

**UTILIZATION OF SHRIMP WASTE FOR THE RECOVERY OF VALUABLE
BIOACTIVE COMPOUNDS**

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DECLARATION

I hereby declare that this dissertation represents my own work. It has not been submitted for any diploma/degree or examination at any other Technikon/University. It is being submitted for the Degree of Master of Science: Biotechnology, in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa.

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DEDICATION

To my dad, Ndabivele

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LIST OF ABBREVIATIONS

C ₃ H ₆ O	acetone
CaCl ₂	calcium chloride
CH	chitosan
Ca	calcium
CaCl ₂	calcium chloride
CaCO ₃	calcium carbonate
COS	chitooligosaccharides
DDA	degree of deacetylation
DP	degrees of polymerization
EDTA	ethylenediaminetetraacetic acid
FTIR	fourier-transform infrared spectroscopy
GlcNAc	<i>N</i> -acetyl glucosamine
GlcN	glucosamine
GLY	glycerol
H	hydrogen
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
kDa	Kilodalton
LC-APCI-MS	liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry
NAcCOS	chitooligomers

NAG	<i>N</i> -acetyl glucosamine
NaOCl	hypochlorite
NaOH	sodium hydroxide
NH ₂	amine
NH ₄ Cl ₂	ammonium chloride
NH ₄ HCO ₃	ammonium bicarbonate
OH	hydroxide
PA	acetylation
PEG	polyethylene glycol
PUFAs	polyunsaturated fatty acids
R^2	coefficient
Rf	retardation factor
TS	tensile strength
TGA	thermogravimetric analysis
TLC	Thin-layer chromatography
XRD	X-ray Powder Diffraction

ABSTRACT

Shrimp waste is a major by-product of crustacean processing and represents an interesting source of bioactive molecules. In addition, its use increases the sustainability of processing fishery products. The present study reports a process developed for recovering bioactive molecules from shrimp waste through the use of chemical methods. The samples of shrimp were confirmed to be from the species *Haliporoides triarthrus*. The recovery of chitin was 30% of the processing waste and 30-60% chitosan (CH) from chitin. CH was characterized by FTIR analysis and exhibited a degree of deacetylation (DDA) of 72%. From the demineralization extract, CaCO_3 was extracted and confirmed by FTIR. Based on a kinetic study of acid hydrolysis, it was demonstrated that chitin can be quantitatively hydrolysed into glucosamine (GIN), *N*-acetyl glucosamine (GlcNAc) and their respective oligomers with 32% hydrochloric acid at 60°C and qualitatively from CH with 32% hydrochloric acid at 80°C. The oligomer mixed fractions were desalted by activated charcoal extraction and the components of each fraction were analysed by TLC and HPLC. Chitooligosaccharides (COS) and *N*-acetyl chitooligosaccharides (NACCOS) with degrees of polymerization (DP) ranging from 2 to 6 were obtained from CH and chitin, respectively. The antimicrobial activities of chitosan, COS and NACCOS were investigated against five gram-negative bacteria and five gram-positive bacteria. Chitosan exhibited stronger bacteriostatic effects against gram-positive bacteria than gram-negative bacteria in the presence of 1% chitosan. The oligomers showed no bacteriostatic or bactericidal effects on all tested bacteria. A total $30.74 \pm 0.078 \mu\text{g.g}^{-1}$ astaxanthin was extracted with 90% acetone from the species; *Haliporoides triarthrus* and TLC analysis indicated that the species contained both astaxanthin and its esters.

Chitosan films were obtained by solution casting of blends of chitosan with glycerol, polyethylene glycol 200 (PEG-200) and polyethylene glycol 600 (PEG-600) as plasticizers. Films were characterized by FTIR, XRD diffraction, TGA, and SEM analysis. The tensile strength and elongation at break properties of the films were also evaluated. CH films and CH/GLY blended films were translucent in appearance and the CH/PEG 200 and CH/PEG 600

films were opaque. The CH films yielded mechanically resistant films without the use of a plasticizer.

These data point to the feasibility of an integrated process for isolating highly bioactive molecules, such as oligosaccharides, with a broad spectrum of applications from shrimp processing waste.

1. INTRODUCTION AND LITERATURE REVIEW

Aquaculture is a fast growing food production sector which provides acceptable protein rich supplements to wild aquatic fauna and flora to which they may also be a substitute for (Prameela et al., 2012). It is diverse with regards to the materials used; the nature and magnitude of the preferred methods and versatile environmental characteristics. On account of increased consumer awareness of the relation between health and diet, the utilization of marine-related foods has increased dramatically. Consumers recognize sea food's as complete and nutritious foods and are therefore considered an exceptional source of quality proteins and beneficial lipids with high quantities of polyunsaturated fatty acids (PUFAs) recognized to contribute in improvement of personal health by various means, such as alleviating hypertension and cardiovascular disorders (Alireza and Mohammed, 2012). A typical example of such food is shrimp.

Shrimp is a high value aquacultural product (Knorr, 1991, Omum, 1992) supplied to shrimp processing industries and is usually exported devoid of an exoskeleton, however in spite of the aforementioned desirable properties, this seafood along with other marine-based food products, is highly susceptible to quality dilapidation. Biodegradation of chitin in the crustacean shell waste is relatively slow and thus encourages the accumulation of discards which have become a major concern for processing industries (Fereidoon et al., 1999). This is largely a result of lipid oxidative reactions, (especially those associated with PUFAs) that are catalysed by the presence of large concentrations of nonheme and heme proteins (Decker et al., 1992).

In present years, the generation of shrimp waste from such industries has dramatically increased and the continuous production of these biomaterials without the means of a utilization technology has ensued in disposal, accumulation of waste and pollution problems (Hengameh and Mehdi, 2009). Shrimp comprises approximately 45% of seafood that is processed.

The waste, composed of the cephalothoraxes and exoskeleton (Ibrahim et al., 1999, Venugopal and Shahidi, 1995) accounts for 50-70% of the raw materials weight (Prameela et al., 2012) and is usually discarded. The limited utilization of shrimp lies with the material

being highly perishable (Anonymous., 1997) that is, the waste quickly becomes colonized by spoilage microorganisms under hot and humid conditions and can rapidly be transformed into biogenic amines with very foul odours (Zakaria et al., 1998). If decaying is not prevented or avoided, the biomaterial then disintegrates into actual waste and the high protein content becomes an environmental hazard and a financial strain if not discarded appropriately (Prameela et al., 2012). It is noteworthy that a suitable technology is necessary to delay or prevent decay of these biomaterials.

In an effort to encourage the utilization of shrimp biomaterials, medium and large scale processing has been generated. This involves drying and mixing the waste with other agricultural raw materials for the production of animal feed, however this sun drying technique has low hygienic control. As a result, the waste is disposed of in landfills, soil and in the ocean, producing large surface pollution and unpleasant odours along coastal areas and thereby causing a serious risk of environmental pollution (Prameela et al., 2012). The standard practice of discarding shrimp by-products not only wastes valuable resources that can be used for other applications but also threatens endangered species and thus the ecology (Morgan and Chuenpagdue, 2003).

Many valuable bioactive compounds like pigments, chitin, amino acids, fatty acids and proteins (Shahidi and Synowiecki, 1991, Roberts, 1992, Al Sagheer et al., 2009) are said to be contained in the biowaste. Therefore, if treated with appropriate processing; these compounds can contribute substantially to overall profitability through their calibre of applications in biotechnology, medical, cosmetic, pulp, paper, textile and food industries (Prameela et al., 2012).

At most, 5% of shrimp waste is mainly spent as animal feed, therefore the remaining waste constituents such as the carotenoid pigment, mainly astaxanthin (Gimeno et al., 2007), chitin (14-30%), minerals (35%) and protein (40%) (Synowiecki and Al-Khateeb, 2000) can be explored for other useful applications. Chitin, a white, hard, inelastic, nitrogenous polysaccharide (Al Sagheer et al., 2009) is especially important because it is the supporting material of the exoskeleton shrimp and other crustaceans such as crabs and lobsters (Pradip et al., 2004), and can therefore be isolated as a cheap, renewable biopolymer (Muzzarelli,

1977). This polysaccharide possess distinct characteristics and structures different from those of typical synthetic polymers (Hengameh and Mehdi, 2009). With over a century since its discovery, chitin has been widely accepted as an important biomaterial in many respects, (Feisal and Montarop, 2010) including agriculture, food technology, microbiology, wastewater treatment, material science, drug delivery systems and tissue engineering (Feisal and Montarop, 2010).

Therefore in the interest of recycling shrimp waste and the potential economic prospects it provides; it is the aim of this project to convert shrimp biomaterials into commercially viable products and in turn address both environmental and economic concerns.

1.1 CHITIN

Of the numerous kinds of polysaccharides available, chitin along with cellulose are the foremost important biomass resources (Hengameh and Mehdi, 2009). Chitin, second only to cellulose is the most abundant biopolymer (Jörg et al., 2011) and through a β (1-4) linkage is comprised of 2-acetamido-2-deoxyglucose (Majeti and Ravi, 2000). Chitin is acquired from lower animals whereas cellulose is produced in plants. Chitin, unlike cellulose, can be both a source of carbon and nitrogen (C:N=8:1) containing 6-7% nitrogen and 7-9.5% nitrogen in its deacetylated form (chitosan), however regardless of nitrogen present, its immunogenicity is astonishingly low (Prameela et al., 2012).

The polymer chain arrangement, crystallinity and purity of chitin will vary depending on its origin, and also determines its properties (Rinaudo, 2006). Depending on its source, it can exist in three allomorphs; namely the α , β and γ forms (Fig.1.1) (Hengameh and Mehdi, 2009). β -chitin is obtained from molluscs such as squid and assumes a monoclinic unit cell wherein the polysaccharides are predominantly composed in parallel chains. The γ -chitin has not yet been completely identified, however the chains are proposed to be in a mixed form, containing one anti-parallel and two parallel strands of chitin (Neetu et al., 2006, Atkins, 1985). Amongst the three allomorphs of chitin, α -chitin is the most abundant form, mainly obtained from shrimp shells and crab (Feisal and Montarop, 2010) and its chains are aligned in an antiparallel form. The β -form can be converted to the α -form, the reverse

however is not possible. The γ -chitin, if treated with lithium thiocyanate can be converted to α -chitin (Feisal and Montarop, 2010).

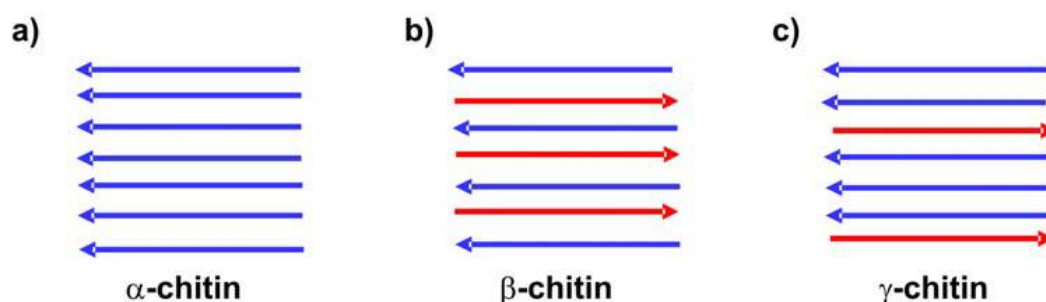


Fig. 1.1 Arrangement of the three allomorphs configurations of chitin; α -chitin (a); β -chitin (b) and γ -chitin (c) (Hengameh and Mehgi, 2009).

On account of its intermolecular hydrogen bonds and compact structure (Al Sagheer et al., 2009), chitin is insoluble in water, resembling cellulose in its low chemical reactivity and solubility (Majeti and Ravi, 2000), however, water-soluble derivatives of chitin such as carboxymethyl chitin or chitosan can be obtained (Majeti and Ravi, 2000). These have a unique ability to be moulded into numerous forms including fibres, beads, hydrogels, membranes and sponges (Mano et al., 2007). Chitin is structurally identical to cellulose with the exception of the hydroxyl group replaced by an acetamide group on the C-2 position (Fig. 1.2). Chitin functions naturally similar to cellulose, as a structural polysaccharide (Majeti and Ravi, 2000).

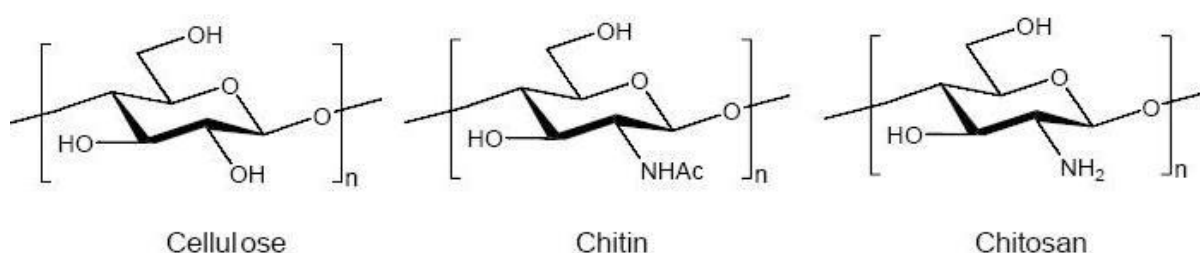


Fig. 1.2 Chemical arrangements of cellulose, chitin and chitosan (Majeti and Ravi, 2000).

There are over 200 estimated potential uses of chitin and chitin derivatives, especially chitosan, (Brzeski, 1987). Its non-toxicity, biocompatibility, absorption and biodegradability properties are supported by many reports in biomedical applications (Jollès and Muzzarell,

1999). Chitin is a functional material with great potential, however underutilized. Therefore the growing interest in chitin and its broad range of applications in many fields has become inevitable (Majeti and Ravi, 2000).

1.1.1 Sources of chitin

Chitin is primarily present in the exoskeleton of crustaceans (Hengameh and Mehdi, 2009) and is the primary constituent of arthropod exoskeleton, tendons and the inner layers of their digestive, excretory, and respiratory systems (Feisal and Montarop, 2010). It is synthesized by a variety of different living organisms found in the lower animal and plant kingdoms, assisting in activities pertaining to strength and reinforcement, if necessary (Marguerite, 2006). It may also be found in worms, insects and mushrooms, although in varying amounts (Prameela et al., 2012). Along with β -glucans, chitin is the dominant component of fungal and yeast cell walls (Jörg et al., 2011). The presence of chitin has been reported in the iridophores; which are the reflective materials in the epidermis and the eyes of cephalopods of the phylum; *Mollusca* and arthropods (Herring, 1979).

A report by Wagner et al., (1993) based on studies of enzymatic degradation, lectin binding, and endo-chitinase binding, confirmed that the vertebrates; *Lipophrys trigloides* (fish) epidermal cuticles are chitinous implicating that not only is chitin an important component of invertebrates but its presence in vertebrates is probable (Feisal and Montarop, 2010).

1.1.2 Processing of chitin from shrimp waste

Numerous techniques for extracting chitin from different sources have been reported (Al Sagheer et al., 2009). Isolation may be from fungal mycelium which is involved with fermentation processes. There over 10^6 species from which chitin is biosynthesized in the three allomorphic configurations (Fig.1.1), however on an industrial scale or in laboratories, isolation is usually from the exoskeletons of crustaceans, especially in crabs and shrimps (Tolaimate et al., 2003).

The isolation from crustacean shell biomass where chitin production is associated with the food industry (Majeti and Ravi, 2000) is the most common technique used, referred to as the chemical procedure (Al Sagheer et al., 2009). It has four conventional steps:

demineralization, deproteinization, decolourization, and deacetylation (Fig.1.3). Extraction of chitin however, is only comprised of the deproteinization step which is involved in protein removal and the demineralization step, responsible for the disintegration of high concentrations of calcium carbonate present in the shells (Feisal and Montarop, 2010).

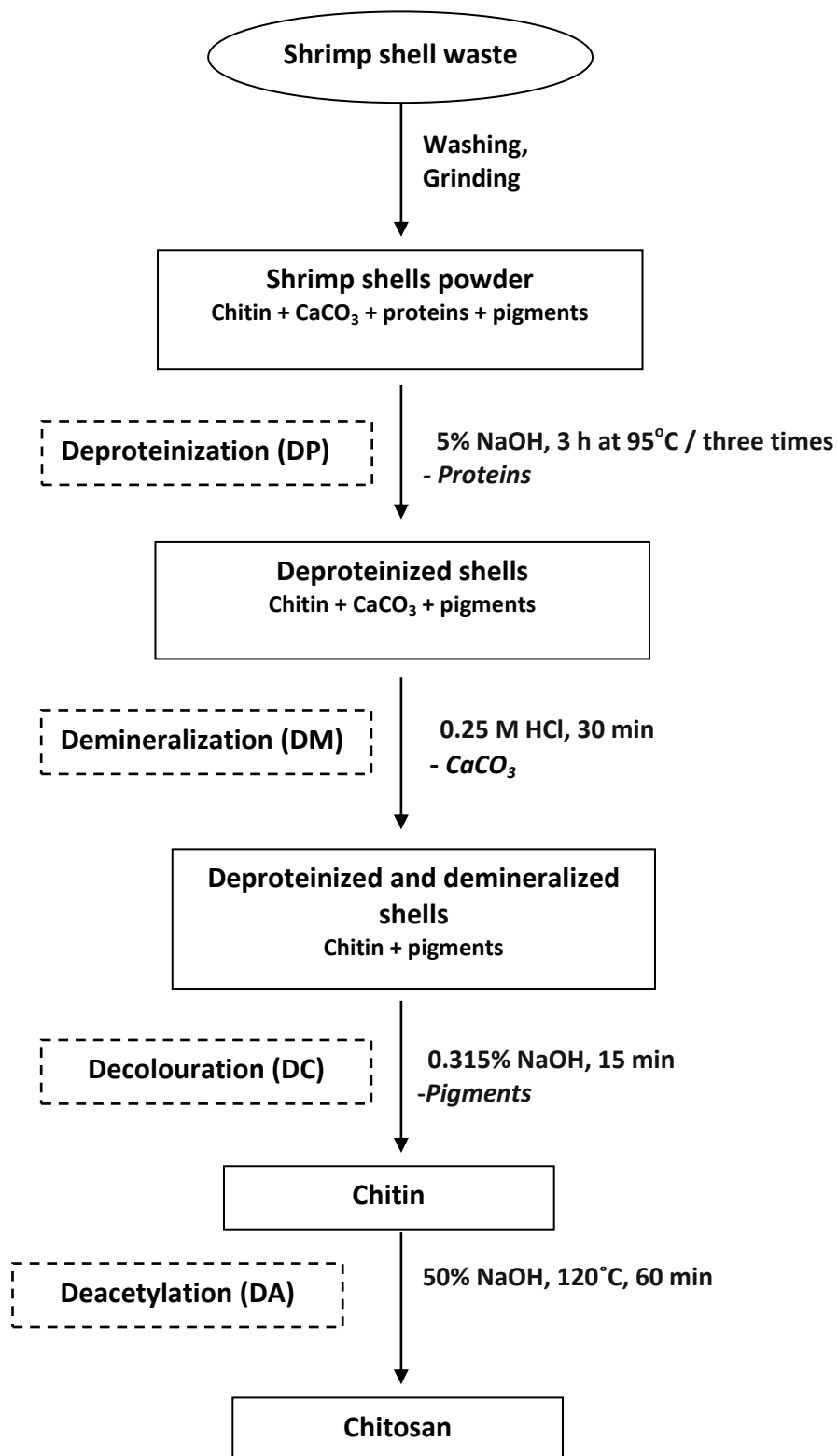


Fig. 1. 3 Modified scheme for the production of chitin and chitosan using a chemical method (Al Sagheer et al., 2009).

1.1.2.1 Deproteinization

The crustacean shell waste is first crushed and treated with sodium hydroxide (NaOH) at high temperatures ranging from 70 to 120°C (Rao et al., 2000). Depending on methods of preparation the reaction time may range from 0.5 to 12 h. Under such conditions, the protein dissolves and is dissociated from the shrimp shell solid component (Prameela et al., 2012). If alkaline treatment is prolonged under severe conditions, depolymerisation and deacetylation may ensue. After deproteinization, solids are separated by filtration from the protein slurry and the protein hydrolysate is easily removed and dried. It can thereafter be used in powder form or as a cake for protein supplement in feed (Prameela et al., 2012).

Alcalase (Maryam et al., 2005, Guerard et al., 2007) and Trypsin (Synowiecki and Al-Khateeb, 2000) are proteolytic enzymes which have been used for the degradation of proteins, however, the residual protein often remains relatively high in the produced chitin and unlike chemical deproteinization, the reaction time is longer and the process is expensive because commercial enzymes are used. These disadvantages of the enzymatic method cause it to be the least favourable for industrial scale lest there are developments made to make the process more economical and proficient (Percot et al., 2003a).

1.1.2.2 Demineralization

Demineralization of crustacean waste is generally achieved with hydrochloric acid (HCl) which converts insoluble calcium carbonate (CaCO_3) in the solid fraction into soluble calcium chloride (CaCl_2) which is consequently removed by washing. If appropriate demineralization and deproteinization is performed, the remaining solid fraction consists mostly of chitin with small amounts of calcium and protein that can be quantified by ashing and a weak Biuret reaction, respectively (Kalut, 2008).

1.1.2.3 Decolourization

The degree to which chitin is associated with pigments varies from species to species among crustaceans. Both alkali and acid treatments will produce a coloured chitin product; however the chitin will still need to be decolourized for commercial acceptability. This decolourization or bleaching step will remove pigments and astaxanthin's to generate cream

coloured chitin flakes (No et al., 1989), however the chemical used during this process, must not alter or change the functional or physicochemical properties of chitin and chitosan. The product of chitin should be insoluble in most acids, organic and alkali solvents.

1.1.2.4 Deacetylation

Solid state chitin remains insoluble in most solvents because it has a compact structure. Chemical deacetylation is therefore performed to produce its most common derivative, chitosan (Roberts, 1992). Deacetylation is the process of removing acetyl groups. It requires strong chemical conditions, usually 50% NaOH, and temperatures as high as 70-90°C. In order to achieve a marketable product, the highest degree of deacetylation is desirable; therefore alkaline treatment may be repeated to reach a sufficient degree for complete deacetylation. Longer reaction times will however produce fragmented molecules, this is known as polydispersion (Dupuis and LeHoux, 2007).

β -chitin contrary to α -chitin can be deacetylated at significantly lower temperatures such as $\pm 80^\circ\text{C}$. This reaction is sufficient for deacetylation as well as for the dissolution of colouration processes, yielding almost colourless chitosan products (Kurita et al., 1993). 30-10% acetylated chitosan (deacetylated by 70-90%) is considered good end product.

1.2 CHITOSAN

Chitosan is a modified, high-molecular weight, natural carbohydrate polymer (Kandasamy, 2005) obtained from partial deacetylation of chitin (Jörg et al., 2011), however, an acute classification regarding the degree of N-deacetylation between chitin and chitosan is not yet characterized (Muzzarelli, 1977, Zikakis, 1984). Chitosan is composed of co-polymers of N-acetyl glucosamine (GlcNAc) and glucosamine (GlcN) connected through α - β (1-4) linkages. GlcN to GlcNAc ratio is referred to as the degree of deacetylation (DDA), a factor of both the origin of chitosan (shrimp, fungi, crab, etc.) and methods of preparation, and can be within the ranges of 30% to 100%. Depending on its DDA, chitosan's physical properties such as crystallinity, degradation, and surface energy will vary (Jayakumar et al., 2008).

In nature chitosan can be found in the green algae *Chlorella*, inside the cell walls of the *Zygomycete* class of fungi, protozoa and yeast and also in the cuticles of insects and

commonly in the exoskeletons of crustaceans (Rudrapatnam and Farooqahmed, 2003). The sources and methods used to chitosan's (Brine and Austin, 1981) will influence their physicochemical properties and functionalities (Rhazi et al., 2004). Therefore it can be expected that both these properties of chitosan derived from shell waste will differ depending on the different extraction protocols employed (Kandasamy, 2005).

Although relatively insoluble in water, chitosan is soluble in dilute HCl at pH below 6 or in organic acids including citric, acetic and lactic acids. Films are easily made by solvent evaporation. Solubilisation is achieved on the D-glucosamine repeating unit, on the C-2 position through the protonation of amine (NH₂) functional group. It is in this position where the polymer is converted into a polyelectrolyte if applied into acidic media (Hengameh and Mehdi, 2009). It is also when the DDA is higher than 50%, that chitosan solubilises in acidic aqueous solutions thus functioning as a cationic polyelectrolyte (Al Sagheer et al., 2009).

Chemical properties of chitosan include: (Pradip et al., 2004).

- Chelates many transitional metal ions.
- Reactive hydroxyl groups available,
- Reactive amino groups,
- Linear polymine

α and β forms of chitin and chitosan are commercialized and have commercial appeal in view of their high nitrogen content (6.89%) unlike synthetic cellulose substitutes (1.25%), making chitin a valuable chelating agent (Muzzarelli, 1976), however, the presence of amine groups in chitosan renders it of greater potential in contrast to chitin and consequently its use in various applications (Hengameh and Mehdi, 2009). Chitosan has been vastly used in various fields, ranging from biotechnological, agricultural, cosmetics, food, and non-food industries (paper, water treatment, and textile), pharmaceutical and biomedical industries (Gupta and Ravi, 2000).

1.2.1 Characterization of chitosan

Chitosan may be characterized on the basis of its intrinsic properties (purity, viscosity, DDA, molar mass), quality and physical forms (Mathur and Narang, 1990). It was reported that chitosan chemical characteristics such as the DDA and molar mass are essential as they affect its performance in many applications (Abdou et al., 2008). The properties and quality of the chitosan product may differ extensively as a result of various aspects involved in the manufacturing processes which impact the final product characteristics (Lee et al., 2003).

Methods of preparing chitosan are different and each result in different DDA, molecular weight, distribution of acetyl groups and viscosity of the end product (Berger et al., 2005). These differences in turn influence the solubility and antimicrobial activity amongst other properties. Commercialized chitosan generally has a molecular weight varying from 50 to 2000 kilodalton (kDa) and a DDA ranging from 70% to 95%, (Rege et al., 2003).

1.2.2 Degree of deacetylation

The DDA over the many characteristics of chitosan, is amongst the most crucial and influential factor determining the functioning of chitosan in the applications for which it is employed (Baxter et al., 1992a). The free amino group content in the polysaccharide are revealed by the DDA (Li et al., 1992a), and can therefore be used to distinguish between chitosan and chitin. The removal of acetyl groups from chitin, are influenced by variables including temperature or concentration of NaOH hence producing a variety of chitosan molecules with varying chemical and physical properties (Baxter et al., 1992a). Since the DDA depends mainly on the reaction conditions and method of purification (Li et al., 1997b), it is important to characterize chitosan by investigating its DDA before utilization. Chitin with a DDA of 70% or higher is referred to as chitosan.

Many methods to determine the DDA have been used, such as infrared spectroscopy (Baxter et al., 1992a), resonance spectroscopy (Hirai et al., 1991), linear potentiometric titration (Ke and Chen, 1990), derivative UV-VIS spectrophotometry (Muzzarelli and Rocchetti, 1985), nuclear magnetic pyrolysis-mass spectrometry (Nieto et al., 1991), and titrimetry (Raymond et al., 1993). Some methods are destructive to the sample or either too costly or tedious for routine analysis (e.g., NMR spectroscopy). Infrared spectroscopy,

however is the most frequently used method due to its simplicity and is a relatively quick technique for qualitatively evaluating the DDA by determining the absorption ratios (Khan et al., 2002).

1.2.2.1 Viscosity

Viscosity as with the DDA is an essential characteristic of chitosan. It is largely reliant on the DDA, concentration of solution, temperature and ionic strength. The chitosan extraction processes employed also affects chitosan viscosity, for instance, Moorjani et al. (1975) reported that increased demineralization time decreased chitosan's viscosity. Bough et al. (1978) found that the viscosity of the final chitosan product decreased when the demineralization step was omitted during chitin preparation. Moorjani et al. (1975) stated that bleaching chitosan with sodium hypochlorite (NaOCl) or acetone (C₃H₆O) during any stage of the isolation process resulted in a significant decrease in viscosity. No et al. (1999) established that chitosan's viscosity is substantially affected by chemical (ozone) and physical (autoclaving, grinding, ultra-sonication, heating) treatments. Increased temperature treatment and reaction times also cause viscosity to decrease.

1.2.2.2 Formation of films

Film forming active biomolecules such as chitosan with a good water barrier, a stable structure, good mechanical properties and a homogeneous matrix (Hengameh and Mehdi, 2009) have proven to be very effective in food preservation (Coma et al., 2002, Myong et al., 2006). According to many reports, the intramolecular and intermolecular hydrogen bonds are responsible for providing this high molecular weight polymer with good film-forming properties (Muzzarelli, 1977). The chitosan film characteristics will differ based on the source of isolation, the solvents utilised, film preparation methods, conditions of drying, and the amounts and types of co-polymers and or plasticizers used (Begin and Van Calsteren, 1999, Cervera et al., 2004, Ritthidej et al., 2002). Conversion of chitin into chitosan has different processing variables which largely influences the properties of chitosan (No et al., 1999) and hence the homogeneity of films produced, such variables include the homogeneity of particle size of initial starting material (shells).

Shelf life of food is extended through the following mechanisms when coated with films (Kester and Fennema, 1986, Labuza and Breene, 1989):

- I. Controlled transfer of moisture between food and enclosing environment;
- II. Controlled delivery of chemical agents like antimicrobial and antioxidant substances;
- III. Decreased rate of metabolism by minimizing oxygen partial pressure inside the packet;
- IV. Controlling rate of respiration, increased impermeability to certain components such as fats and oils;
- V. Temperature regulation, reinforcing coat flavour compounds and food structure

This new tendency in food technology preservation that consists of developing edible and coatings films with antimicrobial properties, seeks to address spoilage of food products by pathogenic bacteria, which has become a serious public concern. The increased consumer demand for quality and longer extended shelf life of foods has also encouraged this research (Pereda et al., 2011).

1.2.2.3 Antimicrobial properties

The precise mechanism of antimicrobial action of chitosan, chitin and their derivatives is yet unknown, but several hypotheses have been proposed (Fereidoon et al., 1999). Some proposed mechanisms in literature include the interaction of microbial DNA with diffused hydrolysis products, thus inhibiting the synthesis of mRNA and protein (Sudarshan et al., 1992) and metal chelating essential nutrients and spore elements (Cuero et al., 1991). The biological properties of chitin and chitosan samples can also be related to the crystallinity, polydispersity and distribution of GlcN and GlcNAc units ahead of the polymeric chain characterized by the sequence of acetylation (*PA*) (Aranaz et al., 2009, Weinhold et al., 2009). Chitosan is usually insoluble in water, however soluble in acid and as a result has greater antimicrobial activity than chitin (Chen et al., 1998a). Chitosan's antimicrobial activity is contingent upon different factors like the kind of chitosan (DDA, viscosity) used; the temperature and the pH of the medium.

Reports in several articles have been made on the antibacterial and antifungal activities of chitinous products (Chen et al., 1998a). Tsai and Su (1999a) reported the ability of chitosan to possess antibacterial effects against *Escherichia coli* by means of cross-linking between anions on the *E. coli* surface and chitosan (as cation).

1.3 APPLICATIONS OF CHITIN AND CHITOSAN

Chitin and chitosan have attracted commercial interest as acceptable renewable resources (Jayakumar et al., 2008). These polymers are a promising new and unique class of physiological materials possessing highly refined functions as a result of their exceptional biocompatibility, thorough biodegradability along with low toxicity and versatile biological activity (Rinaudo, 2006, Arai et al., 1968).

In order to exploit these unique properties and to obtain and realize the complete potential of these multifaceted polysaccharides, attempts are under way to chemically modify them (Jayakumar et al., 2008). Chitosan however, has been the mostly researched derivative of chitin owing to its solubility in dilute aqueous acids rendering chitosan more accessible for chemical reactions and utilization (Eugene and Lee, 2003).

1.3.1 Carotenoids

Carotenoids are a faction of pigments that are soluble in fat which may be found in many animals, plants, algae and microorganisms (Prameela et al., 2012). In crustaceans, carotenoid occurrence is mainly a result of dietary pigment absorption, which is deposited or transferred metabolically to hydroxyl or keto groups (Davies, 1985, Castillo et al., 1982). Natural carotenoids have a potential market use in desserts, soft drinks, ice cream, aquaculture feeds and candies (Delgado-Vargas and Paredes-Lopez, 2003).

Astaxanthin is a carotenoid found in ample amounts within the shells. It has not yet been synthesised and is currently marketed in aquaculture as an additive in fish food especially salmon (Pradip et al., 2004). Astaxanthin and its esters have been reported to be present and recovered from shrimp waste material (Prameela et al., 2012, Shahidi and Synowiecki, 1991). It is formed through oxidative transformation from ingested β -carotene (Latscha, 1990) and is the ketocarotenoid pigment largely responsible for the reddish orange color in

salmon flesh and crustacean (Miki, 1991). This natural ketocarotenoid pigment serves as a powerful biological antioxidant amongst many of its applications (Miki, 1991). Its antioxidant activity is estimated to be ten times higher than that of other carotenoids including canthaxanthin, lutein, β -carotene and zeaxanthin (Naguib, 2000).

Astaxanthin can be used as a cheaper alternative to meet industrial needs in the applications of aquaculture feed formulations or coloration of a number of surimi-based products. Its function in the medical and biomedical industries includes inhibiting prostate cancer, bladder carcinogenesis (Tanaka et al., 1994), and regulating responses of the immune system against tumour cells (Guerin et al., 2003). As a Vitamin A precursor, astaxanthin may have a potential function in human health (Prameela et al., 2012).

Many reports of the extraction of carotenoids have been made (Prameela et al., 2012) but astaxanthin is a principle carotenoid that offers lucrative economic prospects and has thus drawn considerable attention (Jianing and Subramaniam, 2011).

1.3.2 Agriculture

The growing concerns on plant parasitic root-knot nematodes stretches across most crop production regions; tropical and sub-tropical throughout the world (Sikora and Fernandez, 2005). The employment of chemical nematicides, although a simple and effective approach has raised concerns for both human and environmental health (Rich et al., 2004). Therefore, in view of this, the application of organic soil enhancements has been seen as a possible environmentally friendly alternative (Oka, 2010, Tabarant et al., 2011). Products that use organic composites from shell waste are vast therefore chitin and its derivative chitosan, possess immense economic value due to their wide range of biological activities and agrochemical operations (Radwan et al., 2012). Reports of both natural compounds being active against nematodes and other pests upon application to soil or foliage have been made (El Hadrami et al., 2010) and were proven to play a role in the defence mechanisms against microbial invasion of plants (Day et al., 2001).

The four main approaches that have been identified for chitin application in agriculture are (Hirano, 1997):

- I. Protection of plants from pests and diseases before and after harvest;
- II. Enhancing of antagonist microorganisms action and biological controls;
- III. Enhancing the beneficial symbiotic plant-microorganism interactions;
- IV. Regulating plant growth and development;

Chemical agents are associated with impending problems. These include increased number of post-harvest pathogens that have gained fungicide tolerance, public resistance developed against fungicide-treated produce (El Ghaouth et al., 1992), therefore using bioactive substances like chitosan has received a lot of attention because of its ability to control post-harvest fungal disease.

1.4 CHITIN AND CHITOSAN OLIGOSACCHARIDES

Chitooligosaccharides are homo- or heterooligomers of GlcNAc and/or GlcN. By definition, oligosaccharides are polymers of monosaccharides with degrees of polymerization (DP) varying from 2 and 10 (3 and 10 according to nomenclature of the IUB-IUPAC), but DPs up to 20-25 are often associated with them (Barreteau et al., 2006). These oligosaccharides can be produced by either chemical or enzymatic depolymerisation of chitosan or chitin (Einbu, 2007). Chitin and chitosan chemical modification have received considerable attention since these modifications maintain the principle skeleton of both chitosan and chitin and the original biochemical and physicochemical properties are maintained (Jayakumar et al., 2008). Chitin and chitosan chemical modifications would yield new functions in accordance to the nature of the group introduced (Sashiwa, 2006). The primary amino group is responsible for the possibility of salt formation with acids and relatively easy chitosan chemical modification (Roberts, 1992) (Fig.1.4).

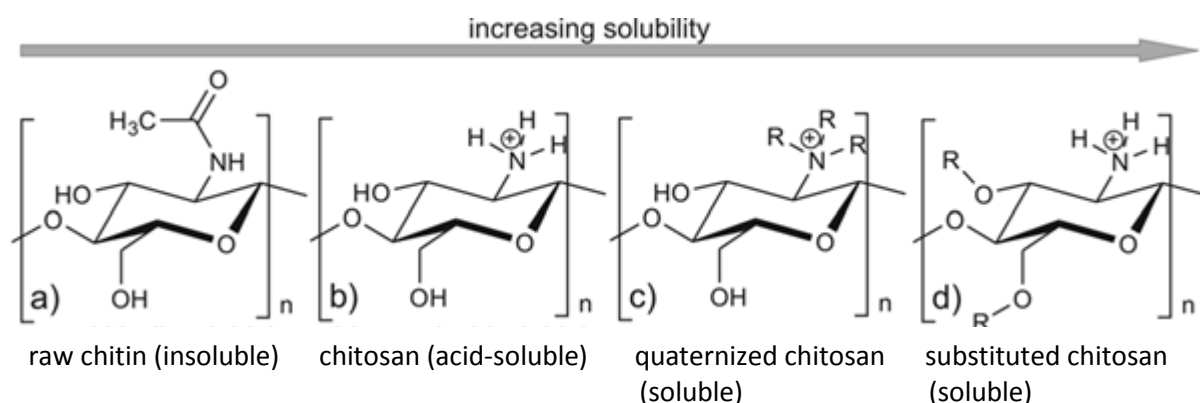


Fig. 1.4 Diagrammatic illustration of chitin and chitosan derivatives. Solubility of nearly insoluble chitin biopolymer can be improved through the chemical structure functionalization. Quaternized and substituted derivatives unlike chitosan, display greater solubility under alkaline conditions (Roberts, 1992).

Chitooligosaccharides have attracted noticeable attention owing to their biological activities (Kandasamy, 2005). Chitosan and its oligomers have been proven to suppress the growth of many bacteria and fungi, especially pathogens. The correlation between the inhibition effect and chitosan DP has revealed that strong physiological activities are displaced by oligomers with six or more residues. Those with DP ranging from 2 to 8 along with low-molecular weight chitosan exhibit growth inhibitory effects stronger than those of high-molecular weight chitosan when tested against different pathogens, including *Alternaria alternata*, *Phomopsis fukushi*, *Fusarium oxysporum*, (Felt et al., 2000). In plants, chitooligosaccharides are believed to evoke the generation of β -1,3-glucanase and chitinase enzymes which degrade fungal cell wall.

Novel classes of chitosan derivatives with greater antimicrobial action have been synthesized (Zhong et al., 2008). The antimicrobial activities of benzoyl, acetyl, thiourea and chloroacetyl derivatives of chitosan were studied against four bacterial species and the results showed that the acyl and thiourea derivatives have better antimicrobial activities than that of the native chitosan. The mechanism of antimicrobial and antifungal action of the derivatives of chitin and chitin itself is yet to be completely understood (Feisal and Montarop, 2010).

Since there isn't a single kind of chitosan or chitosan derivative that can exert all manner of bioactivities, studies on their biological activities have therefore increased (Feisal and Montarop, 2010).

1.4.1 Glucosamine and *N*-acetyl glucosamine

Amino sugars; 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetoamido-2-deoxy-D-glucose (GlcNAc) are the building blocks from which chitin and chitosan are produced (Einbu, 2007). Both are naturally occurring in diverse tissue cells and molecules. GlcN is an amino derivative of glucose and is the important constituent of many natural polysaccharides that can form structural materials for cells analogous to structural proteins. It is manufactured as a nutraceutical product for the treatment of the most regular type of arthritis, called osteoarthritis that can affect the hips, shoulders, hands, knees, and joints in humans and animals (Anderson et al., 2005). It is predominately obtained by acid hydrolysis of chitin. Alternatively, it can be produced by acid hydrolysis of variously acetylated chitosan. GlcN has also been obtained by hydrolysing GlcNAc to GlcN by reacting GlcNAc with an acid (HCl).

GlcNAc is polymerised generally through linearly β -(1,4) linkages. Its molecular weight and molecular formula is 221.21 and $C_8H_{15}NO_6$, respectively. Generally, it is a slightly sweet white powder, with a melting temperature of 221°C. Its solubility in water is 25% and colourless and clear in 1% aqueous solutions (Ashry and Aly, 2007). GlcNAc serves as a structural component and a constituent of homogeneous polysaccharides such as chitin and heterogeneous polysaccharides, like hyaluronic acid (also known as hyaluronate or hyaluronan) (Ashry and Aly, 2007) and murein (Gooday, 1990), respectively. Several clinical trials have already been carried out to help patients suffering from joint ailments, such as osteoarthritis, arthritic disorders, joint injury and cartilage damage. Delivery of GlcNAc containing preparations can be oral, trans mucosal, parenteral and topical administration. Inhibition of joint damage was proven to increase significantly through the delivery of GlcNAc (Rovati et al., 1972, Marcum and Seanor, 2007). GlcNAc moreover, hinders elastase activity and releases superoxide from an individual's polymorph nuclear leukocytes (Kamel et al., 1991, Kamel and Alnahdi, 1992) and through topical administration, it is used to improve and enhance skin quality.

GlcNAc is a minor commercial by-product of chitin obtained by enzymatic hydrolysis (Haynes *et al.*, 1999) and chemical acetylation of GlcN when treated with acetic anhydride (He *et al.*, 2001), and the latter has a greater market price and is approved as a food additive in Japan (Mustaparta, 2006). The sales of GlcN is rapidly increasing mainly for the treatment of osteoarthritis and is the current chief driving force in the chitin-derived product market (Sandford, 2002). GlcN is an accepted dietary supplement with inconsiderable side-effects and in the US health food market; it has become a major commercial product (Hungerford & Jones, 2003). Presently, China manufactures approximately 90% of all available GlcN (Sandford, 2002) hence it could potentially be economically beneficial if South Africa ventured into producing its own.

1.5 ECONOMIC ASPECTS

Present day people being well informed and better educated, prefer to have natural/organic products, whether a drug or food, and are willing to pay a premium price for anything safe and natural. Natural biopolymers therefore possess distinct advantages, such as availability from marine food or renewable agricultural resources, biocompatibility and biodegradability. This leads to environmental safety and the possible preparation of diverse chemically modified derivatives for special end uses. Polysaccharides, being a group of natural macromolecules, tend towards being intensely bioactive and are commonly obtained from crustacean shell wastes or agricultural feedstock (Ramesh and Tharanathan, 2003). Chitin and chitosan are produced from the former while cellulose, pectin, starch, etc. are the biopolymers obtained from the latter. Chitin is the most available biopolymer reaching over 10 gigatons annually (Harish Prashanth and Tharanathan, 2007). Of the produced chitin approximately 75% is used to manufacture nutraceutical products, however the increasing sales of GlcN as a dietary supplement are the main and current driving force in the market (Sandford, 2002). An estimated 65% of the produced chitin is converted into GlcN; $\pm 9\%$ is used for the production oligosaccharides, $\pm 25\%$ is converted into chitosan, and $\pm 1\%$ is used for the production of GlcNAc (Mustaparta, 2006). The 2006 universal estimates of chitin-based product production and market prices are shown in Table 1.1 (Mustaparta, 2006).

Table 1.1 Global production of chitin-based products with respect to chitin utilized during production and their estimated market prices (Mustaparta, 2006).

Product	Annual production (tons)	Chitin consumption (tons)	Market price (USD/kg)
Glucosamine	4500	9000	7-35
Chitosan	3000	4000	10-100 ^a
Oligosaccharides	500	1000	50-100 ^b
<i>N</i> -acetylglucosamine	100	200	20-140 ^c

^aUltra pure/ Quality System-Good Manufacturing Practice product prices as higher than 50.000 USD.kg⁻¹.

^bUltra pure and accurately characterised product prices may be as higher than 10.000 USD.g⁻¹.

^cChemically produced: 20 USD.kg⁻¹ and enzymatically produced: 100-140 USD.kg⁻¹.

The approximated market price variations in Table 1.1 are subject to change depending on the quality of the product and its availability on the market. Presently, the cuticles of various crustaceans, mainly shrimp and crab are the primary industrial sources for the production of chitin (Kim and Rajapakse, 2005) and the estimated market price for average quality chitin is 5-8 USD (Mustaparta, 2006).

1.5.1 Scope of study

Marine shrimp waste accumulates from processing plants as a result of inefficient utilization (Benhabiles et al., 2013), therefore it is imperative to establish and implement an effective and inexpensive method for waste utilization. Shrimp shells are commercially valuable because they are rich in chitin, pigments, protein, lipids and flavour compounds. Through complete utilization of these bioactive compounds present in the shell waste, the economics of crustacean industrial processing can be improved (Heu et al., 2003, Shahidi and Synowiecki, 1991).

The challenge however is in converting these industrial discards into profitable and marketable by-products and alternate specialty materials for food studies and developments assimilated with various functions of chitin-based polymers. In this regard

these biopolymers offer a diverse array of distinctive applications which include production of high quality food products through bioconversion (Shahidi and Synowiecki, 1991, Johnson and Peniston, 1982). This process plays an economical role in both shellfish processing, and in integrated aquaculture systems (Revah-Moiseev and Carroad, 1981). Indirectly, it will serve as a method of waste management by reducing from processing plants, large masses of crustacean waste.

Therefore, this current study focuses on utilizing shrimp waste to recover bioactive compounds and thus their applications in the production of valuable products.

1.5.2 Objectives

- I. To optimize chitin and chitosan extraction from shrimp shell waste by use of chemical methods and the hydrolysis of respective monomers
- II. To isolate chitin and chitosan derivatives and screen for antimicrobial activity
- III. To recover calcium carbonate and the carotenoid, astaxanthin during the isolation of chitin
- IV. To develop chitosan bio-plastic films

2. MATERIALS AND METHODS

Within the crustacean shell, chitin has strong associations with different organic and inorganic components, which must be quantitatively removed to achieve the highly purified chitin required for biological applications (Percot et al., 2003). Protein and chitin merge, producing a protein-chitin matrix in the skeletal tissue, which is calcified extensively, yielding hard shells. Crustacean waste can also consist of muscle residue lipids and carotenoids, generally astaxanthin and its derivatives (Kjartansson et al., 2006). In general, chitin is prepared from various crustacean by-products involving mainly the removal of proteins and minerals with 2-5 % bases or acids at elevated temperatures (Synowiecki and Al-Khateeb, 2003; Percot et al., 2003; Kurita, 2006). The primary inorganic constituent of shrimp shells is calcium carbonate and only dilute HCl is used to decalcify the shells and it also prevents chitin hydrolysis (No and Meyers, 1995). Percot et al. (2003b) expressed that using HCl during chitin demineralization results in adverse effects on the DDA that negatively affects the intrinsic properties of the purified chitin. The authors expanded therefore on the significance of optimizing the isolation process parameters (solids to acid ratio, time, temperature and pH) in an effort to minimize degradation of chitin and reduce impurity levels to satisfactory levels for specific applications. Consequently, a less harmful inexpensive demineralization process is required (Kalut, 2008). Likewise, the effectiveness of the alkali deproteinization step largely depends on the process temperature, solid to solvent ratio and alkali concentration. Optimization is therefore imperative.

Commercialization of the production of chitin has been reported for decades, yet little information regarding the optimization of the isolation process is available in the literature, and there also is no standard method for the extraction of chitin from crustacean by-products (Chang and Tsai, 1997; Percot et al., 2003a).

Hence a fast, cost effective industrial process that is easily controlled is required for isolating chitins with high purity and dependable qualities for unique applications especially in food and biomedicine. This phase of the investigation focused on optimising demineralization

and deproteinization steps for the production of high quality chitin and chitosan and thus the recovery of astaxanthin, protein and calcium.

2.1 OPTIMIZATION OF CHITIN AND CHITOSAN PRODUCTION

2.1.1 Shrimp shell collection

Fresh pink shrimp shell waste comprising the cephalothoraxes (total body weight about 10-12 g) as raw material were provided by Umgeni Fish Market in Durban, KwaZulu-Natal and used in this study. All shells were from a single species of *Haliporoides triarthrus*. Shells were transported to the laboratory in plastic bags and immediately stored at -20°C, until further use.

2.1.2 Raw material preparation

Shrimp shells were thawed and washed with tap water to remove contaminants, adherent proteins, soluble organics and any remaining muscle particles before drying in an oven at 80°C overnight. Dried processing waste was mixed with distilled water (1:1) and then boiled for an hour to detach any tissue. Shells were then dried at 80°C in an oven overnight to break down the crystalline skeleton structure of chitin, thus making them more brittle (Mukherjee, 2002). The dried shells were then homogenized using an electric blender. Chitin and chitosan were prepared by the modified method of Benhabiles et al., (2012).

2.1.3 Demineralization

The shells were treated with HCl (Sigma-Aldrich) at concentrations ranging from 1 M to 6 M. The solid to acid solvent ratio was between 1:10 and 1:60 (w/v). Both optimization steps were conducted at ambient temperature with magnetic stirring at 150 rpm, until no gas bubbles were produced. As a check, 10 ml of hydrochloric acid (7%) was added. The shells were considered decalcified when no further generation of gas occurred. The slurry was then filtered through a sieve (250 µm) and washed with 10 volumes tap water until neutrality. Neutrality was confirmed by assessing a sample of the filtrate with a pH meter. The slurry was then rinsed with deionised water as a final rinse and finally dried in an oven at 80°C overnight. The liquid filtrate obtained following hydrolysis was stored in polyethylene flasks at -20°C until further use for the recovery of CaCO₃. The amount of

calcium removed from the solid residue was analysed by means of an atomic absorption spectrophotometer (Shidmazu 9000, South Africa) following the method of Onwuliri and Anekwe (1992). The extent of demineralization was assessed by calculating the ash content in the solid residue. Degree of demineralization (DDM) was expressed as a percentage using the following equation by Rao et al. (2000):

$$\%DDM = \frac{[(AO \times O) - (AR \times R)]}{AO \times O} \times 100$$

where AO and AR are percentage ash contents before and after demineralization, respectively. O and R denote the mass (g) of initial and final demineralised material respectively, as dry weight.

2.1.4 Deproteinization

The demineralised shells (from section 2.1.3) were treated with NaOH (Merck) at concentrations ranging from 1 M to 6 M using the ratio 1:10. The temperature range tested was between 30 to 65°C and the reaction time was varied from 15 to 150 min. The solids were filtered, washed and dried in a similar manner as the demineralization step (Section 2.1.3). The extract supernatant (3-5 ml) was used to determine protein content (N x 6.25) according to the procedure of Kjeldahl (AOAC., 1998).

2.1.5 Decolourization

The extracted chitin was mixed with acetone (Sigma) using a 1:10 (w/v) ratio for 10 min, filtered and then dried for 2 h at room temperature. After 2 h of drying, the residue was bleached with 0.315% NaOCl (Radchem) using the same ratio for 5 min (No and Meyers, 1995). The decolourized chitin was filtered and washed as mentioned in Section 2.1.3.

2.1.6 Deacetylation

Chitin was converted into chitosan using the deacetylation procedure described by Kurita (2001). The hydrolysis parameters including concentration of NaOH, reaction time, and temperature were as follows: a 1 g suspension of chitin was mixed with 50 ml of aqueous NaOH (50% by weight) at 90-100°C with continuous stirring for 3-5 h. The filtrate was

separated by filtration, washed with 80% (v/v) ethanol (Sigma) and water until it reached neutrality. It was then was dried in an oven at 80°C overnight.

2.2 PHYSICOCHEMICAL CHARACTERIZATION OF SHELLS, CHITIN AND CHITOSAN

2.2.1 Proximate composition analysis

All proximate composition analyses were performed according to the AOAC (1998) methods.

2.2.1.1 Moisture content

Empty porcelain crucibles were kept in an oven for 3 h at $105 \pm 1^{\circ}\text{C}$, transferred into a desiccator to cool and weighed. Samples of shells, chitin and chitosan (3 g) were uniformly placed onto the pre-weighed crucibles and placed into the oven for 3 h at $105 \pm 1^{\circ}\text{C}$. Following drying, the crucibles were transferred into a desiccator to cool and re-weighed. All moisture analyses were performed in triplicate.

2.2.1.2 Ash content

Porcelain crucibles were washed, dried and placed overnight in a furnace at 550°C to remove all impurities. Subsequently, the crucibles were placed in a desiccator to cool for 30 min and weighed. Thereafter, 3-5 g of powdered shells, chitin and chitosan samples were precisely weighed into the crucibles and placed in a muffle furnace (Labcon) at a temperature of $550 \pm 1^{\circ}\text{C}$ until a grey ash was apparent. Crucibles were cooled in a desiccator and re-weighed. Percentage of ash was estimated as ratio of the weight of the obtained ash after heating compared to the mass of the original samples. All ash analyses were performed in triplicate.

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

2.2.1.3 Protein content

Ground shells (3-5 g), chitin and chitosan were weighed and transferred into digestion tubes (Labotech). The samples were digested for 45 min with concentrated sulphuric acid (25 ml) (Radchem) and 4 g of Kjeldahl catalyst (9 part of potassium sulphate (Sigma) mixed with 1 part copper sulphate (Sigma) until a clear solution was obtained. 40 ml of distilled water was added to dilute the digested samples. Following this, 150 ml of 25% (w/v) NaOH solution was also added. The samples were steam distilled, releasing nitrogen in the form of NH₃ into a receiving flask containing 25 ml of 2% (w/v) boric acid (Radchem) solution, to which 5 drops of filtered methyl red were added. Steam distillation proceeded for 4 min and the solution in the receiving flask was titrated with 0.1 N H₂SO₄. The crude percentage protein of the specimens was enumerated from the estimated nitrogen content (after titration) using 6.25 as a conversion factor (AOAC, 1998). All protein analyses were performed in triplicate.

$$\text{Percentage } N = \frac{(V_{\text{sample}} - V_{\text{blank}} \times N \times 14.0067 \times 100)}{W}$$

V_{sample} = volume (ml) used in sample titration

V_{blank} = volume (ml) used in in blank titration

N = normality of H₂SO₄

W = weight (mg) of the sample

Extent of deproteinization was enumerated using the formula:

$$\% \text{ Proteinization} = \frac{\text{Protein in supernatant}}{\text{Protein in waste}} \times 100$$

2.2.1.4 Lipid content

Lipid content was assessed using the method of Bligh and Dyer (1959). Ground shells (3 g), chitin and chitosan were treated with 25 ml of methanol and 25 ml of chloroform over a period of 3 min and then homogenized using a Polytron homogenizer (Janke and Kunkel). Distilled water (25 ml) was added to the mixture and the solution was filtered using a Büchner funnel. The solution was left in a separatory funnel to separate overnight, after

which aliquots of 10 ml of the filtrate was removed and introduced into round bottom flasks which were pre-weighed. The solvent was evaporated using a rotary evaporator (Heidolph). The flask was dried at 80°C for 1 h in an air-forced oven. After drying, the flask containing the lipids was placed in a desiccator to cool and then weighed. The total lipid content was calculated gravimetrically, as follows:

$$\% \text{ Lipid} = \frac{\text{Weight of lipid}}{\text{Weight of sample}} \times 100$$

2.2.2 Mineral content

The minerals analysed included calcium, magnesium, sodium, phosphorus, iron, potassium and zinc. Analyses were conducted following the method of Onwuliri and Anekwe (1992) by means of an atomic absorption spectrophotometer (Shidmazu 9000). Dihydrogen phosphate (Sigma) was employed as the standard test solution to colorimetrically estimate phosphorus content in samples (AOAC., 1998).

2.2.3 Viscosity

The intrinsic viscosity of chitosan (1%) was determined viscometrically in 1% acetic acid (Sigma) with the addition of 0.3 M NaCl (Merck). The measurement was conducted at 25 ± 0.1°C. The chitosan solution was charged into the viscometer (Labotech), set at 100 rpm and the cP (centipoise) value was recorded.

2.2.4 FTIR analysis

The spectra of shells, chitin and chitosan specimens were recorded on a Fourier transform infrared spectroscopy instrument (Varian 800 FT-IR) at room temperature. The frequency range was set from 4000 to 400 cm⁻¹. The KBr disk technique involved mixing 0.8 mg of each sample with 300 mg of pure grade KBr (Merck) powder. The mix was compacted into thin pellets of 13 mm diameter. The degree of deacetylation was calculated according to method of Sabnis and Block (1997). The DDA was enumerated using the following equation:

$$\text{DDA} = 100 - \left[\frac{A_{1655}}{A_{3450}} \times \frac{100}{1.33} \right]$$

A_{1655} = amide-I band absorbance of 1655 cm^{-1} quantifying the content of N-acetyl group

A_{3450} = absorbance of the hydroxyl band at 3450 cm^{-1}

1.33 = signifies the value of A_{1655}/A_{3450} ratio for fully deacetylated chitosan

where A_{1655} is the amide-I band absorbance of 1655 cm^{-1} quantifying the content of N-acetyl group; A_{3450} , the absorbance of the hydroxyl band at 3450 cm^{-1} and 1.33 the value of A_{1655}/A_{3450} ratio for fully deacetylated chitosan. It was presumed that for fully deacetylated chitosan, the value of the ratio was zero and that between the amide-I absorbance band and the N-acetyl group content (Domszy and Roberts, 2003), was a rectilinear relationship.

2.3 HYDROLYSIS OF OLIGOSACCHARIDES FROM CHITIN

2.3.1 Preparation of N-acetyl-chitooligosaccharides (NACCOS)

NACCOS was prepared by acid hydrolysis of chitin. The chitin material (1 g) was hydrolysed with concentrated HCl (25 ml) (60°C ; 2 h), during which samples were collected hourly. The resultant brownish-black material was dissolved in distilled water and decolourized for 1 h with activated charcoal (1 g) and then centrifuged (10 min; $16\,000\times g$) to remove coloured material. 1.5 volumes of ethanol was added to the solution and kept at 4°C for 24 h to increase precipitation of oligomers. The precipitated oligomers were concentrated in a rotary evaporator. The resultant powder/crystals was suspended in ethanol and kept at 4°C for 24 h to further enhance the yield of precipitation. Resultant precipitated oligomers were concentrated, rinsed with ice cold alcohol and dried at 50°C in hot air before measuring the weight (Sibi et al., 2013).

2.3.2 TLC analysis of COS fractions

A series of spots (1 μl) consisted of collected oligomer mixed fraction along with standards were applied on a TLC silica gel plate (5.0 cm x 6.0 cm). The plate was eluted in mobile phase comprising of n-butanol (Associated Chemical Enterprises): methanol: 30% ammonium solution (Radchem): H_2O (5:4:2:1) (v/v). For visualisation of spots, the plate was sprayed with aniline-diphenylamine reagent and heated at 121°C for 5-10 min (Tanaka et al., 1994).

2.3.3 HPLC analysis of NACCOS

(GlcNAc)₂ to (GlcNAc)₆ was procured from Sigma-Adrich. The hydrolysis products (from section 2.2.1) were analysed by HPLC using a Shimadzu LC-20AB HPLC system equipped with Aminex HPX-87H column (Bio-Rad). 10 µl of filtered samples were injected into the HPLC system. 5 mM H₂SO₄ was prepared as mobile phase. The flow rate was fixed at 0.6 ml.min⁻¹ and the column temperature was held at 40°C. The oligosaccharide products were detected by ultraviolet absorption at 210 nm. The standards GlcNAc and (GlcNAc)₂ to (GlcNAc)₆ were prepared at concentrations of 100 ppm to 500 ppm and 100 ppm to 700 ppm respectively. Samples were then filtered into vials using a 0.25 µm syringe filter (Membrane Solutions) for HPLC analysis. Based on the retention time and peak areas acquired from HPLC profiles, standard calibration curves were constructed for each oligosaccharide. Hydrolysis products were identified according to the retention time and quantified from a linear curve generated from each standard amino sugar.

2.4 HYDROLYSIS OF OLIGOSACCHARIDES FROM CHITOSAN

2.4.1 Preparation of chitosan oligosaccharides (COS)

COS was produced by acid hydrolysis of chitosan. This partial hydrolysis reaction proceeded for 1-6 h at 80°C with 1 g suspension of chitosan mixed with 25 ml of HCl (35%). Hydrolysate samples were collected at hourly intervals and diluted with 3 volumes distilled water, followed by centrifugation (10 min; 16 000× *g*) to remove any residual chitosan polymer. To the diluted hydrolysate free of residual polymer, 1 g of activated charcoal was added and mixed for 3 h and then centrifuged (10 min; 16 000× *g*) to remove coloured material. The supernatant was kept at -20°C for 24-48h to generate and precipitate chitosan oligomers. If needed, 0.25-1.5 volumes of methanol were mixed to the previously diluted hydrolysate to increase the yield of precipitate. Precipitated oligomers were concentrated on a rotary evaporator and washed with ice cold ethanol. The powdered products were then dried for 3 h at 70°C and weight (Lee et al., 1999).

2.4.2 TLC analysis of COS fractions

TLC analysis of COS fractions was performed as described in section 2.3.2.

2.5 RECOVERY OF CALCIUM CARBONATE

2.5.1 Synthesis of CaCO₃ polymorphs

Synthesis was achieved by the double injection method (Chen and Xiang, 2009): 100 ml of solution obtained from section 2.1.3 and 100 ml of 1 mol.L⁻¹ NH₄HCO₃ (Sigma) solution was injected simultaneously into distilled water (90 ml). The solution was mixed at a stirring rate of 450 rpm at 30°C. The precipitate product was filtered using filter paper, washed with distilled water and dried for 12 h at 105°C.

2.5.2 FTIR analysis

FTIR analysis was conducted as described in Section 2.2.4.

2.5.3 Mineral analysis

Mineral analysis for calcium content was conducted as described in Section 2.2.2.

2.6 ISOLATION OF ASTAXANTHIN

2.6.1 Astaxanthin extraction

Acid and alkaline washes obtained during chitin purification were mixed and centrifuged (Eppendorf) (10 min; 10 000× *g*) at room temperature and the precipitate was dried at 70°C and stored until further use. To extract carotenoids, 1500 ml of 90% (v/v) ethanol was mixed to the precipitate in three 500 ml portions. The pigmented extract was concentrated (100 rpm, 40°C) in a rotary evaporator and re-dissolved with hexane (5 ml) and the resultant solution was centrifuged (3000× *g*; 5 min) (Mezzomo et. al., 2011).

2.6.2 Identification of compounds by thin-layer chromatography

Analysis of components in the shrimp shell extract was done using thin layer chromatography (TLC) based on the method of Kobayashi and Sakamoto, (1999). A small volume of the extract was spotted on silicagel G plates and developed with acetone: hexane 3:7 (v/v) as mobile phase. The separated bands were identified using standard astaxanthin (Sigma) and internationally accepted R_f values for astaxanthin monoester and astaxanthin diester (Renstrom and Liaaen-Jensen, 1981).

2.6.3 HPLC analysis

HPLC separations were performed according to the methods of Kuhn et al. (2009). Chromatographic analysis was carried out using a Shimadzu LC-20AB HPLC system equipped with a thermostated (40°C) C18 reverse-phase column (Phenomenex, 100 X 1.6 mm). A UV-Visible detector (λ -460 nm) was used. The mobile phase comprised of a mixture of acetonitrile (Merck): methanol (90:10) (v/v) and the flow rate was fixed at 0.8 ml.min⁻¹. Calibration curves were constructed from the retention time of five concentrations (100 ppm-500 ppm) of standard astaxanthin (Sigma). Carotenoid concentration were thus calculated directly from the peak areas. The HPLC conditions for both the standard and extracts analysis were the same.

2.7 ANTIMICROBIAL ACTIVITY OF CHITOSAN, CHITO-OLIGOSACCHARIDES AND NACOS-OLIGOSACCHARIDES

2.7.1 Strain and culture conditions

Antimicrobial activity was tested against ten strains of bacteria including five Gram-positive bacteria (*Micrococcus luteus* ATCC4698, *Bacillus subtilis* ATCC17728, *Staphylococcus epidermidis* ATCC12228, *Staphylococcus aureus* ATCC22061 and *Bacillus cereus* ATCC9634) and five Gram-negative bacteria (*Serratia marcescens* ATCC14756, *Salmonella typhimurium* ATCC700720D, *Enterobacter aerogenes* ATCC13048, *Escherichia coli* ATCC8739 and *Klebsiella pneumoniae* ATCC13883). All cultures were obtained from the Department of Biotechnology and Food Technology, Durban University of Technology. These strains were maintained on Luria Bertani (LB) agar plates, containing 5 g.L⁻¹ yeast extract (Fluka); 10 g.L⁻¹ tryptone (Oxoid); 10 g.L⁻¹ sodium chloride; 17 g.L⁻¹ bacteriological agar (Pronadisa), and stored at 4°C. For the antimicrobial tests, the organisms were sub-cultured onto LB agar plates and incubated at 37°C for \pm 24 h.

2.7.2 Preparation of chitosan and COS solutions

A solution of chitosan was prepared by suspending chitosan (1 g.100 ml⁻¹) into 1% acetic acid solution and sterilised (121°C, 15 min). Solutions of COS and NACOS were made up by dissolving 1% (w/v) of each oligomer in deionized water and filter sterilised using a 0.45 μ m filter. In both cases, the pH was adjusted to 5.8 with 1 M NaOH (the most suitable to solubilize chitosan without having any antibacterial effect) (Fernandes et al., 2008).

2.7.3 Antibacterial tests

Two colonies of each strain was sub-cultured from a freshly prepared plate culture and inoculated into 20 ml of Muller Hinton Broth (MHB) in 50 ml sterile centrifuge tubes (Lasec) and incubated in an orbital shaker (Infors HT Multitron) (37°C; 180 rpm). After approximately 24 h of growth, optical density was adjusted to meet a McFarland standard of 0.5 (1×10^8 cfu.ml⁻¹). Conical flasks (150 ml) containing MHB were inoculated with a 1% inoculum at a final volume of 100 ml. 1 ml of sterile chitosan, COS and NAcCOS was added into the respective bacterial suspensions in MHB and incubated with shaking at 37°C and 180 rpm. As a negative control, flasks were inoculated with 1% acetic acid solutions and as a positive control; the organism was grown only in MHB media. Samples were taken at 1 h intervals and the turbidity was measured. Growth curves were constructed from the optical density (600 nm) versus the growth period for each test organism. Inhibitory effects of chitosan, COS and NAcCOS as a result of their antibacterial activities were indicated by altered growth curves. All experiments were performed in triplicate.

2.8 DEVELOPMENT OF CHITOSAN EDIBLE FILMS

2.8.1 Preparation of films

Chitosan solutions (1 g.100 ml⁻¹) were prepared by dissolving chitosan powder in 1% solution of acetic acid with constant stirring at 23°C (Xu et al., 2005). To ensure dispersion of chitosan, the solution was kept overnight under constant agitation at room temperature and centrifuged thereafter to remove impurities. Glycerol (Merck), polyethylene glycol (PEG) 200 (Merck) and PEG 600 (Sigma) were added and tested as plasticizers to achieve flexible coatings that would be easily manipulated and folded without breakage. Each plasticizer was added to obtain a plasticizer/chitosan weight ratio of 0.04 (Alvarez et al., 2013). The emulsions were then left overnight at 4°C to eliminate bubbles, after which they were poured into petri dishes and placed in an oven (35°C, 24 h) for drying. The dried films were then peeled off and arranged in a desiccator before further testing.

2.8.2 Characterization of the films

2.8.2.1 Thickness

Film thickness (mm) was measured at four random locations of each film using a digital micrometer (Mitutoyo) (Abugoch et al., 2011).

2.8.2.2 Optical properties of films

Colorimetric properties of films were determined using a ColorFlex EZ (HunterLab) with an 8 mm diameter measuring area. Colour parameters calculated were: “L”- lightness, “a”- red-green and “b”- yellow-blue). Snap shots were taken in triplicate and values were taken directly from a digital print. The readings were averaged, computed and reported (Pereda et al., 2008).

2.8.2.3 Water solubility

The water solubility of the films was determined using the method of Gontard et al. (1992). Each film was cut (1 x 4 cm²), weighed and dried at 105°C for 24 h in an air-circulating oven. Films were recovered after drying, re-weighed and their initial dry weight (M_i) was determined. Thereafter, the films were placed in distilled water (30 ml) with occasional shaking (100 rpm) at 22-25°C for 24 h. Afterwards the films were filtered through pre-weighed filter paper (Whatman 1). Then the unsolubilised fraction together with the filter paper was dried in an air-circulating oven (105°C, 24 h) and weighed (M_f). Using the below equation the solubility of each film was determined:

$$FS(\%) = \frac{M_i - M_f}{M_i} \times 100$$

M_i =initial weight of the sample

M_f = final weight of the sample

2.8.2.4 Film microstructure

Film microstructure characterization was evaluated by using a Scanning Electron Microscope (Ultra Plus FE-SEM). The films were mounted into a cylindrical die of 10 mm diameter using double sided tape. The films were rendered electrically conductive by coating them with gold for 3 min. The captured images were recorded on photographic film (Abugoch et al., 2011).

2.8.2.5 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to identify the chemical structure of the composite films and possible interactions between their components. The film spectra was recorded on an FTIR instrument (Varian 800 FT-IR) at room temperature, using a spectral range of 4000 to 400 cm^{-1} . The film samples were mounted on the IR specimen holder and analysed (Abugoch et al., 2011).

2.8.2.6 Thermogravimetric analysis (TGA)

TGA was analysed to determine the degradation of pure chitosan films compared to the series of composite films at different heating rates. It was carried out on the films using on a SDT Q600 V20.9 Build 20 controller over temperature range of 25-590°C and 10°C/min as heating rate. Approximately 5 and 10 mg weights of sample were tested. During analysis, nitrogen gas was passed over the samples placed in open crucibles. The percentage weight loss was assessed using the associated software throughout the heating cycle (Abugoch et al., 2011).

2.8.2.7 X-ray diffraction

X-ray diffraction analysis was used to measure the percentage of crystallinity of chitosan films. The diffraction patterns were obtained using the method of Abugoch et al., (2001), with slight modifications. Samples were loaded into XRD sample holders and then run in the PANalytical Empyrean XRD Diffractometer with an X'Celerator detector and Co $\text{K}\alpha$ radiation. Samples were scanned at a theta range of 4 to 60 (2θ)° and at a scanning speed of 0.06°/min, the crystal structure was then analysed from the obtained XRD sequences.

2.9 STATISTICAL ANALYSIS

Variance analysis (ANOVA) was carried out to obtain differences amongst the means (SAS, 1988).

3. RESULTS AND DISCUSSION

3.1 OPTIMIZATION OF CHITIN AND CHITOSAN PRODUCTION

Chitin was produced from a species of shrimp called *Haliporoides triarthrus* through successive chemical treatments. A suitable extraction method was developed and optimized to recover a white chitin product equivalent to commercial chitins. Demineralization consisted of mineral removal, mainly calcium in the form of calcium carbonate which was converted into calcium salts soluble in water, releasing carbon dioxide in the process. The remaining minerals in the shellfish cuticle were easily separated by filtration of the chitin solid phase followed by washing using tap water and deionized water for the final rinse. Various concentrations of HCl (1-6 M) were tested at room temperature with continued shaking. Demineralization was accomplished with 2 N HCl at room temperature with a demineralization extent of 98% (Fig. 3.1). A maximum of 184.43 g.kg⁻¹ Ca was removed at this point of extraction. Increasing acid concentration (4-6 M) yielded no significant increase in the conversion or amount of calcium removed. A broad range of shells to acid ratios (1:2 to as low as 1:40) has been reported in literature for crustacean shell demineralization (No and Meyers, 1995). In this study, the ratios ranged from 1:10 to 1:60 (w/v). Varying the solid to solvent ratios did not significantly affect the extent of demineralization (Fig. 3.2). In fact, the results show an approximately 100% decrease in the original ash content within the range tested. This is noticeably substantial and worth mentioning that earlier studies have established that chitosan of high quality should have an ash content of less than 1% (No and Meyers, 1995). These findings were comparable to those of Benhabiles et al. (2012) who also reported a \pm 100% decrease in ash content at solid-solvent ratios of 1:10 to 1:30 (w/v). The initial concentration of Ca in the dried shrimp shells used in this study was determined to be 274.29 g.kg⁻¹, after optimising parameters, a maximal 223.96 g.kg⁻¹ Ca was removed (81% Ca removal).

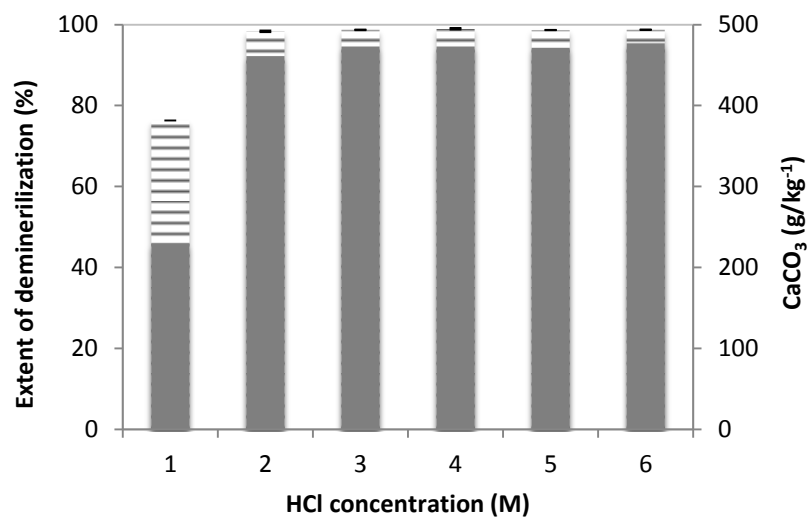


Fig. 3.1 Effect of HCl concentration on the extent of demineralization (\equiv), at room temperature, 1:10 solid-solvent ratio and the respective amount of calcium carbonate (\blacksquare) removed with each concentration. Each point represents the mean of triplicate determinations with \pm SD. [Note: error bars are not visible as they are small]

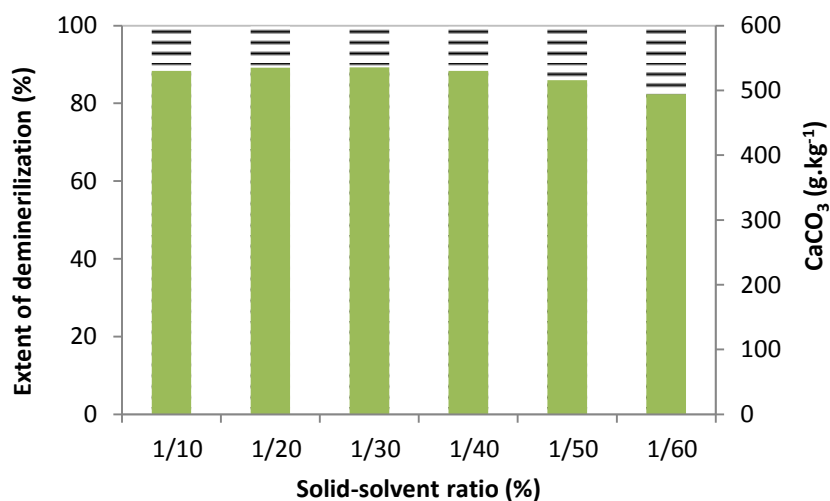


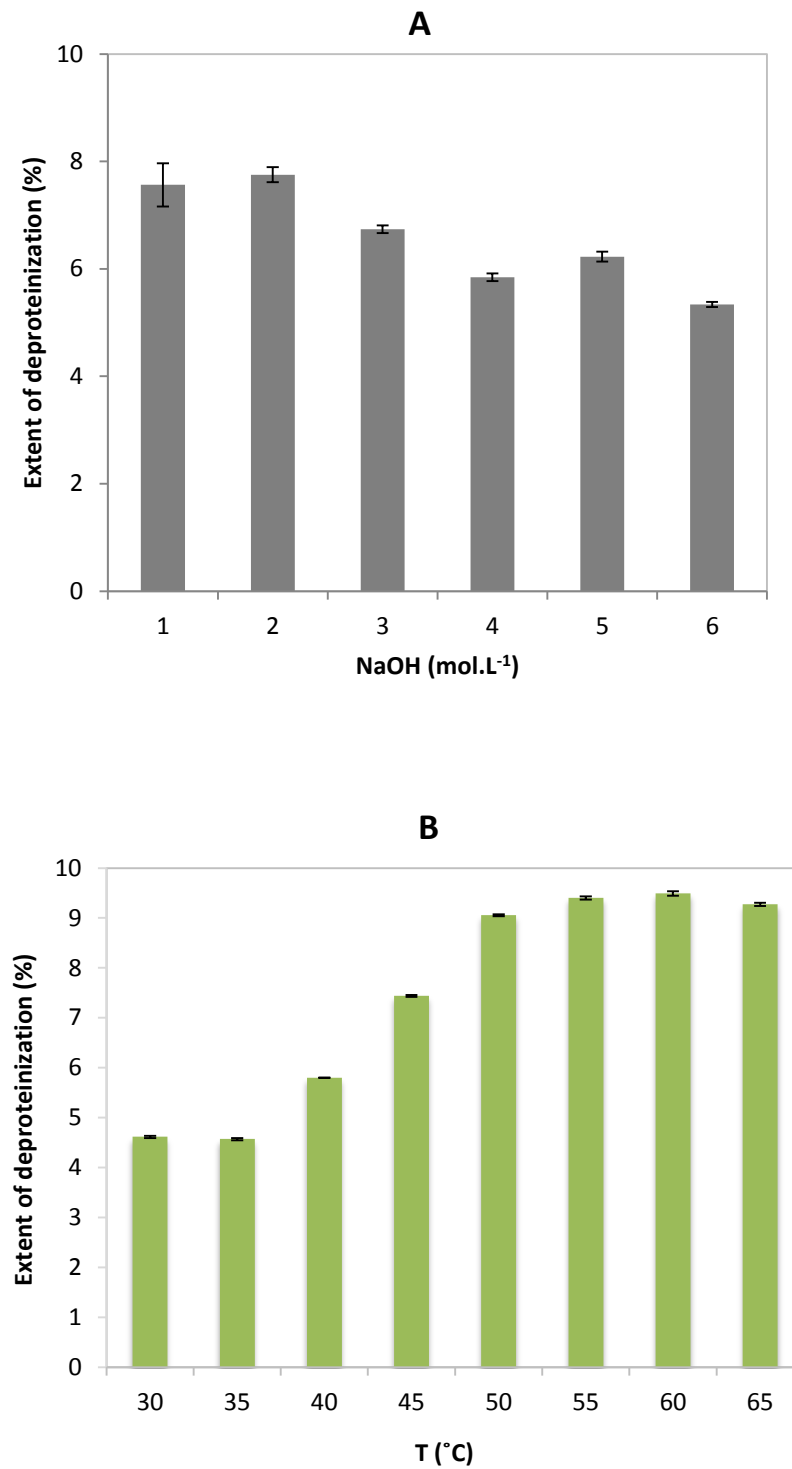
Fig. 3.2 Effect of solid-solvent ratio on the extent of demineralization (\equiv), at room temperature with 2N HCl and the respective amount of calcium carbonate (\blacksquare)

removed with each concentration. Each point represents the mean of triplicate determinations with \pm SD. [Note: error bars are not visible as they are small].

An aqueous solution of NaOH was used for the removal of proteins, pigments and lipids from the demineralized shells. This step serves to alter the chemical covalent bonds between the protein-chitin complexes. Contrary to Benhabiles et al. (2012) who reported 40% deproteinization with 2 N NaOH, a maximum of 7.8% (Fig 3.3a) deproteinization was achieved in this study, using the same NaOH concentration. As the concentration of NaOH increased, the degree of protein removal did not exceed 7.8%. Poor protein recovery can be due to proteins being bound to the chitin by covalent bonds through histidyl or aspartyl residues or both, producing stable aggregates such as glycoproteins (Nakagawa et al., 2015). Disruption of this chemical bond is difficult thus making it hard to achieve high protein yield. Near complete protein removal is most desirable since it enables greater chitosan solubility after the deacetylation step. Similarly, optimization of deproteinization time displayed the same trend observed with the optimization NaOH concentration. Maximum protein removal was obtained at 60 min (11.7%) (Fig 3.3b) after which no further increase in protein removal was apparent. Rather, the protein concentrations decreased after 60 min. The temperature and solid to solvent ratio parameters had a greater impact on the extent of deproteinization. A temperature increase from 35°C to 55°C increased the degree of protein removal from 4.6% to 9.4%, respectively (Figure 3.3b), reaching a maximum 9.5% at 60°C. The elevated temperature most likely encouraged an increase in protein solubility in the alkali solution however, beyond that; the solution may have reached saturation. These results are similar to the results obtained by Khanafari et al. (2008).

Optimizing the shells to solvent ratio showed maximum protein removal (13.7%) when the ratio was 1:10. Raising the ratio from 1:15 to 1:35 (w/v) resulted in a sharp decline in protein yield from 11.5% to 3.8%. This yield (i.e. 13.7% removal of protein) was the highest value obtained in this study. Benhabiles et al. (2013) however reported an increase in deproteinization from 30% to 96% at a solid to solvent ratio of 1/10 (w/v) and 1:20 (w/v), respectively. The author reported a final 96% protein removal after optimization. In this study all methods of preparation afforded a total of 13.7% removal of protein by varying the reaction time, temperature, NaOH concentration, and solid to solvent ratio. The percent

protein removal in these findings was inferior with the above study. These could be due to variations in the type of shrimp species used and methods employed.



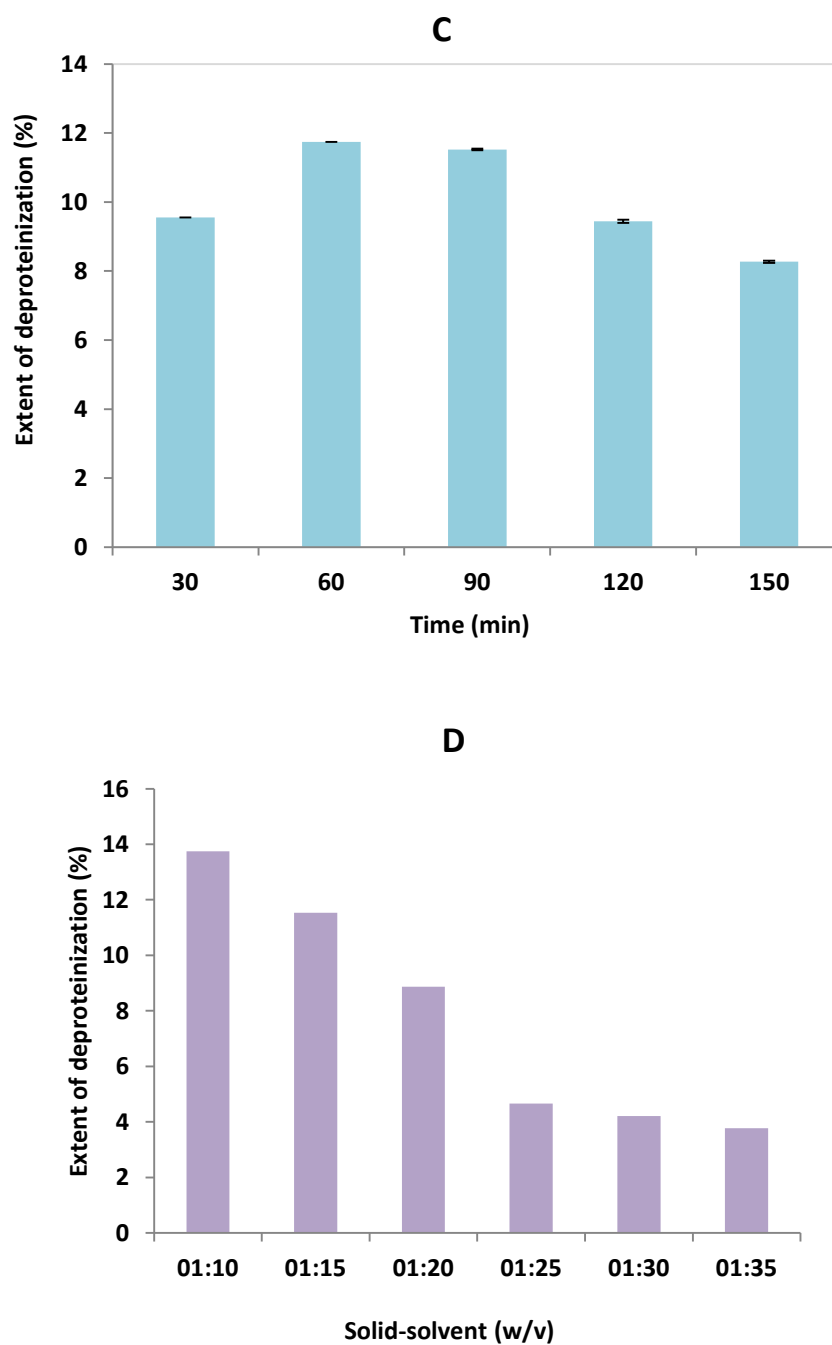


Fig. 3.3 The effect of NaOH concentration (A) on the extent of deproteinization at 50°C, 2h and 1:20 solid-solvent ratio. The effect of temperature (B) using 2N NaOH, 1:20 solid-solvent ratio in 2 h. The effect of reaction time (C) using 2N NaOH and 1:20

solid-solvent ratio at 60°C. The effect of solid-solvent ratio (D) at 60°C, 2h using 2N NaOH. Each point represents the mean of triplicate determinations with \pm SD.

3.2 PHYSICOCHEMICAL CHARACTERIZATION OF SHELLS, CHITIN AND CHITOSAN

3.2.1 Proximate composition analysis

Proximate composition of shrimp shell waste varies with each species and many other factors. Chemical analyses showed that shrimp waste consisted of 20-30% chitin (dry weight) and a relatively high protein content of $44.60 \pm 0.34\%$ and $35.15 \pm 0.31\%$ ash on a dry basis. These results were consistent with those of Cosio et al. (1981) who reported values of 18.1% and 34.9 for chitin and protein content, respectively. Bough et al. (1981) obtained 31-36% chitin in shrimp-shell waste, while Carroad and Tom (1978) reported 24.4% in the same type of waste. These findings show that shrimp shell waste can be considered a good source of chitin and protein, through either bioconversion or direct extraction. It is likely that these findings would be inconsistent because qualitative and quantitative changes in chitin may be due to seasonal variations or the organism's physiological state as well as species variation, (Fernandez-Kim, 2004). After deacetylation of chitin and decolourization treatment with ethanol, the chitosan yield obtained was 60-80%. The yield and degree of deacetylation of the chitosan are highly influenced by the reaction time (Chang et al., 2003). Evidently, the produced chitin had similar appearance compared to commercial chitin and (Fig. 3.4). The results further showed that the shells had the highest moisture content of $4.18 \pm 0.99\%$, followed by chitin (2.50 ± 0.43) and chitosan (0.53 ± 0.17 -Table 3.1). Chitosan is hygroscopic in nature (Khan et al., 2002), therefore it is very likely this value is higher than when initially extracted due to moisture absorption during storage. According to Li et al. (1992) commercial chitosan products should contain less than 10% moisture content. The quantity of ash indicates the efficiency of the demineralization step for calcium carbonate removal. Commercial chitosan products contain less than 2% ash (No et al., 1995). In the present study, the optimized demineralization parameters reduced the mineral content to acceptable limits in the chitin (0.25 ± 0.31) and chitosan ($0.19 \pm 0.06\%$). It can be deduced that the produced chitosan was of high quality since it had % ash of less than 1% (No et al.,

1995). This low ash content for both polymers indicated a satisfactory removal of minerals and calcium carbonate from the starting material. Results also show low lipid content of 0.28 ± 0.11 , 0.05 ± 0.02 , and 0.03 ± 0.02 for the shells, chitin and chitosan, respectively.

Accurate estimation of the DDA of chitosan is an important parameter that indicates the molar percentage of monomeric units that have amino groups and differ from zero (chitin) to 100 for fully deacetylated chitosan (Jayakumar et al., 2008). N-deacetylation conditions entailed treating isolated chitin with 50% NaOH for 3-5 h, after which the solid was filtered, rinsed with water until the filtrate was neutral. After removal of the chitin acetyl groups, DDA was determined to be 72%. Chitosan deacetylated by 70-90% is considered a good end product (Kurita et al., 1993) and when the DDA is higher than 50% chitosan solubilises in weak acidic solutions. Along with the DDA, the physiochemical characteristics of chitosan also included a viscosity of 83.7 ± 0.49 cP in 1% acetic acid solution. In literature, the reported viscosity of chitosan solutions commonly ranges from 60 to 780 cP (Alimuniar and Zainuddin, 1992; Anderson et al., 1978). This range in viscosity was also observed by Cho et al. (1998) with five commercially available chitosan preparations. Therefore, chitosan with viscosity comparable to commercial preparations can be produced by the chemical method used in this study.

Table 3.1 Proximate compositions of shells, chitin and chitosan

	Prawn Shells	Chitin	Chitosan
% ash (dw/dw)	35.15 ± 0.31	0.25 ± 0.31	0.19 ± 0.06
% protein (dw/dw)	44.60 ± 0.34	36.17 ± 1.07	24.67 ± 0.26
Moisture	4.18 ± 0.99	2.50 ± 0.43	0.53 ± 0.17
Lipids	0.28 ± 0.11	0.05 ± 0.02	0.03 ± 0.02
Viscosity (cP)	NT	NT	83.7 ± 0.49
Degree of deacetylation (%)	NT	NT	72

data are expressed as mean \pm standard deviation (n = 3). NT-not tested



Fig. 3.4 Crude chitin isolated from shrimp shells (A) compared to commercial chitin (Sigma-Aldrich) (B).



Fig. 3.5 Crude chitosan after deacetylation of chitin isolated from shrimp shells (A) compared to commercial chitosan (B).

3.2.2 Mineral content of shrimp shells

The most abundant minerals in the shell waste were Ca, Mg, P, Na and Fe, with Ca being the dominant mineral (Table 3.2). Hansen and Illanes (1994) reported that calcium is the main mineral constituent of shellfish waste and Mahmoud et al. (2007) reported it to be 23 and 6 times greater than the amount of magnesium and phosphorus, respectively. The average calcium content observed in this study was 274 287 mg.kg⁻¹, which is 12 times higher than magnesium. Calcium is necessary for solid tissue structure, nerve transmission, blood clotting, muscle contraction, osmoregulation and as a cofactor for enzymatic processes (Lovell, 1989), but in exoskeletons it serves a purely structural function. Iron was determined at minor levels. Iron is a vital microelement (micronutrient) for healthy normal reproduction and growth. These results show that the shells can be utilized as an important alternative source of mineral supply for feed formulation for animals and human consumption because of the high content of functional minerals (Cao et al., 2009).

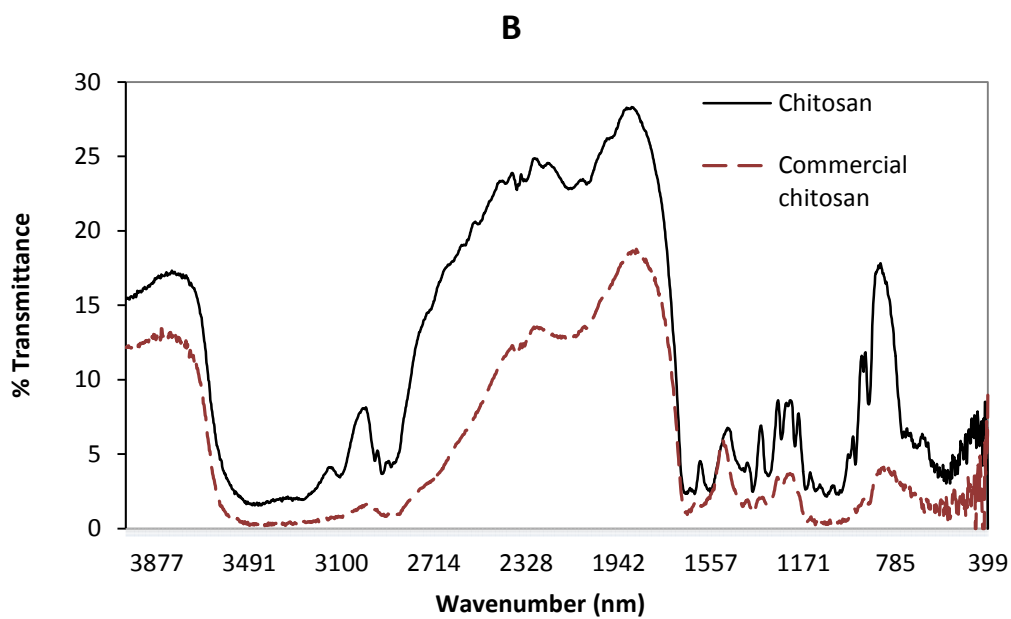
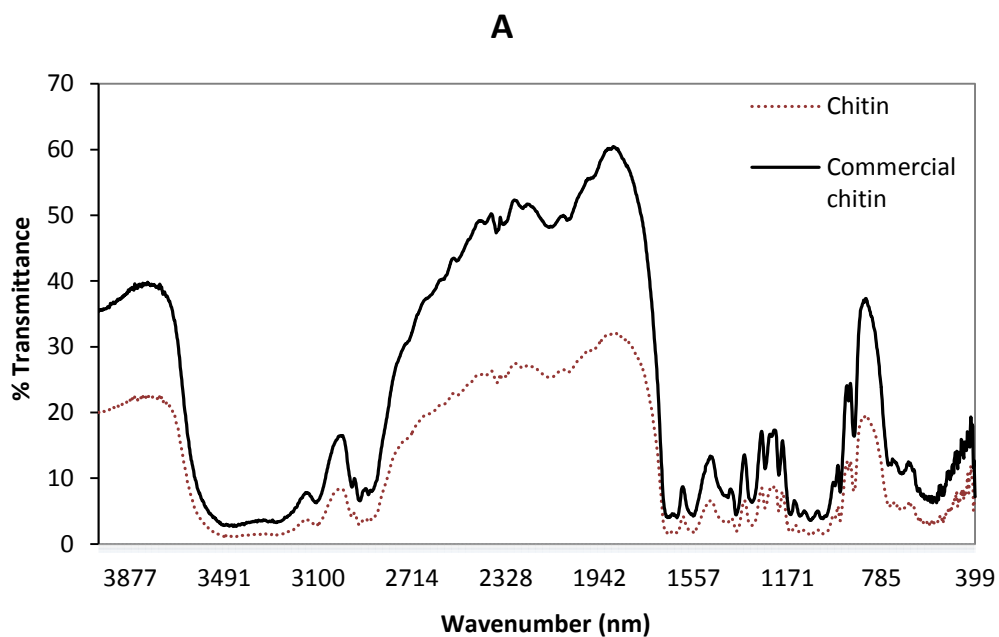
Table 3.2 Mineral analysis of shrimp shell raw material

Minerals		g/kg
Calcium	(Ca)	274.29
Magnesium	(Mg)	218.87
Phosphorous	(P)	1.38
Potassium	(K)	1.27
Sodium	(Na)	0.80
Iron	(Fe)	0.22

3.2.3 FTIR analysis of chitin and chitosan

The FTIR spectra results for chitin are displayed in Fig. 3.6A and the spectrum of Fig. 3.6B corresponds to the deacetylated sample now referred to as chitosan. Chitin displayed a peak trough at 1562 cm^{-1} representative of the N-H deformation of amide II (Duarte et al., 2001, Ravindra et al., 1998). Common to all samples is a broad peak in the region of $3500\text{--}3175\text{ cm}^{-1}$, attributed to OH stretching (Ottenhof et al., 2003) as a result of the moisture content of samples. The peak identified at 1654 cm^{-1} decreases as a result of chitin deacetylation, while an increase at 1551 cm^{-1} occurs, indicating the predominance of NH_2 groups (Bordi et al., 1991). This increase in intensity at 1551 cm^{-1} than at 1654 cm^{-1} , suggested effective deacetylation of chitin. All spectra show the characteristic chitosan bands around the 1000 cm^{-1} range denoting the presence of the *N-acetyl* glucosamine group unique to chitosan. Fig. 3.6B shows the chitosan spectrum isolated from shrimp. FTIR characterization showed that the overall extraction efficiency and the level of chitosan purity were satisfactory and similar to that of commercially available chitosan. The peak at 1647 cm^{-1} and 1654 cm^{-1} are ascribed to the amide I band vibrations, and the peak at 1654 cm^{-1} are indicative of the amide I elongation of $\text{C}=\text{O}$. The band at 1618 cm^{-1} can be assigned to the stretching of C-N vibrations of the overlapping $\text{C}=\text{O}$ group, linked by H bonding to the OH group. These bands are clearly seen in all three samples. The peak at

1552 cm^{-1} correlates to the N-H distortion of amide II and at 2940 cm^{-1} correlates to a symmetrical distortion of the CH_3 group (Duarte et al., 2001; Ravindra et al., 1998).



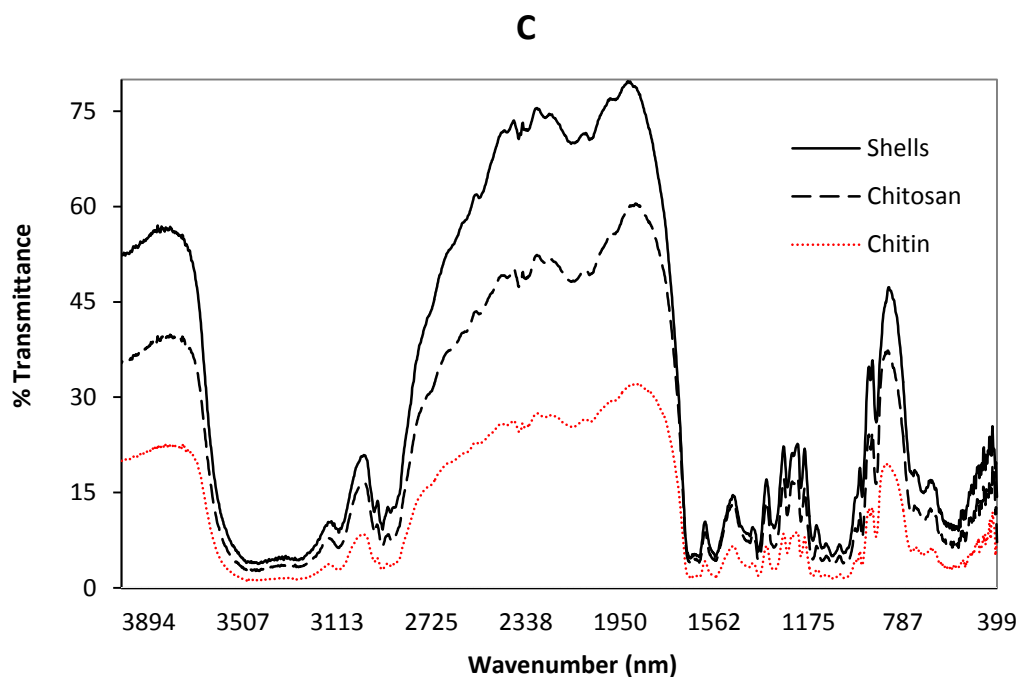


Fig. 3.6 FTIR spectra of chitin (A) and chitosan (B) isolated from shrimp shells in comparison to commercial chitin and commercial chitosan, respectively. FTIR spectra of the shell, chitin and chitosan are superimposed in (C).

3.3 HYDROLYSIS OF CHITIN AND CHITOSAN

Chitin and chitosan produced a series of oligomers after partial depolymerisation with concentrated HCl. Strong acid hydrolysis was chosen over other available approaches, like enzymatic or nitrous acid treatments (Allan and Peyron, 1997), because during acid treatment depolymerisation is accompanied by the cleavage of *N*-acetyl groups. The produced oligomers were purified by de-colourization, precipitated with cold acetone, filtered and dried.

TLC analysis provided a preliminary insight on the separation and number of oligomers present in the NAcCOS mixed fraction obtained after acid hydrolysis (Fig. 3.7). Separation of GlcN (DP 2–6) oligomers and GlcNAc was achieved by TLC. Seven definite and well-separated fractions of NAcCOS were detected. GlcN and GlcNAc elution times were confirmed by the commercial standards against which they were tested.

The prepared NAcCOS were analysed by HPLC and the retention times of the five GlcNAc oligomers standards (GlcNAc dimer, GlcNAc trimer, GlcNAc tetramer, GlcNAc pentamer and

GlcNAc hexamer) were 6, 6.3, 6.7, 7.4, and 8.7 min, respectively (Fig. 3.8A). Li et al. (2012) reported retention times of 10.4, 13.4, 15.7, 17.3 and 18.5 min for GlcNAc oligomers (DP 2-6). These retention times differed to those of this study; however, this is to be expected since the authors chromatography was carried out on a Click Maltose column. Fig. 3.8B depicts the chromatogram curve of GlcNAc with a retention time of 11.2 min.

The NACCOS components hydrolysed from chitin are illustrated in Fig. 3.8C. There were in total, 8 fractions identified in the HPLC system. The dimer, trimer, tetramer, pentamer and hexamer were all present in the NACCOS mixed fraction and were in accord with the oligomer standards in relation to their retention times. The precipitated chitin oligomers were largely composed of GlcNAc, the dimer (GlcNAc)₂, and the trimer (GlcNAc)₃ in that order. The hexamer (GlcNAc)₆, tetramer (GlcNAc)₄ and pentamer (GlcNAc)₅ had the lowest concentrations. GlcNAc was the major component, with a yield of 142.41 mg.ml⁻¹, twice the value reported by Bohlman et al. (2004). GlcN could not be detected or quantified simultaneously with the oligomers by the HPLC system used, as it generally requires a Zorbax SB-C18, 5 µm, 150 mm x 4.6 mm column and all chromatographic conditions necessary for its detection were different from the conditions used for the oligomers.

According to Bohlman et al. (2004), the reaction conditions should be high enough to adequately degrade chitin without destroying the products. Such parameters include a HCl concentration of 15-36% and a temperature of 40-80°C. Under these conditions, up to 6.42 g.L⁻¹ GlcNAc can be produced in 1 h. Ryosuke et al. (2002), in an attempt to produce natural GlcNAc, combined ion-exchange membrane electrophoresis and the acid hydrolysis of chitin. Therefore, it is possible to use chemically-modified GlcNAc as a low-cost resource in pharmaceuticals, food additives and cosmetics in future. It has also been revealed that *Rhodotorula glutinis* is capable of converting GlcNAc into biofuel (Yoon and Rhee, 1983, Hamme et al., 2006). The bioethanol could be produced with the GlcNAc from chitin waste as a C6 carbon source (Wendland et al., 2009). In conclusion, this investigation revealed that the distribution of GlcNAc in NACCOS ranged from 2-to 6-mers and NACCOS could be used as materials for the preparation of GlcNAc with single DP, upon fractionation

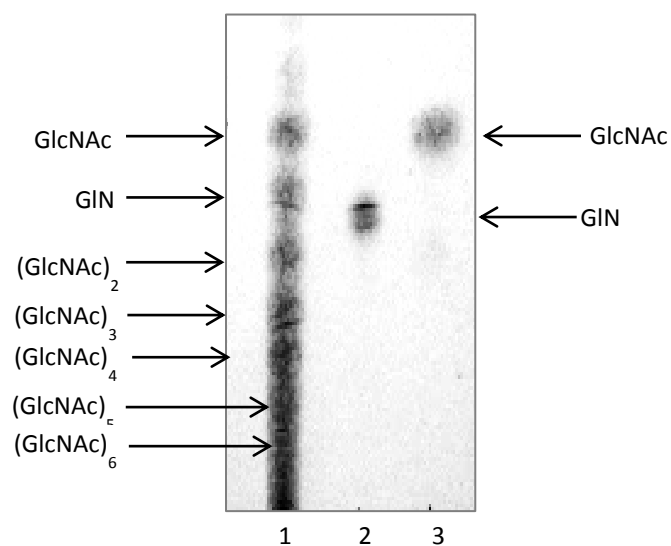
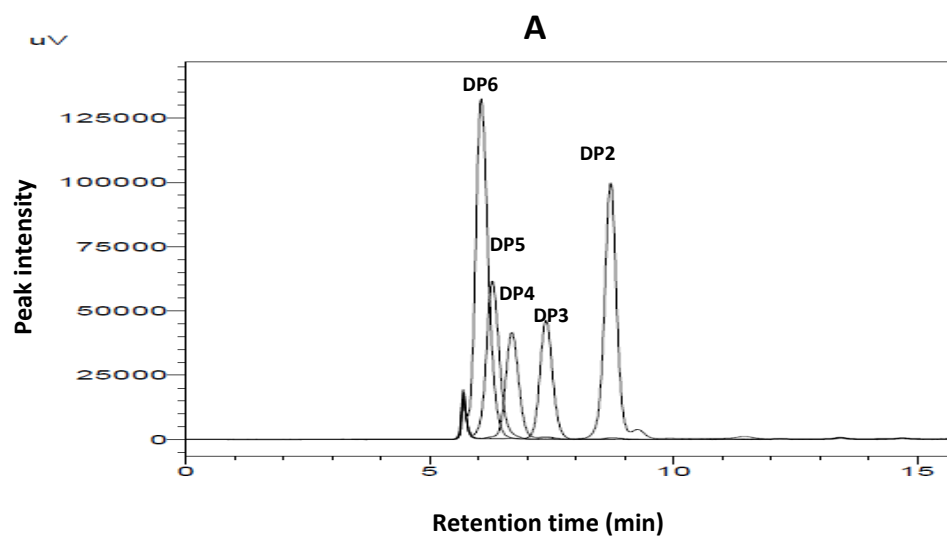


Fig. 3.7 Chromatographic profile of NAcCOS mixed fraction (Lane 1) hydrolysed from chitin and standards GlcN (Lane 2) and GlcNAc (Lane 3).



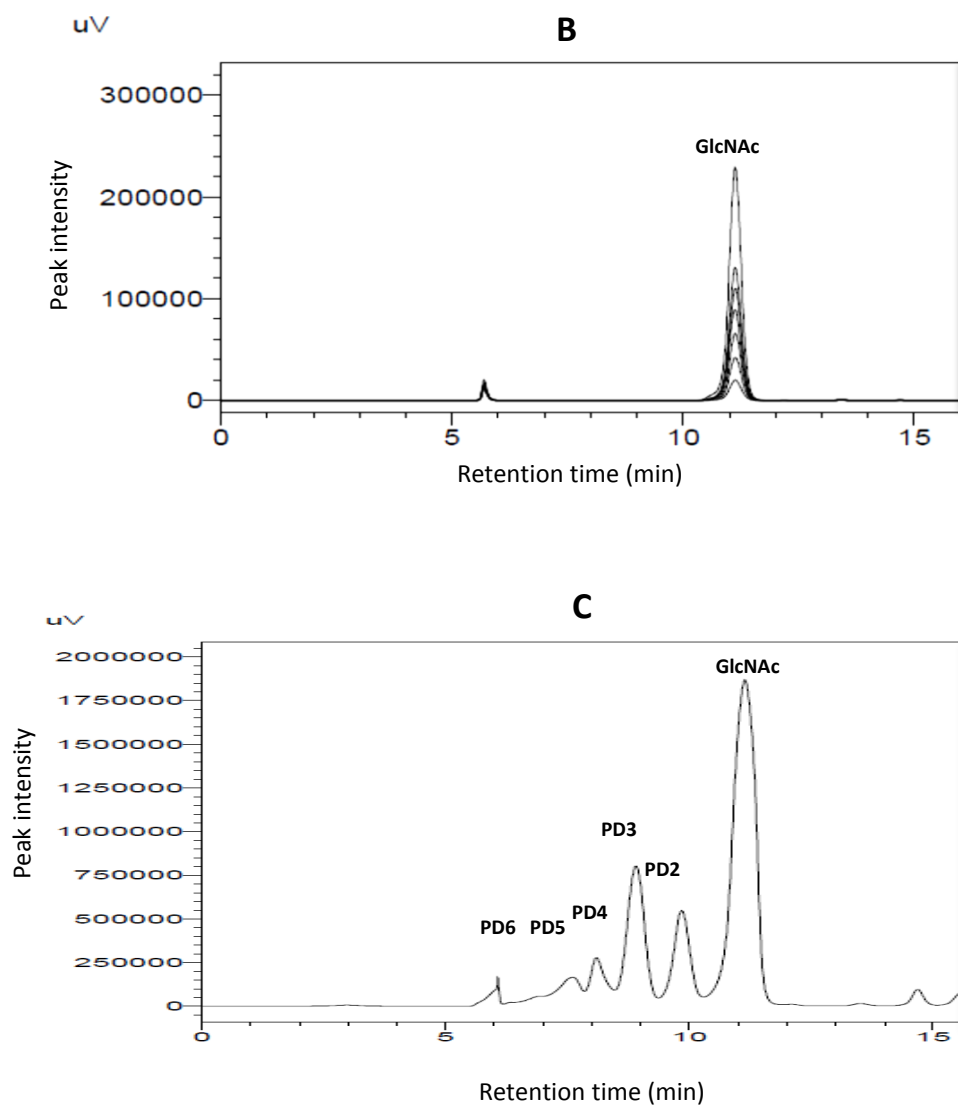


Fig. 3.8 HPLC spectra of NAcCOS (DP 2-6) and GlcNAc. Five main peaks with the retention times of 6, 6.3, 6.7, 7.4, and 8.7 min corresponding to the GlcNAc oligomers standards (GlcNAc dimer, GlcNAc trimer, GlcNAc tetramer, GlcNAc pentamer and GlcNAc hexamer) (A). GlcNAc standard with a retention time of 11.2 (B) and HPLC spectrum of NAcCOS produced from chitin after 2 h with concentrated HCl (C).

Table 3.3 Yield of chitin oligomers

Retention time (min)	Component	Yield (mg.ml ⁻¹)
11.2	GlcNAc	142.41
8.6	(GlcNAc) ₂	24.96
7.4	(GlcNAc) ₃	7.24
6.7	(GlcNAc) ₄	1.97
6.3	(GlcNAc) ₅	0.29
6	(GlcNAc) ₆	2.66

Chitosan hydrolysis in concentrated HCl involves two specific steps, that is, depolymerising chitosan into smaller oligosaccharides and deacetylating GlcNAc units into GlcN units (Einbu and Varum, 2008). In this event, a well deacetylated chitosan was used as starting material and acetyl groups were detached during the hydrolysis. As is illustrated in Fig. 3.9, chitosan oligomers consisted of DP 2-6 and GlcN were visible on the TLC plate. In this system, oligosaccharide retention depends on the hydrophilic interactions of amino groups and oligosaccharide hydroxyl groups with the stationary phase. In general, the oligosaccharides with more hydroxyl and amino groups will have longer retention times (Fu et al., 2010).

Prior studies have depicted separation of several chitooligosaccharides with narrow or single DP by capillary electrophoresis, size exclusion chromatography and immobilized metal affinity chromatography (Ledevdec et al., 2008). However in this study, the COS were not quantified on an HPLC system due to unavailability of standards. TLC analysis was sufficient in providing initial insight on the separation and number of oligomers present in the COS mixed fraction obtained after acid hydrolysis of chitosan. It is noteworthy that the oligomers (DP 2-6) and GlcN are present in all the samples after being collected and precipitated at 1 h intervals. We can speculate that the yield of the oligomers decreases with time. Lee et al., (1999) investigated the production of oligomers against time and his data revealed that 0.5 and 2 h reactions precipitated 10.1 and 7.3% oligomers, respectively, but after a 4 h

reaction, only 3.1% of the oligomers were obtained. A majority of the higher MW oligomer in 4 h is degraded into lower MW oligomers. Therefore, the authors strongly recommended a hydrolysis time less than 2 h if hydrolysing at 80°C with 35% HCl to obtain only higher MW oligomer. The previous work of Yabuki (1995) supports these findings wherein similar conditions were used and obtained oligomers with DP 1-4 after 6 h of depolymerisation. Although oligomers with DP 2-6 are available on the TLC chromatogram, we can also assume that the quantity after a couple of hours of depolymerisation will decrease.

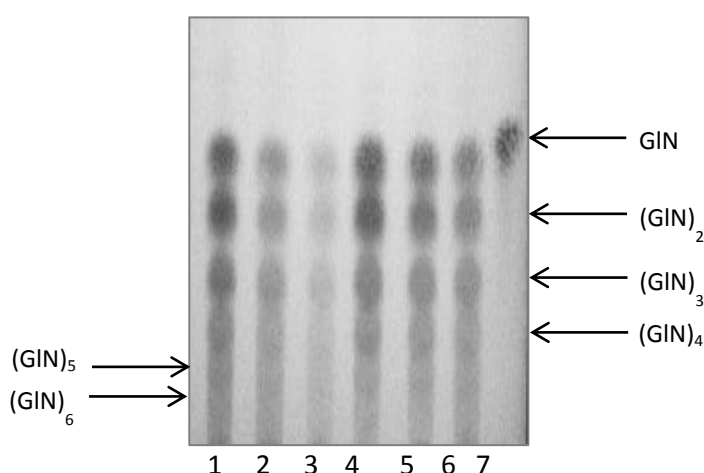


Fig.3.9 Chromatographic profile of chitosan hydrolysis products taken at 1 h intervals for 6 h (represented by lanes 1-6) during acid hydrolysis and standard GlcN in lane 7.

3.4 ANTIMICROBIAL ACTIVITY OF CHITOSAN, COS AND NACcOS

The extracted chitin was converted into the more practical, soluble chitosan. Both products along with their respective oligomers were characterized for their biological activity in terms of antimicrobial properties. The antimicrobial properties of chitosan and COS were studied against different cultures of gram positive and gram negative bacteria and the variations in the organisms degree of susceptibility to the compounds were observed. Chitosan was prepared by deacetylation of chitin, while COS was prepared by chemical hydrolysis.

Chitosan displayed a bacteriostatic effect on all tested bacteria while COS possessed no antibacterial activity. The bacteriostatic effect, however, differed with regard to the kind of

bacterium tested. In general, as was ascertained by Jeon et al. (2001), chitosan exhibited stronger bacteriostatic effects against Gram positive bacteria than Gram negative bacteria. Antibacterial activity of chitosan has been reported by several investigators (Uchida et al., 1989; Jeon et al., 2001) as being superior to that of chitosan oligomers. For example, Uchida et al. (1989) discovered that chitosan inhibited the growth of *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa*, whereas chitosan oligomers at levels as high as 0.5-1% showed weak or no antibacterial activity. Several authors have established that chitosan generally has stronger effects on Gram-positive bacteria (e.g. *Lactobacillus plantarum*, *Listeria monocytogenes*, *L. bulgaris*, *Bacillus megaterium*, *Staphylococcus aureus*, *B. cereus*, *L. brevis* etc.) than on Gram-negative bacteria (e.g. *Vibrio parahaemolyticus*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *E. coli*, etc.) (No et al., 2002; Coma et al., 2003). The cell surface charge density is a determining factor to decide the amount of chitosan adsorbed. Evidently, more adsorbed chitosan would result in greater changes in the cell membrane's permeability and in the structure. This would indicate that the antibacterial mode of action is dependent on the host microorganism (Másson et al., 2008).

The effect of chitosan (1%) against *E. coli* and *S. marcescens* was evident after 4 h and 3 h of growth within the log phase, respectively, and growth declined after 6 h for both organisms (Fig. 3.10 and Fig. 3.11). The susceptibility of *E. coli* was lower than expected. In a similar manner, Allan and Peyron (1997) reported that *E. coli* was only slightly affected using the same concentration. Cho et al. (1998) reported that chitosan's antibacterial activity against *E. coli* and *Bacillus sp.* was better improved when viscosity was within the ranges of 1000 to 10 cP. The viscosity of chitosan reported in this study was within the range specified (83.7 ± 0.49 cP). Chitosan caused a sharp decline after 4 h of growth against *S. aureus* and NACCOS exhibited no effect. Benhabiles et al. (2012), however reported complete inhibition of *S. aureus* after 2 h of incubation in the presence of 0.1% NACCOS.

Chitosan exhibited inhibitory growth activity against *E. aerogenes* throughout the 1-7 h incubation period, thus being the most susceptible of the entire gram negatives tested (Fig. 3.11), however, *S. typhimurium* showed to be the least susceptible organism against the effects of chitosan (Fig. 3.16), similar to the findings of Wang (1992) who reported that 0.5% chitosan was ineffective for suppressing *S. typhimurium*. Chitosan was effective against

S.epidermis, completely suppressing growth from 1-5 h after which the organism's growth was stimulated by the NAcCOS. Along with *S.epidermis*, *K. pneumoniae* was also growth stimulated by COS after 3 h of growth (Fig. 3.12) and *B. cereus* for the entire period of incubation (1-7 h-Fig. 3.18). Benhabiles et al., (2012) in similar research concluded that 0.1% (w/v) NAcCOS displayed higher antibacterial activities than native polysaccharides and notably suppressed the growth of all tested bacteria. The opposite was however true for the results in this study. Furthermore, they reported complete growth inhibition of *B. subtilis* and *B. cereus* was achieved within 3 h of inoculation whereupon the optical density declined to zero. NAcCOS had neither positive nor negative effects against *M.luteus*, however, chitosan's growth inhibition was best exhibited against *M.luteus*, suspending the organism in the lag phase for 6 h and growth ensuing thereafter.

The proposed and most acceptable mode of action of chitosan is synergy between chitin/chitosan positively charged molecules and the negatively charged bacterial cell membranes. This interaction is mediated by the electrostatic forces between the negative residues and the protonated NH^{3+} groups (Tsai and Su, 1999), most likely by competing on the membrane surface with Ca^{2+} for electronegative sites (Young et al., 1982). This electrostatic interaction results in a bifold interference:

- I. by encouraging changes in the functions of the membrane wall permeability, hence inciting internal osmotic imbalances and thereupon inhibiting the growth of organisms (Hadwiger et al., 1981; Shahidi et al., 1999)
- II. by the hydrolysis of peptidoglycans in the organisms wall, causing leakage of intracellular electrolytes like potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, glucose and nucleic acids) (Chen et al., 1998).

There are several reports discussing the antimicrobial activity of chitosan in different or similar conditions with opposing results (Eaton et al. 2008; Helander et al. 2001 ; Simunek et al. 2006). However we can conclude that they all confirm that chitosan has strong antimicrobial effects and is safe for human utilization. Therefore, the antimicrobial

properties of chitosan present a lucrative potential for producing natural food preservatives for functional-food additives and food-processing applications.

Chitosan solvents also affect the antibacterial activity of chitosan. More often than not, the organic acid, acetic acid is used for solubilizing chitosan (Bough et al. 1975; No and Meyers, 1989) because chitosan is soluble in most organic acid solutions with pH less than 6 (Muzzarelli, 1973). Wang, (1992) found that antibacterial activity of chitosan against five species of foodborne pathogens (*Y. enterocolitica*, *E. coli*, *S. aureus*, *S. typhimurium*, *L. monocytogenes*) was higher at pH 5.5 than at pH 6.5. These findings clearly suggest that applying chitosan to acidic foods will improve its efficacy as a natural preservative.

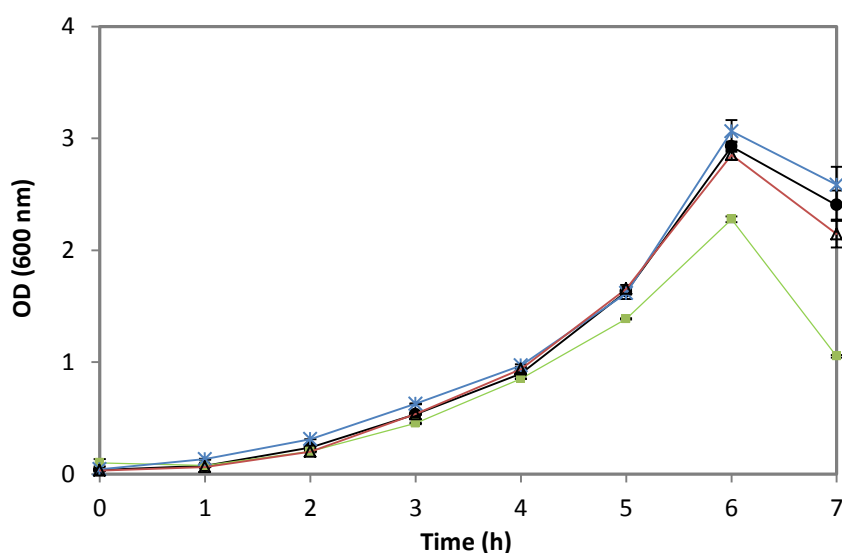


Fig. 3.10 Growth curves of *E.coli* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NAcCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

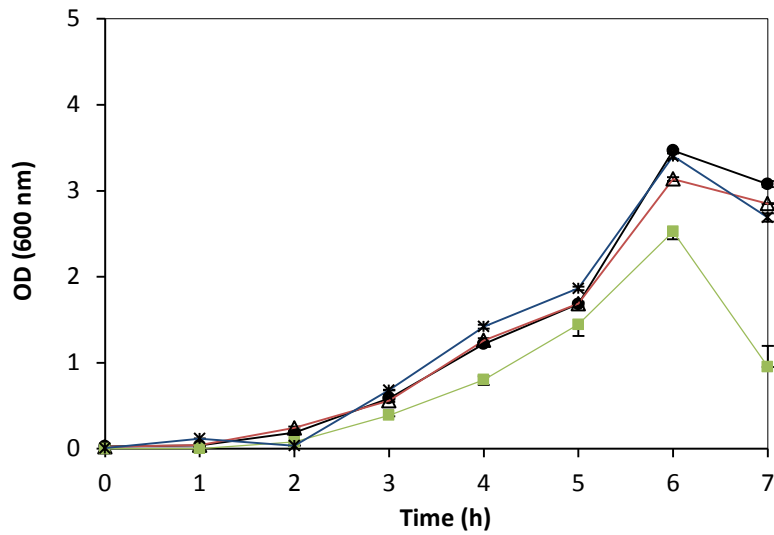


Fig. 3.11 Growth curves of *S. marcescens* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

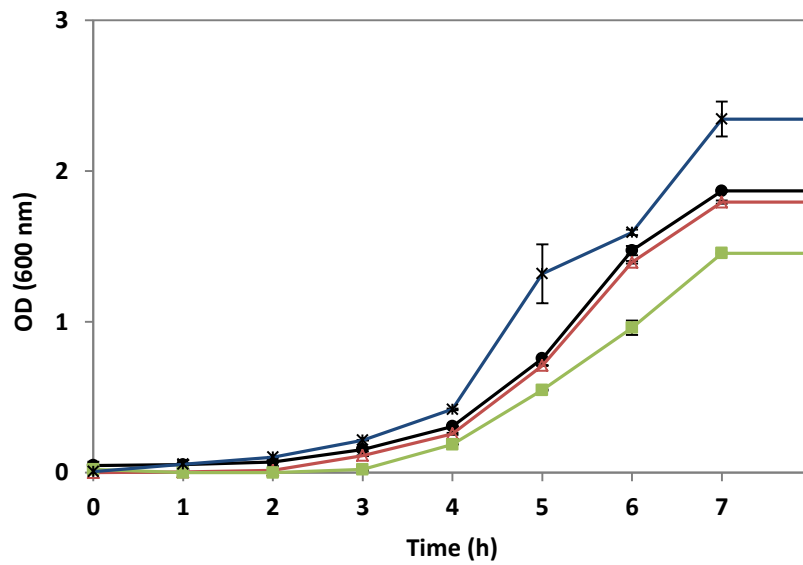


Fig. 3.12 Growth curves of *K. pneumoniae* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

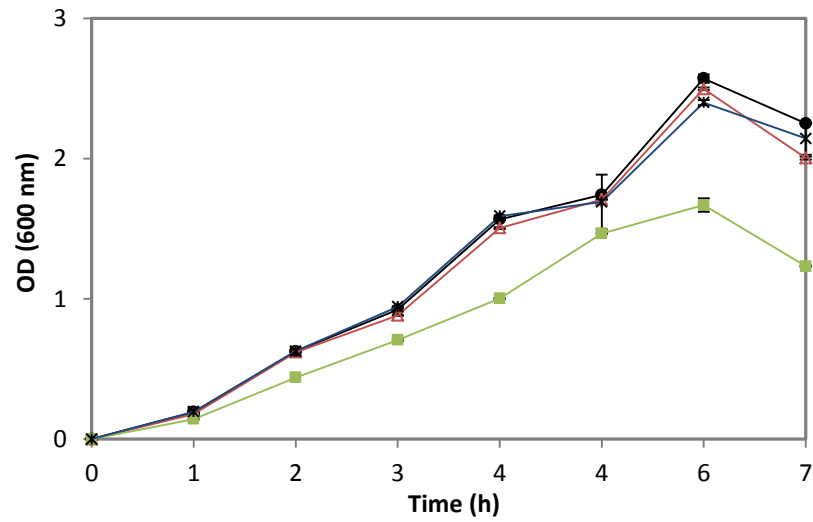


Fig. 3.13 Growth curves of *E. aerogenes* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

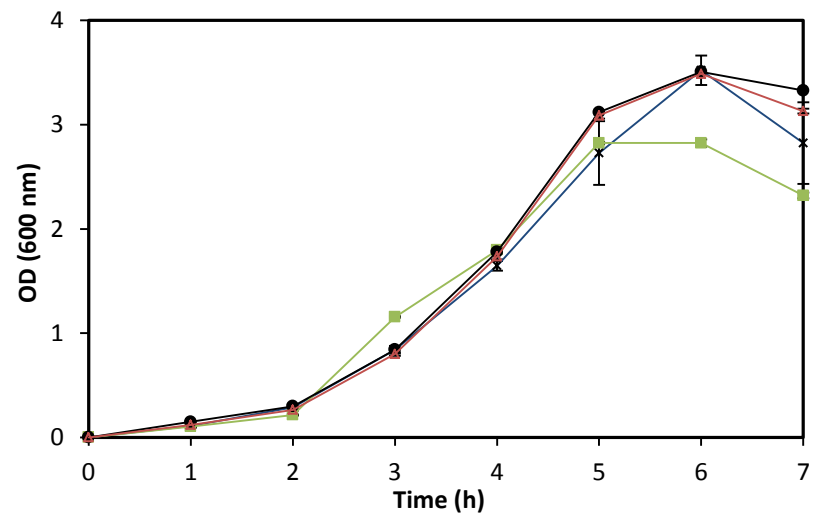


Fig. 3.14 Growth curves of *S. typhimurium* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

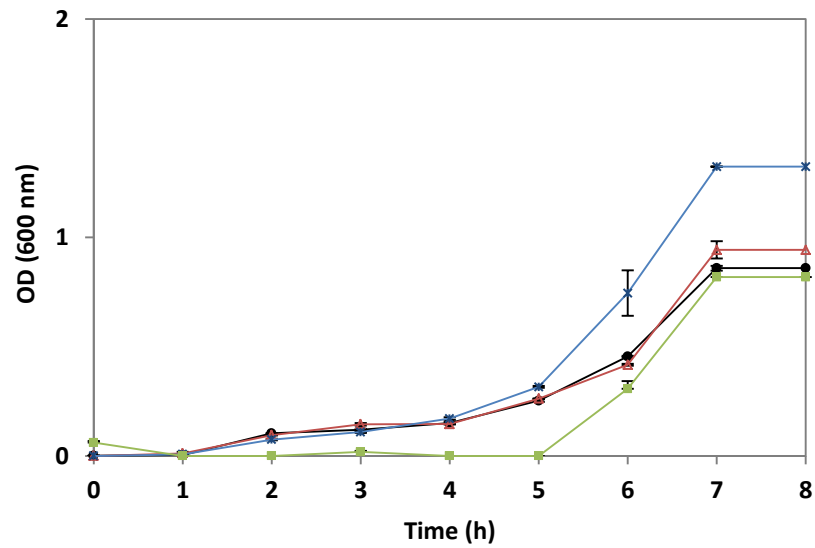


Fig. 3.15 Growth curves of *S. epidermidis* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants

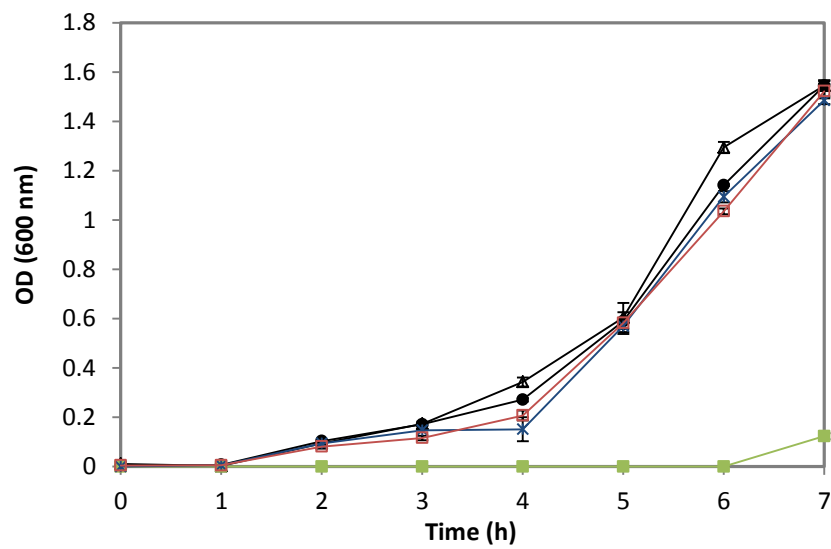


Fig. 3.16 Growth curves of *M. luteus* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

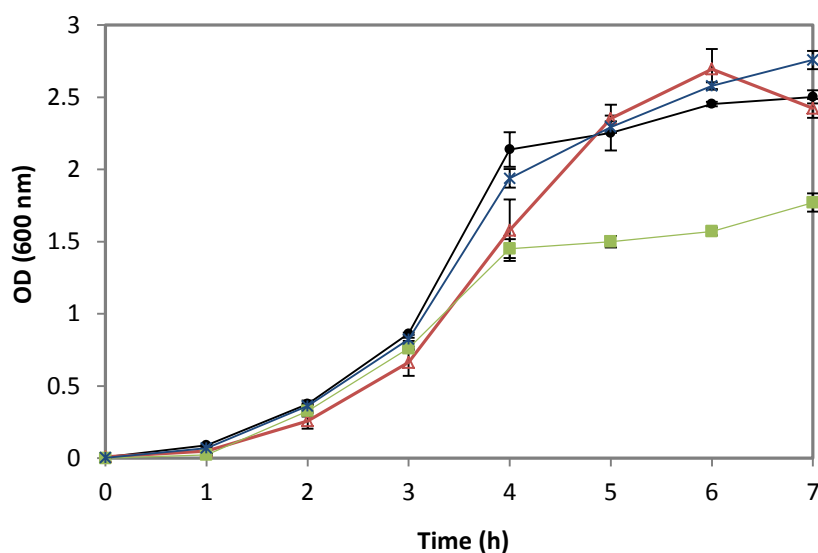


Fig. 3.17 Growth curves of *B. subtilis* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NAcCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

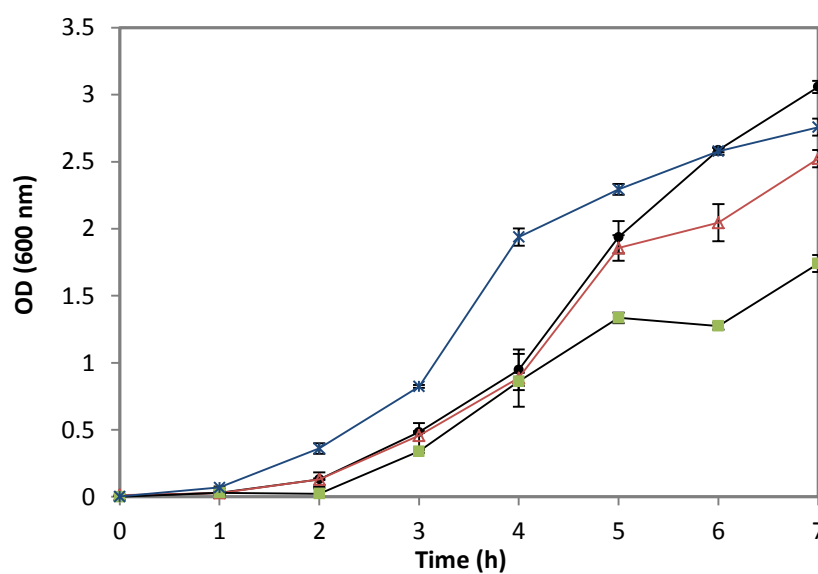


Fig. 3.18 Growth curves of *B. cereus* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NAcCOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

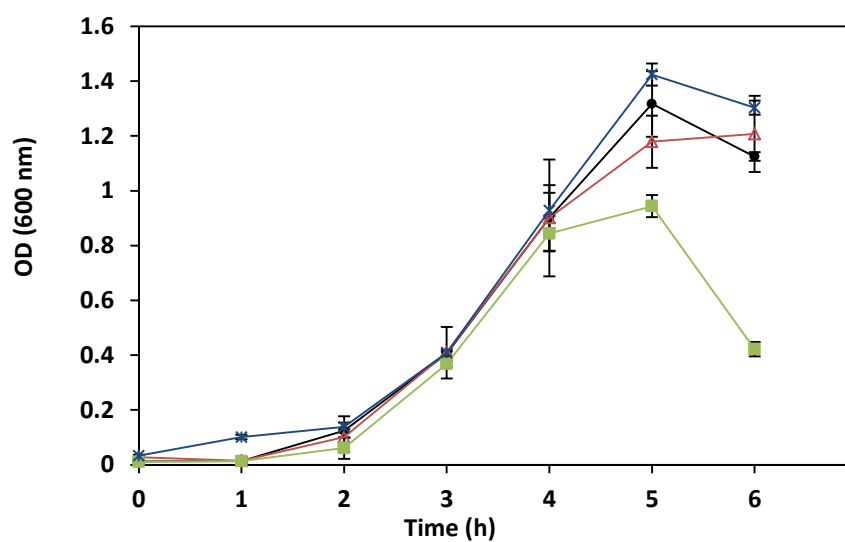


Fig. 3.19 Growth curves of *S. aureus* at 37°C and 180 rpm in MHB media (●), containing 1% chitosan (■), 1% NACOS (*) and 1% acetic (Δ). Each point is represents a mean of triplicate determinants.

3.5 RECOVERY OF ASTAXANTHIN AND CALCIUM CARBONATE

3.5.1 Astaxanthin content

The pigment extracted using 90% acetone was reddish-orange in appearance (Fig. 3.20). The total astaxanthin extracted in shrimp was $30.74 \mu\text{g.g}^{-1}$. The carotenoid content in shrimp by-products generally varies between $119\text{--}148 \mu\text{g.g}^{-1}$. Sachindra et al. (2006) reported that a 50:50 blend of hexane and isopropyl alcohol gave the highest yield ($43.91 \mu\text{g.g}^{-1}$) of carotenoid compared to acetone ($40.60 \mu\text{g.g}^{-1}$). This variation may be due to differences in species or due to the high polarity of acetone. However, Vimla and Paul, (2009) have reported that maximum yield of carotenoids from *Penaeus monodon* waste was obtained with acetone, compared to other solvents used for extraction. Polar solvents are generally favourable extraction solvents for xanthophylls whereas non polar solvents are not commended because they have limited penetration through the hydrophobic mass that encloses the pigment (Vargus et al., 2000). It was also observed that for any dry sample (deproteinized or non deproteinized), 90% acetone was found to be the best solvent for carotenoid extraction. Several reports are available on the yield of astaxanthin from different species of deep sea shrimps. Of these, the highest yield of $148 \mu\text{g.g}^{-1}$ dry waste was reported by Shahidi and Synowieki (1991) from the waste of *Pandalus borealis* shrimp. Several studies have shown that the astaxanthin yield depends on the extraction technique, media or solvents and other factors including the waste particle size (Sachindra et al., 2006). Furthermore, the difference of extractable astaxanthin in the waste and variations in species can also influence the yield of astaxanthin extraction. The quality is affected by storage conditions and processing i.e., exposure to air, heat treatment, intensity of exposure light and oxygen and length of exposure time, (Meyers and Bligh, 1981). In order to improve the extraction yield in this study, it may be beneficial to (i) reduce pre-treatment time of raw material prior to extraction, and/or (ii) carry out the extraction in the absence of air and light).

TLC and HPLC of carotenoid extracts revealed that the shrimp shells consist of astaxanthin and its esters. TLC is a method extensively used to isolate and purify carotenoids owing to its flexibility, low cost and simplicity. In marine crustaceans, astaxanthin and its esters have

been reported to be the most dominant carotenoids (Shahidi et al., 1998). In this study, TLC separation yielded four distinct bands (Fig. 3.21) at R_f 0.33 (orange), 0.40 (orange), 0.50 (orange) and 0.99 (yellow). The orange band at R_f 0.33 corresponded to free astaxanthin, R_f 0.99, the yellow band corresponded to β-carotene while the yellow bands at R_f 0.50 and 0.40 corresponded to astaxanthin monoester and canthaxanthin, respectively (Lorenz, 1998). The R_f values obtained for astaxanthin were in agreement with Sindhua and Sherief (2011). Astaxanthin was also analysed by reversed phase HPLC using a C18 column. Reversed phase liquid chromatography with typical eluents, including methanol and acetonitrile were used for elution of carotenoids in the shrimp shell material (Su et al., 2002; Verdoes et al., 2003). The standard curve linearity was expressed with reference to the determination coefficient (R^2) from plots of the integrated peak area vs. standard concentration (in mg.ml⁻¹). Calibration lines were constructed, and showed linear correlation of $R^2 = 0.9998$, having the following regression equation: $y = 6E+06X + 6E+07$. The carotenoid peak at the retention time of 2.993 (Fig. 3.22A) was recognised as astaxanthin based on the identical retention time on HPLC with authentic astaxanthin standard (Fig. 3.22B). The results revealed that the peak resolution of astaxanthin was not satisfactory, and the astaxanthin peak was tailing. The analytical conditions displayed poor separation efficiency (overlapping peaks). Weber et al. (2007) developed a reversed phase HPLC method with acetone/water as solvent system to analyse and identify some carotenoids by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). However, it was often found that astaxanthin failed to yield a symmetrical peak, and the carotenoids peak resolution was poor.

Nonetheless, the present study does reveal that deep sea shrimps may be used as a carotenoid source for pen-reared salmonids and other applications that have demanding requirements for carotenoid pigment.

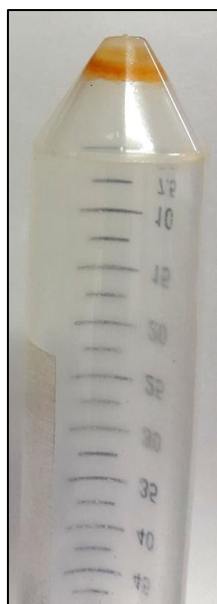


Fig. 3.20 Dried astaxanthin produced from 90% acetone.

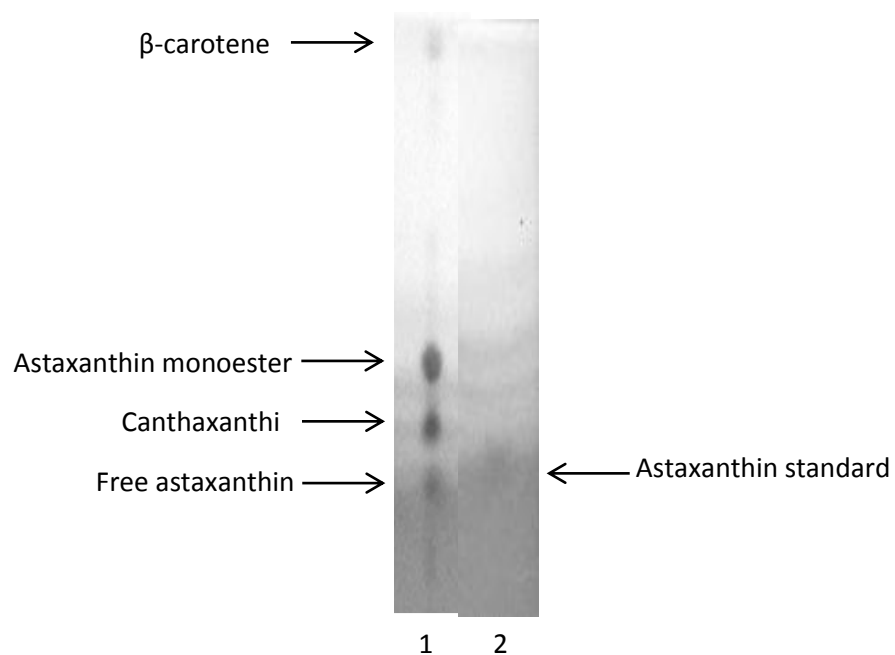


Fig. 3.21 Chromatographic profile of astaxanthin and its esters (Lane 1) isolated during chitin isolation and standard astaxanthin (Lane 2).

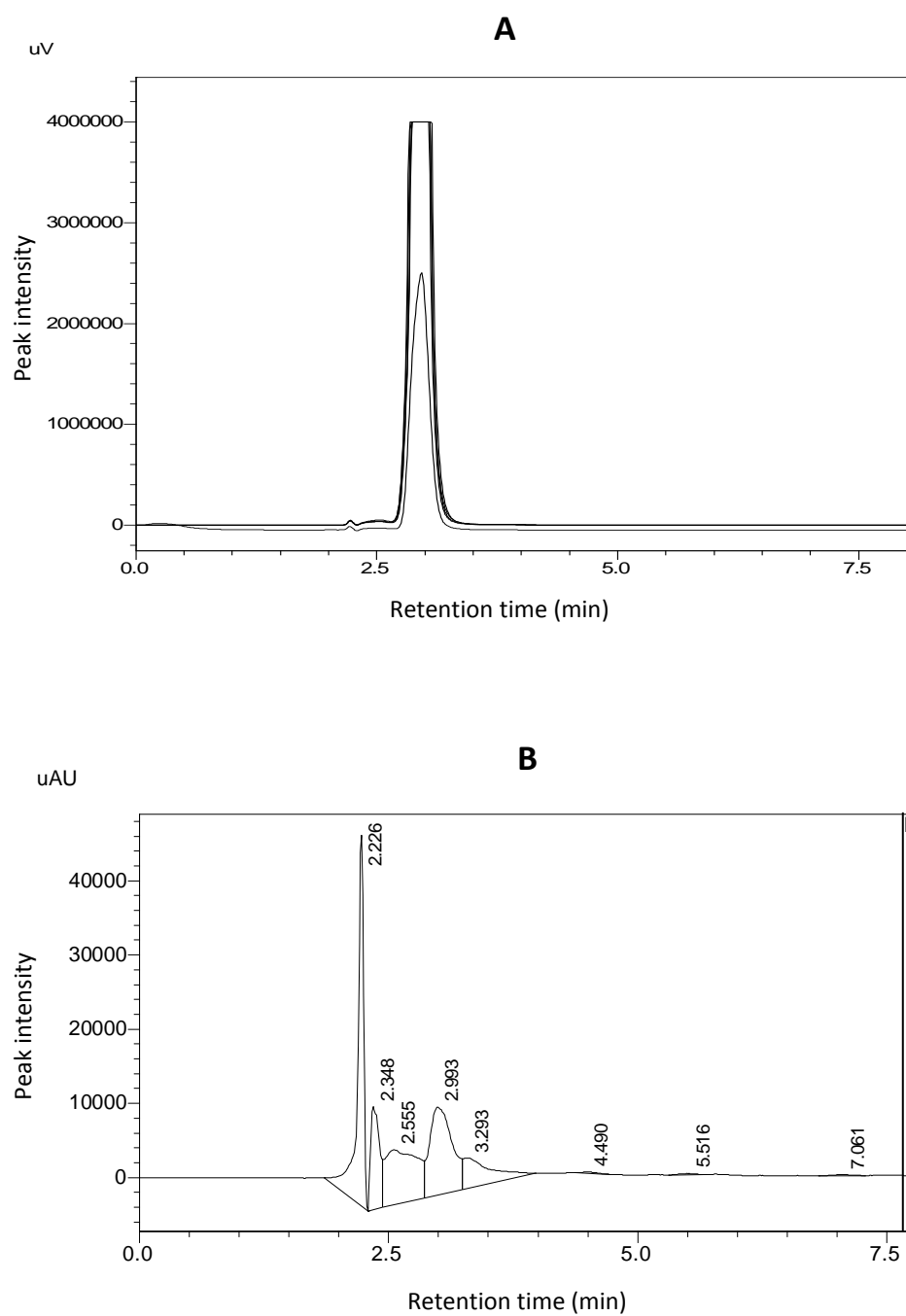


Fig. 3.22 HPLC chromatogram of carotenoids in shrimp waste obtained with 90% acetone (A) and (B) astaxanthin standard with the retention time of 2.993 min.

3.5.2 Synthesis of CaCO_3

Of the carbonate minerals, CaCO_3 is so far the most prominent in nature and industry (e.g. scale and filler formation, pigment) and consequently this is a well-studied mineral system. CaCO_3 was re-synthesised by mixing CaCl_2 solution from the demineralisation extract with NH_4HCO_3 ($\text{CaCl}_2 + \text{NH}_4\text{HCO}_3 = \text{CaCO}_3 + \text{NH}_4\text{Cl}_2$). 40g.100ml⁻¹ of white CaCO_3 precipitate was recovered (Fig. 3.23).

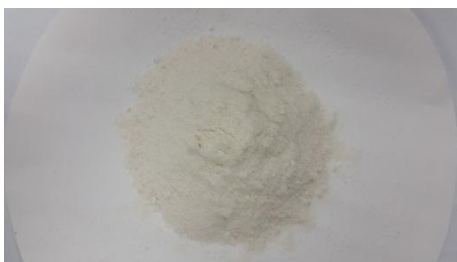


Fig. 3.23 CaCO_3 precipitate isolated from demineralization extract of shrimp shells

CaCO_3 is one of the most abundant minerals in nature and can exist in polymorphic forms: vaterite, aragonite and calcite. It is abundant in geological scales but also in biominerals, primarily as exoskeleton in cell walls of shells or in spines and spicules as mechanical support (Manoli and Dalas, 2000).

3.5.2.1 FTIR patterns of the CaCO_3

The FTIR spectrum of CaCO_3 powder showed an organic matrix and a mineral phase with many bands from 4000 cm^{-1} to 400 cm^{-1} (Fig. 3.24). The spectra of the produced CaCO_3 synergised well with the commercial spectra. The region at 3179 to 3522 cm^{-1} was attributed to OH absorption (Sigma, 2012). An important characteristic peak of carbonate was identified at 2997 cm^{-1} (Wang et al., 2007) for commercial CaCO_3 and CaCO_3 synthesized from the shells demineralization extract. The other functional groups, represented by the smaller absorption bands in both the commercial CaCO_3 and CaCO_3 powder, were carboxylic acid (2650 cm^{-1}) and amide (2423 cm^{-1}). The FTIR results also showed the absorption peak of calcite at 895 cm^{-1} for synthesized CaCO_3 . These are in agreement with reported findings of Choi and Kim, (2000) where it was observed that absorption peaks of calcite was at 875 cm^{-1} and the absorption peaks of aragonite were at 667 cm^{-1} and 857 cm^{-1} of CO_3^{-2} .

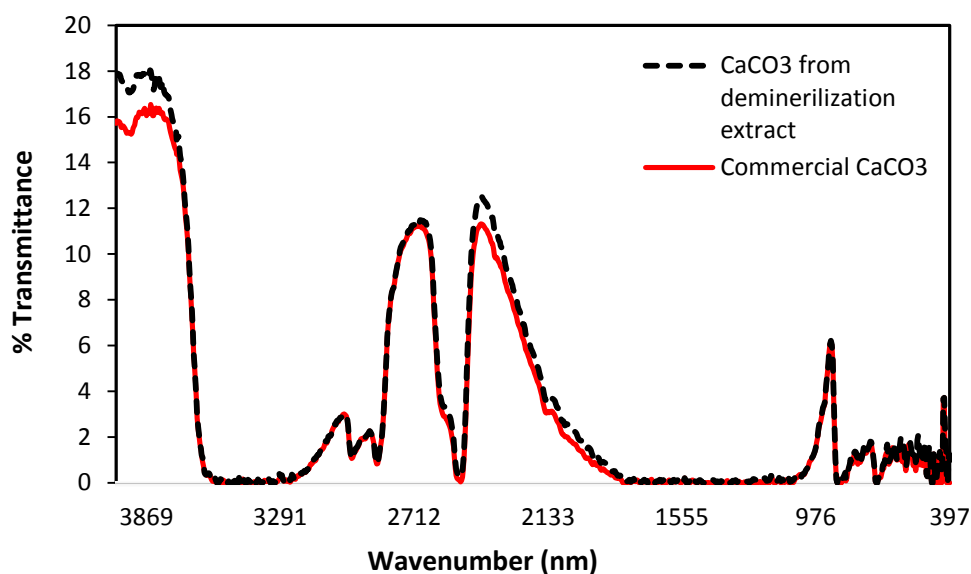


Fig. 3.24 FTIR spectra of CaCO_3 produced from shrimp shells in comparison to commercial CaCO_3 .

The industrial applications of CaCO_3 are broad ranging, including paints, paper, plastics, inks, feedstuff, medicines, rubbers and adhesives (Xiang et al., 2004). There is ample opportunity for developing this beneficial natural mineral substance and its polymorphs from inexpensive mineral resources of shrimp shells and can be applied in many fields, including medicine, bone biomaterials and industry.

3.6 DEVELOPMENT OF EDIBLE FILMS

Chitosan based films were successfully produced from viscous solutions of chitosan dissolved in 1% acetic organic acid. CH was blended with GLY, PEG 200 and PEG 600 and the interactions between these biopolymers was established, showing different physicochemical properties compared to CH films. At a macroscopic scale, all films were homogeneous and showed smooth surfaces. The incorporation of plasticizers increased the film thickness and flexibility than those prepared from CH solution alone, however this addition greatly reduced the TS of the films. Film transparency was also reduced with the addition of plasticizers. The blended films tended towards a more hydrophilic behaviour than the CH films. Crystallinity was highly dependent on chemical composition of films, which had a critical influence on the properties of the resulting films. The structural

properties of CH blended films determined by TGA, FTIR and XRD analyses further showed weak compatibility between both polymers proving CH to have superior properties and adequate for packaging purposes.

3.6.1 Film appearance and thickness

The CH films (Fig. 3.25) (A) were transparent and were easiest to peel off from the Petri dishes. These films dried within 24 hours whilst the other film blends took over 72 h. All plasticizers were added at a 0.04 weight ratio. CH films (A) were the most transparent followed by CH/GLY films (B). The films containing PEG (C, D) appeared opaque and brittle. Since film transparency is crucial, especially in food application, we can assume that the films with PEG content may not be suitable.

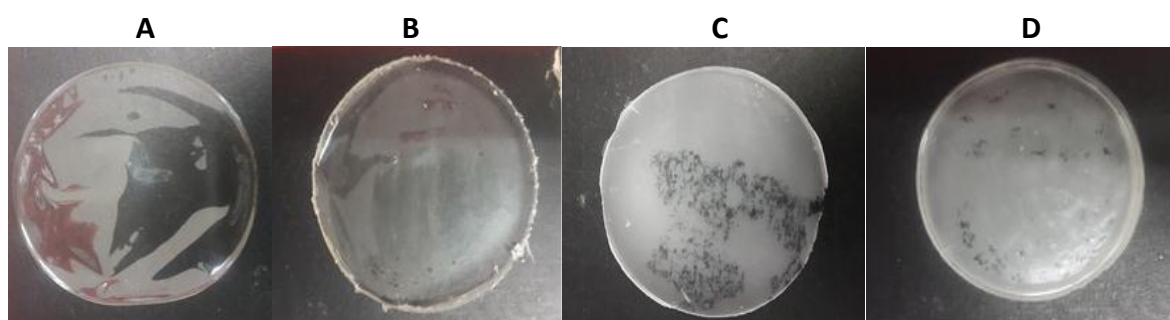


Fig. 3.25 Images of chitosan film (A) and chitosan blends with plasticizers (B) CH/GLY (C) CH/PEG 200 and (D) CH/PEG 600 added at a 0.04 weight ratio.

All films were measured for thickness and the findings are shown in Table 3.4. Film thickness was dependent upon the film's composition and nature. This observation was consistent with that of Di Pierro et al. (2006) and Sebti et al. (2007) who found a dependent affiliation between film thickness and the nature and content of the film-forming polymer. The measured thickness for CH films was 0.05 mm. The incorporation of plasticizers on the CH films resulted in a significant increase in film thickness ranging from 0.33 to 0.38 mm. The CH/GLY film exhibited a thickness of 0.38 mm. These results support those reported by Sebti et al. (2007).

Table 3.4 Thickness (mm), elongation at break (%), tensile strength (MPa), and film solubility of chitosan and chitosan blended films

Film composition	Thickness (mm)	Tensile strength (MPa)	Elongation at break (%)	Film solubility (%)
Chitosan	0.050± 0.002	0.170± 0.002	15	10± 1.087
CH/GLY	0.380± 0.002	7.757± 0.002	2.1	52± 2.665
CH/PEG 200	0.330± 0.005	55.810± 0.008	10.09	41± 0.402
CH/PEG 600	0.350± 0.001	13.335± 0.002	/	10± 1.958

*plasticizers were added at a 0.04 weight ratio

/ result inconclusive

3.6.2 Mechanical properties of films

Adequate mechanical elasticity and strength are generally necessary for a packaging film to maintain its integrity and withstand external stress similar to barrier properties during packaging applications (Rao et al., 2010). Tensile strength (TS) is a measure of the energy needed to break the film by stretching and the elongation at break is the extent the film can be stretched. Both parameters for the films developed in this study are listed in Table 3.6. The CH/GLY films (7.757 MPa) and CH films (0.17 MPa) had the lowest TS values compared to the PEG blended CH films. The TS value of CH film was much lower than that reported by Srinivasa et al. (2007) (11.58 MPa). This could be due to a number of factors such as chitosan source and composition, film preparation and storage (Sánchez-González et al., 2009). The authors further reported a TS value of 4.14 MPa for chitosan films with glycerol as a plasticizer, which was half the value obtained in this study (7.757MPa). CH/PEG 200 had the highest TS value of 55.81 MPa. The PEG seemingly led the CH films to exhibit high inorganic characters such as being brittle making the %E low. CH films however had the highest elongation at break (15%). This was also reported by Perez-Gago et al., (2002) where chitosan films showed a higher elongation at break than the blended films, albeit at a much higher level (69.84%). As soon as glycerol decreases chitosan chain intermolecular attractions, polymer mobility increases and consequently the films' elongation (Perez-Gago et al., 2002). Overall, the tensile strength increased with the addition of plasticizers and the

opposite was true for the elongation at break. This effect may be ascribed to the heterogeneity introduced into the film structure and the negative effect on the cohesion forces of the CH matrix by the addition of GLY and PEG (Perez-Gago et al., 2002). The low TS of CH film reflects the plastics stiffness whilst the TS increased upon the incorporation of plasticizers into the CH matrix. These observations strongly suggest that the presence of GLY and PEG in the film significantly enhanced the flexibility and elasticity compared to the corresponding films made from CH alone, however, they did not improve the mechanical properties of chitosan films as expected since the elongation break was considerably low. We can conclude that the CH films though stiff are much stronger and tougher which is appropriate for applications in food packaging. The addition of PEG led to formation of hydrogen bonds between CH and PEG which crosslinks the blend. The poly blend may have not been compatible due to the nonattractive intermolecular interactions. This is also suggested by the SEM micrographs of this film blend (Zeng et al., 2004).

3.6.3 Water solubility

Solubility of films is a measure of water resistance and it also provides insight on the nature of the film in an aqueous environment. Of all the tested films, CH/GLY exhibited the highest percentage of solubility (52%, Table 3.4), similar to the value of 54.75% reported by (López-Mata et al., 2013). This high solubility may be due to glycerol's water binding capacity and chitosan's functional groups (Ojagh et al., 2010). Second to CH/GLY, CH/PEG 200 also had a high solubility of 41%. These observations for both films correlates with the FTIR results (Fig. 3.27). The shift in the peak ascribed to C-O-H group when plasticizers were incorporated to the film can be related to the quantity of the water molecules absorbed in the chitosan film. Moreover, the acetic acid used to solubilize chitosan has been reported to increase its protonation, thus increasing their affinity for water, as soon as the film is formed (Casariego et al., 2009). The lowest water solubility was observed for CH films (10%) and the film was not affected by the incorporation of PEG 200, maintaining the 10% water solubility. Chitosan is soluble in dilute aqueous acidic solutions ($\text{pH} < 6.5$) however, it is insoluble in water, whilst PEG polymer is water soluble. Even though all tested films maintained the original shape after 24 h immersion in water at room temperature, the blended films dissolved most

easily. High water resistance of plastic films is desirable for many industrial applications and CH films displayed superior properties than the blended films.

3.6.4 Optical properties of films

The optical properties of tested films are displayed in Table 3.5. CH and CH/GLY films showed a tendency towards lightness (L^*) while the CH/PEG 200 and CH/PEG 200 films were opaque. The incorporation of glycerol and PEG 200 and 600 in the chitosan based films showed no significant effect on the lightness of the films ($p < 0.05$), however the slight decrease in L^* suggested a decrease in the transparency for both CH/PEG 200 and CH/PEG 600 films. This is apparent in the appearance of the films (Fig. 3.24). The CH/PEG 600 blend showed a tendency toward greenness (a^*). The value of b^* (representing yellow-blue) on the CH/PEG 200 blend indicated an increase of the yellowness of the film. The CH/PEG 600 blend showed a slight tendency toward greenness (a^*).

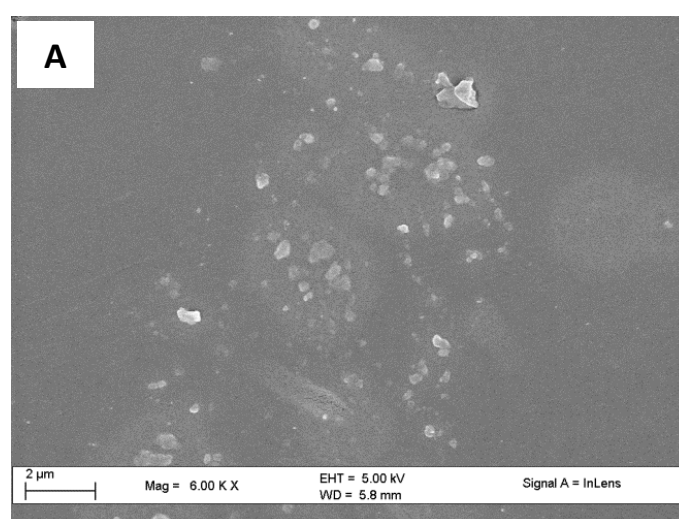
Table 3.5 Optical properties of chitosan and chitosan blended films

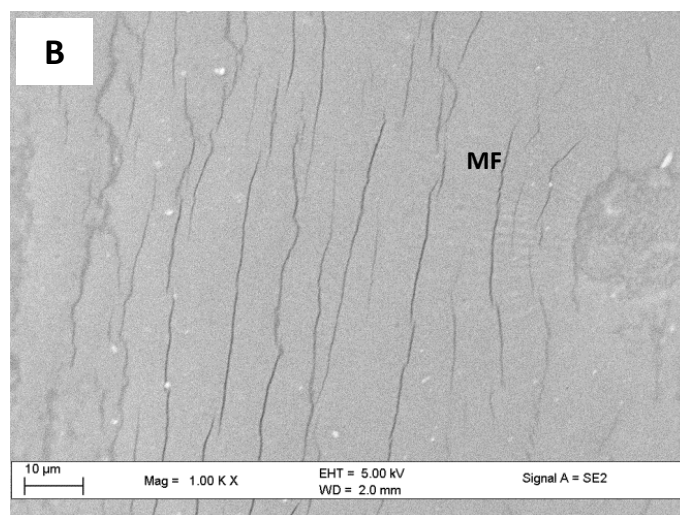
Colour	Edible films			
	CH	CH/GLY	CH/PEG 200	CH/PEG 600
L^*	91.4±0.4	91.0±0.2	90.6±0.0	90.0±0.0
a^*	-0.9±0.0	-1.0±0.0	-1.3±0.1	-1.8±0.1
b^*	5.7±0.5	4.3±0.0	12.71±0.6	5.7±0.4

3.6.5 Characterisation of film microstructure

The micrographs of the films obtained from scanning electron microscope are illustrated in Fig. 3.26. Images of the superficial arrangement of the CH film (Fig. 3.26A) revealed that the film was continuous, compact, and nonporous with irregularly shaped folds. These findings were comparable to those of Bhuvaneshwari et al. (2003) whose analysis revealed that pure chitosan film was nonporous and the texture plain without pores. A nonporous CH

morphology has been reported (Casariego et al., 2009). Other authors have however confirmed the presence of evenly distributed pores on the film surface (Pereda et al., 2011). This phenomenon is typically contingent on the nature of the chitosan used (molecular weight and degree of deacetylation). From the micrograph, however, a different superficial structure is revealed after the incorporation of glycerol into the chitosan blend (Fig. 3.26B). The film surface was modified in that randomly distributed microstructure spaces that looked like cracks were evident. The addition of PEG intensified the film's heterogeneity, giving the material surface heterogeneous, rough, irregular surfaces in character, with discontinuances associated with the formation of two phases in the matrix (Fig. 3.26C) (Sánchez-González et al., 2009). This suggested that the PEG and the chitosan film particles may have failed to completely homogenize, suggesting low compatibility between the components. The CH/PEG 600 film however displayed more pronounced scattered and larger patch like holes. It was clear that the addition of PEG significantly altered the morphologies of the films suggesting weak physical interaction between active sites in the film constituents. The addition of PEG did not enhance the smoothness of the films as reported by Mahatmanti et al. (2014), whose findings established that the incorporation of PEG causes a reduction of the pores in chitosan-silica films by virtue of the PEG being physically trapped on the solid surface. In a related effort, Zeng et al., (2004) confirmed our findings by proposing that the pore size increases with the addition of crosslinking agents.





*MF, microfractures

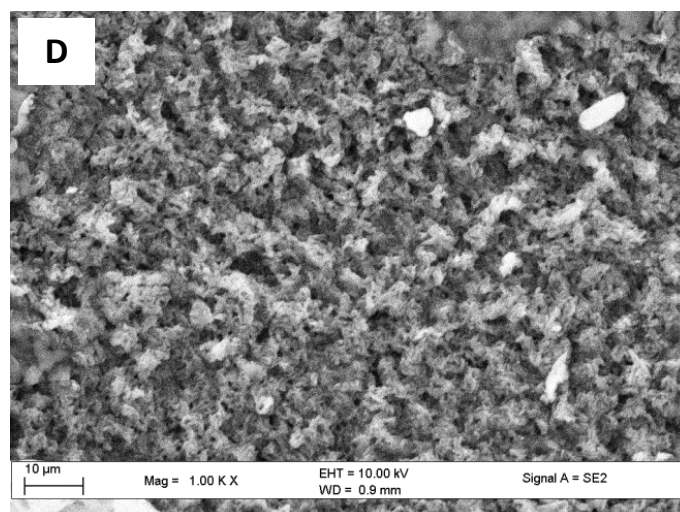
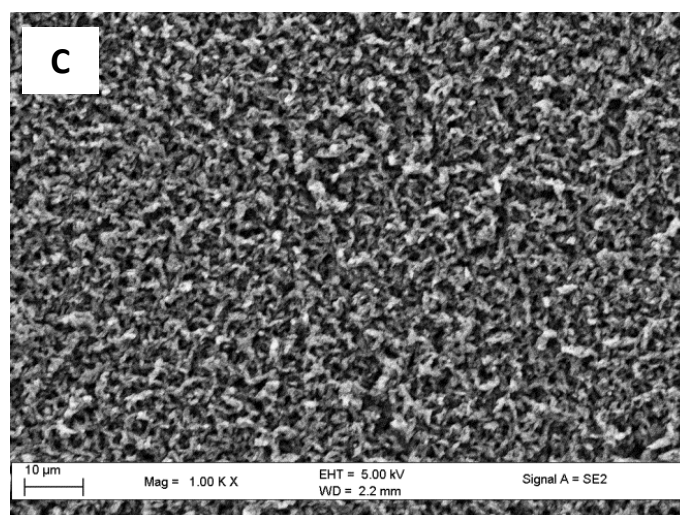


Fig. 3.26 SEM micrograms of chitosan films and blends. CH (A), CH/GLY (B), CH/PEG 200 (C) and CH/PEG 600 (D).

3.6.6 Fourier transform infrared spectroscopy of films

FTIR spectra of CH, CH/GLY, CH/PEG 200 and CH/PEG 600 showed broad absorption bands between 3478 and 2529 cm^{-1} for all films, indicative of the stretching vibration of O-H bond (Fig. 3.27). The absorption peaks at 1659 cm^{-1} and 1595 cm^{-1} common to all films are related to the CH amide I and amide II bands, respectively. The assignment of these absorption bands are in agreement with those reported for chitosan (Pastor et al., 2004). The peak at 1148 cm^{-1} for chitosan corresponds to the symmetric stretching of the C-O-C bond.

When two or more polymers were mixed, the characteristic spectra peaks of the blends should be considerably different from the spectra of each component due to the chemical interactions, resulting in band shifts, intensity changes and broadening (Xiao et al., 2003). The characteristic peak at 1550 cm^{-1} for CH/PEG 200 blend film was ascribed as $-\text{NH}_2$ group, which decreased after the incorporation of PEG 200. This observation suggested that the hydrogen-bonding interactions between hydroxyl groups and amino groups of chitosan become weak (Zhang et al., 2009). A significant shift is observed in the absorption bands indicative of O-H vibration for the blended films were much broader than that of chitosan, the absorption band at 3524 cm^{-1} shifted to higher frequencies at 3663 cm^{-1} , 3657 cm^{-1} and 3611 cm^{-1} for CH/GLY, CH/PEG 200 and CH/PEG 600, respectively. Furthermore, their intensity decreased with the added plasticizers, indicating that parts of the H₂ bonds in chitosan were broken thereafter. Both the amide I and amide II bands moved to higher wavenumbers for CH/GLY films from 1635 cm^{-1} to 1657 cm^{-1} , and for amide II band from 1550 cm^{-1} to 1552 cm^{-1} . The peak intensities however, including those of CH/PEG 200 and CH/PEG 600 films were significantly reduced almost to the point of negligibility. Since amide I and II bands represented the structure of GlcNAc, the results suggested that the non-deacetylated groups in chitosan were forming more with the hydroxyl bonds with PEG than with the hydrogen groups (Abugoch et al., 2011). The interaction between PEG and $-\text{NH}_2$ groups of chitosan could not be recognized by the present results. FTIR analysis suggested the existence of hydrogen bonding interactions between PEG polymers and chitosan. The broad band at approximately 1029 cm^{-1} for PE may be assigned to a contribution of different groups such as out-of-plane C-H bending (from aromatic structures) (Schmidt et al., 2005, Su et al., 2002).

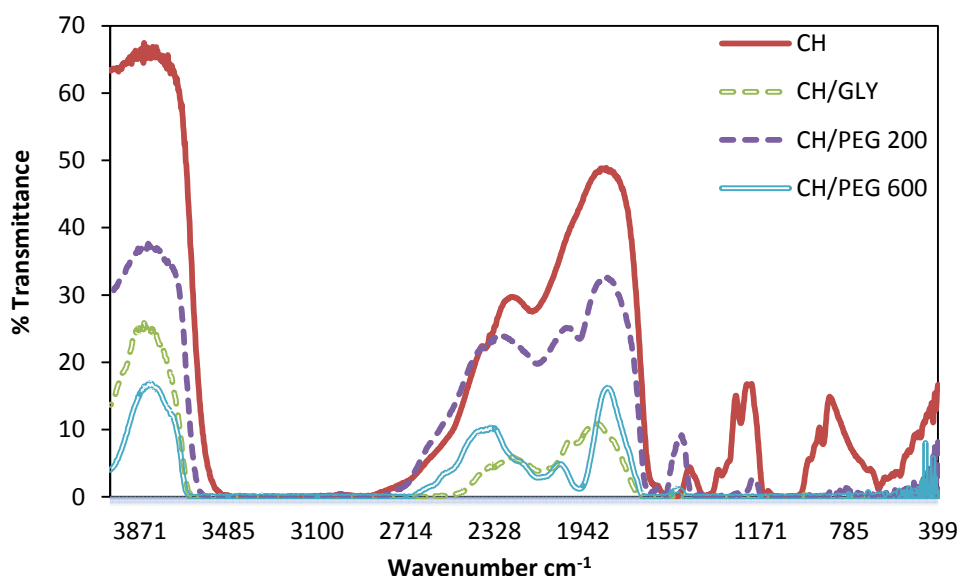


Fig. 3.27 FTIR spectra of chitosan films and blends.

3.6.7 Thermogravimetric analysis of films

Thermogravimetric properties were determined by analysing changes in weight of films with increase in temperature. Fig. 3.28 shows two stages of decomposition for CH films and CH/PEG 600 blend films. The first stage for CH films begins at 45°C with an 11% weight loss and the second stage starts at 270°C and arrives at a maximum at 380°C with a weight loss of 27°C. For CH/GLY blend films, trinomial degradation peaks were observed. The first peak began at 45°C to 75°C, losing 19% of its initial weight, the second and third stages at 140°C and 240°C accounted for 27.1 and 17.86% weight loss, respectively. This was probably due to degradation of glycerol, since the flashpoint of glycerol is around 180°C, and nitrogen gas environments mass loss ensues at 199°C (Castelló and Dweck, 2009). CH/PEG 600 blend films lost 8% weight at 35°C to 65°C, followed by a 46.52% weight loss between 270 and 415°C. This weight loss for PEG blended films is credited to the decomposition of the polymeric network (Ma et al., 2009; Neto et al., 2005). For CH/PEG 200, the first peak at 35°C was the initial decomposition accompanied by a 14.99% weight loss. The second and third peaks at 170°C and 270°C account for 44.64% and 16.44% weight loss, respectively. All three plasticizers significantly affected weight loss of plastics with CH/PEG 200 and CH/PEG

600 having the lowest degradation onset temperatures. This was most likely due to moisture increase in the plastic leading to destructure. Upon the incorporation of PEG, the intra-molecular interactions between the terminal hydroxyl groups of PEG predominate, inhibiting the attractive bonds. The PEG blended films become incompatible and consequently their thermal stability is lesser than that of chitosan. This observation was contrary to that of Reiad et al. (2012) who found that PEG blended chitosan had higher thermal stability than that of chitosan, but decreases if mass ratio of chitosan to PEG is too high.

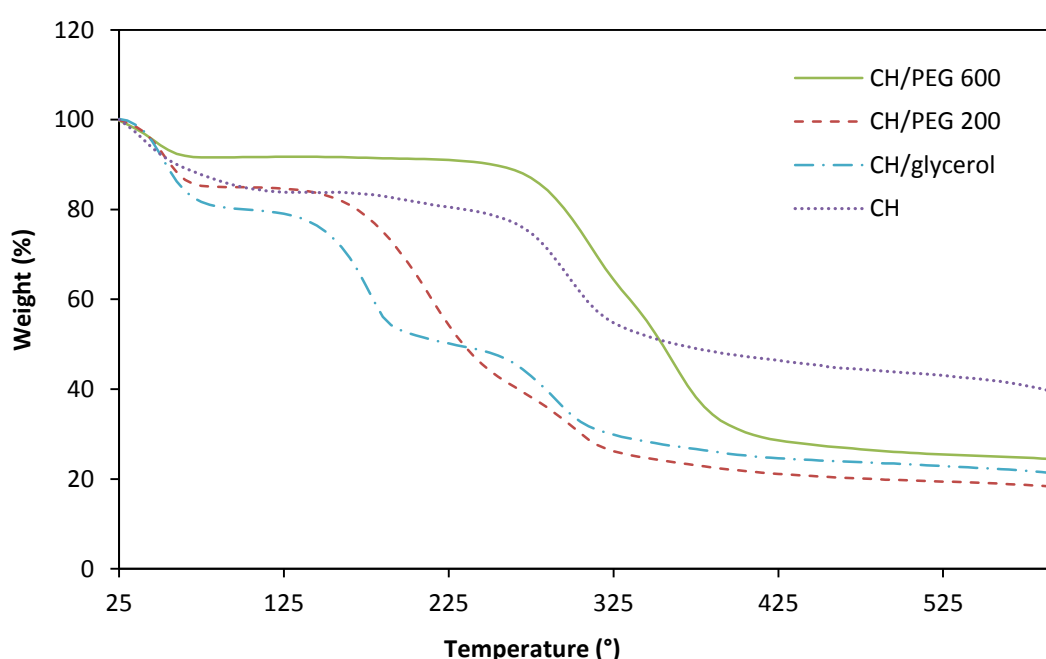


Fig. 3.28 TGA thermograms of chitosan films and blends.

3.6.8 X-ray diffraction

CH/GLY showed well-defined and strong characteristic peaks at 16.4° , 19.9° and 30° (Fig. 3.28) while CH/PEG 600 film blend showed spectral reflections at 19.6° , 23.7° and 29.7° 2θ . These diffractograms were characteristic of a semi-crystalline nature indicating that intermolecular interactions between chitosan and CH/PEG 600 exist, implicating positive compatibility between the two polymers. The intensification of spectral peaks of CH/PEG 600 blend film at 23.7° and 29.7° 2θ also indicate the existence of intermolecular

interactions between PE 600 and CH. CH/PEG 200 exhibited a broad peak between 20° to 35° 2θ , suggesting an amorphous nature of the film. This can be ascribed to the fact that chitosan content in the blends limited the growth of crystallinity. The stiff molecular chitosan chains altered the overall movements in the blend and hindered the crystallisation process (Zhao et al., 1995). The spectral peaks for chitosan films were, however, less intense, broader and flatter, suggesting that chitosan had a predominantly amorphous form in the films. The spectral peaks at 2θ 9.7° , 13.2° and 21.2° were consistent with previously published results (Abugoch et al., 2011). The diffraction peak at 2θ 21.2° is the typical fingerprint for chitosan films (Zhong et al., 2011) and was observed in all films (Fig. 3.28). This diffraction peak at around 20° is reported for chitosan films prepared using acetic acid as a solvent (Bangyekan et al., 2006). The peak at around 13° was assigned to chitosan anhydrous crystal.

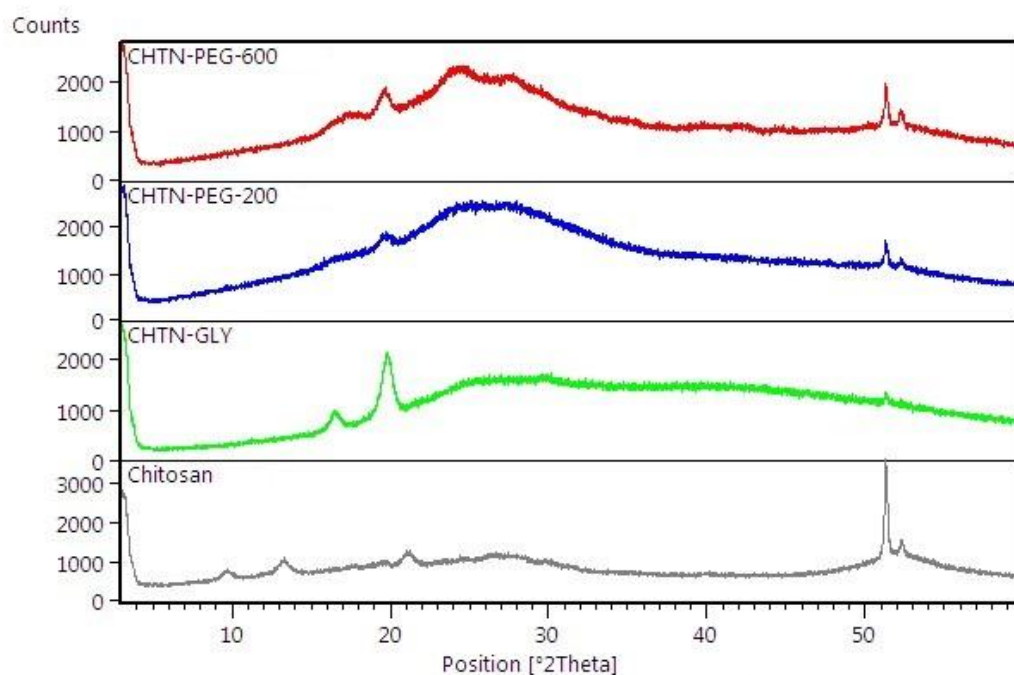


Fig. 3.29 X-ray diffraction patterns of chitosan film and CH/GLY, CH/PEG 200 and CH/PEG 600 blend films.

4. CONCLUSIONS

Many countries rely on the seafood industry as the major source of income. 65% of shrimp is edible and the remaining percentage is considered inedible waste (exoskeleton and cephalothorax) and is therefore discarded. The efficient consumption of resources from crustacean biomass not only functions in converting food wastes into valuable products, but has also developed into an environmental priority (Rudrapatnam and Farooqahmed, 2003). Therefore it is necessary to exploit valuable biopolymers and recover the by-products thereof (Ferrer et al., 1996). In this study, chitin and chitosan were produced by chemical treatments of shrimp shells. This research has revealed that varied chitosan possessing antibacterial activity can be generated and high antibacterial activity is characteristic of chitosan. This suggests the possibility of manipulating chitosan as an effective bacterial inhibitor and possibly fungi upon further research. This research puts forth opportunities for developing valuable products by processing the crustacean by-products with possible biological activity. Chitosan is approved in both Japan and Korea as an additive in food since 1983 and 1995, respectively (Weinhold et al., 2009). The need for using chitosan possessing elevated antibacterial activity is evident especially as a preservative to avoid health risks associated with consuming foods contaminated with bacterial pathogens or to prolong food shelf-life by suppressing spoilage bacteria from growing (Cho et al., 1998). The effectiveness of chitosan as a natural preservative can be enhanced upon its addition on acidic foods; this is implicated by its increased antibacterial activity at lower pH, however, before application in foods, further research on other factors that remarkably influence effectiveness as an inhibitor is required; such factors include storage temperature of food, degree of contamination and water activity (No et al., 2002).

As a result of desirable protective features displayed in the antibacterial activity tests, we can conclude that chitin and chitosan are natural biopolymers that can be employed in the food industry. The future of bioactive by-product research is very bright especially with the enormous expanding market worldwide. The application versatility of both chitin and chitosan increases in being a great challenge to the scientific community and various industries. The availability of highly-pure commercial forms of both polymers and the continued arrival of new derivatives of chitin/chitosan with more effective and unique

properties has prompted unlimited efforts on R&D of these multifaceted amino polysaccharides. It can be anticipated that, in the near future, additional specialized applications will be developed.

4.2 Recommendations for future work

Based on the results obtained in this study, the following should be addressed in future investigations:

- I. Investigation of the efficacy of antimicrobial activity of edible films on fruits and vegetables.
- II. Antioxidant activity of astaxanthin should be quantified and investigated for possible applications in industry.
- III. How bacteriostatic effects of chitosan can be enhanced against gram-negative bacteria.

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