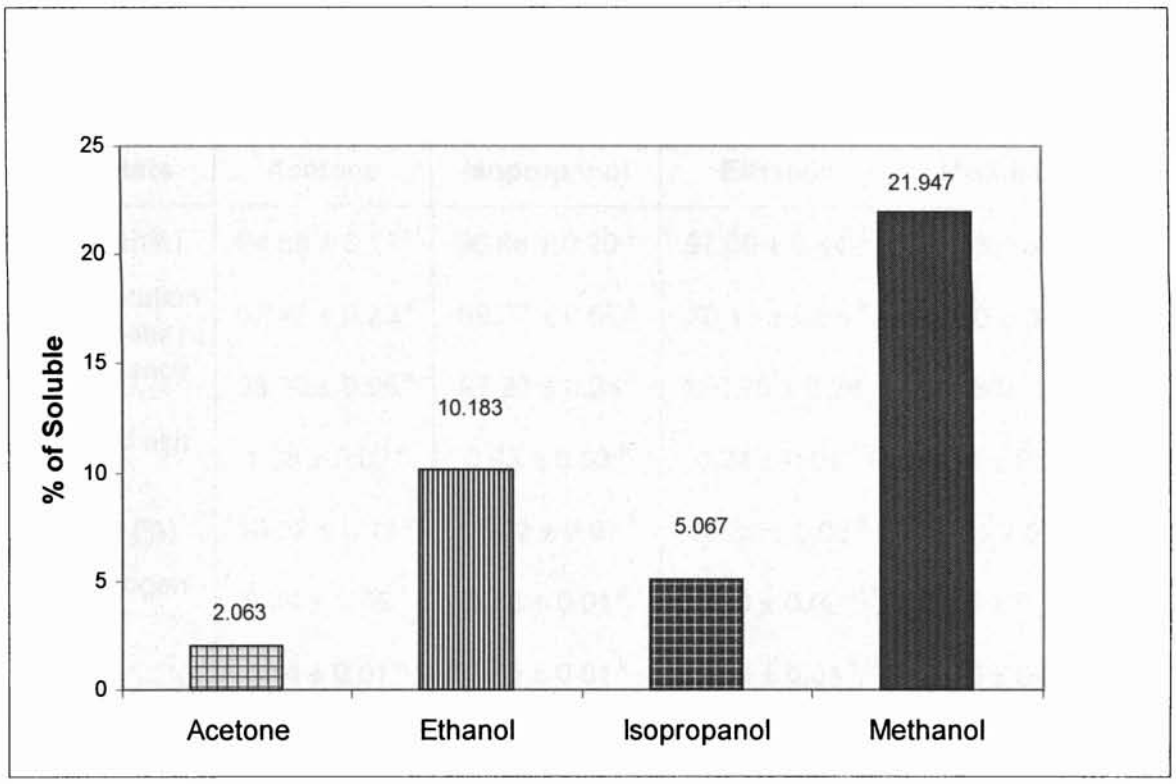


Fig. 15 Solubles in different solvents after glucosamine purification

Table-15 Role of solvents on glucosamine quality parameters

Parameters	Acetone	Isopropanol	Ethanol	Methanol
Assay (gm%)	94.68 ± 0.17[a]	96.68 ± 0.20[b]	97.09 ± 0.44[c]	99.23 ± 0.25[d]
Specific rotation (C=10,Water)	67.47 ± 0.23[a]	69.07 ± 0.67[b]	70.13 ± 0.65[c]	72.20 ± 0.25[d]
Transmittance %	98.30 ± 0.66[a]	97.20 ± 0.26[b]	100.20 ± 0.26[c]	101.80± 0.20[d]
Sulphated ash (%)	1.28 ± 0.02[a]	0.93 ± 0.03[b]	0.24 ± 0.01[c]	0.11 ± 0.01[d]
Chloride (%)	18.02 ± 0.11[a]	17.52 ± 0.07[b]	16.89 ± 0.08[c]	16.51 ± 0.02[b]
Total Nitrogen (%)	6.74 ± 0.05[a]	6.73 ± 0.01[a]	6.50 ± 0.02[b]	6.49 ± 0.01[b]
pH	3.74 ± 0.01[a]	3.85 ± 0.01[b]	3.89 ± 0.01[c]	4.20 ± 0.02[d]

Different superscript letters in the same row indicate significant differences($P < 0.01$).

4.7.1 Quality of Glucosamine hydrochloride

Table-15 represents the quality parameters of Glucosamine hydrochloride purified with different solvents. Among the quality parameters glucosamine assay, specific rotation, transmittance, sulphated ash, chloride, total nitrogen and pH were determined. Glucosamine assay on the basis of hexosamine determination reveals that content of glucosamine ranges from 94.68% to 99.23%. Among the different solvents used for purification acetone washed glucosamine results 94.68% assay. This value is the least among the four solvents used for purification. Crude glucosamine hydrochloride on purification with isopropanol gives 96.68% purity while ethanol gave only 97.99% glucosamine assay. Methanol washed glucosamine hydrochloride is having maximum purity with 99.23% glucosamine assay. From these results it is clear that methanol is the best solvent for glucosamine hydrochloride purification. The ANOVA on glucosamine assays reveals that the results are highly significant at 1% level when different solvents are used for glucosamine hydrochloride purification. Duncan's test indicates their values belong to different subsets.

Specific rotation of glucosamine hydrochloride in water decreased with time and changes from initial 100 to 72.5 and stabilizes after 3 hours. Specific rotation value was taken as an important factor for the determination of purity. Sigma grade glucosamine hydrochloride has specific rotation of 72.5. Glucosamine hydrochloride purified with acetone gives 67.47 specific rotation and their value is 7% less than standard. Glucosamine hydrochloride purified with isopropanol results 69.07 and this value was 4.73% less than standard value. Glucosamine hydrochloride on purification with ethanol gave a value of 70.13 and it was 3.27% lesser than standard value. Among different solvents used for the purification glucosamine hydrochloride purified with methanol is having a specific rotation value of 72.20. This value was almost equal to standard. ANOVA of specific rotation of Glucosamine samples indicates that they are significant at 1% level. Duncan's test also reveals that they belong to different subsets.

Transmittance of glucosamine hydrochloride purified with different

solvent ranges from 97.20% to 101.8%. Transmittance usually expressed as optical density which is the fraction of radiant energy that passes through a substance. The color of glucosamine hydrochloride samples might have interfered transmittance. The differences in transmittance among the samples are mainly due to the colour of impurities in glucosamine hydrochloride. In the present study, minimum transmittance was shown by glucosamine samples prepared after acetone washing, where as isopropanol washed glucosamine hydrochloride is having 97.2% transmittance. Ethanol washed glucosamine sample does have 100.2% transmittance. Among the different glucosamine samples maximum value for transmittance was observed in methanol washed glucosamine hydrochloride. This is justified by the apparent color difference of the samples purified with the different solvents used for the study. This higher value of transmittance may be due to reflection or scattering of light. ANOVA reveals that transmittance of glucosamine hydrochloride samples purified with different solvents are highly significant ($P<0.01$).

Minerals present in glucosamine sample were measured as sulphated ash. Acetone washed crude glucosamine hydrochloride is having 1.28% total sulfated ash. Isopropanol washed glucosamine hydrochloride sample possess 0.93% ash content. This value is 27% lesser than acetone washing. Glucosamine hydrochloride samples purified with ethanol removes 82% of minerals. While methanol washed sample is having only 0.11% minerals and the removal was 92%. ANOVA reveals that these values are highly significant at 1% level.

Chloride content of glucosamine hydrochloride purified with different solvents is given in Table-15. Theoretically the glucosamine hydrochloride contains 16.47% chloride. Crude glucosamine purified with acetone possess 18.02 % chlorides and their value is 9.4% higher than standard value. Glucosamine hydrochloride purified with isopropanol contains 17.52 % chlorides and it is 6.38% higher than reference value. Ethanol washed glucosamine shows 16.89% chloride value and this was 2.5% higher than standard value. Among the different samples used for purification, the least value for chloride was obtained with methanol washed glucosamine and this

value was 0.24% higher than sigma grade glucosamine. In this experiment the highest value for chloride was obtained in acetone washed glucosamine. Zhou et al (2000) reported that crude glucosamine hydrochloride on purification with 732-Cation exchange column gives a chloride content of 16.74%.

Nitrogen content in glucosamine sample varies from 6.39%-6.74%. Glucosamine hydrochloride theoretically contains 6.49% Nitrogen. Occurrence of deacetylated acetyl amino groups i.e. glucosamine residues and the presence of protein fractions increase the nitrogen value in samples. Glucosamine hydrochloride purified with acetone and isopropanol contains 6.74 and 6.73 gm/100 gm nitrogen respectively. These values are 3.85% and 3.70% higher than theoretical value. Glucosamine hydrochloride purified with ethanol was shown that their values in the range of 6.50. This value is slightly higher than reference value. Glucosamine hydrochloride purified with methanol maintains the theoretical nitrogen content. ANOVA reveals that the nitrogen content among different glucosamine samples are significant at 1% level ($p<0.01$). Duncan's multiple range test indicates that values of acetone washed and isopropanol washed samples belong to same subset while ethanol and methanol washed sample belongs to different subset.

The pH value of glucosamine hydrochloride sample, ranges from 3.74 to 4.20 Crude glucosamine hydrochloride purified with acetone had a pH value of 3.74 while ethanol washed glucosamine hydrochloride possess 3.89 Crude glucosamine on purification with isopropanol has 3.85 pH. Among the different glucosamine hydrochloride samples, the higher pH value was observed in methanol washed glucosamine. In general, the pH specification of glucosamine hydrochloride ranges from 3 to 5. All the samples are with in the range but lower pH values are undesirable from the consumer's point of view. Statistical analysis results showed that there was significant difference between pH of glucosamine hydrochloride samples purified with different solvents ($p<0.01$).

FTIR spectrum of sigma grade glucosamine and FTIR spectrum of glucosamine hydrochloride prepared from shrimp chitin purified with

methanol hydrochloride were presented in Figure-16 and Figure-17. Spectrum shows a strong hydrogen bonded (O - H) stretching absorption at 3453 cm^{-1} in standard glucosamine hydrochloride while the same peak was found at 3415 cm^{-1}in glucosamine hydrochloride from shrimp chitin purified with methanol. A prominent (C – H) stretching absorption around 2941 cm^{-1} was found in both the cases. Characteristic primary amine (N – H) bending vibration frequency is found at 1616 cm^{-1} and 1540 cm^{-1} in both samples. The less pronounced NH_{3+} peak is due to the hydrogen bonding with O – H group present in the molecule. C – O –C stretch vibration was found at 1070 cm^{-1} –1136 cm^{-1} for glucosamine hydrochloride sigma grade and glucosamine hydrochloride from shrimp chitin purified with methanol. The results suggest that the FTIR spectrum obtained from purified glucosamine hydrochloride is comparable with standard glucosamine hydrochloride supplied by the Sigma.

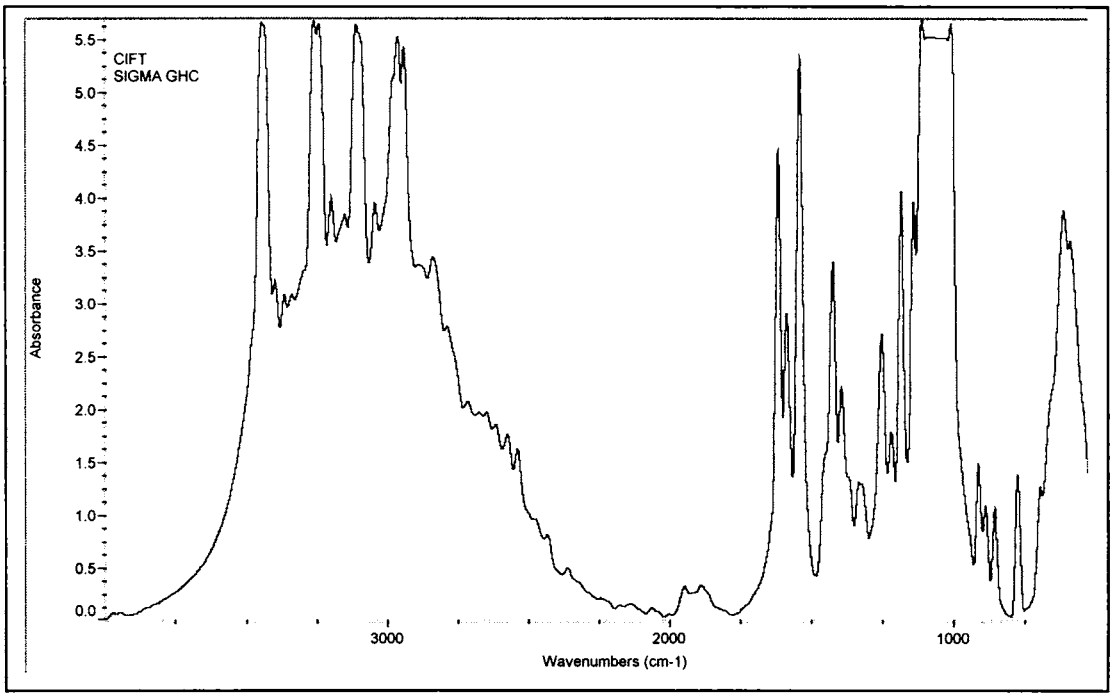


Fig. 16 FTIR Spectrum of Sigma grade Glucosamine hydrochloride

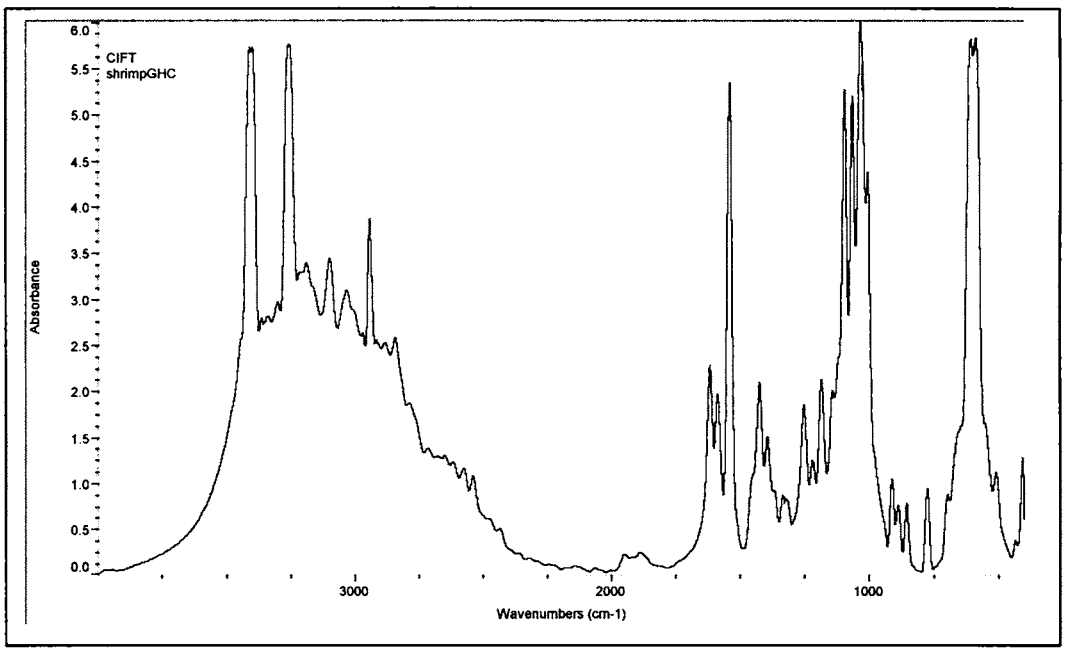


Fig. 17 FTIR Spectrum of Glucosamine hydrochloride prepared from Shrimp chitin

4.8. Storage Characteristics and Maillard Reaction of Glucosamine Hydrochloride

During storage the color of glucosamine hydrochloride turns from white to brown depending up on the purification process, i.e. selection of solvent. Compared to food and biological system only limited information is available on Maillard reaction in pharmaceutical products.

4.8.1. Change in color

The extent of Maillard reaction was measured by selected wavelength absorption. Table 16 shows the color of glucosamine samples purified with different solvents. The extent of browning was usually associated with the increase in yellowness. The intensity of red color was higher in isopropanol washed sample followed by acetone washed sample, lowest red value of 0.1 was observed in methanol washed sample. Yellow intensity was more in acetone washed sample followed by isopropanol washed sample. Blueness of fresh glucosamine sample varied from 0 to 0.7. Among the different glucosamine hydrochloride samples purified more blueness was observed in isopropanol washed glucosamine hydrochloride. Acetone washed Glucosamine hydrochloride showed lower value compared to glucosamine hydrochloride purified with isopropanol, ethanol and methanol. During storage blueness increased to 2.4 for glucosamine purified with isopropanol. Isopropanol washed glucosamine hydrochloride sample showed maximum value for blueness followed by acetone washed glucosamine hydrochloride. Ethanol washed glucosamine hydrochloride sample showed 0.6 blueness. Among the different Glucosamine hydrochloride samples least value for blueness was observed in methanol washed.

4.8.2. Change in Transmittance

Fig. I8 show the change in transmittance of fresh glucosamine hydrochloride during storage. Maximum transmittance was found in methanol-purified glucosamine followed by ethanol, acetone, and isopropanol indicating the purity of the product. Acetone washed

glucosamine hydrochloride having 98.3% to transmittance during preparation and it reduced to 89.5% during storage due to Maillard color development. Similarly the reduction in transmittance due to purification using isopropanol, ethanol and methanol were 14.5%, 6%, and 5.1% respectively. The isopropanol showed maximum reduction in transmittance indicating maximum Maillard reaction. The reason behind this fact was that during chitin hydrolysis protein present in chitin get hydrolyzed into amino acids and these compounds associated with crude glucosamine hydrochloride cannot be removed by isopropanol.

4.8.3. Change in Specific rotation

Change of specific rotation of glucosamine under Maillard reaction condition is indicated in Fig.19. Standard Glucosamine hydrochloride had a specific rotation value of 72.5 in water. Specific rotation value of glucosamine hydrochloride samples purified with different solvents ranges from 67 to 72.5. Among the different Glucosamine hydrochloride samples minimum value of specific rotation was found in glucosamine purified with acetone followed by isopropanol. During Maillard color development the specific rotation value changes from 67.47 to 66.48 in glucosamine hydrochloride purified with acetone. This reduction was 1.46% of the initial value. Glucosamine hydrochloride purification with isopropanol gave 69.7 specific rotation. After 1 year the change was1.78%. In the case of glucosamine hydrochloride washed with ethanol initial specific rotation value was 70.13 and it diminished to 69.65 during Maillard color development and their reduction was 1%. Glucosamine hydrochloride purified with methanol gave relatively higher specific rotation values i.e. 72.25. During Maillard reaction their specific rotation value was slightly changed to 72. This reduction was only 0.34%. In general the specific rotation of glucosamine hydrochloride samples was reduced up to 1.78% during storage. Maximum reduction was found in isopropanol washed glucosamine hydrochloride followed by acetone. Minimum change in specific rotation was observed in glucosamine hydrochloride purified with methanol.

4.8.4. Changes in Glucosamine assay

Changes in Glucosamine assay due to Maillard reaction are given in Fig. 20. Glucosamine content of samples purified with different solvents range from 94.68 to 99.23%. After color development this reduced to 92.2%. Glucosamine washed with acetone gave 94.68% assay during initial condition and on storage it reduced to 92.2% with 2.62% reduction. Isopropanol washed Glucosamine hydrochloride samples initially had 96.17% purity in assay and it reduced to 94.33% subsequently. This change was 2% less than the initial value. Ethanol washed glucosamine hydrochloride initially had 97.09% glucosamine assay. It reduced to 96.35% on color development. Change in assay of glucosamine hydrochloride washed with methanol ranged from 99.23 to 99.02. This change was only 0.21% than the initial value.

The present study indicates that during storage glucosamine hydrochloride undergoes Maillard reaction and the intensity of the color of glucosamine hydrochloride varies depending on the solvents used for purification. Among different glucosamine hydrochloride samples, Maillard reaction was more pronounced in isopropanol and acetone washed samples compared to the other two. Least Maillard reaction was observed in glucosamine hydrochloride purified with methanol as indicated by low reduction in transmittance and less change in specific rotation. Thus glucosamine hydrochloride purified with methanol retains its white color and characteristic properties during storage due to the removal of components easily susceptible to Maillard reaction.

Table-16 Change in Color of Glucosamine during Maillard reaction.

Solvent	Fresh Glucosamine	Stored for 1 Year
Acetone	1.0 R + 2.2 Y + 0.6 B	2.9 R + 6.3 Y + 1.5 B
Isopropanol	1.2 R + 2.1 Y + 0.7 B	3.7 R + 5.2 Y + 2.4 B
Ethanol	0.6 R + 1.0 Y + 0.3 B	1.85 R + 2.1 Y + 0.6 B
Methanol	0.1 R + 0.2 Y + 0.0 B	0.6 R + 0.7 Y + 0.3 B

(R=Red, Y=Yellow, B=Blue)

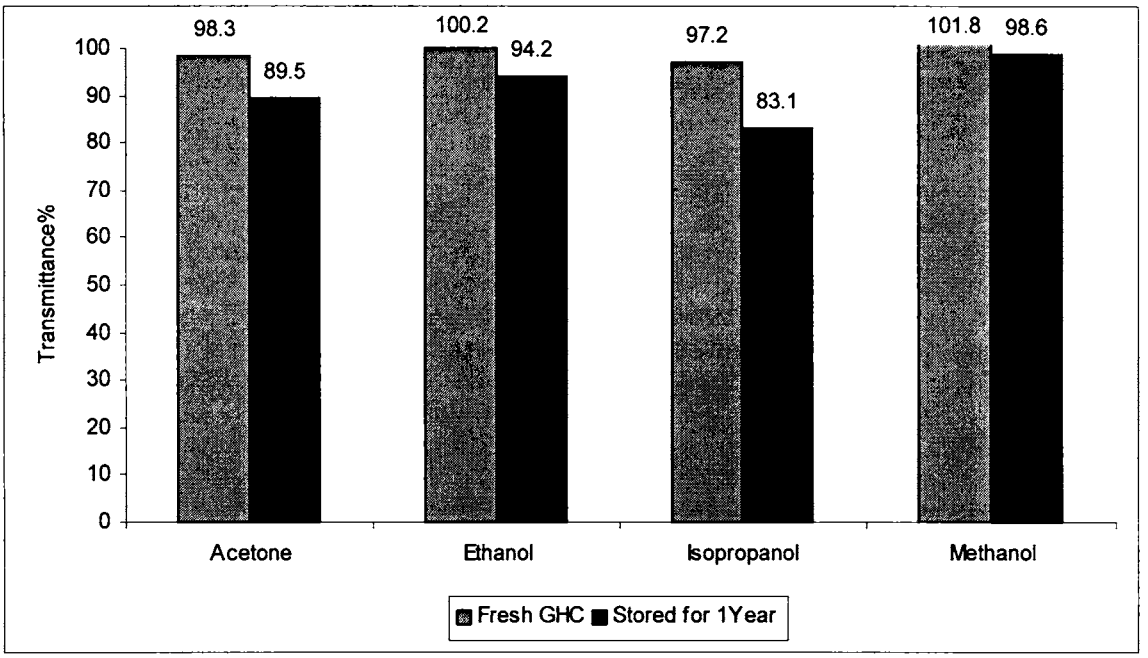


Fig. 18 Transmittance change of Glucosamine hydrochloride during Maillard reaction

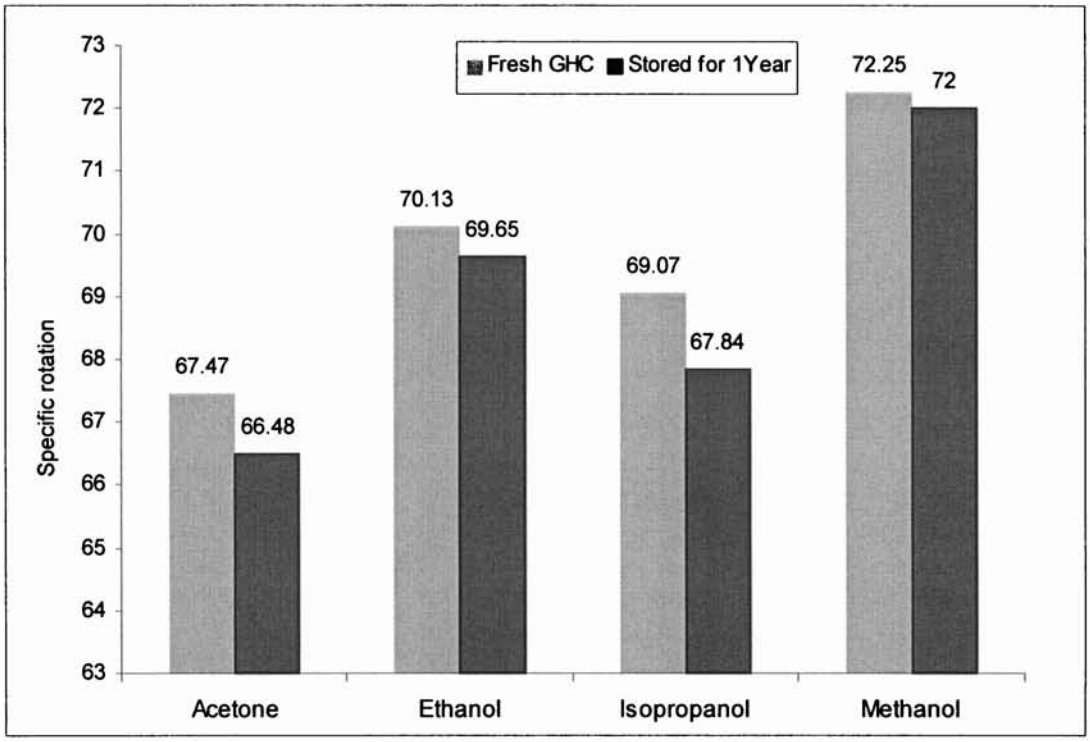


Fig. 19 Change in specific rotation of Glucosamine hydrochloride during browning reaction

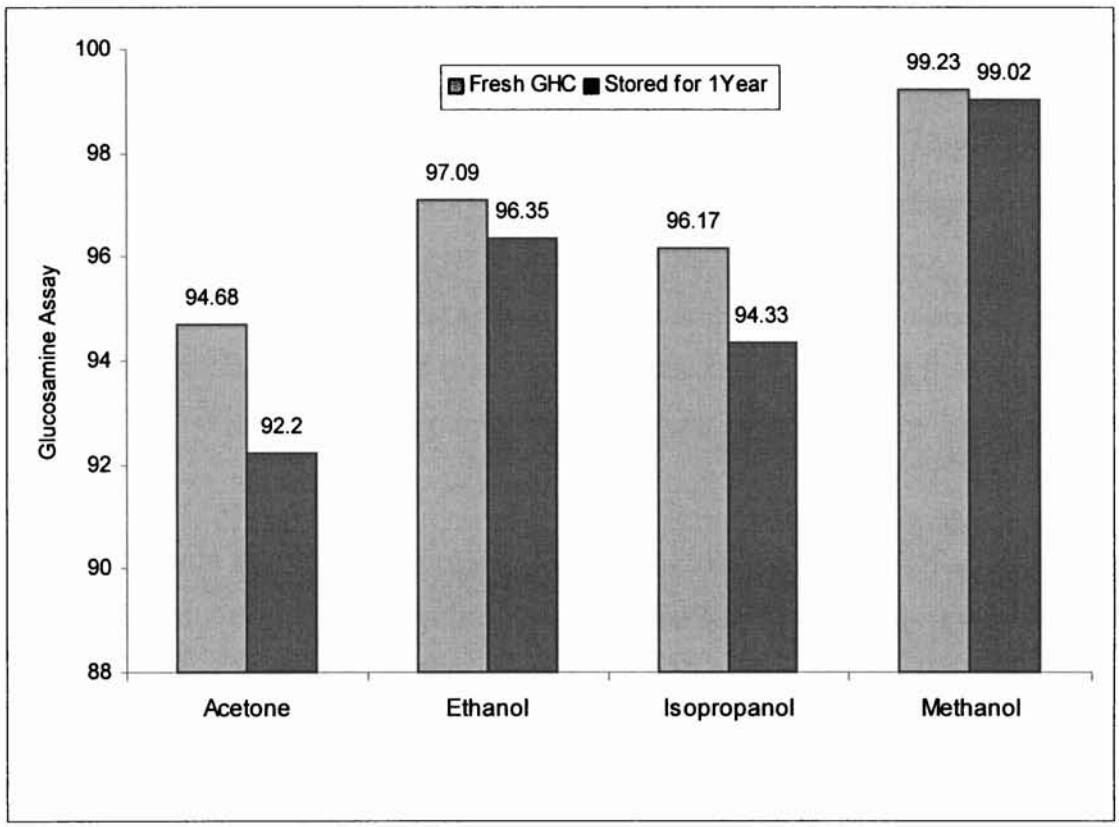


Fig. 20 Change in Glucosamine assay of glucosamine hydrochloride during storage.

4.9. Solvent Recovery

Solvents are used to improve the yield in the synthesis of active pharmaceuticals substances besides imparting characteristic crystal form, purity and solubility. The use of solvents may therefore have a critical role in the synthetic process. Depending upon the safety data and there risk to the human health the solvents is classified into three classes. For the purification of crude glucosamine hydrochloride difference solvents like acetone, isopropanol, ethanol and methanol are used. Among the solvents used in the study methanol comes under class -2 and others belongs to class-3.

Solvents obtained after purification of glucosamine hydrochloride were acidic in nature and are neutralized and distilled for obtaining reusable solvents. Fig. 21 represents the recovery of solvents. Acetone washed for the purification of glucosamine gives the maximum reusable solvents followed by Isopropanol. Glucosamine purified with ethanol on neutralization and distillation gives 86.66% reusable solvents while crude glucosamine hydrochloride washings with methanol on distillation gave 74.33% reusable methanol. The recovery of solvents can be nearly 100% when perfect solvent recovery systems are employed in the commercial production. Crude glucosamine hydrochloride contains glucosamine hydrochloride, D-glucosamine, protein fractions, minerals, oligosaccharides etc. During purifications solvents remove some of these fractions. Methanol solubles obtained from crude glucosamine on evaporation of methanol gives a slightly viscous material. Other solvents like ethanol, acetone and isopropanol could not remove these materials when used for purification of glucosamine hydrochloride because these are not soluble in these solvents.

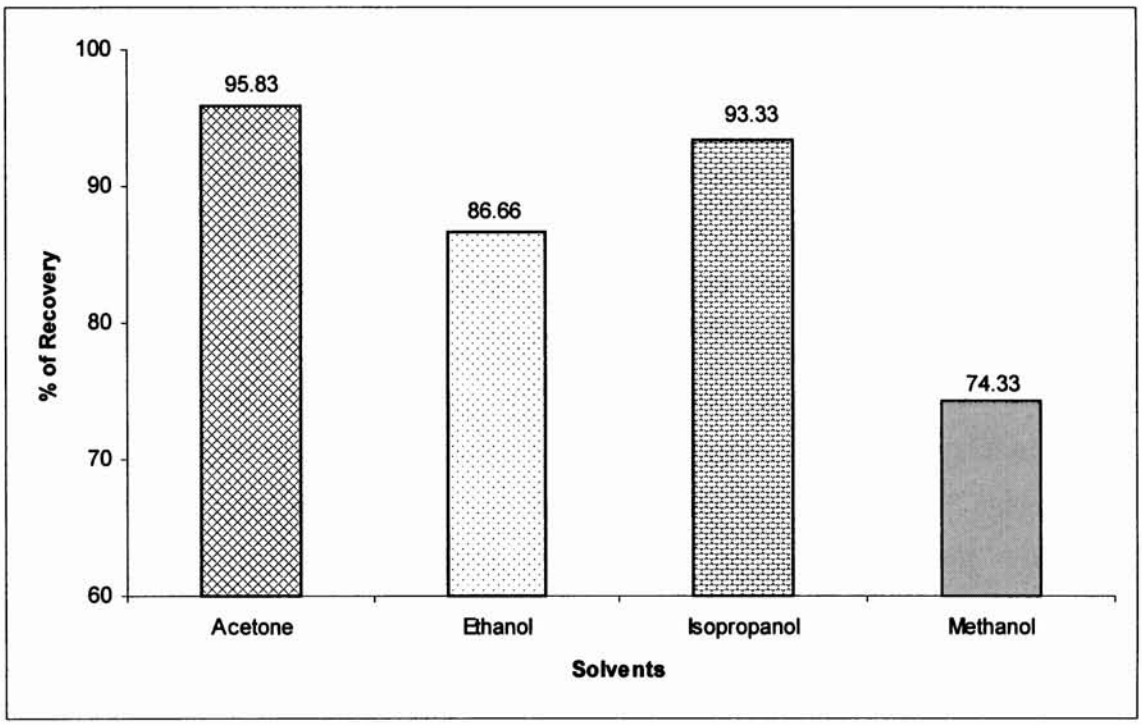


Fig. 21 Recovery of solvents

4.9.1. Residual solvents

Residual solvents are defined as organic volatile impurities that are used or produced in the manufactures of active pharmaceuticals substances or excipients or in the preparations of medicinal products. During the manufacturing processes the solvents are not completely removed. The residual solvents do not have therapeutic effects. As such effort should be made to remove them to the extent possible to meet the specifications prescribed in the individual monograph and good manufacturing practices or other quality based requirements. Safety limit of methanol in pharmaceuticals product was 3000 ppm and in others it was 5000 ppm. Non-specific method like loss on drying may be used for monitoring the content of class -3 solvents. Glucosamine purified with methanol on vacuum drying for 45 minutes was analyzed for residual methanol level. In all the samples the residual level was less than 2000 ppm. Thus the methanol washing followed by vacuum drying the products for 45 minutes resulted a safe product.

4.9.2. Acid recovery and reuse

During chitin hydrolysis acetic acid was produced along with glucosamine hydrochloride thus the acid obtained are not suitable for hydrolysis. Hydrochloric acid was recovered by the concentration of decolorized hydrolysates to the extent of 65 to 70 % as an aqueous solution, and its strength ranged from 7 - 8 N. Part of this acid can be utilized for the decalcification of prawn shell. The remaining recovered acid can be concentrated in a suitable distillation units and the maximum boiling azeotrope can be further fortified with fresh hydrochloric acid gas to obtain required strength (approximately 10 N) for hydrolysis of chitin. During hydrolysis at 95°C and 100°C more acid fumes generated than 80°C to 90°C. This acid fumes can be passed through the water to obtain 1 to 1.5 N strength. This solution can be directly used for demineralization of prawn shell.

Charcoal required for the decolourisation process was 10 % of the chitin. Thus its requirement in the process is based on the use of fresh

charcoal for each treatment.

4.10. Methanol Soluble Components

Methanol solubles were collected during the purification of crude glucosamine hydrochloride and their characteristics were listed in Table-17. They are hygroscopic in nature, which may be due to the presence of calcium chloride. The mineral content in methanol soluble residue was ranged from 3 - 5%. Analysis reveals that the contents are mainly calcium chloride. Methanol soluble fraction was soluble in water, partially soluble in ethanol and insoluble in acetone, isopropanol and ether. Budavari (1996) and Lewis (1993) reported that glucosamine is partially soluble in methanol or ethanol. Specific rotation values of methanol solubles range from 45 - 50. This variation in specific rotation value was due to the contamination of other components. Specific rotation value of D-glucosamine is 47.5. Drying the solubles above 110^0C cause decomposition. The chloride content estimated was due to the presence of calcium chloride. Total nitrogen content of methanol solubles are between 6.5 - 7.5% and this value was higher than glucosamine hydrochloride. Theoretically glucosamine contains 7.81% nitrogen, where as in methanol soluble this value was reduced due to the minerals. FTIR spectrum of methanol soluble gives an indication that the component is glucosamine (Fig.-22). FTIR-spectrum of methanol soluble contains limited peaks than glucosamine hydrochloride. The broad O-H stetch was found at 3442cm^{-1} Primary amine (N – H) bending vibration frequency is found at 1628 cm^{-1} . The presence of free amino group in D-glucosamine or oligomers causes removal of minerals. Glucosamine when dissolved in methanol also carries trace minerals present in hydrolyzed chitin.

Table17 Characteristics of Methanol solubles

1	Nature of product	Highly Hygroscopic
2	Solubility	Soluble in Water, Methanol, Partially soluble in Ethanol, Insoluble in Acetone, Isopropanol, Petroleum ether
3	Mineral content	3 - 5%
4	Specific rotation	45 - 50
5	Total Nitrogen	6.5 - 7.5%
6	Chloride content	5 - 7 %
7	Acetyl groups	Nil

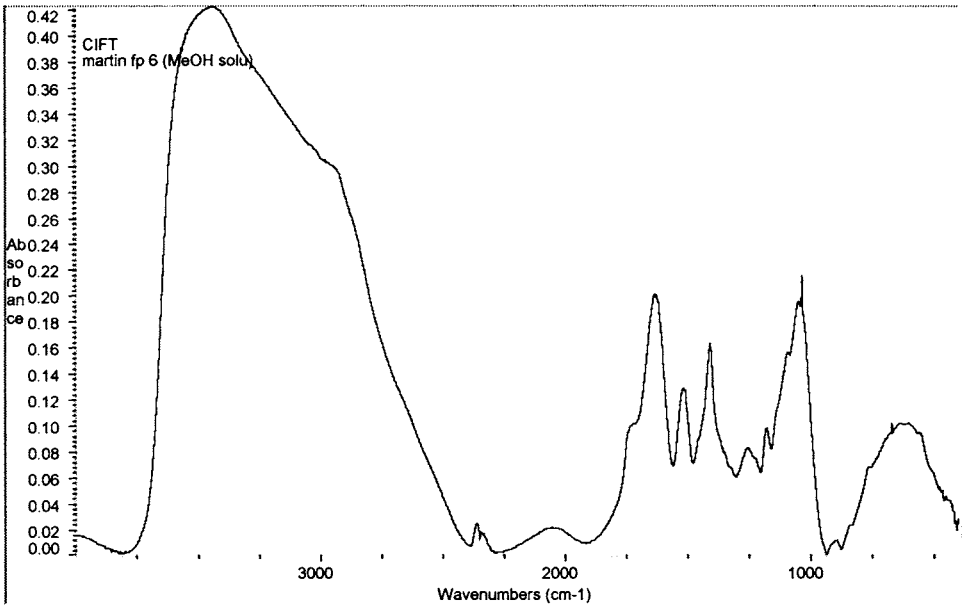


Figure-22 FTIR Spectrum of Methanol solubles

5. CONCLUSIONS

Deproteinisation of shrimp shell had significant effect on quality of chitin. Amount of alkali used for deproteinisation affects the chitin quality. Higher alkali concentration results deacetylation of chitin, which results reduction in glucosamine hydrochloride yield. Concentration of alkali used for deproteinisation increased the amount of methanol solubles. This is an indication of deacetylation thus chitin prepared with 3% NaOH for 30 minutes was suitable for hydrolysis ie glucosamine hydrochloride production.

Demineralization of shrimp shell also influences chitin quality. Demineralization in less concentrated acid results more ash content in chitin. Higher acid concentration results more deacetylation and depolymerization. Demineralization with 1N Hydrochloric acid for 60 minutes results good quality chitin, which on hydrolysis gives more glucosamine hydrochloride.

Concentration of Hydrochloric acid affects the glucosamine hydrochloride yield. When 38% Hydrochloric acid was used for hydrolysis, the yield was lower than chitin hydrolysis with 32%. Amount of methanol solubles was higher at 38% concentrations Faster glucosamine hydrochloride conversion was observed at higher acid strength and glucosamine hydrochloride obtained had high quality by chitin hydrolysis with 38% Hydrochloric acid. When high quality glucosamine hydrochloride was envisaged, it is better to use 38% Hydrochloric acid for hydrolysis. For medical purposes, chemical purity of hydrochloric acid was not aimed. Thus we can go for hydrolysis with 32% Hydrochloric acid.

1:2 chitin Hydrochloric acid ratio was found to be ideal for chitin hydrolysis. The ratio below this was not sufficient for hydrolysis and 1:3 and 1:4 ratios are unworthful. The ratios above 1:2 should not improve the glucosamine hydrochloride yield. Glucosamine hydrochloride quality was not affected by chitin-acid ratios.

Hydrolysis of chitin at 95^0C for 45 minutes gives glucosamine hydrochloride having high purity thus this temperature was recommended for

glucosamine production. At 80^0C the conversion was slower and the purity was lesser than other temperatures while, at 100^0C the yield obtained was less than other temperatures.

Solvents used for glucosamine hydrochloride affects the final yield and purity. But their quantity varied from solvent to solvent. High quality glucosamine hydrochloride was obtained by purification with methanol followed by ethanol Non glucosamine matter (solubles) obtained from the solvents during purification was also varied. Highest soluble content was obtained from methanol followed by ethanol.

During storage, glucosamine hydrochloride undergoes color change. The change of color intensity was related to the purity of glucosamine hydrochloride. Non-glucosamine hydrochloride matter accelerated browning. During storage maximum color was retained by methanol washed glucosamine hydrochloride followed by ethanol washed sample. It was observed that during Maillard browning all the quality parameters were reduced, but the intensity of quality reduction was minimum for glucosamine hydrochloride purified with methanol.

Methanol solubles are non-glucosamine hydrochloride matters obtained during purification, which contains D-glucosamine units, oligomers, calcium chloride, hydrolyzed protein etc.

Recovery of reusable solvents from spent solvent ranges from 75-95%. Among the solvents used Acetone gives maximum recovery followed by isopropanol and the least from methanol. Reduction in recovery is due to the solubles in this solvent.

Hydrochloric acid was recovered by the concentration of decolorized hydrolysates to the extent of 65 to 70% as an aqueous solution of hydrochloric acid and acetic acid and its strength ranged from 7 - 8 N. Part of this acid can be utilized for the decalcification of prawn shell during chitin production thus the problem of acid waste can be solved in a eco-friendly manner.

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