CHAPTER 2

LITERATURE REVIEWS

2.1 Crab shell

Fish info network market reported in 2007 that world fisheries and aquaculture production of crab increased from 407,800 tons in 1970 to more than 2 million tons in 2005. The main crab fishing countries are China, the United States and Canada, the three of them producing more than 70 % of world crab production altogether. World crab imports totaled more than 400,000 tons, equivalent to nearly 3 billion US dollars in 2005. The top world crab markets are the United States and Japan, whose imports account for circa 60 % of world crab trade (Fish info network, 2007).

Crabs products are 6.11% of all export products from Thailand and exported crabs product in 2007 is 13,234 tons. Crab products from Thailand are whole frozen, as frozen sections, and pasteurized crabmeat (Fish info network, 2007). In the preserved segment which includes crab in cans, pouches and jars, French imports increased by 17% to over 4,100 tones for the eleven month period last year. The value increase was significantly less at just 4% suggesting a volume boost based on cheaper euro import prices. The strongest increase was from Thailand, French imports increasing by 50% to 1,200 tons. Thailand share has increased over the 2006 – 2007 period from 23 to 29% (O'Sullivan, 2008). Figure 2.1 shows monthly crab products import of USA in June 2011.

Since the biodegradation of chitin is very slow in crustacean shell waste, accumulation of large quantities of discards from processing of crustaceans has become a major concern in the seafood processing industry. Out of the total solid waste landing in the USA, 50±90% is from shellfish processing discards, the total global annual estimates of it is around 5,118,000 tons. Out of the different species of crustaceans, shrimp and crab shell wastes have been widely used for the isolation of chitin (Fereidoon *et al.*, 1999).

Table 2.1 Monthly crab products import of USA (In June 2011)

Country	Import product	
	Kilos	**Dollars**
Argentina	128,305	2,088,639
Canada	7,879,144	99,921,559
Chile	134,638	1,575,516
China	710,332	7,551,223
Colombia	23,433	394,683
Dominican Republic	735	4,303
Ecuador	5,111	74,688
Greenland	57,558	714,569
India	123,673	2,008,8884
Indonesia	898,442	19,425,854
Italy	2,470	26,807
Japan	2,096	33,268
Malaysia	14,726	153,522
Maxico	210,173	2,716,946
Nicaragua	12,942	52,190
Peru	1,588	30,052
Philippines	159,110	3,376,375
Russian Federation	645,613	12,475,923
Singapore	1,620	11,340
South Korea	70,088	551,188
Sri Lanka	36,076	596,376
Sweden	691	5,505
Thailand	**207,853**	**2,511,951**
Venezuela	298,709	3,125,188
Viet Nam	179,428	2,819,473
Grand Total: June 2011	**11,804,524**	**162,246,022**

Source: http://www.aquafind.com (2 October 2011)

Crustacean shell waste consists of protein (20-40%), calcium and magnesium salts mainly carbonate and phosphate (30-60%), chitin (20-30%) and lipid (0-14%). These propotion vary with species and season (Goycoolea *et al.*, 2000; Seo *et al.*, 2006). Utilization of marine crustacean waste as substrate and optimal condition was show in Table 2.2.

Table 2.2 Utilization of marine crustacean waste as substrate and optimal condition

Substrate	Optimal condition	yield	Reference
Crustacean waste	30°C for 3 days in 100 ml of medium (pH 6) containing 7% shrimp and crab shell powder, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.0% arabinose, 1.5% $NaNO_3$, and 1.5% $CaCl_2$.	Protease activity 20.2 U/ml	Yang *et al.*, 2000
Shellfish waste	37°C for 7 days in medium containing 5 g of 10% lactic acid-treated crab shell, 0.028% KH_2PO_4,0.007% $CaCl_2 2H_2O$, 0.025% $MgSO_4 7H_2O$ and 0.3% peptone	Hydrolyzed chitin at least 33% with chitinolytic enzyme	Rattanakit *et al.*, 2003
Shellfish chitin waste	37°C pH 6 for 1 day	Chitinase activity 5.6 U/ml 100% inhibit the grow of *Fusarium oxysporum*	Wang *et al.*, 2006[b]
Shellfish waste	Shrimp waste 14 g/l, $FeCl_3$ 0.035 g/l, $ZnSO_4$ 0.065 g/l, and pH 8	Protease activity 129.02 ± 2.03 U/ml protein removal was 85.12 ± 4.7%	Singh and Chhatpar, 2010
Shrimp and crab shell waste	25°C for 2 days in medium (pH 8) containing 5 g of 5% shrimp and crab shell powder, 1% lactose, 0.5% NH_4NO_3, 0.1% K_2HPO_4, 0.007% $CaCl_2 2H_2O$, 0.5% $MgSO_4$ and 0.5% $FeSO_4 7H_2O$ initial	Protease activity 2.2 U/ml protein removal was 72%	Oh *et al.*, 2000

Table 2.2 (Cont.) Utilization of marine crustacean waste as substrate and optimal condition

Substrate	Optimal condition	yield	Reference
Squid pen	37°C for 3 days in 100 ml of medium (pH 7) containing 0.1% K_2HPO_4, 0.05% $MgSO_47H_2O$, and 1% squid pen powder	The protease retains 21% and 91% activity in the presence of Tween 20 (2% w/v) and SDS (2 mM), respectively	Wang *et al.*, 2008
Squid pen	30°C for 4 days in 100 ml of medium (pH 6) containing 2% squid pen powder, 0.1% K_2HPO_4, and 0.5% $MgSO_4$	Metalloprotease activity 0.065 U/ml protein removal was 73%	Wang *et al.*, 2006[a]
Red crab shell	30°C for 7 days in MRS medium (*Lactobacillus paracasei* subsp. *tolerans* KCTC-3074) and LB medium (*Serratia marcescens* FS-3)	Deproteinization was 52.6%	Jung *et al.*, 2006
Crab shell waste	Highest protease activity after 3 days and protein removal after 7 days	Protease activity 60.7 U/ml protein removal was 84%	Jung *et al.*, 2008
Red crab shell waste	-	demineralization rate increased to 97.62% deproteinization rate increased to 67.49%	Jung *et al.*, 2007
Shrimp waste	shrimp wastes powder 30 (g/l), KCl 1.5 (g/l), K_2HPO_4 0.5(g/l), and KH_2PO_4 0.5 (g/l). 3 hours hydrolysis at 60°C	The percent of protein removal at an enzyme/ substrate (E/S) ratio of 0.5 and 5 (Unit of enzyme/mg of protein) were about 68 and 81%, respectively	Hadder *et al.*, 2011

2.2 Chitin

Chitin can be found in a variety of species in both the animal and plant kingdoms (Figure 2.2). The traditional source of chitin is shellfish waste from shrimp, Antarctic Krill, crab and lobster processing. It is present in amounts varying from trace quantities up to about 40% of the body weight of the organism. The crustacean waste is the most important chitin source for commercial use due to its high chitin content and ready availability. However, chitin present in the crustacean waste is associated with proteins, minerals (mainly calcium carbonate) and lipids including pigments (Khanafari *et al.*, 2008). In Figure 2.1 show characteristic of chitin powder.

Figure 2.1 Chitin powder.
Source: www.CNN.com (4 February1999)

Chitin was discovered in 1811 by Branonnot who called its fungine. The name chitin was conferred by Odier in 1823 "for what has subsequently been found to be the same compound in insects". In early 1843 Payan had noted that contains nitrogen. Later publications of Ledderhose in 1876 reported obtaining a crystalline compound he named glucosamine; he also collected acetic acid but failed to draw the inference that acetyl might have been linked to the amino group *in situ* (Mattheus, 1997).

The chemical structure of chitin is described as a straight homopolymer composed of ß-1,4-linked N-acetylglucosamine units, with a three-dimensional α-helical configuration stabilised by intramolecular hydrogen bonding (Oh *et al.*, 2000; Tsigos *et al.*, 2000; Yang *et al.*, 2000; Wang *et al.*, 2002; Vânia *et al.*, 2003; Wang *et al.*, 2006; Franca *et al.*, 2008). The structure of chitin, chitosan and cellulose are shown in Figure 2.2.

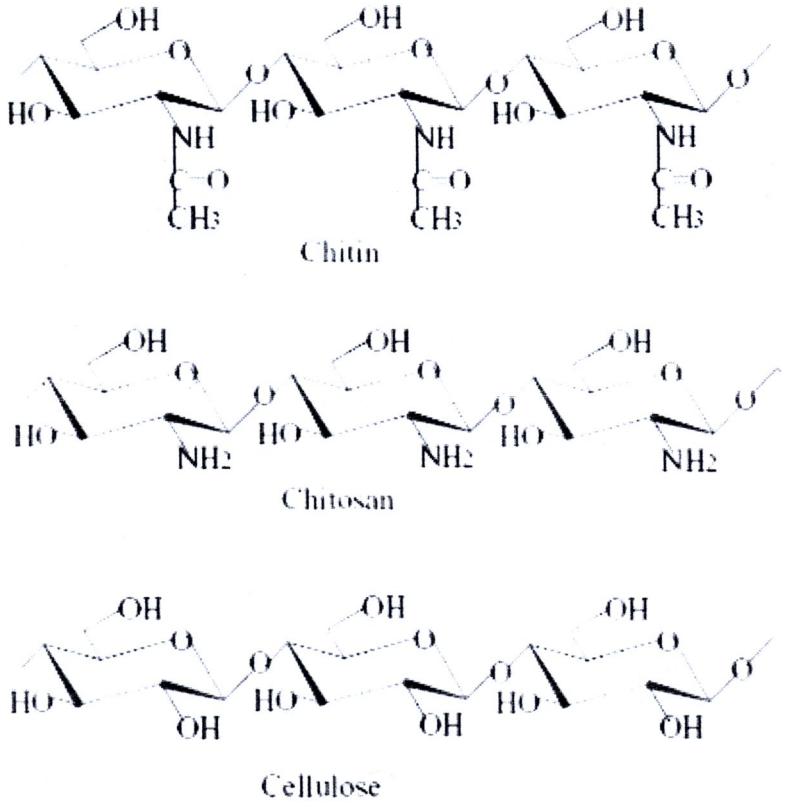

Figure 2.2 Structure of chitin, chitosan and cellulose (Barbara, 2004).

Depending on its source, chitin occurs as two allomorphs, namely the α and β forms, which can be differentiated by infrared and solid-state NMR spectroscopy together with X-ray diffraction. A third allomorph γ-chitin has also been described but from a detailed analysis, it seems that it is just a variant of the a family. α-Chitin is by far the most abundant; it occurs in fungal and yeast cell walls, in krill, in lobster and crab tendons and shells, and in shrimp shells, as well as in insect cuticle. It is also found in or produced by various marine living organisms. In this respect, one can cite the harpoons of cone snails the oral grasping spine of *Sagitta* and the filaments ejected by the seaweed *Phaeocystis*, etc. These exotic α-chitins have proved particularly interesting for structural studies since, in comparison with the abundant arthropod chitin, some of them present remarkably high crystallinity together with high purity (they are synthesized in the absence of pigment, protein, or calcite). In addition to the

native chitin, α-chitin systematically results from recrystallization from solution in vitro biosynthesis or enzymatic polymerization (Rinaudo, 2006).

Chitin is one of the most important biopolymers in nature (Table 2.3). It is mainly produced by fungi, arthropods and nematodes. In insects, it functions as scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium. Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures. For this purpose, insects repeatedly produce chitin synthases and chitinolytic enzymes in different tissues. Coordination of chitin synthesis and its degradation requires strict control of the participating enzymes during development (Hans and Lars, 2003).

Table 2.3 Sources of chitin and chitosan

Sea animals	Insects	Microorganisms
Annelida	Scorpions	Green algae
Mollusca	Spiders	Yeast (b-type)
Coelenterata	Brachiopods	Fungi (cell walls)
Crustaceans:	Ants	Mycelia Penicillium
Lobster	Cockroaches	Brown algae
Crab	Beetles	Spores
Shrimp		Chytridiaceae
Prawn		Ascomydes
Krill		Blastocladiaceae

Source: Mathur and Narang, 1990

2.2.1 Chitin in solid state

Crystallography of chitin

Natural chitin has three anhydrous crystalline polymorphs, α-, β-, γ-chitins, in the native state, which is found in the skeletal structure of crustaceans, insects, mushrooms, and the cell wall of fungi. The structure of α and β forms differ only in that the piles of chains are arranged alternately antiparalel in a α-chitin, whereas they

are all parallel in β-chitin. The γ-chitin form has characteristics of both α and β forms, where two chains run in one direction and another chain in the opposite direction; however, it is considered only a variant of the α family, because it has the same properties as the α-chitin. α- Chitin is the most abundant and also the most stable thermodynamically and the β- and γ-chitin forms can be irreversibly converted into the α-form. Chitosan has anhydrous and hydrated forms, with piles chains arranged in an antiparallel fashion (Franca et al., 2008)

At first glance the powder X-ray diagrams of chitins from shrimp shell (α-chitin) and anhydrous squid pen (β-chitin) appear nearly the same, but in a refined analysis, they can be differentiated in two ways: (i) a strong diffraction ring, often quoted as the α-chitin signature is found at 0.338 nm whereas a similar ring occurs at 0.324 nm in β-chitin; (ii) an inner ring at 0.918nm in β-chitin is sensitive to hydration, moving to 1.16 nm in the presence of liquid water, whereas a similar strong inner ring at 0.943 nm in α-chitin is insensitive to hydration (Rinaudo, 2006).

2.2.2 Soluble chitin and characterization

Chitin occurs naturally partially deacetylated (with a low content of glucosamine units), depending on the source; nevertheless, both α and β forms are insoluble in all the usual solvents, despite natural variations in crystallinity. The insolubility is a major problem that confronts the development of processing and uses of chitin. An important mechanism previously mentioned is that a solid-state transformation of β-chitin into α-chitin occurs by treatment with strong aqueous HCl (over 7 M) and washing with water. In addition, β-chitin is more reactive than the α form, an important property in regard to enzymatic and chemical transformations of chitin (Rinaudo, 2006).

Because of the solubility problem, only limited information is available on the physical properties of chitin in solution. The first well-developed study was by Austin, who introduced the solubility parameters for chitin in various solvents. He obtained a complex between chitin and LiCl (which is coordinated with the acetyl carbonyl group). The complex is soluble in dimethylacetamide and in N-methyl-2-pyrrolidone. We recall that the same solvents and, especially, LiCl/DMAc mixtures, are also solvents for cellulose, another β (1-4) glucan. In addition, Austin also used

formic, dichloroacetic and trichloroacetic acids for dissolution of chitin chains (Rinaudo, 2006).

2.2.3 Chitin derivative

The most important derivative of chitin is chitosan, obtained by (partial) deacetylation of chitin in the solid state under alkaline conditions (concentrated NaOH) or by enzymatic hydrolysis in the presence of a chitin deacetylase. Because of the semicrystalline morphology of chitin, chitosans obtained by a solid-state reaction have a heterogeneous distribution of acetyl groups along the chains. In addition, it has been demonstrated that β-chitin exhibits much higher reactivity in deacetylation than α-chitin. The influence of this distribution was examined by Aiba (1991), who showed that the distribution, random or blockwise, is very important in controlling solution properties. Reacetylation, up to 51%, of a highly deacetylated chitin in the presence of acetic anhydride gives a water soluble derivative, whereas a heterogeneous product obtained by partial deacetylation of chitin is soluble only under acidic conditions, or even insoluble. It was demonstrated from NMR measurements that the distribution of acetyl groupsmust be random to achieve the higher water solubility around 50% acetylation (Rinaudo, 2006).

Homogeneously deacetylated samples were obtained recently by alkaline treatment of chitin under dissolved conditions (Cho *et al.*, 2000). After chitosan, the most studied derivative of chitin is carboxymethylchitin (CM-chitin), a water-soluble anionic polymer. The carboxymethylation of chitin is done similarly to that of cellulose; chitin is treated with monochloracetic acid in the presence of concentrated sodium hydroxide. The same method can be used for carboxymethylation of chitosan . The method for cellulose derivatization is also used to prepare hydroxypropylchitin, a water-soluble derivative used for artificial lachrymal drops (Rinaudo, 2006).

Other derivatives such as fluorinated chitin (Chow and Khor, 2001) N- and O-sulfated chitin (Murata *et al.*, 1991), (diethylamino) ethylchitin, phosphoryl chitin (Andrew *et al.*, 1998), mercaptochitin and chitin carbamates have been described in the literature. Modification of chitin is also often effected via water soluble derivatives of chitin (mainly CMchitin). The same type of chemical modifications (etherification and esterification) as for cellulose can be performed on the available C-6 and C-3 – OH groups of chitin (Rinaudo, 2006).

Chitin can be used in blends with natural or synthetic polymers; it can be crosslinked by the agents used for cellulose (epichlorhydrin, glutaraldehyde, etc.) or grafted in the presence of ceric salt or after selective modification (Rinaudo, 2006).

Chitin is partially degraded by acid to obtain series of oligochitins. These oligomers, as well as those derived from chitosan, are recognized for their bioactivity: including anti-tumor, bactericidal and fungicidal activity, eliciting chitinase and regulating plant growth. They are used in testing for lysozyme activity. They are also used as active starting blocks to be grafted on protein and lipids to obtain analogs of glycoproteins and glycolipids (Rinaudo, 2006).

Table 2.4 Chitin derivatives and their proposed uses

Derivative	Examples	Potential uses
N-Acyl chitosans	Formyl, acetyl, propionyl, butyryl, hexanoyl, octanoyl, decanoyl, dodecanoyl, tetradecanoyl, and lauroyl, myristoyl, palmitoyl, stearoyl, benzoyl, monochloroacetoyl, dichloroacetyl, trifluoroacetyl, carbamoyl, succinyl, acetoxybenzoyl	Textiles, membranes medical aids
N-Carboxyalkyl *N*-(*N*- (aryl) chitosans	Carboxybenzyl, glycine-glucan carboxy-methyl chitosan), alanine glucan, phenylalanine media glucan, tyrosine glucan, serine glucan, glutamic acid glucan, methionine glucan, leucine glucan	Chromatographic and ion metal collection
N-Carboxyacyl chitosans	From anhydrides such as maleic, itaconic, acetyl- thiosuccinic, glutaric, cyclohexane 1,2-dicarboxylic, phthalic, *cis*- tetrahydrophthalic, 5-norbornene- 2,3-dicarboxylic, diphenic, salicylic, trimellitic, pyromellitic anhydride	?
o-Carboxyalkyl	*o*-Carboxymethyl, crosslinked *o*-carboxymethyl	Molecular sieves, chitosans viscosity builders, and metal ion collection
Sugar derivatives	1-Deoxygalactic-1-yl-, 1-deoxyglucit-1-yl-, 1-deoxymelibiit-1-yl-, 1-deoxylactit-1-yl-, 1-deoxylactit-1-yl-4(2,2,6,6-tetramethylpiperidine-1-oxyl)-, 1-deoxy-69-aldehydolactit-1-yl-,1-deoxy-69-aldehydomelibiit-1-yl-, cellobiit-1-ylchitosans, products obtained from ascorbic acid	?

Table 2.4 (cont.) Chitin derivatives and their proposed uses

Derivative	Examples	Potential uses
Metal ion chelates	Palladium, copper, silver, iodine	Catalyst, photography, health products, and insecticides
Semisynthetic resins of chitosan	Copolymer of chitosan with methyl methacrylate, polyurea-urethane, poly(amideester), acrylamidemaleic Anhydride	Textiles
Natural polysaccharide complexes, miscellaneous	Chitosan glucans from various organisms	Flocculation and metal ion chelation
	Alkyl chitin, benzyl chitin	Intermediate, serine protease purification
	Hydroxy butyl chitin, cyanoethyl chitosan	Desalting filtration, dialysis and insulating Papers
	Hydroxy ethyl glycol chitosan	Enzymology, dialysis and special papers
	Glutaraldehyde chitosan	Enzyme immobilization
	Linoelic acid–chitosan complex	Food additive and anticholesterolemic
	Uracylchitosan, theophylline chitosan, adeninechitosan, chitosan salts of acid polysaccharides, chitosan streptomycin, 2-amido-2,6-diaminoheptanoic acid chitosan	

Source: Kumar, 2000

2.2.4 Application of chitin

Chitin and its deacetylated form are widely used in food, cosmetic industries medical products, agriculture, wastewater treatment, and membranes because of its biodegradability, biocompatibility and non-toxicity (Jung *et al.*, 2007).

2.2.4.1 Application of chitin and chitosan in the ecological and environmental fields

Chitin and Chitosan are environmentally and ecologically active polymers. They are fertilized into farming soils, and the soil microbial flora are improved. The total number of the useful microorganisms increases, and that of harmful microorganisms decreases (Mattheus, 1997).

Chitin, chitosan and their derivatives are usable as a new wound-healing material. Several wound dressing as an artificial skin have been manufactured from chitin and chitosan and are commercialized for the healing of both human and animal wounds (Mattheus, 1997). Chitin, chitosan and their derivatives are usable as an absorbable, implantable material in animal and plant tissue for control release of drugs and as carrier material for the targeting of drugs to specific cells, tissue, or organs. A commercial product is a chitin suture absorbable in human and animal tissues. It is not necessary to take out the suture after a clinical operation because the suture is digested by lysozyme in the tissues (Mattheus, 1997).

2.2.4.2 Nutritional effect of chitin and chitosan in foods

Multiple action of chitin and chitosan in food systems relate to their effects as dietary fiber and as functional ingredients. The effect of chitin, chitosan and cellulose as dietary supplements on the growth of cultured red sea bream, Japaneese eel, and yellow tail has been investigated by Kono *et al.*, (1987) The growth rate of all fishes fed with a 10% chitin supplement was the highest, thus indicating its applicability in feed. Feed efficiency in the red sea bream and Japaneese eel fed a 10% chitin supplemented diet was also the highest. Austin *et al.*, (1981) reported the effect of chitin as a feed additive on the growth of bifidobacteria in the guts of chickens. Addition of chitin increased the growth of bifidobacteria which are important as they inhibit the growth of other types of microorganisms (Fereidoon *et al.*, 1999).

2.2.4.3 New application of chitin and its derivatives in plant protection

The usefulness of chitin and its derivatives in agricultural applications is in the following areas: biostimulation of plant growth; plant protection against fungi, bacterias, and viruses; and post harvest protection. Biostimulation of the plant growth is mainly realized by seed coating, root deepening, and plant dressing; whereas plant protection against fungi, bacteria, or viruses can be achieved by seed coating, plant dressing, and postharvest treatment. A special interest for the use of chitin and its derivative is postharvest treatment subjected to harvest protection (especially against fungi) and harvest freshness protection (Mattheus, 1997).

2.3 Chitin production

2.3.1 Conventional processes

Conventional chemical processes to accomplish each of operations include: protein removal by treatment with dilute alkali and removal of mineral salts by acid treatmentbut the demineralization step can also precede the deproteinisation step (Goycoolea *et al.*, 2000). In the recovery of chitin from shellfish sources, the removal of associated minerals and proteins normally requires the use of HCl and NaOH. These reagents can cause deacetylation and depolymerization of chitin (Wee *et al.*, 2001). Chitin is now produced commercially from crab and shrimp shells by treatment with dilute NaOH solution for deproteinization, followed by treatment with dilute HCl solution for demineralization (Mattheus, 1997) Figure 2.5 show Overall process for the preparation of chitin.

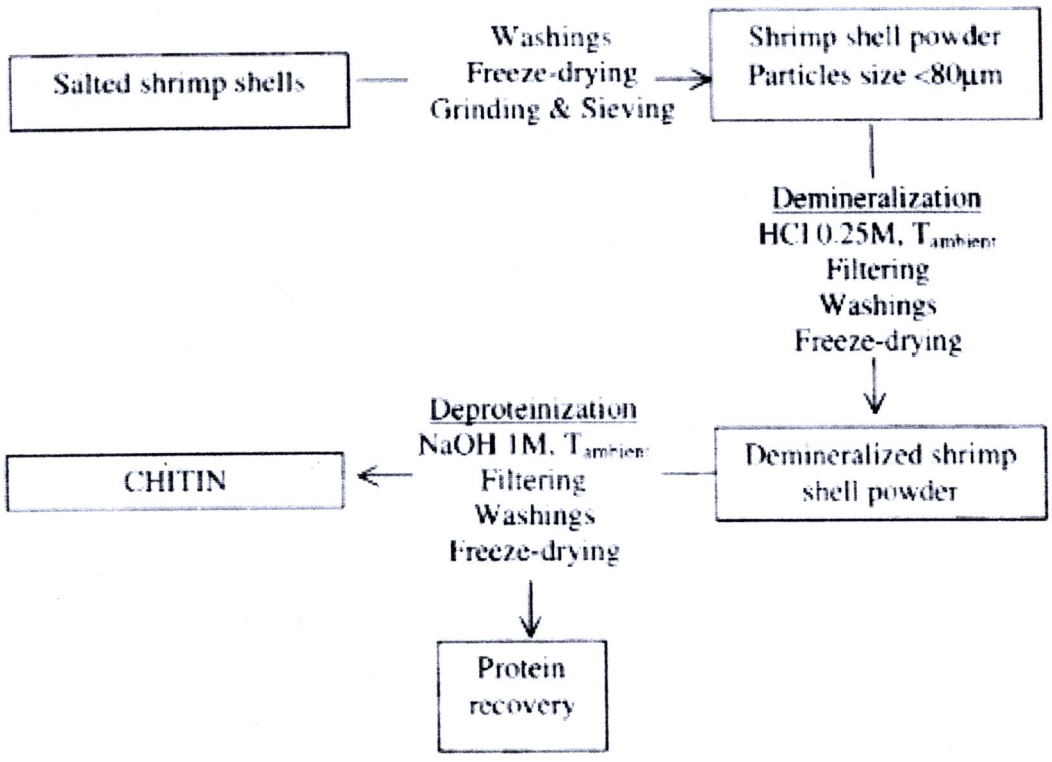

Figure 2.3 Overall process for the preparation of chitin from salted shrimp shells (Percot *et al.*, 2003).

Deproteinization. When the removed protein is to be marketed, for instance as a high grade additive to livestock starter feeds, its extraction before the demineralisation step is preferred so as to maximise both yield and protein quality. In the chemical process, crustacean shells are usually treated with NaOH solution (1-10%) at elevated temperature (65-100°C) in order to dissolve the protein present. Early patents also claim the use of a wide range of agents for this step including NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $Ca(OH)_2$, Na_2SO_3, $NaHSO_3$, $CaHSO_3$, Na_3PO_4 and Na_2S. The mildest alkaline treatments reported are those utilizing Na_2CO_3 at concentrations of up to 0.1 M, together with soap, at ~100°C for 4 hours. However, most conventional chemical treatments have involved the use of NaOH, but with a wide variation in concentration (0.25-2.5 M), temperature (65-100°C duration of treatment (0.5-72 hours) and number of operations (Goycoolea *et al.*, 2000).

Demineralization. Removal of calcium carbonate from crustacean waste is normally accomplished by dilute HCl at room temperature. The concentration of HCl, time and ratio of solids to solution of HCl vary with source, and a wide range of such

conditions have been reported in different studies. Most of these, however, agree in that the removal should be carried out at room temperature to prevent polymer degradation. Pinelli *et al.* investigated various demineralisation conditions using HCI of various concentration, temperature and solution:chitin ratio, for treatment of shell waste of blue shrimp *(Penaeus styh'rostris)* or brown shrimp *(P. californiensis)*. The lowest ash contents (~14%) were found with 1.4N HCl at room temperature, 24 hours and with a shell:solution ratio 1:7. Exceptions to the use of HCl include use of HNO_3, H_2SO_4, CH_3COOH and HCOOH. Austin (1981) used EDTA at alkaline pH in an attempt to minimise degradation. It is important that the amount of acid be stoiehiometrieally equal to, or greater than, all minerals present in the shell to ensure complete reaction (Goycoolea *et al.*, 2000).

Decolouration. The exoskeletons of erustaeea contain colouring matter whose recovery can be integrated as a part of the chitin manufacturing process, since they have a high commercial value. Chemically, the major pigment compounds of erustaeea are astaeene, astaxanthin, eanthaxantin, lutein and 13-carotene. Various extraction and bleaching conditions used to remove pigments form chitin have been reported. These valuable compounds may be removed by extracting the shell with ethanol, ether, acetone, chloroform or oil atter demineralisation by either acid or EDTA treatment. Bleaching agents conventionally used to oxidise the pigments are 0.5-3% H_2O_2 or 0.32% NaClO. It has also been claimed that warm 50% aqueous acetic acid simultaneously demineralises the shell and extracts the earotenoids. The oxidative chemical changes that bleaching treatments could entail should be worth considering (Goycoolea *et al.*, 2000).

However the use of these chemicals may cause a partial deacetylation of chitin and hydrolysis of the polymer, resulting in a final inconsistent physiological properties (Wang *et al.*, 2006). Chemical process were not easy to controlled, leading to broad and heterogeneous range of product. These chemical treatments also create waste disposal problems, because neutralization and detoxification of the discharged waste water may be necessary (Wang *et al.*, 2006).

Goycoolea and co-worker (2000) suggest that in the industrial manufacture of chitin must consider as major criteria lower consumption of water, recycling and treatment of their effluents. Figures of water consumption required to wash chitin

from blue crab shell after acid/alkali treatments registered in pilot plant trials in their laboratories, gave approximately 420 litres of water per kilogram of chitin obtained. This is certainly a drawback to chitin large scale recovery, particularly in regions with relatively large amounts of raw materials such as lobster waste *(Panlirus argus)* in the western part of Cuba and of blue crab *(Callinectes sapidus)* and shrimp *(Penaeus* spp.) in the coast of Northwest Mexico.

2.3.2 Bioprocesses

An alternative to these harsh chemical treatments is the use of proteolytic microorganisms, in particular extracellular proteases secreted from fungi. Hall and Silva reported that the fermentation of crustacean shells with lactic acid bacteria lowered the pH of the medium to approximately pH 4, facilitating the hydrolysis of proteins while leaving the associated chitin intact. Bustos and Healy (1994) demonstrated that chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganisms including *Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae*, had higher molecular weights compared to chemically prepared shellfish chitin (Wee *et al.*, 2001).

Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes and further enhance the utilization of chitin-containing marine crustacean waste (Wang *et al.*, 2006).

Recently, studies of biological process for chitin production have reported using organic acids producing bacteria and enzymes for demineralization and deproteinization of crustacean shells (Jung *et al.*, 2007).

2.3.3 Proteolytic enzyme for deproteinization

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta *et al.*, 2002)

In recent years, certain proteolytic enzymes such as Alcalase, chymotrypsin, and papain have been used to extract the protein and chitin parts of shrimp waste separately and therefore, it has been possible to use protein hydrolysate and also chitin with more desirable physico-chemical properties and with smaller amounts of chemical contaminants. Also, in order to improve protein extraction and to achieve a higher degree of purity in chitin, lactic acid fermentation has been used before enzymatic hydrolysis (Mizani *et al.*, 2005).

2.4 Proteases

Proteases are by far the most important group of enzymes produced commercially and are used in many areas of applications. In recent years, ample successes in degradation of proteinaceous waste into useful biomass by proteases have also been demonstrated. Proteases produced by *Bacillus* spp., including *Bacillus* spp., *B. subtilis*, *B. firmus*, are by far the most important group of enzymes being exploited (Jen, 1999).

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sale. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications (Rao *et al.*, 1998).

Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Gupta *et al.*, 2002)

Among the enzymes employed in food processing, proteases are most extensively used for improving the quality, stability and functionality of products. Most proteases used in the food industry are derived from microbial and animal sources. In recent years, interest has been expressed in isolating enzymes from marine species (Han and Shahidi, 1995).

Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Gupta *et al.*, 2002)

2.4.1 Microorganism using for deproteinisation in chitin production

For alternative approaches to overcome the shortage of the chemical treatments, microorganisms and proteolytic enzymes for the deproteinization of crustacean wastes has been applied. For example, Yang and coworker (2000) reported that shrimp shells was fermented with *Bacillus subtilis* and recorded 88% of protein removal (Jo *et al.*, 2008). The bioconversion of shellfish chitin wastes for the production of proteases to further enhance the utilization of chitin-containing marine crustacean. Protease producing microorganisms using marine crustacean waste as substrate were show in Table 2.5.

Table 2.5 Protease producing microorganisms using marine crustacean waste as substrate

Microorganism	Substrate	Optimal condition	Product	Reference
Bacillus subtilis	Crustacean waste	30°C for 3 days in 100 ml of medium 7% shrimp and crab shell powder (SCSP), 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.0% arabinose, 1.5% $NaNO_3$, and 1.5% $CaCl_2$	protein removal 88% (shrimp shell), 67% (crab shell), and 83% (lobster shell)	Yang *et al.* 2000
Bacillus sp. TKU004	Squid pen	30°C for 4 days in 100 ml of medium (pH 6) containing 2% squid pen powder, 0.1% K_2HPO_4, and 0.5% $MgSO_4$	Protease activity 0.065 U/ml protein removal was 73%	Wang *et al* 2006
Pseudomonas aeruginosa K-187	Shrimp and crab shell waste	25°C for 2 days in medium containing 5 g of 5% shrimp and crab shell powder, 1% lactose, 0.5% NH_4NO_3, 0.1% K_2HPO_4, 0.007% $CaCl_2 2H_2O$, 0.5% $MgSO_4$ and 0.5% $FeSO_4 7H_2O$ initial pH 8	Protease activity 2.2 U/ml protein removal was 72%	Oh *et al.* 2000
Streptomyces sp. A6	Shellfish waste	Shrimp waste 14 g/l, $FeCl_3$ 0.035 g/l, $ZnSO_4$ 0.065 g/l, and pH 8	Protease activity 129.02 ± 2.03 U/ml protein removal was 85.12 ± 4.7%	Singh and Chhatpar 2010

Table 2.5 (Cont.) Protease producing microorganisms using marine crustacean waste as substrate

Microorganism	Substrate	Optimal condition	Product	Reference
Lactobacillus paracasei subsp. *tolerans* KCTC-3074 and *Serratia marcescens* FS-3	Red crab shell	30°C for 7 days in MRS medium (*Lactobacillus paracasei* subsp *tolerans* KCTC-3074) and LB medium (*Serratia marcescens* FS-3)	Deproteinization was 52.6%	Jung *et al.* 2006
Serratia marcescens FS-3	Crab shell waste	Highest protease activity after 3 days and protein removal after 7 days	Protease activity 60.7 U/ml protein removal was 84%	Jung *et al.* 2008
Bacillus licheniformis RP1	Shrimp waste	shrimp wastes powder 30 (g/l), KCl 1.5 (g/l), K_2HPO_4 0.5(g/l), and KH_2PO_4 0.5 (g/l), 3 hours hydrolysis at 60°C	The percent of protein removal at an enzyme/substrate (E/S) ratio of 0.5 and 5 (Unit of enzyme/mg of protein) were about 68 and 81%, respectively	Hadder *et al.* 2011

Bacteria The study of Jo and co-workers in 2008 was about the screening of *Serratia marcescens FS-3* from a soil sample in the southwestern area of Korea and use it for the deproteinized of natural crab shell waste after 7 days of fermentation.

In 2006, Wang and co-workers investigated the deproteinization of shell waste by protease enzyme from *Bacillus* sp. TKU004. Under the optimized condition, both the production of protease and the resulted protein removal attained the optimum. They are 0.065 unit/ml and 73%, respectively. In 2008, they studied the production of protease by *Lactobacillus paracasei* subsp *paracasei* TKU012. The protease retains 21% and 91% activity in the presence of Tween 20 and SDS, respectively.

While the biological operations apparently minimise chemical degradation of chitin and lead to environmentally cleaner operations, protein removal is not complete so they can be regarded only as a pre-treatment if a high purity chitin is to be obtained. In a recent study, a purified pig pancreatic peptidases mixture was tested to eliminate protein from various sources of chitinous waste (at 49 ±1°C, pH ~ 7.8, 24 h, ratio enzyme:substrate, 1:100). Namely, from shrimp *(Penaeus* spp.*)* shell (58% protein), blue crab *(Callinectes sapidus)* carapace (13% protein) and from giant squid *(Dosidicus gigas)* pen (55% protein). In the case of shrimp shell and squid pen, ~ 80% of the protein present was removed, while in crab carapace only~ 30% of the protein was removed his may reflect differences in the type of proteins associated to the species as well as in the porosity of the chitin-protein matrix in each case (Goycoolea. *et al.*, 2000).

Most commercial proteases, mainly neutral and alkaline, are roduced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their

activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al.*, 1998).

Table 2.6 Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers. *n.s.* Not specified

Supplier	Product trade name	Microbial source	Application
Novo Nordisk,	Alcalase	*Bacillus licheniformis*	Detergent, silk degumming
Denmark	Savinase	*Bacillus* sp.	Detergent, textile
	Maxatase	*Bacillus* sp.	Detergent
	Biofeed pro	*B. licheniformis*	Feed
	Durazym	*Bacillus* sp.	Detergent
	Novozyme 471MP	n.s.	Photographic gelatin hydrolysis
	Novozyme 243	*B. licheniformis*	Denture cleaners
	Nue	*Bacillus* sp.	Leather
Genencor	Purafact	*B. lentus*	Detergent
International, USA	Primatan	Bacterial source	Leather
Gist-Brocades, The	Subtilisin	*B. alcalophilus*	Detergent
Netherlands	Maxacal	*Bacillus* sp.	Detergent
	Maxatase	*Bacillus* sp.	Detergent
Solvay Enzymes,	Opticlean	*B. alcalophilus*	Detergent
Germany	Optimase	*B. licheniformis*	Detergent
	Maxapem	Protein engineered variant of *Bacillus* sp.	Detergent
	HT-proteolytic	*B. subtilis*	Alcohol, baking, brewing, feed, food, leather, photographic waste
	Protease	*B. licheniformis*	Food, waste
Amano	Proleather	*Bacillus* sp.	Food
Amano	Collagenase	*Clostridium* sp.	Technical
Pharmaceticals, Japan	Amano protease S	*Bacillus* sp.	Food
	Enzeco alkaline	*B. licheniformis*	Industrial
Enzyme	Protease		
Development, USA	Enzeco alkaline protease-L FG	*B. licheniformis*	Food
	Enzeco high alkaline protease	*Bacillus* sp.	Industrial
	Bioprase	*B. subtilis*	Cosmetic, pharmaceuticals
Nagase	concentrate		

Table 2.6 (Cont.) Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers. *n.s.* Not specified

Supplier	Product trade name	Microbial source	Application
Biochemicals, Japan	Ps. protease	*Pseudomonas aeruginosa*	Research
	Ps. elastase	*Pseudomonas aeruginosa*	Research
	Cryst. protease	*B. subtilis* (K2)	Research
	Cryst. protease	*B. subtilis* (*bioteus*)	Research
	Bioprase	*B. subtilis*	Detergent, cleaning
	Bioprase SP-10	*B. subtilis*	Food
	Godo-Bap	*B. licheniformis*	Detergent, food
Godo Shusei, Japan	Corolase 7089	*B. subtilis*	Food
Rohm, Germany	Wuxi	*Bacillus* sp.	Detergent
Wuxi Synder Bioproducts, China	Protosol	*Bacillus* sp.	Detergent
Advance Biochemicals, India			

Source: Gupta *et al.*, 2002

Treatments by enzymatic digestion or fermentation with proteolytie bacteria have also been used to partially remove proteins. In the former ease, various proteases such as tripsin, pepsin, ehymotripsin and papain have been used successfully. Residual protein levels in the waste atter deproteinisation with enzymes vary in the range from 1 to 5%. Also, it has been demonstrated that incubation of krill waste with an excess of a bacterial protease (NOVA) for 120 hours at pH 6.5 at 55°C facilitated the extraction of astaein with chloroform. In turn, silage treatments offer the possibility of recovering value-added products such as endogenous proteases, lipids and pigments. In the enzymatic process the quality of the hydrolysed protein is bound to be retained. It is well known that alkali hydrolysis of protein can induce racemization of aminoaeids from the L- to the D- form, as well as to the formation of toxic compounds such as lysinoalanine, leading to detriment of the nutritional quality (Goycoolea *et al.*, 2000).