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TEL: 0116 260 2917
FAX: 0116 269 6639

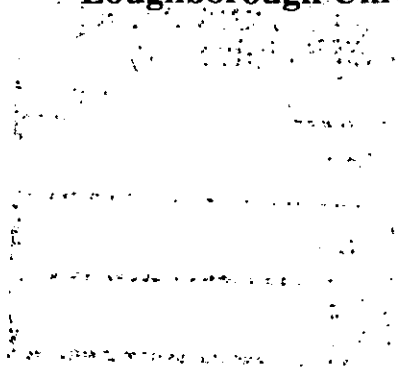
LACTIC ACID PURIFICATION OF CHITIN FROM PRAWN WASTE USING A HORIZONTAL ROTATING BIOREACTOR

By

ZAINOHA ZAKARIA

A DOCTORAL THESIS

Submitted in partial fulfilment of the requirements
for the award of **Doctor of Philosophy** of
Loughborough University



ABSTRACT

Shellfish waste obtained from seafood processing plants contains chitin, protein and calcium carbonate. Chitin is a versatile biopolymer with many applications. Conventionally, chitin is separated from calcium carbonate and protein by acid and alkali respectively. In this project, a biotechnological approach was applied to recover chitin from scampi (*Nephrops norvegicus*) waste using lactic acid bacteria (LAB) to produce lactic acid from glucose which lowers the pH of the mixture, thus preserving the waste from spoilage. The acid also dissolves the calcium carbonate and under these conditions native enzymes breakdown the protein (autolysis), thus affording a substantial amount of purification of chitin. LAB were isolated and identified from various shellfish waste fermentations. Studies on their acid-producing ability revealed a few potentially good strains, identified as *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Pediococcus* sp. The strain of *Lactobacillus paracasei* was used as a starter culture in the fermentation of shellfish waste in a horizontal rotating bioreactor in order to evaluate the feasibility of the process. The design of the bioreactor was such that it enabled separation of solid and liquid end products during fermentation. Several important fermentation parameters were studied including mode of rotation, concentration of glucose, temperature, rotation rates, loading capacity, type and particle size of waste. Partial purification of the scampi waste was achieved using both batch and fed batch operation, but in the latter, improved purification was achieved at the cost of increased glucose consumption and extended fermentation times. Whilst higher temperatures increased the rates of fermentation, higher rotation rates seemed to have the reverse effect. Mincing the waste helped to increase breakdown of protein whilst larger particles tended to undergo rapid spoilage. Analysis of the chitin product enabled this method to be compared with the conventional method. The results obtained showed that this method is capable of saving large volumes of chemicals and besides producing chitin, the protein liquor by-product could also be used as an ingredient in an animal feed which is not possible by the conventional method.

Keywords: Shellfish waste, lactic acid bacteria, lactic acid fermentation, chitin, horizontal rotating bioreactor, silage.

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To my husband, Azman Hassan and my three sons, Haniff, Hadi and Amin, you were a constant source of strength throughout these years.

PAPERS AND COMMUNICATIONS

Results from this research have produced the following works:

1) Hall, G.M., C.R. Reid and Z. Zakaria (1994) "Fermentation of prawn waste by lactic acid bacteria." Paper presented at the 6th Int. Conf. Chitin and Chitosan, Poland.

2) Guerrero Legarreta, I., Z. Zakaria and G.M. Hall (1995) "Lactic fermentation of prawn waste: Comparison of commercial and isolated starter cultures." *In* **Advances in Chitin Science**, Vol 1, (ed. A. Domard, C. Jeuniaux, R. Muzzarelli and G. Roberts), 399-406.

3) Hall, G.M., Z. Zakaria and C.L. Reid (1995) "Recovery of value-added products from shellfish waste by a lactic acid fermentation process." Paper presented at WEFTA 25th Annual Conf., Holland.

4) Zakaria, Z., G.M. Hall and G. Shama (1996) "Purification of chitin from shellfish waste by lactic acid fermentation." *In* Proc. The 1996 IChemE Research Event/Second European Conf. for Young Researchers in Chemical Engineering, Leeds, U.K., Vol. 1, 169.

5) Zakaria, Z., G. Shama and G.M. Hall (1996) "Lactic acid purification of chitin from scampi waste using a horizontal rotating bioreactor." *Enzyme Microb. Technol.* (submitted).

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ABBREVIATIONS

LAB	Lactic acid bacteria
SSF	Solid state fermentation
LAF	Lactic acid fermentation
CN	Chitin nitrogen
PN	Protein nitrogen
SPN	Soluble protein nitrogen
TN	Total nitrogen
NPN	Non-protein nitrogen
Liquor	Liquid produced during fermentation
Sediment	The insoluble chitin fraction obtained after fermentation
Silage	Silage is the product of fermentation which contains liquor & sediment
w/w	weight per weight
w/v	weight per volume
m.c.	moisture content
min	minute
h	hour
APT agar	All Purpose agar with Tween
MRS agar	De Man, Rogosa and Sharpe agar
cfu/g	colony forming unit per gram
TTA	Total titratable acidity
HRB	Horizontal rotating bioreactor

CHAPTER 1

INTRODUCTION

1.1 Background to project.

As more countries throughout the world produce shrimp for export, the seafood industry will face an escalating problem of how to dispose of shellfish waste (Meyers, 1986; Shahidi and Synowiecki, 1992). It is estimated that, nearly 2.6 million metric tons of shrimp and prawns are landed annually, three quarters of which is of Asian origin (Csavas, 1988). However, nearly 35 percent of the raw shrimp catch is discarded as waste when processed into "head-less shell-on" products, whilst removing the shell further increases the waste production to 40 - 45 percent (Subangsinghe, 1995). The environmental problems faced by the seafood industry over the years have resulted in efforts to find uses for these waste materials. Currently, most of the shellfish waste which is rich in chitin, protein, pigments, and flavour compounds is either incorporated into animal feed or is disposed of as waste. For many producers, the problem of utilisation of the waste is its sheer perishability under normal ambient conditions. Due to the extremely high levels of microbial and enzymatic activity in the head tissue, such material is extremely labile and subject to rapid decomposition. This is further complicated by factors of high temperature, such as are prevailing in the Asian producing countries, poor transportation and minimal cooling/icing facilities in the production areas. Therefore cheaper techniques of preservation must be sought if the shellfish waste is to be maximally utilised on a global scale.

Chitin, a nitrogen-containing polysaccharide, forms a significant fraction of the waste products of the seafood industry. It is estimated that the processing waste of shrimp and prawn contains anywhere from 10 - 55 percent of chitin on a dry weight basis (Subangsinghe, 1995). Natural chitin is bound by protein and calcium carbonate, removable to a greater or lesser degree by different purification methods. Although chitin was first discovered by Henri Braconnot in 1821, research on chitin has only gained momentum in the last 25 years (Domard, 1996). This is because, in pure form (free of protein and calcium carbonate), chitin has very limited applications due to its

insolubility in water and many commercial solvents. Chemically treated, its uses are extended and the most widely used form, chitosan, is made by the chemical deacetylation of chitin.

Over 200 current and potential applications of chitin, chitosan and other derivatives have been identified (Brzeski, 1987). Those with a relatively high market potential include applications such as seed treatment in agriculture, as a clarifier and a dietary fibre source in the food industry, as a retention aid and coating in the paper and textile industry and for treating drinking water and process water in the food industry. However, in spite of the diversity of applications, widespread industrial use of chitin is still limited mainly because of the high price for technical grade chitin and chitosan (Peter, 1995). The costs of purification of chitin from shellfish waste are such that normal commodity applications, especially in the food industry, are more economically met by other materials such as cellulose, alginates and starches (Knorr, 1984). This situation has forced potential suppliers of chitin products to look at speciality markets where chitin's unique properties make it indispensable. Japan is considerably advanced in the technology and commercialisation of chitosan. The total global production of chitin and chitosan has been estimated at 1600 metric tons with Japan and USA as main producers (Brzeski, 1987). Other less important producers are India, Italy and Poland.

Although the availability of chitin-rich species is considerable, there are practical difficulties in gaining access for commercial chitin/chitosan extraction. As most of the landings are sold without processing, or are processed by small or medium scale processing plants, the efficient collection of waste from such sources is difficult (Subangsinghe, 1995). The need for coordinated collection and transport of carefully collected waste from several sources would add considerably to the cost of the raw material. The seasonality of the resources and the geographical distribution of the processing industries in many countries are factors which can restrict the availability of a regular supply of raw material. The relatively bulky nature of waste and its high perishability can also make sourcing of raw material more difficult. Therefore, only waste produced at large scale processing facilities, would potentially serve as a

reliable, regular source of good quality raw material. However, in recent years, the seasonality problem of the traditional shellfish industry has been overcome by the steadily growing cultured shrimp industry, especially in Asian countries, where production has increased steadily and reached 600,000 metric tons by 1990 (Csavas, 1993).

At present, the traditional chitin purification process uses alternating acid and alkali treatment stages to remove, respectively, calcium carbonate and proteins. The disadvantages of this process are that it requires the use of hazardous, corrosive chemicals and generates relatively large volumes of aqueous wastes while discarding many useful components (Ornum, 1992). In a brief analysis of the main costs of the process: transportation and handling, chemical treatment and generation of potentially polluting wastes (which would have to be treated before being discarded) have been identified as the most important factors determining the high price of chitin and chitosan products (Muzzarelli, 1990). Therefore, an alternative, simple method of purification that minimises added costs and pollution of the environment, would appear to offer interesting prospects for the seafood industry.

Since the 1970's, research on chitin has increased progressively and is now widespread as can be seen from papers appearing in many international conferences held during the past 25 years (Pariser and Lombardi, 1989; Skjak-Braek *et al.*, 1989). However, research has mainly focussed on applications of chitin and chitosan and few changes have been made to the traditional purification methods. Recently, some workers have tried to improve the chitin purification method by using enzymes or proteolytic bacterial cultures, but this has proved to be either costly or time consuming (Shimara *et al.*, 1982; Gagne and Simpson, 1993).

A biotechnological approach to chitin extraction from shellfish waste has been proposed by Hall and De Silva (1992). They worked with tropical waste on a laboratory scale and their approach was based on ensilation which has been applied to fishery waste products for many years. Ensilation is a process of preservation of material or waste material by the use of acids to prevent the growth of spoilage

organisms. During the process, enzymes naturally present in the viscera digest the proteins giving a liquid product which can be used as a constituent of an animal feed. Acid can be added or can be generated *in situ* by bacterial fermentation. In an added-acid silage (normally containing formic, propionic and inorganic acids), the product must be neutralised before it can be fed to animals. In fermented-acid silage, acids, which are relatively expensive, are not needed as acid is generated during fermentation. The acid is normally lactic acid and the process involved is known as "lactic acid fermentation" due to the fermentative action of lactic acid bacteria. In the fermentation of shrimp waste, the lactic acid is produced, dissolves the calcium carbonate in the shells while the indigenous enzymes solubilise the proteinaceous material forming a protein-rich liquor which could be separated from the insoluble chitin (Hall and De Silva, 1992). If the protein could be recovered free of chitin, it could be an excellent ingredient for animal feed as feeds made from whole shrimp waste (heads, meat, viscera and hulls) tend to have a high fibre content, because of the presence of chitin, and low protein content. However, maximal utilisation of this waste requires a good quality material.

This type of fermentation is the basis of many fermented sauces and pastes found in South East Asia such as belachan, trassi-udang and pla-ra in Malaysia, Indonesia and Thailand respectively (Jay, 1992; Steinkraus, 1996). Therefore it is a technology which is understood and acceptable to many people who could adapt it to an industrial process for the production of chitin and feed. Many small-scale shellfish enterprises could benefit from this method as it does not require expensive equipment or corrosive chemicals. The high ambient temperature in tropical countries is an advantage as the fermentation normally proceeds well between 25°C - 35°C. Thus, instead of tranferring highly perishable and bulky waste, the dried, chitin product obtained, could be easily collected and transferred to the main chitin and chitosan processing plants. The chitin product, which is partially purified from protein and calcium carbonate, requires only a very minimal acid and alkali wash, if necessary. It is a new technology worth considering especially in hotter countries where cooling facilities for preservation of waste material may be inadequate and the protein by-products can be sun-dried.

Lactic acid bacteria (LAB) are naturally occurring in and on fish and shellfish but in low concentrations. If a source of carbohydrates is added, LAB will dominate the microbial flora within a short space of time (Jay, 1992). Nowadays, in fermented foods, a starter culture of LAB is added to initiate the fermentation. It is an advantage to use starter cultures which have been isolated from the products to be studied as these bacterial strains are well adapted to the conditions (Jepperson, 1993). LAB isolation from shellfish waste is therefore considered vital in this project. Vuyst and Vandamme(1994a) listed several advantages of using lactic acid bacteria in industrial fermentations :

i) they have been ingested throughout history, implying that they are non-pathogenic
ii) they do not form toxins or toxic products
iii) they are microaerophilic and aerotolerant, requiring a simple fermentation process
iv) they grow rapidly, requiring a short fermentation process
v) they have been used in the food industry for years, implying that methods for their cultivation on a large scale already exist
vi) they can ferment cheap substrates such as milk, whey, plant wastes and hydrolysed starch
vii) their growth discourages spoilage and contamination with other microorganisms

As with any fermentations, reactor design is a vitally important factor which determines the efficiency of the process. The fermentation of shellfish wastes may be considered a "solid state fermentation", as initially only minimal quantities of free water are present. A rotating horizontal bioreactor was considered particularly appropriate for this work as this type of reactor has been used in many solid state fermentations (Pandey, 1991). Since the fermentation products of the shellfish waste are in two phases, the insoluble chitin sediment and a liquified protein fraction, a bioreactor that could assist in the separation of these phases at the end of the fermentation was considered an important criterion.

1.2 Research objectives:

The overall objectives of the project are divided as follows:

I) Isolation of lactic acid bacteria

a) To isolate lactic acid bacteria from different shellfish waste sources.

b) Screening of isolates

c) Identification of isolates

II) Feasibility studies using a horizontal rotating bioreactor

a) Waste pretreatment and characterisation studies

b) Bioreactor modification and requirements

c) Optimisation studies

d) Comparison between chitin purification by fermentation and by conventional method.

CHAPTER 2

LITERATURE REVIEW

2.1 Sources of chitin.

Currently all the chitin produced commercially is derived from the exoskeleton of crustaceans (mainly crab, shrimp and prawn) obtained as waste from the seafood industry. The amounts available are sufficient to meet the present demand for chitin and chitosan and commercial exploitation of other potential sources (mentioned later in this section) is unlikely to take place for some years (Roberts, 1992).

2.1.1 Crustacea.

The crustaceans have a segmental structure enclosed within an exoskeleton or cuticle composed mainly of chitin. They also have jointed appendages. All crustaceans have an enlarged forward portion of the exoskeleton called the carapace which covers the head and the thorax (Early and Stroud, 1982). There is considerable variation in the body form; in some species, for example, lobster and shrimp, the well developed abdomen situated behind the carapace gives a relatively large amount of tail meat. In other species, for example crab, the abdomen is greatly reduced giving no edible part at all; here the carapace dominates and yields a relatively large amount of brown meat, which consists of digestive gland and gonads (Early and Stroud, 1982). In some species there is considerable amount of meat in the claws and legs. The crustaceans increase in size by moulting, that is shedding the rigid exoskeleton periodically, to allow for growth.

2.1.1.1 Classification of crustacea.

The class Crustacea, under the Phylum Arthropoda of the Sub-kingdom Invertebrata, is grouped in eight sub-classes, each of which is divided into a number of orders. However, the majority of the larger and better known Cructacea belong to the sub-class Malacostraca, and in particular to the order Decapoda (Holthuis, 1980).

The Decapoda (meaning that they have ten feet) not only vastly exceeds any other orders of the Crustacea in the number of known species, in the range of their

structure, but also includes all the larger and more familiar examples of the class. This includes the commercially important prawns, shrimps, lobsters and crabs. Regrettably there is no biological definition of what is a prawn and what is a shrimp. The Oxford and Webster's dictionaries describe a shrimp as a small prawn or a prawn as a large shrimp. Consequently, both names, shrimp and prawn, are commonly used, frequently interchanged and equally valid (Ruello, 1976; Early and Stroud, 1982).

The classification of the Decapoda is somewhat complicated. The very numerous species and genera are arranged in some forty-six families, which again are grouped into superfamilies, infr-aorders or tribes, and sub-orders (Holthuis, 1980). For the sake of simplicity, however, it is convenient to subdivide these species into sub-orders Natantia (prawns and shrimps) and Reptantia (crawfish, lobsters and crabs). Prawns and shrimps (Natantia) may be separated into two major tribes, *Penaeidea* and *Caridea*. Most of the world's commercial prawns consist of penaeid prawns of which there are more than 100 species and also many hundreds of carid species. Table 2.1 summarises the names of families included in the sub-orders Natantia and Reptantia (Holthuis, 1980).

Table 2.1: Families belonging to the suborder Natantia and Reptantia

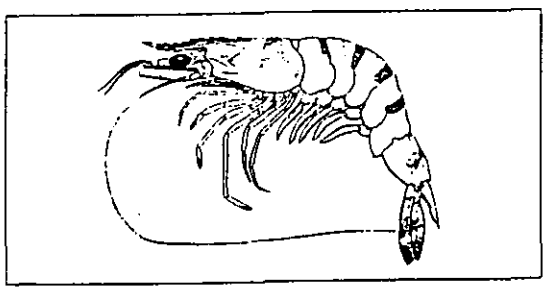
Natantia		Reptantia
Penaeidae	[*Penaeidea*]	*Palinuridae*
Aristeidae	"	*Astacidae*
Sicyoniidae	"	*Paguridae*
Sergestidae	"	*Cancridae*
Solenoceridae	"	*Xanthidae*
Palaemonidae	[*Caridea*]	*Portunidae*
Pandalidae	"	*Grapsidae*
Atyidae	"	*Scyllaridae*
Crangonidae	"	*Galatheidae*
		Lithodidae
		Majidae
		Parastacidae
		Nephropsidae
		Geryonidae

The Tribe *Penaeidea,* which comprises most of the larger prawns of the tropical seas, can be generally recognised by having the first three pairs of legs provided with pincer claws and being nearly alike in size. The penaeid prawns inhabit three main regions in the tropical and subtropical oceans; (a) The Atlantic and Mediterranean, (b) Pacific America and (c) Indian and Pacific Oceans. *Penaeus* is the largest of the families of *Penaeidea* and it contains the greatest number of commercially important species of Natantia, among which are those that are economically of greatest value (Holthuis, 1980). Many of the larger species of *Penaeus*, which are common in the warmer seas and may reach the dimensions of small lobsters, are used for food, are of considerable economic importance in India, Japan, and the Southern United States. Some examples are *Penaeus caramote* which is a common Mediterranean species highly esteemed for the table and *Penaeus monodon* (Figure 2.1a) more commonly known as "crevette" in France and "tiger prawn" in Malaysia and is of considerable commercial importance in Bangladesh, India and the South East Asia. In Malaysia and Thailand, *Penaeus monodon*, is fished in offshore waters, but is also obtained by pond fishing and inshore fishing in Malaysia, Singapore, Indonesia, Phillipines and Taiwan (Holthuis, 1980).

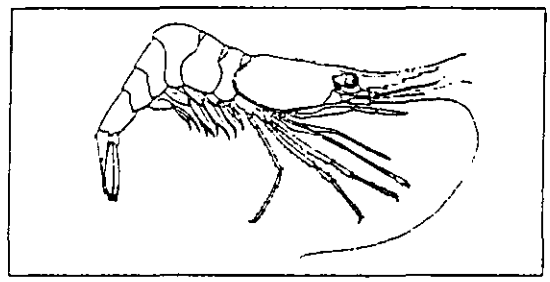
The tribe *Caridea* including the common prawns and shrimps of the British coasts is a large and highly diversified group. Its members may usually be distinguished by having only the first two pairs of legs provided with pincer claws. The Common Prawn (*Leander serratus*) of British coasts is a typical example of the *Caridea.* It is easily recognised by the great length of the sabre-shaped, saw-edged beak or rostrum which projects from the front of the carapace. *Pandalus montagui* is very much like the Common Prawn, but is a little smaller and is also known as the Aesop shrimp (Figure 2.1b). In the North Eastern Atlantic region, the species is only fished commercially by Britain (Holthuis, 1980). Another commercially important caridean of the North Atlantic is *Pandalus borealis* which is commonly known as Pink Shrimp in Great Britain. There are many other kinds of Caridea even in British waters, while in the warmer seas their numbers are endless. In tropical countries, freshwater prawns of many kinds are abundant. The larger kinds belong to the genus

Palaemon. *Palaemon rosenbergii* (commonly known as "udang galah" in Malaysia and Indonesia) is well known for its large size and fine taste. In South East Asia experiments have been started to investigate the possibility of raising this species in ponds for commercial purposes. In Malaysia and Indonesia, the species is economically exploited on a considerable scale.

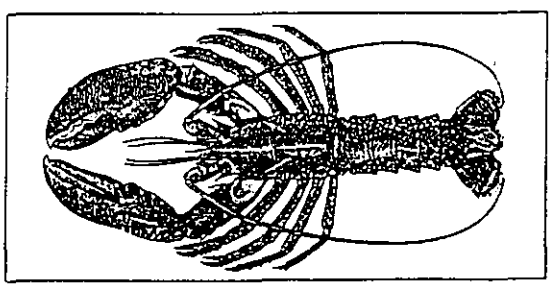
The lobsters consist of three tribes, the Eryonidea, Scyllaridea and the Nephropsidea. The large Spiny Lobsters or Sea Crawfish (*Palinurus vulgaris*) is an example of Scyllaridea and is common on the Southern and Western coasts of British Isles. The tribe Nephropsidea includes the true Lobsters and the freshwater crayfishes. These are distinguished by having the first three pairs of legs provided with pincer-claws, the first pair being large and massive, the rostrum short and more or less flattened and the second antennae slender. The Common Lobster (*Homarus gammarus*) is well known (Figure 2.1c). The so-called Norway Lobster (*Nephrops norvegicus*) also called Dublin Bay Prawn or scampi (see Figure 2.1d), is chiefly caught in the North Sea, the North East Atlantic and the Mediterranean (Early and Stroud, 1982). It is distinguished by its long slender claws and large kidney-shaped eyes. When alive it is of an orange colour, beautifully marked with red and white. Closely allied to the Lobsters are the freshwater crayfishes inhabiting the rivers and lakes of the Northern and Southern Hemispheres respectively.



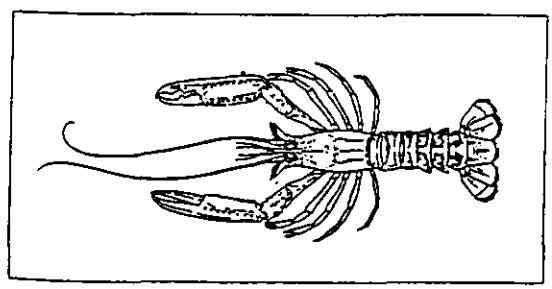
(a) *Penaeus monodon*



(b) *Pandalus montagui*



(c) *Homarus gammarus*



(d) *Nephrops norvegicus*

Figure 2.1 : Different types of prawns and lobsters

2.1.1.2 Exoskeleton of crustacea.

The crustacean exoskeleton, which functions as a support, protection, sites for attachment of muscles and organs and for locomotion, has played a significant role in the evolutionary success of this phylum (O'Brien *et al.*, 1993). This is certainly true of the crustaceans which have become dominant members of the marine, aquatic, and terrestrial environments. The exoskeleton of crustacea consists of a multilaminate construction, the secretory products of a single layered epithelium (Neville, 1975). In general, it has four distinct layers, the epicuticle, exocuticle, endocuticle and membranous layer (Figure 2.2). The outermost layer, the epicuticle covers the whole body and provides a route for the excretion of various glandular products. It is the thinnest layer but is the most complex. It lacks chitin and is thought to be composed of tightly bound proteins, lipids, and calcium salts (O'Brien *et al.*, 1993). It has been suggested that an important function of the epicuticle is to render the exoskeleton impermeable; this would not only prevent water loss, but solubilisation of exoskeletal components as well. Another function suggested is as an important mechanical barrier preventing invasion by parasitic fungi that degrade the cuticle of arthropods by chitinolytic and proteolytic activities (O'Brien *et al.*, 1993). The exocuticle and endocuticle layers contain chitin and protein bound within a calcified matrix (O'Brien *et al.*, 1993). The membranous layer is the innermost layer of the exoskeleton and is in contact with epidermal cells. It contains chitin and protein but is not calcified. The exoskeletons of crabs and lobsters have long since attracted attention as a source of raw material for chitin production as the dry arthropod exoskeleton contains from 25 to 72 % chitin (Skinner *et al.*, 1992). This value varies between species of the decapods. Where flexibility is required, the exoskeleton contains a higher proportion of chitin and its protein is easily solubilised while in species such as crabs which require rigid structures, the chitin content is low and the cuticular proteins are relatively insoluble.

Only portions of the exoskeleton are hardened or sclerotized, for if it is uniformly hard no movement would be possible. The hardened body areas form a series of plates, or sclerites, between which the body wall is soft and flexible. This admirable

arrangement allows the development of exterior parts which serve for protection; it also gives rigidity while at the same time allowing great freedom of movement but must be periodically shed in a process known as ecdysis, or moulting to allow for growth. During the period prior to ecdysis, a moulting fluid containing chitinases and proteinases degrades the old exoskeleton while integumentary tissues synthesize epicuticle and exocuticle of the new exoskeleton which forms the soft shell of the recently exuviated crustacean (O'Brien *et al.*, 1993).

Many decapods, especially those living in environments poor in calcium, reutilise the byproducts of the chitin and calcium salt degradation (Skinner, 1962). As soon as the new cuticle is formed, the arthropod breaks its way through the old one by expanding portions of the body (Goodnight *et al.*, 1964). Following ecdysis, synthesis of the new exoskeleton is completed by the deposition of the endocuticle and membranous layer followed by hardening of the proteins and precipitation of calcium salts.

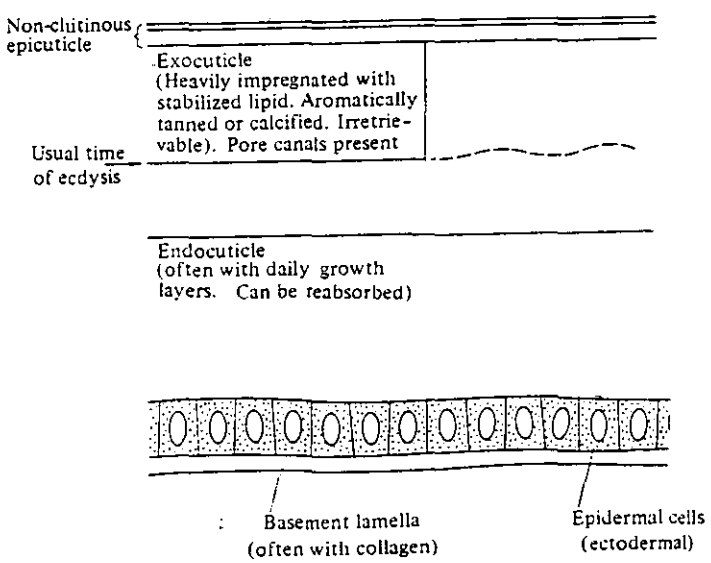


Figure 2.2: The basic structure of the crustacean exoskeleton (after Neville, 1975).

2.1.1.3 Chitin.

The outstanding success of crustaceans in adapting to numerous environments is due to two primary causes: the evolution of the structural material called 'chitin' and the utilisation of this chitin to form a movable protective skeleton. In the crustacean shell, chitin forms interesting composites with polypeptides or proteins, and an inorganic filler, calcium carbonate. Further, chitin is an important component of tendons and other stress-bearing fibrous portions of marine animals, where the chitin molecules adopt a highly oriented structure (Roberts, 1992).

Active systems obviously exist for the synthesis and destruction of chitin, the relative and absolute amounts of which change during the life of the animal (Nicol, 1967). Chitin in the new cuticle is synthesized from sugars derived ultimately from glycogen reserves of the animal. In the epidermis, glucose molecules are aminated and acetylated, leading to the formation of chitin with the aid of an enzyme, chitin synthetase (Neville, 1975). The complete hydrolysis of chitin requires the activity of two enzymes in sequence. Chitinase converts the long chain chitin polymers into smaller oligosaccharides and chitobiose that are in turn hydrolyzed by chitobiase into N-acetyl-D-glucosamine (Jeuniaux, 1966). See section 2.2 for more detail on chitin.

Chitin from animal sources usually occurs in association with protein which functions as a lower modulus (greater deformability) matrix surrounding the chitin to form a composite material (Neville, 1975). Composite materials which normally consist of a combination of a high tensile strength substance dispersed in a lower tensile strength matrix, are stronger than bulk samples of pure substances. This is explained by the fact that pure materials contain many structural defects and under tension, stresses focus on such defects. If instead a crack reaches a weaker, more deformable region, the stress is spread more evenly and the material survives (Neville, 1975). In nature other examples of such composite materials are wood, consisting of cellulose fibres in a lignin matrix and bone consisting of hydroxyapatite crystals in a matrix of collagen and mucopolysaccharide. Thus the cuticle

ultrastructure serves equally well for offence or defence; it has good impact resistance, tensile and compressive strength (Neville, 1975).

2.1.1.4 Protein.

The biological role of the exoskeletal protein is thought to be that of a defence mechanism against the action of chitinases (O'Brien *et al.*, 1993), and Neville (1975) has reported a membrane containing approximately 75 % chitin and 15 % protein that is resistant to the action of chitinases unless the protein is removed, after which the chitin is rapidly attacked. The epicuticular (outermost layer of the exoskeleton) proteins of crabs were much different from proteins in any other layers, having molecular weights ranging from 54 to 42 kDa (O'Brien *et al.*, 1993) and is the only layer devoid of chitin. This kind of protein increases significantly during periods of proecdysis when the new exoskeleton is synthesized but little degradation of this high molecular weight protein from the old epicuticle occurs during moulting. In contrast to proteins of the epicuticle, many proteins in extracts of exocuticle, endocuticle and the membranous layer were of similar size, especially those smaller than 31 kDa. Moreover, the latter may be associated with chitin (O'Brien *et al.*, 1993). Unlike the epicuticular proteins, these smaller proteins had been preferentially degraded during the moulting period. The extent of interaction between chitin and protein molecules differs within a given sample. Austin *et al.*(1981) reported that approximately 3-28 % of total dry crab shells is accounted for by covalently bound protein (total protein 12-73 %) and that protein residues remain with the chitin even after the most drastic alkali treatment. While the composite material structure of hard cuticles demands covalent bonds between chitin and protein, there are other situations where weaker, more labile, bonds are required. Hackman and Golberg (1958) introduced a sequence of extractions with aqueous solutions of increasing extractive power and noted the different percentages of bonding occurring within the molecule:

a) cold water at pH 7.0 for 48 hours, removes soluble, unbound protein,
b) cold 0.17M Na_2SO_4 at pH 7.0 for 48 hours, removes protein bound by weak forces such as van der Waals' forces
c) cold 7M urea at pH 7.0 for 48 hours, removes hydrogen-bound protein

d) cold 0.01M NaOH for 5 hours, removes electrostatically bound protein

e) 1.0M NaOH at 50 - 60°C for 5 hours, removes strongly bound protein, presumably covalently bonded to the chitin.

It can be concluded from the above procedures that boiling with NaOH will ensure complete or almost complete removal of protein from chitin as this treatment disrupts the strongest covalent bond that might occur within the chitin-protein matrix. This result also suggested the existence of different kinds of protein bonding in the exoskeleton.

Although many of the roles of the exoskeleton of crustacea are structural in nature, there are proteins within the exoskeleton whose functions are unrelated to structure but to enzymatic activities. During the proecdysial period for example, the old exoskeleton is permeated by proteinases that disrupt the protein-chitin-calcium components. Although not much is known about the specific proteinases active in the degradation of the crustacean exoskeleton, O'Brien and Skinner (1988) partially purified two sets of proteinases from Bermuda land crab. One set was active at alkaline pH while the other at acidic pH. Almost all the membranous layer proteins (< 24 kDa) were hydrolysed by the alkaline proteases which are thought to play a more important role in the degradation of the exoskeleton than the acidic proteases (Skinner *et al.*, 1992; O'Brien and Skinner, 1988).

Another important enzymatic activity is that of chitinases, chitin-degrading enzymes. The enzyme is thought to be constitutive in nature, activated only during the proecdysis period and displayed optimal activity near pH 5.0, a value typical of chitinases in general (O'Brien *et al.*, 1993). Little is known of the mechanisms that control the degradation of the exoskeleton during the moulting period but cyclic changes in the pH of the extracellular matrix may be one means. The degradation of the cuticle by chitinases proceeded much more rapidly following exposure of the cuticle to proteinases than if the cuticle were exposed to chitinase alone. This fact suggests that chitin is shielded from chitinases by a protective covering of proteins. Since all the chitinases isolated from integumentary tissue exhibit activities with

acidic pH optima and the dissolution of calcium carbonate occurs under acidic conditions, it was suggested that the acidic proteinases are responsible for the degradation of the calcified layers while that with an alkaline pH optimum degrades the membranous uncalcified proteins (O'Brien *et al.*, 1993). However, it must be remembered that the high molecular weight-epicutilar, tightly-bound proteins are least affected by all the above enzymatic processes and that may only be removed by the chemical means in part (e) above.

2.1.1.5 Calcification of crustacea.

Calcification as a means of stiffening cuticle is found in crustaceans and millipedes and in a very small number of insects. In crustaceans, calcification enables them to withstand considerable hydrostatic pressure and so to live in great depths of water. Calcium carbonate is the major inorganic constituent, others are magnesium carbonate and calcium phosphate Absolute values of inorganic constituents vary with age and species (Nicol, 1967). For example, shrimps contain 60 % ash while lobsters and crayfish show values around 74 - 78 % ash.

Calcification of the cuticle takes place rapidly after the moulting period (Nicol, 1967) where calcium is reabsorbed from the environment and also from calcium stores within the body. The degree of calcification stands in inverse proportion to the amount of protein present in the cuticle. In newly formed cuticle prior to calcification, there are roughly equivalent quantities of chitin and protein. With progress of calcification the quantity of protein added to the cuticle decreases. Calcification thus replaces a large proportion of protein that would otherwise be needed for hardening the cuticle, and is an economical process in an environment rich in calcium (Nicol, 1967).

2.1.2 Other sources of chitin.

i) Fungal fermentations.

This source of chitin seems to be quite promising for the future. The pharmaceutical industries of most countries already exploit fungal fermentation for the production of vitamin C and penicillin and large quantities of chitinous waste are produced (Nicol,

1991). The advantage of fungal chitin over crustacean sources is that, this source of chitin is consistent in composition, is available throughout the year and does not require a demineralisation step (Roberts, 1992), although it is usually present in association with other polysaccharides which must also be removed.

ii) Squids and krill.

The pens of squids contain between 30 - 35 percent chitin and largely free of minerals (Kurita *et al.*, 1991). So far, an application for β-chitin from squid which is softer than α-chitin, has yet to be established (Kurita *et al.*, 1994). Krill resembles a very small shrimp in appearance and has been the food of whales for centuries (Martin, 1979). Although krill contains chitin (25 percent dry basis), the primary product of the krill industry is mainly protein which is destined for human consumption or is used whole for aquaculture (Nicol, 1991). After peeling, 85 percent by weight remains as waste. It is expected that the krill catch will expand from its current levels and will dominate the total world crustacean production and would be a major potential source of chitin (Nicol, 1991).

iii) Molluscs (clams and oyster shell).

The use of clams and oyster shells is inhibited by the large quantities of inorganic material that must be removed, up to 90 % dry weight (Roberts, 1992).

iv) Insects.

Insects contain chitin but it is quinone tanned, which makes it difficult to extract and there is no consistant source (Nicol, 1991).

v) Biotechnology.

Currently, the chitin and chitosan industry is thriving by using cheap supplies of waste material. However, if demand increases in future, manufacturers could develop genetically engineered microorganisms to produce these useful molecules (Nicol, 1991). Some species of fungi produce up to 14 percent by weight of chitosan and this would eliminate the deacetylation step which at present makes chitosan nearly twice as expensive to produce as chitin (Crestini *et al.*, 1996). Certain algae

produce pure chitin in the form of extracellular fibres which can be 10 to 15 percent of the dry weight of the cells, but these algae grow slowly under normal conditions. Advances in biotechnology may produce fast growing strains that retain large amounts of chitin (Nicol, 1991).

2.2 Chitin and chitosan.

Chitin is one of the most abundant organic materials, being second only to cellulose in the amount produced annually by biosynthesis (Roberts, 1992). Chitin is an important structural component of the exoskeleton of crustaceans, molluscs and insects (Neville, 1975). It also makes up parts of the jaws and body spines of certain worms, but the occurrence of chitin in the plant kingdom is confined to fungi and some algae (Nicol, 1991; Roberts, 1992). Henri Braconnot was the first to describe chitin, as long ago as 1811 (Knorr, 1991). In 1859, a chemist called Rouget found that heating chitin with very concentrated sodium hydroxide converted it to a related and much more useful chemical, called chitosan (Roberts, 1992). This reaction removes some of the acetyl groups from the molecular chain, leaving behind complete amino (NH_2) groups, making chitosan one the most versatile biopolymers. Chitosan also occurs naturally in the cell wall of some fungi (e.g. *Mucor rouxii, Phycomyces blakesleeanus* and *Aspergillus niger*) but its occurrence is much less widespread than is that of chitin (Simpson *et al.,* 1994). The criterion for distinguishing between chitin and chitosan is the solubility of the polymers in dilute aqueous acid: chitin is insoluble while chitosan forms viscous solutions.

2.2.1 Structure and chemical properties.

2.2.1.1 Chitin.

Chemically, chitin is poly[β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose] (Fig 2.3a). It is structurally similar to cellulose (Fig 2.3c), except that the C2-hydroxyl group of cellulose is replaced by an acetamido group, and this similarity in structure is reflected in the similar roles played by the two polymers in nature, both acting as structural and defensive materials (Roberts, 1992). In a small number of residues the acetyl group may be missing, leaving glucosamine, so that the chitin chain as a whole may be positively charged (Neville, 1975).

Chitin has a highly ordered, crystalline structure as evidenced by X-ray diffraction studies (Roberts, 1992). It has been found in three polymeric forms, α-, β- and γ-chitin, which differ in the arrangement of the chains within the crystalline region. In α-chitin the chains are antiparallel, in β-chitin, they are parallel, and in γ-chitin, they

are mixed parallel and antiparallel orientations. The existence of these three polymorphic forms is related to the diversity of function: α-chitin is normally found where extreme hardness is required, as in the arthropod cuticle, and is frequently associated with sclerotised protein or inorganic materials or both, whereas the β and γ -chitin are found where flexibility and toughness are required such as in the squid pen (β-chitin) and γ-chitin in its stomach lining (Roberts, 1992).

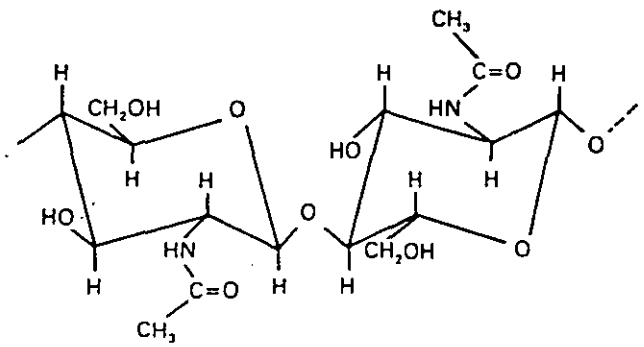
Chitin is insoluble in water and in ordinary organic solvents such as alcohol (Muzzarelli, 1977; Knorr, 1984; Ornum, 1992; Roberts, 1992). However, it is soluble in strong mineral acids and in anhydrous formic acid, but insoluble in alkali. Its high degree of crystallinity enables chitin to be cast into films or membranes (Muzzarelli, 1977). It forms complexes with transition metal ions but to a much lesser extent than chitosan and adsorbs dyes mainly through an ion exchange mechanism. It also adsorb proteins and enzymes possibly through a non-covalent bonding (hydrogen bond or Van der Waals forces). Pyrolysis of chitin at high temperatures (about 900°C) produces a number of flavour compounds such as butyric acid (butter flavour) and pyrazines which contribute significantly to the characteristic flavour of toasted and roasted foods (Simpson *et al.*, 1994).

2.2.1.2 Chitosan.

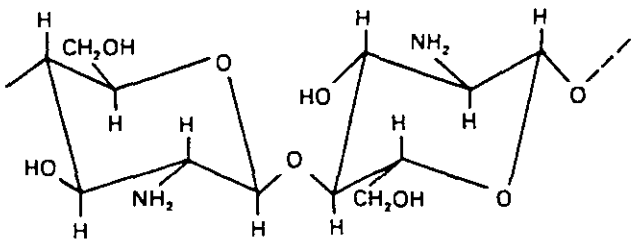
Chitosan is a poly[β-(1→4)-2-amino-2-deoxy-D-glucopyranose] (Figure 2.3b), made either by chemical or by biological means which remove the acetyl groups from chitin, leaving a polymer with varying levels of free amine groups capable of forming strong hydrogen bonding and ionic interaction. Chitosan is thus not a precise chemical species, but a polymer with varying levels of free amine groups. The free amine group gives chitosan its primary properties useful in application development. In both fungi and invertebrates, there are varying degrees of deacetylation (Roberts, 1992), giving a continuum of structure between chitin (fully acetylated) and chitosan (fully deacetylated).

Unlike chitin, chitosan is readily soluble in most dilute organic acids, such as formic and acetic acid, and its salt is soluble in water thus finding wider applications in

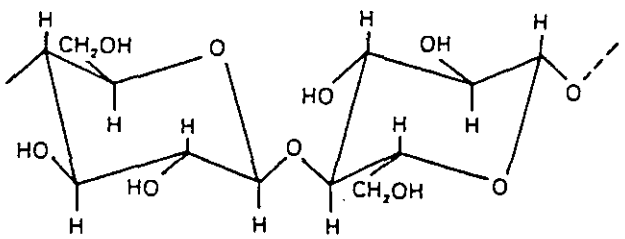
industry than chitin (Simpson *et al.,* 1994). However, chitosan is also insoluble in organic solvents and alkali (Roberts, 1992). At acidic pH, chitosan is a linear polyelectrolyte with a high charge density of $-NH_3^+$, making it a high positively-charged polyelectrolyte, thus is able to attract negatively charged particles (e.g. proteins, anionic polysaccharides, nucleic acids). Chitosan is able to form complexes with transition metal ions (e.g. Cu, Co and Ni) through the free amine group and is also able to adsorb dyes mainly through an ion exchange mechanism. Chitosan can also be modified by derivatisation, producing polymers with enhanced performance.



(a)



(b)



(c)

Figure 2.3: The structure of (a) chitin, (b) chitosan and the structurally related polysaccharide (c) cellulose

2.2.2 Purification of chitin.

Chitin as obtained from crustacean waste is closely associated with proteins, inorganic material (mainly calcium carbonate) pigments and very small amounts of lipid. Therefore in general, the purification of chitin consists of two basic steps: 1) deproteinisation or protein separation, and 2) demineralisation or the removal of minerals (Muzzarelli, 1977). Deproteinisation can be carried out either chemically or enzymatically and demineralisation is purely an acid-based reaction.

2.2.2.1 Chemical methods.

The general scheme for preparing chitin and chitosan from shellfish waste is summarized in Figure 2.4. The demineralisation process is most frequently carried out by treatment with HCl and the deproteinisation process usually by treatment with NaOH or KOH. The order in which these two steps are carried out has varied with different workers and some of the conditions used can be seen in Table 2.2 and 2.3 (Roberts, 1992). The choice of processing conditions may be governed to some extent by the purpose for which the chitin is required. For example, ultrapure chitosan is generally required for medical purposes and pharmaceuticals whilst for industrial applications, such as waste water treatment, agriculture and metal recovery, the quality requirements are not as stringent (Sandford, 1988). Partial deacetylation and depolymerisation of the chitin chain does occur during these two processes (Muzzarelli, 1977). However, since chitin is usually converted to chitosan, partial deacetylation during the deproteinisation step is not a disadvantage whereas depolymerisation affects the viscosity of the final product (Muzzarelli, 1977).

The main disadvantage of using the traditional chemical purification method is that these procedures not only require disposal of waste liquors (containing calcium chloride) or solids for which no market can be found (Roberts, 1992), it also requires high energy input (temperatures in the region of 90°C) for the deproteinisation stage. Hydrochloric acid is also a relatively expensive reagent. Apart from these adverse environmental aspects of the traditional treatment of waste shells, the process does not allow for the recovery of other added-value products such as protein and pigments (see section 2.3). If the protein is recovered, it has to be neutralised before

being used as an animal feed ingredient. Furthermore, protein becomes racemated by alkali treatment and as such it is not nutritionally valuable (Vieira *et al.*, 1995). The processing plant also requires special tanks to avoid corrosion by the chemicals employed.

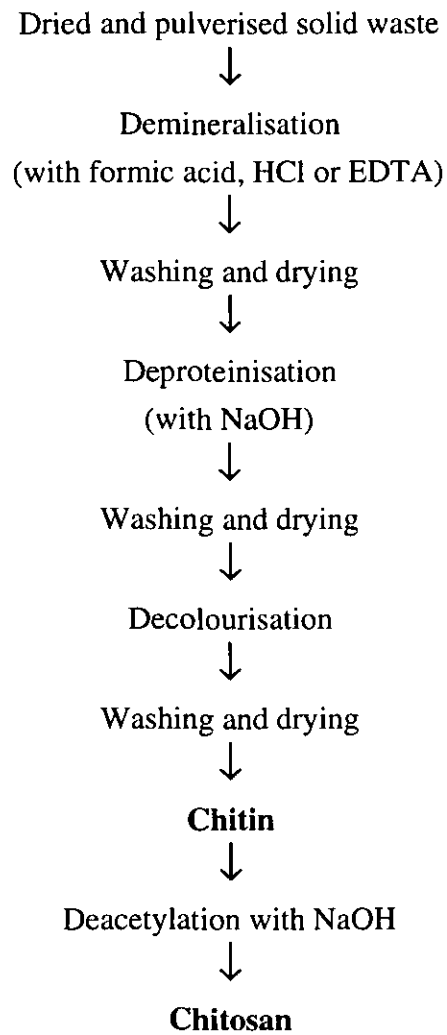


Figure 2.4: General scheme for preparation of chitin and chitosan (Simpson *et al.*, 1994).

Table 2.2: Conditions employed for deproteinisation of chitin-containing waste materials (after Roberts, 1992).

Material source	NaOH concentration(M)	Temperature (°C)	Number of treatments
Shrimp	0.25	65	1
Crab	0.5	65	1
Prawn	i 0.125	100	1
	ii 0.75	100	1
Krill	0.875	90-95	1
Crab	1.0	80	1
Crab	1.0	100	1
Lobster	1.0	100	5
Crab	1.0	100	3
Lobster	1.25	80 - 85	2
Crab	1.25	85 - 90	3
Prawn	1.25	100	1
Crab	1.25	100	1
Crab	2.5	room temp.	3
Lobster	2.5	100	1

Table 2.3: Conditions employed for demineralisation of chitin-containing waste materials (after Roberts, 1992).

Material source	HCl concentration (M)	Temperature (°C)
Shrimp	0.275	RT
Shrimp	0.5	ns
Krill	0.6	RT
Crab	0.65	RT
Crab	1.0	RT
Crab	1.0	RT
Prawn	1.25	RT
Crab	1.57	RT
Lobster	1.57	RT
Prawn	1.57	20 - 22
Crab	2.0	RT
Lobster	2.0	RT
Crab	11.0	-20

ns=not stated, RT= room temp.

2.2.2.2 Biological methods.

Deproteinisation can also be carried out by enzymatic methods. The harshness of the chemical method as well as the environmental and safety implications of this technique, has prompted researchers to find alternatives. Two methods of biologically removing protein from shellfish waste exist: 1) proteolytic bacteria which can secrete extracellular proteases into the medium or 2) by using added proteolytic enzymes.

The first method has been adopted Shimahara *et al.* (1982) and makes use of proteolytic bacterial cultures of *Pseudomonas maltophilia.* This species was found to be highly proteolytic but neither chitinolytic nor chitin deacetylating. The system adopted, however, had a very high water to solid ratio (99:1) and is therefore not economically attractive for the production of a low-cost deproteinised chitin (Healy *et al.*, 1994). The extent of removal of protein using this strain varied with the species of crustacea and with the carapace of *Penaeus japonicus* giving the best result. Although samples were incubated for up to 240 hours at 30°C, no further decrease in protein content occurred after 72 hours even if the chitin was transferred to a freshly innoculated batch. This indicates that the residual protein, ranging from 1 % to 7 % approximately, is inaccessible to the proteinase involved (Roberts, 1992).

In the second method, Takeda and Abe (1962) added proteolytic enzymes such as tuna proteinase, papain or a bacterial proteinase for removal of protein from shell material. This treatment resulted in chitin containing 5 % protein. Although the enzymatic method does not cause any depolymerisation and deacetylation of chitin, complete elimination of protein could not be attained. Furthermore, the use of enzymes results in increased costs particularly if purified preparations are employed.

Recent work by Hall and De Silva (1992) described an interesting and promising application of the ensilation technique to the purification of chitin from shellfish waste. Ensilation is simply the use of acid conditions to prevent the growth of spoilage organisms and has been applied to trash fish for many years (Raa and Gilberg, 1982). Acid conditions can be produced by the addition of mineral or

organic acids or can be generated *in situ* by bacterial fermentation as demonstrated by Hall and De Silva (1992) using a commercial inoculum, "Stabisil". The production of acid by lactic acid bacteria lowers the pH and allows the indigenous proteases in the shellfish waste to initiate autolysis and carry out the breaking down of protein from the waste. At the same time, the acid conditions dissolve calcium carbonate, which is analogous to the demineralisation step in the traditional chitin recovery process. Thus, the lactic acid fermentation, besides acting to preserve the raw material, also partially purifies the chitin from protein and calcium carbonate. The advantage of the lactic acid fermentation is that, this method preserves indigenous enzymes which initiates the protein breakdown from the waste as well as removing the minerals. Vieira *et al.* (1995) reported that protein hydrolysed enzymatically has desirable functional properties compared to those treated by chemicals. They employed proteases such as papain, pepsin and a fungal protease (*Aspergillus niger*) to hydrolyse meat obtained from lobster heads (*Panulirus laevicauda*) and found that the the hydrolysate possessed a distinct, pleasant smell which could lead to its use as a flavour enhancer, as well as a protein supplement.

2.2.2.3 Characteristics of purified chitin.

The purity of chitin varies enomously depending on the procedures adopted and the source material (Muzzarelli, 1977). Even chitin from the same animal varies in the length of its molecular chain, its crystallinity, and in the number of acetyl groups present on the chain (Nicol, 1991). Table 2.4 shows the characteristics of chitin reported by several workers.

Table 2.4: Characteristics of chitin[a] prepared from various sources

Specification	Shrimp[b]	Crab[b]	Tiger shrimp[c]	Crawfish[d]
Moisture	3.00	0.60	4.89	0.1
Ash	0.09	0.10	0.54	not determined
Lipid	0.00	0.00	0.49	not determined
Chitin	not determined	not determined	97.04	not determined
Total nitrogen	not determined	not determined	6.77	not determined
Corrected protein[e]	2.32	0.49	0.75	not determined
Chitin nitrogen	6.29	6.42	6.65	7.01

[a] = % dry wt. basis, [b] = Shahidi & Synowiecki, 1991, [c] = Sophanodora & Hutadilok, 1995, [d] = No *et al.*, 1989, [e] = corrected protein calculated as (total N - chitin N) x 6.25

The chitin nitrogen of the four examples quoted in Table 2.4 all differ from the theoretical value of 6.89 % for chitin ($C_8H_{13}NO_5$). No *et al.* (1989) reasoned that protein might still be present even after vigorous acid and alkali treatment, resulting in high values for chitin nitrogen content of 7.01 %. Deacetylation, if it occurs during purification process will increase the nitrogen content to a maximum of 8.69 % for pure chitosan ($C_6H_{11}NO_4$). Commercial chitin is normally sold as flakes or as a fine powder with a moisture content < 10 %, ash < 2 %, and deacetylation > 15 % (Ornum, 1992).

2.2.3 Production of chitosan.

A Japanese company claimed to be the world's first to produce chitosan industrially in 1971 and in 1986, a total of fifteen companies in Japan were producing chitin and chitosan (Hirano, 1988). These products are supplied in the form of flakes, powders, beads, fibres, sheets and films. In Japan, consumption of chitosan was mainly for flocculants, cosmetics, feeds and foods with an increasing amount being used in the biotechnology industry.

Chitosan can be made by treatment of chitin with hot concentrated sodium hydroxide solution (40 - 50%) for 30 minutes under nitrogen gas to reduce chain cleavage (Bough *et al.*, 1978). Chitin is first treated with very strong NaOH (Figure 2.4) to hydrolyze the N-acetyl linkage, then rinsed, pH adjusted and dewatered. At this stage, the chitosan can be dried to give what is called 'flaked chitosan'. This is a coarse mesh product whose particle size can be reduced by milling to give a finer mesh powder. Increasing the temperature or the strength of the sodium hydroxide solution removes more acetyl groups. Commercial chitosan is typically 70 % - 80 % deacetylated (Kurita, 1986; Ornum, 1992).

2.2.4 Applications.

The challenge for the future of chitin and chitosan is its market development. There are numerous potential applications of chitin and chitosan, but questions remain over the cost-effectiveness of chitosan versus alternative materials and technologies (Ornum, 1992). However, chitin and its derivatives today appear as substances of

much greater significance and relevance than would have been believed ten years ago. Most of the applications discussed below are still largely experimental with only a few commercial products in the marketplace. Several countries, including the US, Japan, Norway, Italy and India, already have chitin/chitosan plants based on shellfish waste as their source (Nicol, 1991). The little that they produce is used primarily by the pharmaceutical industry and in the treatment of waste waters. However, chitin and chitosan have several interesting characteristic features which makes them suitable for use as an industrial material (Knorr, 1984, Simpson *et al.*, 1994). These are that:

i) they represent a major component of biomass which is biologically reproducible
ii) they are biodegradable, breaking down slowly to harmless carbohydrates, carbon dioxide and water
iii) they are biocompatible not only in animal but also in plant tissues
iv) they are non-toxic biopolymers
v) they can be fabricated into gels, beads, fibres, colloids or film
vi) they have amino and hydroxyl groups which are chemically modifiable

2.2.4.1 Applications of chitin.

Chitin itself has relatively few applications compared to chitosan, its most familiar derivative, which has many current and potential uses in several industries. Compared to chitosan, chitin has a lower degree of deacetylation, i.e. a lesser number of free amine groups which are greatly responsible for most of the reactivities of chitin and chitosan and thus their applications. The applications of chitin are discussed under the various properties of chitin.

i) Complex formation with metal ions.

Since the adsorption of metal ions is reported to depend upon the amine group of the molecule, it is expected that pure chitin will not complex with metal ions (Roberts, 1992). Studies on the interactions in a solution of D-glucosamine and N-acetyl-D-glucosamine with Cu(II) ions, using potentiometric and spectroscopic techniques,

revealed that N-acetyl-D-glucosamine did not complex with Cu(II) ions (Roberts, 1992). It is therefore expected that chitin will not have a major application in areas where complexation with metal ions is required. If chitin does complex with metal ions at all, it will do so to a much lesser extent than chitosan, and possibly does so through any available free amine groups that might exist throughout the biomolecule.

ii) Adsorption on chitin.

a) Adsorption of dyes.

It is reported that the adsorption of dyes takes place through an ion exchange mechanism, through both the amino and acetyl amino group on chitin (Roberts, 1992). Because of its high capacity for anionic dyes, chitin has been proposed as an adsorbent of dyehouse effluent and a detailed study has been reported (McKay *et al.*, 1982).

b) Adsorption of proteins.

Chitin is able to adsorb proteins through non-covalent bonding, either via Van der Waals forces or by hydrogen bonds (Roberts, 1992). These proteins, once adsorbed, can be released by various chemicals depending upon the bonding formed. This ability of chitin to adsorb proteins has been made use of in affinity chromatography where wheat germ agglutinin was purified on a chitin column and this approach has also been extensively used for purification of lysozyme (Roberts, 1992). A major market for chitin and chitosan is the water purification industry, where the biodegradable polysaccharides can serve as flocculents.

Since enzymes are proteins, chitin provides a good supporting material for immobilisation of enzymes either through covalent bonds or via physical adsorption (Roberts, 1992). Amylase immobilised on chitin showed almost the same digestion rate for raw corn starch and gelatinised potato starch as did the free enzyme and is reported to be suitable for recycling (Roberts, 1992). Chitin also provides a solid support system for immobilisation of glucose isomerase (Stanley *et al.*, 1976), lactase, α-chymotrypsin, and acid phosphatase (Stanley, *et al.*, 1975) by reaction with gluteraldehyde.

iii) Other uses of chitin.

Chitin has been used in a bioconversion scheme to produce yeast single-cell protein (Cosio *et al.*, 1982; Sabry, 1992). The authors used shrimp shell chitin waste pretreated with acid and alkali. The pretreated chitin was then mixed with chitinase to hydrolyse the chitin to the monomer N-acetyl glucosamine which served as the substrate for production of single-cell protein (SCP) which has been shown to be an acceptable component of aquaculture feed formations (Cosio *et al.*, 1982). Perhaps the greatest potential application of chitin is in paper manufacture. Addition of chitin increases strength, water retaining capacity and makes the paper easier to print on (Nicol, 1991). Paper that incorporates chitin has greatly improved wet strength - an advantage for disposable nappies, shopping bags and paper towels. Any move towards the general use of chitin in paper manufacture would require a huge increase in chitin production. To date most applications make use of either purified chitin or chitosan and its various derivatives. These applications have been made possible due to our present understanding of the underlying chemical mechanisms that might occur, as already explained above. However, an interesting application of a partially purified chitin has been reported by Coughlin *et al.* (1990) in using crab shell waste to purify electroplating wastewater. They excluded the normally-used deproteinisation step but instead applied a minimal deacetylation treatment of only a few minutes to develop chitosan at the outer periphery of the shell particles without substantially converting the interior regions of the particles. This is indeed interesting because if partially purified chitin can find a role in the future this may cut tremendously the cost of production of chitin, including more highly purified forms.

2.2.4.2 Applications of chitosan.

Since both existing and potential uses of chitosan have been thoroughly reviewed by Skjak-Braek *et al.* (1989), only a brief survey of the main areas of applications will be given here.

i) Waste water treatment.

The principal use of chitosan has been as a 'flocculating agent' for clarification of industrial effluent and other waste products, e.g. sludges from breweries, sewage

works, stick water from processing plants, slaughterhouses (No and Meyers, 1989) and as chelator of toxic (heavy and radioactive) metals (Knorr, 1991, Hansen and Illanes, 1994). One of the earliest uses of chitosan was to purify waste water from the processing of shellfish (Nicol, 1991). A study of one crawfish processing plant in Lousiana in 1989 showed that chitosan derived from the waste could be used to remove 97 percent of the solids suspended in waste water (Nicol, 1991). No and Meyers (1989) used crawfish chitosan as a coagulant in the recovery of organic compounds from seafood processing streams. Their results showed that chitosan was found to be superior to synthetic polymers in reducing the turbidity of crawfish wastewater. If chitosan were used to recover protein solids from the food processing industry, the chitosan-protein coagulant could be directly included in animal feed. It is estimated that only 0.05 to 0.1 % of chitosan will be present in animal diets when a chitosan-protein coagulant is used and that this small amount of chitosan has no adverse effects on the nutritional value of proteins and is non-toxic to livestock. Japanese firms such as Kurita Industries sell chitosan as a flocculant (Nicol,1991), as does the Norwegian company Protan, which recommends it for the clarification of swimming pools and spas as it flocculates microbes and removes metals (Nicol, 1991).

iii) Medical care.

Higher technology applications in the medical field include use as a blood anticoagulant, in wound healing and in artificial kidney membranes (Muzzarelli, 1983). Chitosan beads have been used as a biocompatible matrix to deliver drugs (Hirano, 1988). Chitosan has also been shown to be suitable as an eye bandage material and may even have utility as a contact lens material (Hirano, 1988). Chitosan can be made into strong fibres that may have use as suture material and as a wound dressing (Muzzarelli, 1977). Chitosan is also said to have cholesterol-lowering ability (Hirano, 1988) and to have potential for use as dietary fibre (Simpson *et al.*, 1994).

iv) Cosmetics.

Chitin was first used in cosmetics in 1969 (Nicol, 1991). Chitosan has proved to be an excellent raw material for the synthesis of customised polymers for all kinds of purposes in the field of cosmetics (Lang and Clausen, 1988). In Japan, chitosan and carboxymethylchitin (CM-chitin) have been approved as ingredients for hair and skin products (Hirano, 1988). CM-chitin is soluble in water and its aqueous solution is viscous and moisture retaining. This property is essentially similar to that of hyaluronic acid present in human skin. The products synthesized on the basis of chitosan are suited as film-forming agents for setting lotions, blow-dry lotions, hair sprays and nail vanishes, humectants for skin cosmetics, thickening agents for gels and emulsions, substantive polymers for shampoos and hair conditioners as well as other hair and skin care preparations. Clear solutions form clear films that adhere to skin or hair, primarily due to chitosan's cationic character. Several cosmetic products containing chitosan are on sale from Wella in Germany and now in Japan (Nicol, 1991).

v) Agriculture.

This area of application is one of the most recent and promising (Hansen and Illanes, 1994). Chitosan appears to have multiple physiological effects. Chitosan can act as a fungicide, growth enhancer, and protective agent for plants and trees (Sandford, 1988). Ramachandran Nair *et al.*, (1986) has also reported prolonged ripening period of fruits like mangoes and bananas by surface coating.

vi) Biotechnological uses.

Due to its non-toxic nature, biocompatibility, variety of useful forms, and its unique properties, chitosan is used commercially to immobilized enzymes and cells. Chitosan mixed with alginate has been used in the preparation of membranes for the encapsulation of tissue and bacterial cells (Rodriguez-Sanchez and Rha, 1981). The biological material remained viable and protected by a membrane fully permeable to nutrients and metabolic products.

vii) Food industry.

In the food processing industry, there exist three key future applications of chitinous polymers (Bough, 1977; Knorr, 1984; Ramachandran Nair *et al.*, 1986, Knorr, 1991, Hansen and Illanes, 1994):

a) As an agent for treatment of food processing wastewater. The chelating properties of chitosan prove advantageous in removing heavy metals, dyes, pigments and proteins (mentioned in detail in part i).

b) As a functional ingredient to improve texture. Chitosan can be used as a food thickener, adhesive and gel-forming agent for the production of sauces, ice cream and fruit juices. It can also be used as an emulsifier because of its ability to bind water and fat.

c) As a new polymer for improving packaging materials. Based on the fact that chitosan has film forming properties and that chitosan degrading microorganisms are abundant, use of chitosan polymers as packaging materials provide protection for the environment.

2.3 Shellfish wastes.

In general, the shellfish industry discards up to 60 % of the shellfish waste which includes parts such as the heads (cephalothorax), claws (including the inner meat), shells and adhered meats (Hansen and Illanes, 1994). This waste material contains many valuable components, among the most prominent of which is the chitin. The extraction and uses of chitin have been covered in section 2.2. Other useful components are the protein, enzymes and pigments. Today, finding uses for these waste components is gaining in popularity as a means to improve profitability in the seafood industry.

2.3.1 Composition of shellfish waste.

2.3.1.1 Chitin, protein and calcium content.

The high protein content reported in shellfish waste actually comes from the adhered meat, meat in the claws and protein from the head. In additon, the exoskeleton also contains a small amount of protein besides calcium carbonate and chitin. The composition of shellfish waste in terms of chitin, protein and calcium carbonate as reported by various workers is given in Table 2.5:

Table 2.5: Typical values of chitin, protein and calcium content of shellfish wastes

Source of waste	Chitin (%)	Protein (%)	Calcium (%)	References
Crawfish	14.2	32.2	18.1	Chen & Meyers, 1983
Shrimp shells	27.2	22.8	11.1	Barratt & Montano, 1986
Shrimp heads	11.1	53.5	7.2	Barratt & Montano, 1986
Crawfish	15.9	35.8	12.3	No *et al.*, 1989
Shrimp	17.0	41.9	ns	Shahidi & Synowiecki, 1991
Shrimp	21.4	27.9	16.0	Sabry, 1992

ns = not stated

The protein if recovered is an excellent ingredient for animal feed. Shrimp silages have been prepared by the addition of organic acids (Barratt and Montano, 1986). The acid conditions allow the enzymes to breakdown protein forming a liquid protein (silage). The liquid silage can be mixed with other substances such as malt wastes, rice husk, corn, bananas and other carbohydrate-rich substances. Once dried, it could be included in formulations in poultry, cattle as well as shrimp-feeding.

2.3.1.2 Enzymes.

The shellfish wastes contain many valuable enzymes, either from the digestive system in the viscera which includes proteases and carbohydrate-degrading enzymes, the muscles or from chitin metabolism (O'Brien *et al.*, 1993, Hansen and Illanes, 1994). All these enzymes play a role in the growth of crustacea. However, during processing of seafood, many of these valuable enzymes may be discarded as wastes or are washed away in the processing wash water (Hansen and Illanes, 1994). Enzymes from marine organisms are reported to have unique properties such as having high molecular activity at low temperatures (Simpson and Haard, 1987) and have many applications in the food industry (Haard and Simpson, 1994).

Olsen *et al.* (1990), was able to recover four types of enzyme from the effluent of the shrimp processing plant which were: alkaline phosphatase, hyaluronidase (a carbohydrate-degrading enzyme), acetylglucosaminidase and a chitinase. Another study on the proteolytic enzymes in grass shrimp (*Penaeus monodon*) revealed the existence of trypsin-like and chymotrypsin-like enzymes (Jiang *et al.*, 1992). Crustacea and other invertebrates have generally been considered to be able to hydrolyse protein with an enzyme complement similar to that of vertebrates except for the lack of a peptic enzyme (Vonk, 1960). The lack in pepsinolytic activity in crustacea has also been reported by Baranowski *et al.* (1984). It has also been suggested that crabs and amphipods use catheptic and tryptic enzymes whereas shrimps and barnacles have only catheptic components (DeVillez and Buschlen, 1967). The existence of cathepsins has also been reported by Jiang *et al.* (1992). However, trypsin from fish tends to be stable in alkaline medium and very unstable in acidic conditions whereas trypsin from mammals is most stable in an acidic medium (Haard and Simpson, 1994). Cathepsins from different aquatic species display maximum activity over a broad pH range, from pH 3.5 to pH 8.0 (Haard and Simpson, 1994). If shrimp wastes are to preserved by a lactic acid fermentation it would therefore be expected that the enzymes that might be active during autolysis of waste protein are mostly those that are active at acidic conditions. To date, no such data has been published. The enzymes involved in chitin metabolism have been covered in section 2.1.1.4.

2.3.1.3 Pigments.

The exoskeleton of crustaceans contain carotenoid pigments, mainly astaxanthin, which is suitable for incorporation into fish feed for aquaculture of salmonid species (Guillou *et al.*, 1995). The presence of carotenoid pigments (astaxanthin) in fish feed influences the red colouration of salmonid fish flesh and favours consumer acceptability of the product (Bough, 1977, Meyers and Bligh, 1989). The benefit of using pigments in diets of broiler chicken has also been reported (Chawan and Gerry, 1974). The pigments are said to impart desirable coloration to egg yolks in laying hens and quails. The isolation of carotenoids from shellfish wastes in a suitable form for fish feeding can be achieved by oil extraction (Chen and Meyers, 1982). It has also been reported that ensiling crawfish waste with mixtures of acids, results in an increase in the concentration of astaxanthin extracted compared to the non-ensiled product (Chen and Meyers, 1983; Guillou *et al.*, 1995). In addition, the acid also removed 70 percent of the calcium carbonate in the shells and reduced spoilage.

2.3.2 Spoilage of shellfish.

Microbiological spoilage of foodstuffs results in the production of ill-smelling compounds and in chemical changes which result in the formation of toxic compounds (Hobbs, 1982). In fish, most of the undesirable odour and flavour changes associated with spoilage result from bacterial action on the soluble, low molecular weight, components of fish muscle (Hobbs, 1982). Compounds produced by bacteria that are typical of spoilage include trimethylamine, which has a characteristic "fishy" or ammoniacal smell, sulphur compounds which give a smell of bad eggs, indole which gives faecal smells, and carbonyl compounds which give rancid or fruity smells. The occurrence of microorganisms associated with spoilage in seafood such as shrimp, oysters, and clams depends greatly on the quality of the water from which these animals are harvested. Assuming good quality waters, most of the organisms are picked up during processing (Jay, 1992). Not all the bacteria thus acquired are able to produce these changes; spoilage odours and flavours are produced in the main by *Pseudomonads* (Hobbs, 1982). Many of the organisms from fresh fish have also been reported on crustaceans, with *Pseudomonads*, *Acinetobacter-Moraxella*, and yeast spp. being predominant on microbially spoiled

crustacean meats (Jay, 1992). The presence of higher quantities of free amino acids in particular and of higher quantities of nitrogenous extractives in crustacean meats in general makes them quite susceptible to rapid attack by the spoilage flora (Jay, 1992). Initial spoilage of crustacean meats is accompanied by the production of large amounts of volatile base nitrogen, which includes ammonia, dimethylamine and trymethylamine. Total volatile nitrogen (TVN) which can be obtained by steam distillation of samples has been employed in Australia and Japan for testing shrimps where a maximum level of acceptable quality in products is 30 mg TVN/100 g (Cobb and Vanderzant, 1985).

However, these changes in the nitrogenous compounds may also be the result of its own autolytic activity (Cobb *et al.*, 1973). Shrimp has been reported to have a higher content of free amino acids than fish and contain cathepsin-like enzymes that rapidly break down proteins (Early and Stroud, 1982; Jay,1992). It is suspected that a release of the digestive enzyme from hepatopancreas occurs upon death of the prawn and catalyzes muscle degradation that causes the prawn meat to exhibit a mushy texture after cooking (Baranowski *et al.*, 1984).

2.4 Lactic acid preservation.

Lactic acid fermentation is one of the oldest preservation techniques used in the food industry (Table 2.6). It represents a low-cost method for preparing food and feed products with enhanced storage properties (Table 2.6) and also reduces the need for refrigeration or other energy-intensive operations (Platt; 1987; Gould, 1989). Lactic acid fermentations are also becoming more popular due to a trend towards more natural, less additive-based preservation techniques (Gould, 1989). The organisms that are associated with this type of fermentation, the lactic acid bacteria, have had an important role in preserving foods, preventing food poisoning, and indirectly feeding the hungry on every continent (Platt, 1987). Some of the most important nutritional and therapeutic effects ascribed to these bacteria can be summarised as follows (Vuyst and Vandamme, 1994a):

i) improvement of the nutritional quality of food and feed, e.g. lysine enrichment of fermented cereals
ii) stimulation of the overall metabolism by producing vitamins e.g. folic acid
iii) stabilization of the intestinal microflora, excluding colonization by pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp. and enteropathogenic *E. coli* strains, via adhesion to the intestinal wall and competition for nutrients
iv) protection against intestinal and urinary tract infections, e.g. by production of antibacterial substances
v) decreased risk of colon cancer by detoxification of carcinogenic compounds and toxic substances
vi) tumour suppression via an aspecific stimulation of the immune system to produce macrophages

2.4.1 Lactic acid bacteria.

Lactic acid bacteria (LAB) are typically described as Gram-positive, non-sporing, catalase negative, microaerophilic cocci or rods. They are strictly fermentative with lactic acid as the major end product of hexose fermentation, thus giving the name to the group (Ingram, 1975). They are generally associated with habitats rich in

carbohydrate, such as various food products (dairy products, fermented meat and vegetables, fruits, silage and beverages) but some are also members of the normal flora of the respiratory, intestinal and genital tracts of humans and animals (Hammes and Vogel, 1995). LAB do not require oxygen for growth, are resistant to inhibition by carbon dioxide, nitrite and smoke, are able to grow at relatively high salt concentrations and can tolerate lower pH values than the Gram-negative bacteria (Egan, 1983). LAB constitutes what may be termed the 'natural flora' of many spontaneous food or feed fermentation processes, in addition they may also be deliberately incorporated as starter cultures (Steinkraus, 1996).

Table 2.6: Major Food Preservation Technologies

Objective	Factor	Mode of achievement
Slowing down or inhibition of microbial growth	Lower temperature	Chill storage Freeze and frozen-storage
	Reduced water activity	Drying and freeze drying Curing and salting Conserving with added sugars
	Decreased oxygen	Vacuum and nitrogen-packaging
	Increased carbon dioxide	Carbon dioxide-enriched 'controlled atmosphere' storage or modified atmosphere packaging
	Acidification	Addition of acids Lactic or acetic fermentation
	Alcoholic fermentation	Brewing
	Use of preservatives	Addition of preservatives: -inorganic (e.g. sulphite, nitrite) -organic (e.g. propionate, sorbate, benzoate, parabens) -antibiotic (e.g. nisin, pimaricin)
Inactivation	Heating	Pasteurisation Sterilisation
	Ionising irradiation	Radurisation Radicidation Radappertisation
Restriction of access of microorganisms to foods	Decontamination	Treatment of ingredients (e.g. with ethylene oxide) Treatment of packaging material (e.g. with hydrogen peroxide, and/ or heat, irradiation)
	Aseptic processing	Aseptic thermal processing and packaging without recontamination

Adapted from Gould (1989).

2.4.1.1 Classification.

The classification of LAB into the different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, steric configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (Ingram, 1975). The genera that, in most respects, fit the general description of the typical LAB as they appear in the latest edition of Bergey's Manual are *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus.* However, recent taxonomic revisions of these genera suggest that the LAB now comprise the following genera (Axelsson, 1993, Vuyst and Vandamme, 1994a, Holzapfel and Wood, 1995):

Table 2.7: The different genera of lactic acid bacteria

Current	Former	Morphology
Enterococcus	*Streptococcus*	*coccus*
Lactococcus	*Streptococcus*	*coccus*
Streptococcus sensu stricto	*Streptococcus*	*coccus*
Vagococcus	*Streptococcus*	*coccus*
Carnobacterium	*Lactobacillus*	*rod*
Lactobacillus		*rod*
Pediococcus		*coccus*
Tetragenococcus	*Pediococcus*	*coccus*
Aerococcus		*coccus*
Leuconostoc		*coccus*

With the advent of new sophisticated tools for identification and classification methods, further revisions are to be expected especially with regard to the genera *Lactobacillus, Leuconostoc, and Pediococcus* (Axelsson, 1993).

LAB exhibit an enormous capacity to ferment different carbohydrates through several different pathways, principally to generate energy, and in the process they produce weak acids as byproducts, into the environment (Vuyst and Vandamme, 1994b). However, LAB are also able to adapt to various conditions and change their metabolism accordingly and this may lead to significantly different end-product

patterns (Axelsson, 1993). The levels and relative proportions of these fermentation end-products which accumulate in the microbial environment depend on the species of the organism(s) involved, the chemical composition of the environment, and the physical conditions encountered during the fermentation process (Lindgren & Dobrogosz, 1990). The following section describes briefly the more common fermentation pathways and how a simple sugar such as glucose is fermented to produce their end products, which forms the basis of classification.

There are two major pathways for hexose (e.g. glucose and fructose) fermentation occurring within LAB (Axelsson, 1993). Under normal conditions, i.e., excess sugar and limited access to oxygen, glucose is fermented via the Embden-Meyerhof pathway or Glycolysis, mainly to lactic acid (Figure 2.5) and this metabolism is referred to as a "homolactic fermentation". Those LAB that use the glycolytic pathway are then termed the "homofermentative LAB" and this is due to the presence of the constitutive enzyme, Fructose diphosphate aldolase (FDP).

The other main pathway, known as the pentose phosphate pathway (Fig 2.5), leads to the formation of CO_2 and ethanol in addition to lactic acid and is referred to as "heterolactic fermentation" and those that use this pathway as "heterofermentative LAB". This pathway is used by those LAB that possess the constitutive enzyme, phosphoketolase.

In theory, homolactic fermentation of glucose results in 2 moles of lactic acid and a net gain of 2 ATP per mole of glucose consumed. Heterolactic fermentation gives 1 mole each of lactic acid, ethanol, and CO_2 and 1 mole of ATP per mole of glucose. Thus, the production of carbon dioxide gas is a typical characteristic of heterofermentative LAB.

However, it should be noted that glycolysis may lead to a heterolactic fermentation under certain conditions and that some LAB regarded as homofermentative, use the pentose phosphate pathway when metabolising certain substrates. Hence the existence of a third group, the "facultatively heterofermentative LAB". These

bacteria resemble the obligately homofermentative LAB in that they possess a constitutive aldolase, but the synthesis of phosphoketolase is inducible by the presence of pentoses, resulting in a heterolactic fermentation. These LAB are thus homofermentative with regard to hexoses and heterofermentative with regard to pentoses (Axelsson, 1993).

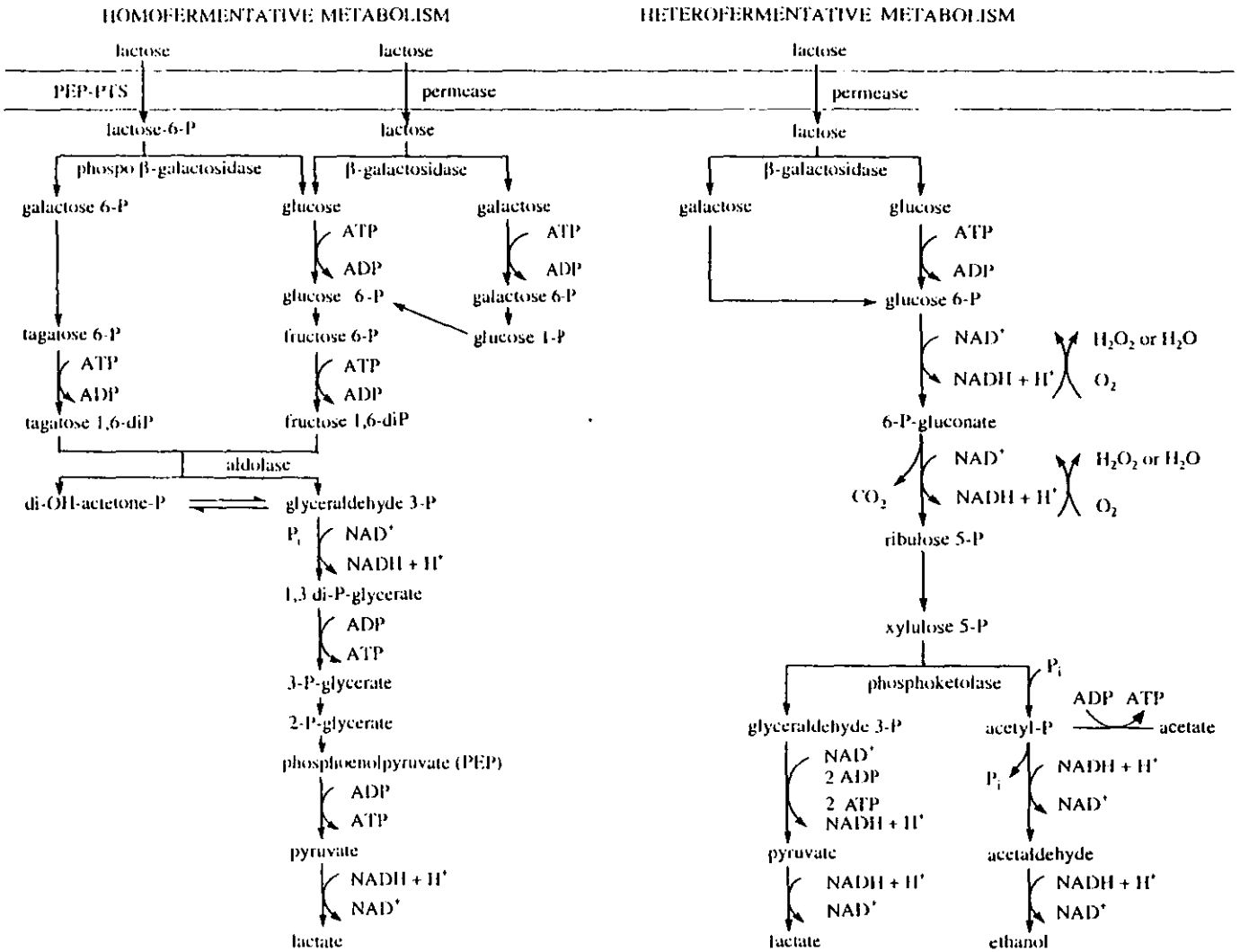


Figure 2.5: Carbohydrate metabolism in homofermentative and heterofermentative lactic acid bacteria (Vuyst and Vandamme, 1994b).

There are several compounds other than glucose that are degraded by the glycolytic pathway, although in general two or three special reactions are needed to bring the compound into the pathway. Hexoses, other than glucose, such as mannose, galactose, and fructose are fermented by many LAB via the glycolytic pathway. Fermentation of disaccharides such as lactose has also been reported by strains of *Lactococcus lactis* and *Lactobacillus casei* (Axelsson, 1993). The fermentation of other disaccharides such as cellobiose, melibiose and trehalose requires a specific transport system and hydrolases before the monosaccharide can enter the common pathways (Axelsson, 1993). Fermentation of pentoses is possible in heterofermentative and facultative LAB and fermentation of pentoses, unlike the fermentation of glucose, leads to the formation of equimolar amounts of lactic acid and acetic acid (Axelsson, 1993). Other minor pathways are also used by certain species leading to the production of diacetyl, acetoin, and hydrogen peroxide (Axelsson, 1993).

Table 2.8 shows the groupings of LAB according to their metabolic capabilities. The genus *Lactobacillus* contains species which can be placed in all three categories and only the more well-characterised species have been included (Schleifer and Ludwig, 1995).

Table 2.8: Metabolic categories of LAB

Obligately homofermentative	Facultatively heterofermentative	Obligately heterofermentative
Lb acidophilus	Certain Lb [a]	*Lb. brevis*
Lb. delbrueckii	*Enterococci*	*Lb. buchnerii*
Lb. helveticus	*Lactococci*	*Lb. fermentum*
Lb. salivarius	*Pediococci*	*Lb. reuteri*
	Streptococci	*Leuconostocs*
	Tetragenococci	
	Carnobacterium	
	Vagococci	

a = *Lb. casei, Lb. curvatus, Lb. plantarum and Lb. sake, Lb. paracasei, Lb. pentosus.*

The ability of different LAB to produce different end products is especially important in fermented foods, as these fermentation end products produce changes to taste and texture of the substrate, besides preserving its shelf life.

2.4.1.2 Methods of Isolation of LAB.

In lactic acid fermentation, isolation of potential LAB from the food source to be fermented is an important step towards achieving a successful fermentation processes (Jepperson, 1993; Tanaka *et al.*, 1994). Strains for investigation are selected when present in large numbers in the product which shows no sign of spoilage and gives a rapid drop in pH. The advantage of using these bacterial strains will be their known ability to grow well in the products to be fermented.

However, considerable problems exist in counting and isolating LAB. This is because they give poor growth on most ordinary laboratory culture media, with very little surface growth on any medium. Many strains of LAB form only pin-point colonies on media such as Plate Count Agar (Egan, 1983). On other nutrient media they may fail to grow aerobically or may be overgrown by other organisms. Evans and Niven (1951) developed All Purpose agar with Tween (APT) for the isolation of *Lactobacillus viridescens*. However, this medium is non-selective, but supports good growth of LAB (Kitchell and Shaw, 1975). De Man, Rogosa and Sharpe (MRS) agar (De Man *et al.*, 1960), pH 6.0 - 6.5, which was originally developed for the cultivation of lactobacilli of dairy origin, is widely used and has been used to enumerate LAB from vacuum-packed bacon (Kitchell and Shaw, 1975). At present, these two agars are widely used with LAB (Kandler and Weiss, 1986; Collins *et al.*, 1989a; Vignolo *et al.*, 1993).

2.4.1.3 Methods of identification of LAB.

At genus level. LAB can be divided into rods and cocci. Tetrad formation is used as a key characteristic in the differentiation of the cocci. Another important characteristic used in the differentiation of LAB genera is the mode of glucose fermentation under standard conditions, i.e., non limiting concentrations of glucose and limited oxygen availability. Under these conditions, LAB can be divided into

two groups: homofermentative, which convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO_2 (Sharp, 1979). A summary of the differentiation of the LAB genera is shown in Table 2.9.

At species level. Classification at species level is somewhat more difficult to achieve. The classical biochemical characterization is important for a preliminary classification as well as learning about the properties of the strains. Some of the characteristics listed in Table 2.9 are useful also in the classification at species level. The fermentation behaviour on different carbohydrates can be conveniently carried out using the API kit system. However, with this kit, certain species are more easily recognised but others may need more sophisticated tools, based on a molecular approach. New tools for classification and identification of LAB are under development and the most promising ones are nucleic acid probing techniques, partial rRNA gene sequencing using the polymerase chain reaction, and soluble protein patterns. These developments will almost certainly become an important aid in the classification of LAB, as exemplified by the descriptions of new genera (Collins *et al*, 1989b).

2.4.2 Preservative components of LAB.

One of the earliest food biopreservation methods recognised by our ancestors was the ability of weak organic acids, produced by LAB, to reduce spoilage and extend the life of many foods. These acids reduce the pH of foods to the extent that undesirable microorganisms present in the raw materials, or introduced during subsequent handling, either fail to multiply or do so at a reduced growth rate. In addition, some strains of LAB also produce other inhibitory substances although in much smaller amounts. These include hydrogen peroxide, diacetyl and bacteriocins which have potential to inhibit a variety of other microorganisms (Daeschel, 1989; Vuyst and Vandamme, 1994b).

Table 2.9: Differential characteristics of lactic acid bacteria[a](from Axelsson, 1993).

	Rods		Cocci						
Characteristic	*Carnob.*	*Lactob.*	*Aeroc.*	*Enteroc.*	*Lactoc. Vagoc.*	*Leucon.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*
Tetrad formation	–	–	+	–	–	–	+	–	+
CO_2 from glucose[b]	–	±	–	–	–	+	–	–	–
Growth at 10°C	+	±	+	+	+	+	±	–	+
Growth at 45°C	–	±	–	+	–	–	±	±	–
Growth in 6.5% NaCl	ND[d]	±	+	+	–	±	±	–	+
Growth in 18% NaCl	–	–	–	–	–	–	–	–	+
Growth at pH 4.4	ND	±	–	+	±	±	+	–	–
Growth at pH 9.6	–	–	+	+	–	–	–	–	+
Lactic acid[c]	L	D,L,DL[e]	L	L	L	D	L, DL[e]	L	L

[a]+, positive; –, negative; ±, response varies between species; ND, not determined.
[b]Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.
[c]Configuration of lactic acid produced from glucose.
[d]No growth in 8% NaCl has been reported.
[e]Production of D-, L-, or DL-lactic acid varies between species.

In this section, the preservative components of LAB are divided into two types, fermentation end products and bacteriocins and are considered separately.

2.4.2.1 Fermentation end products.

i) Organic acids.

The major metabolite of LAB is lactic acid which is primarily responsible for the lowering of environmental pH. In addition to lactate, other organic acids are also formed, for example acetic acid, formic acid and succinic acid (Schleifer and Ludwig, 1995). The high amounts of weak acids produced by these bacterial genera reduce the pH of the environment considerably and inhibit or reduce the growth of many bacteria, including those that cause food spoilage and foodborne diseases (Earnshaw, 1992; Ray and Sandine, 1992; Mayra-Makinen and Bigret, 1993), Gram negative bacteria being the most susceptible to the inhibitory effect of lactic acid (Ray and Sandine, 1992).

The antimicrobial effects of the organic acids are produced through the destabilisation of different functional and structural components of cells (Ray and Sandine, 1992). Their antimicrobial action appears to be the result of the ability of the undissociated weak organic acid molecules to penetrate the bacterial plasma membrane (Adams and Hall, 1988). The lipid bilayer membrane of bacteria is quite permeable to lipophilic acetic, propionic and lactic acids and this facilitates their diffusion into the cytoplasm. The internal pH of the bacteria being relatively alkaline, and the acids being highly soluble in the internal aqueous environment, the undissociated molecules will dissociate, producing protons and anions. The buffering capacity of the cytoplasmic materials will tend to prevent reduction of internal pH by neutralising some protons. When protons exceeding the buffering capacity are produced, they are transported out through the membrane-bound proton pumps, thus depleting the energy reserves. As the energy reserve is limited, proton extrusion through proton pumps stops, causing a lowering of internal pH, which in turn denatures proteins and destabilises other structural and functional components of cells and interferes with viability and growth (Ray and Sandine, 1992). Thus, the antimicrobial effect of the weak acids is principally produced by the undissociated

molecules through the acidification of cytoplasm, destruction of the transmembrane proton motive force, and loss of active transport of nutrient through the membrane. The antimicrobial effect of a weak organic acid depends upon the dissociation constant (pKa value) and the pH of the external medium (Vuyst and Vandamme, 1994b). For example acetic acid (pKa = 4.74) is generally a more effective inhibitor than lactic acid (p*K*a = 3.85) at the same pH value, since a greater proportion would be undissociated (Adams and Hall, 1988; Davidson and Hoover, 1993, Vuyst and Vandamme, 1994b). The antimicrobial efficiency of weak acids also increases as the pH is reduced. The majority of spoilage bacteria and pathogens that are present in meat mixes are inhibited more effectively by the rapid attainment of low pHs by the use of starter cultures (Adams *et al.*, 1987). In general, fermentative bacteria such as lactic acid bacteria, have the ability to grow and survive at a lower pH than do the respiring bacteria (Ray and Sandine, 1992).

ii) Hydrogen peroxide.

Hydrogen peroxide (H_2O_2) is generated by different mechanisms by certain lactobacilli during growth (Mayra-Makinen and Bigret, 1993). Many fermentative bacteria, including lactic acid bacteria produce hydrogen peroxide as a mechanism for protecting themselves against oxygen toxicity (Daeschel and Penner, 1992). In the presence of oxygen, lactic acid bacteria produce hydrogen peroxide through electron transport via flavin enzymes (Figure 2.6). Thus hydrogen peroxide may be synthesized from NADH, pyruvate, and even lactate by NADH oxidase, pyruvate oxidase and lactate dehydrogenase respectively (Vuyst and Vandamme, 1994b). The observation of hydrogen peroxide production and accumulation was most often associated with the lactobacilli in contrast to the other lactic genera (Daeschel and Penner, 1992). For example, *Lactobacillus bulgaricus* and *Lactobacillus lactis* inhibited the growth of *Staphylococcus aureus* (Davidson and Hoover, 1993) and inhibition of *Pseudomonas* spp. by *Lactobacillus plantarum* (Vuyst and Vandamme, 1994b). Hydrogen peroxide is an effective antimicrobial by virtue of its oxidising potential. The mechanism is thought to be of the generation of a toxic hydroxyl radical which is very active and will react and damage essential cellular components such as membrane lipids and DNA (Daeschel and Penner, 1992). However, since

lactic acid bacteria are catalase negative (i.e. unable to remove hydrogen peroxide) there will be accumulation of hydrogen peroxide in the growth medium of such bacteria which will eventually reach autoinhibitory levels.

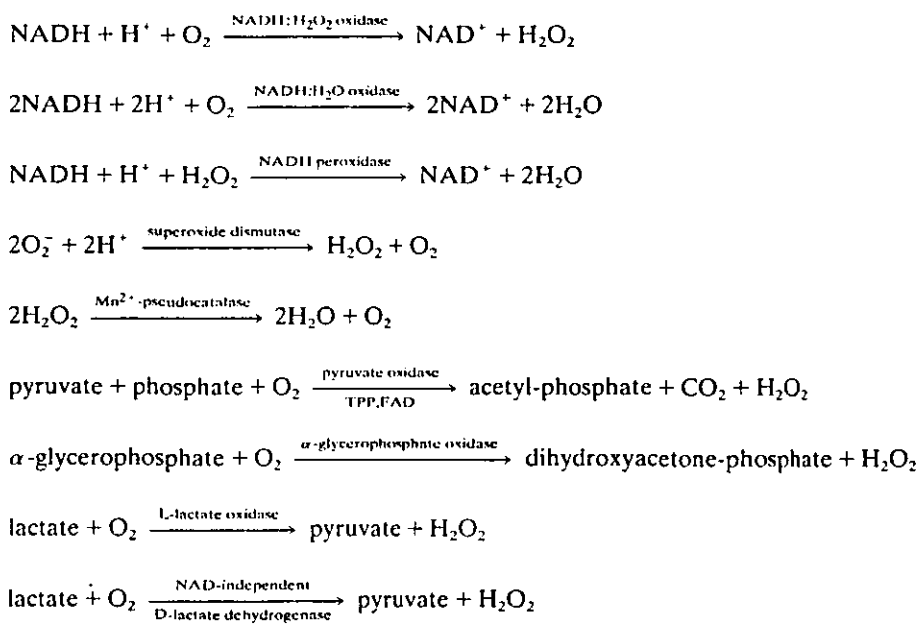


Figure 2.6: Enzymes of lactic acid bacteria involved in oxygen metabolism (from Vuyst and Vandamme, 1994b).

iii) Carbon dioxide.

The preservative effect of carbon dioxide-enriched atmosphere on plant products has been known since 1917 and was first put into commercial use in 1928 (Jay, 1992). Since then it has been used to prevent fungal rotting in stored fruits. Carbon dioxide is also inhibitory to bacterial growth. Gram-negative bacteria are more sensitive to carbon dioxide than Gram-positive, with pseudomonads being among the most sensitive and the LAB and anaerobes being the most resistant (Jay, 1992). The use of carbon dioxide to preserve other food products has also been reported and these include, meat carcasses, vacuum packed meat, fish and shellfish products (Jay, 1992). Although carbon dioxide has long been known to be an inhibitor of certain microorganisms there is still no clear understanding of how inhibition is achieved (Dixon, and Kell, 1989).

iv) Diacetyl.

Citrate fermentation produces diacetyl (2,3 butanedione), a common property amongst lactococci (*Lactococcus lactis*) and lactobacilli (*Lactobacillus brevis*)and leuconostocs (*Leuconostoc cremoris*) and is an attribute of dairy starter cultures which confers a desirable buttery flavour in certain products (Earnshaw, 1992; Ray, 1992). The compound has long been known to be inhibitory and Jay (1992) reported that 200 µg/ml diacetyl inhibits yeasts and Gram-negative bacteria. However, the effective concentration for diacetyl is too high for it to play a major role in natural preservation or as an added biopreservative (Davidson and Hoover, 1993). Its role in the combination of inhibitors produced by LAB, however, is undoubtedly important (Ray, 1992).

2.4.2.2 Bacteriocins.

Bacteriocins are microbially produced proteins with a lethal effect on other, often closely related bacteria (Earnshaw, 1992). Lactic acid bacteria have been found to produce a variety of such compounds, many of which have been isolated and characterised (Vuyst and Vandamme, 1994b). The most widely known is nisin, a polypeptide produced by strains of *Lactococcus lactis.* Nisin is perhaps one of the best natural preservatives and has been proven to be non-toxic (Frazer *et al.*, 1962).

In the UK, nisin is now permitted as a preservative in certain foods, eg. cheese and clotted cream, and canned foods (Earnshaw, 1992; Jay, 1992) where it is known to effectively prevent the growth of *Clostridium botulinum* spores and *Listeria monocytogenes* (Mayra-Makinen and Bigret, 1993). Nisin has a narrow spectrum of inhibitory activity affecting only Gram-positive bacteria including lactic acid bacteria, streptococci, and bacilli. It does not generally inhibit Gram-negative bacteria, yeast, or moulds (Davidson and Hoover, 1993). Commercial production of nisin started in 1950s and it is currently available under the trade name Nisaplin (Earnshaw, 1992).

Although not so intensely studied, and not used as food preservatives, many other lactic bacteriocins have also been described, for example, diplococcin produced by *Lactococcus lactis* ssp. *cremoris*, lactocin by *Lactobacillus* sp., pediocin by *Pediococcus* sp. and plantaricin by *Lactobacillus plantarum* (Earnshaw, 1992).

Mechanisms controlling the synthesis and production of bacteriocins are generally unknown, however, it can be hypothesised that they are produced under conditions of ecological stress to function as a survival mechanism (Earnshaw, 1992). In the coming decade it is likely that progress in bacteriocin research will result in a whole generation of food preservatives and processing aids that provide economic and safe alternatives to the current range of chemical preservatives.

2.4.3 Applications of lactic acid fermentation (LAF).

Fermented foods have been produced for hundred of years by the use of LAB. Historically, the primary role of LAB in fermented foods was one of preservation. With the advent of modern processing and preservation techniques, their modified role is now to provide variety in the food supply. They carry out this function by altering the flavour, texture, and appearance of raw commodities in a desirable way (Platt, 1987; Davidson and Hoover, 1993; Steinkraus, 1996). Most of these fermentations have been spontaneous due to indigenous LAB or have been initiated by the addition of small portions of already fermented products known as "back slopping". Nowadays the production of fermented foods is generally much more

industrialised using commercially produced starter cultures. Products which have low concentrations of naturally occurring LAB such as fish and shellfish, will require the addition of starter cultures to ensure successful preservation (Jepperson, 1993). Nonetheless, the following conditions have to be met if optimization of the LAF is to be obtained (Deschamps, 1993):

i) rapid anaerobiosis
ii) the use of strains with high acidification capacity such as homofermentative ones
iii) concentration of starter cultures must be high enough to balance and compete with the endogenous microflora (usually 10^5 to 10^8 cfu/ml or g)
iv) decreased water activity
v) maintenance of temperature suitable for the microorganisms
vi) control of the nutritional requirements or accessibility to fermentable sugars by the LAB which may be obtained by adding fermentable sugars, enzymes, growth factors (vitamins or amino acids) or mixing.

The applications of LAF are widely distributed ranging from food to animal feed production. Most of the fermentations are conducted to preserve fresh, surplus commodities and to increase shelf life and flavour while others make use of LAF to preserve and upgrade waste products to produce suitable ingredients for animal feeds. Some may involve one particular strain of LAB while others may be fermented by a series of LAB. Some of the main applications of LAF are shown in Table 2.10:

Table 2.10: Applications of lactic acid fermentation.

Substrate	Products	References
Fish silage	Animal Feed	Beddows, 1985; Wyk & Heydenrych, 1985; Lindgren & Pleje, 1983;
Dairy products	Fermented Food	Mayra-Makinen & Bigret, 1993
Fish	Fermented Food	Twiddy *et al.*, 1987
Meat	Fermented Sausages	Egan, 1983; Vignolo *et al.*, 1993
Plant Straw	Animal Feed	Hrubant, 1985; Woolford, 1985; Tanaka *et al.*, 1994

2.4.4 Lactic acid fermentation of shellfish waste.

Shellfish waste, produced via various commercial processing operations, comprises mainly heads and claws, is rich in chitin and protein and contains a substantial amount of calcium carbonate. The heads may contain many active and useful enzymes if the waste is kept in a well preserved state. Traditionally such by products are usually discarded or kiln-dried into a meal for use into a variety of livestock and aquaculture diets (Meyers and Benjamin, 1987).

The adoption of LAF to shellfish waste in an attempt to purify chitin is a unique example of the application of LAF processes. This is because, in the fermentation of shellfish waste, the production of acid will have a dual effect on the substrate: that of preserving the waste material by lowering the pH, and of dissolving the calcium carbonate, thus enabling a certain degree of purification of the chitin solids. The acid conditions also promote the indigenous enzymes to break down proteinaceous material from the waste, forming a protein-rich liquor (Hall and De Silva, 1992). These two main processes, i.e. the removal of calcium carbonate and protein from the shellfish waste represent the two main processes involved in the traditional chitin purification industry (Simpson *et al.*, 1994). These processes which are traditionally carried out by a chemical method, utilise large amounts of acid and alkali which are discarded as effluents from the process. By using the biological approach of LAF in a solid state fermentation, it is envisaged that this method of purification or partial purification will reduce the use of vast amounts of corrosive chemicals and by-products such as the protein liquor could be recovered.

Pioneering work on LAF of shellfish waste for chitin recovery has been initiated by the Food and Biotechnology Group at Loughborough University and has produced some encouraging results with tropical species, *Peneaus monodon* (Hall and De Silva, 1992). In this process, glucose and LAB were added to minced waste in a closed container, and a rapid acidification occurred bringing the pH down to 5.0 within 48 hours. The acid environment preserved the waste, and allowing indigenous enzymes to solubilise the protein, forming a protein-rich liquor in a relatively short time. The lactic acid also dissolves the calcium carbonate from the waste, producing

copious amounts of carbon dioxide. The carbon dioxide-rich environment further helps to reduce spoilage and stimulates growth of LAB (Jay, 1992). The protein liquor can be sun-dried and used as an animal feed ingredient and the chitin can be recovered for further processing.

2.5 Bioreactors in solid state fermentation.

Solid state fermentation (SSF) is one of the oldest, and remains one of the most economical, methods of producing and preserving foods (Steinkraus, 1983; Tangerdy, 1985). Such processes have been used extensively in Oriental and African countries for the production of fermented foods, starter inocula for fermented brews, mushrooms, dough fermentations, silages and compost. In recent years, a new awareness of the importance of biological materials as renewable resources for the production of energy and feed, and as an important source of chemical feedstocks has revived the interest in this most ancient form of fermentation (Tangerdy, 1985). Substrates traditionally fermented in the solid state include a variety of agricultural products, such as rice, wheat, millet, barley, corn and soya beans as well as non traditional substrates which include agricultural, forest and food-processing wastes.

2.5.1 Definition of a solid state fermentation.

The term "solid-state fermentation" is generally defined as any fermentation process in which the moist, water-insoluble solid substrate is fermented by microorganisms in the absence of excess water (Mitchell and Lonsane, 1992). While Hesseltine (1972) simply used the term SSF to describe any fermentation in which the substrate is not liquid, Aidoo *et al.* (1982) in their review reserved the term "solid-state fermentation" for any fermentation that takes place on solid or semisolid substrate or that occurs in a nutritionally inert solid support which provides some advantage to the microorganisms with respect to access to nutrients. The term cannot be applied to fermentation on solid materials suspended in a liquid phase or to fermentation in media comprising a high concentration of insoluble particles (Durand and Chereau, 1988). SSF are distinguished from submerged fermentations (SF) by the fact that microbial growth and product formation occur at or near the surfaces of solid materials with low moisture contents. This kind of growth on solid surfaces is a characteristic of fungi that decay organic matter, food and feed materials, and of some bacteria involved in composting and ensilation (Tangerdy, 1985).

2.5.2 The advantages and disadvantages of a solid state fermentation.

Several papers have reviewed the numerous economical and practical advantages of SSF over SF (Hesseltine, 1972; Lonsane *et al.*, 1985; Mitchell and Lonsane, 1992). The advantages of SSF include the following:

i) fermentation vessels remain small, since little water is used and the substrate is concentrated.
ii) low moisture reduces the problem of contamination.
iii) conditions for growth are similar to those in natural habitats.
iv) aeration is facilitated by spaces between substrate particles and particle mixing.
v) product yields may be much higher than those in liquid media and are reproducible.
vi) products may be incorporated directly into animal feeds.
vii) relatively low level of technological complexity and corresponding low capital investment.
viii) reduced energy requirement.
ix) low wastewater output.
x) elimination of foam problems.

On the other hand, Lonsane *et al.* (1985) and Durand and Chereau (1988) point out some of the limitations that may be encountered in SSF such as:

i) difficulties in fermentation control (heat build-up, control of moisture level of substrate, control of aeration).
ii) lack of microbiological knowledge relating to the physiological and growth mechanisms on solid materials.
iii) types of organisms are limited to those which can grow at reduced moisture levels (fungi, some yeasts and bacteria).
iv) lack of technological knowledge of suitable equipment even at the laboratory scale.
v) problems in scaling-up.

Some of these problems such as i) and v) may be overcome by using an appropriate bioreactor. Although many SSF involve the use of fungi and yeasts, bacteria such as the LAB have been used in many SSF processes.

2.5.3 Factors affecting the design of a bioreactor.

In any fermentation processes, the bioreactor provides the environment for the growth and activity of the microorganisms which carry out the biological reactions. An ideal fermenter should have several characteristics; in particular the material of construction should be non-toxic and able to withstand hydraulic and pneumatic pressures (Pandey, 1991) and it should not be affected by chemical corrosion. There should be proper arrangements for aeration/agitation, with sampling, charging and discharge ports. Although there are numerous designs for industrial fermenters using liquid or submerged fermentations (SF), only limited developments have been made in processes using SSF. Process parameters are crucial in the design of SSF bioreactors. Mitchell *et al.*, (1992) described some of the important considerations as follows:

i) whether mixing is required and how it will be achieved.
ii) the degree of aeration necessary.
iii) the substrate type and its properties and further handling of products.
iv) sterilisation and the prevention of contamination.
v) the mode of process operation.

In this section, those considerations that are considered relevant to the 'lactic acid fermentation' are further elaborated, based on other SSF processes.

2.5.3.1 Agitation.

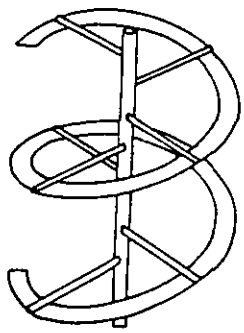
SSF processes can be divided into three groups based on the mixing regime that is used: static, periodically agitated and continously agitated. The requirement of agitation in SSF systems is governed by the type of process, reactor design and the product concerned. For example, tray fermentations are usually carried out in static

conditions while the production of aflatoxins, and enzymes are greatly enhanced in agitated systems (Lonsane *et al.*, 1992). Processes involving filamentous organisms will require the use of intermittent rather than continous agitation to prevent damage and disruption of mycelial attachment to solids.

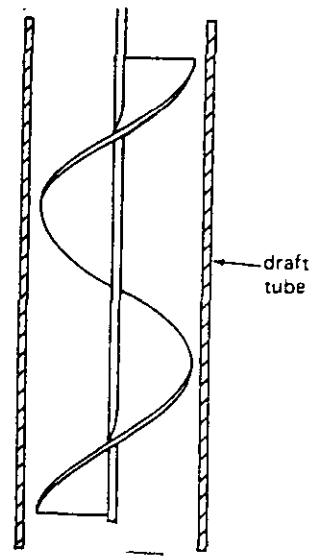
In general, agitation facilitates the maintenance of homogeneous conditions, preventing any localised changes within the bioreactor, especially with respect to the temperature and the gaseous environment (Mitchell *et al.*, 1992). In addition, agitation facilitates the uniform distribution of any additives which may be introduced during the process. Depending on the substrate properties, agitation may prevent or encourage the agglomeration of solids. In periodically agitated SSF, the agitation serves to replenish the interparticle spaces with fresh air. The frequency of mixing required depends on the substrate properties such as particle size and shape. It also governs the rate of growth of organisms and the associated production of metabolic heat. Shear forces in SSF are complex and are therefore difficult to characterize and this might explain why relatively few studies have been devoted to this area.

In SSF, where the substrates are either solid or semi solid in nature, paddle impellers which work well with liquids, are unable to provide sufficient mixing of the substrates. Paddle impellers are largely rotational and fail to provide top to bottom mixing (Godfrey, 1985). For this reason, more complex design such as the helical ribbon (see Figure 2.7a) and helical screw (see Figure 2.7b) which are generally used in mixing highly viscous liquids (Godfrey, 1985), were adopted by various workers. These types of mixers can also minimize the deleterious effects of mixing. A promising design is the helical screw fermenter, used by Tangerdy (1985) for batch and continous fermentation of wheat straw by *Chaetomium cellulolyticum.* The impeller used was a combination of helical screw and helical ribbon (see Figure 2.7c). This configuration apparently avoids compacting the substrate and does not damage the mycelia by shearing and provides a thorough mixing. Helical screws can also be used horizontally (refer Figure 2.7d) and in this way, instead of moving the substrate from bottom to top, it merely relocates the substrate from one end to the

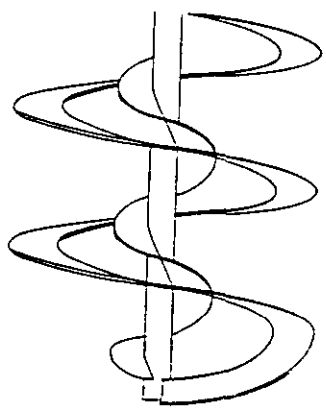
other (Edwards, 1985). The balance between the advantageous and the disadvantageous effects of mixing will differ in different substrate-microorganism-reactor SSF systems. In any case, agitation should only be provided as necessary, and periodic agitation is usually sufficient (Mitchell *et al.*, 1992).



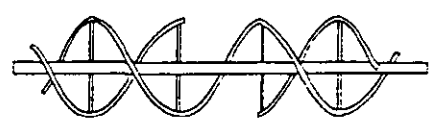
(a) Helical ribbon



(b) Helical screw with draft tube



(c) Combination of helical ribbon & screw



(d) Helical ribbon mounted horizontally

Figure 2.7: Different types of mixers

2.5.3.2 Mode of reactor operation.

Most SSF applications involve batch culture (Mitchell *et al.*, 1992). Effective implementation of fed-batch or continuous techniques at large scale will require the application of automated solids-handling techniques. Continuous processes are usually stirred tanks where fresh substrate is mixed homogenously in with cultured substrate with equal amounts of this mixture removed at an outlet. These processes are obviously only possible in agitated systems.

2.5.3.3 Substrate Type.

In general, substrates for SSF are relatively unprocessed agricultural products or by products. For many processes substrates are chosen because they are cheap, or because they would otherwise be discarded as wastes (Mitchell *et al.*, 1992). For SSF at the rural level the production site is usually situated close to the source of the substrate to minimise transportation costs.

Pretreatment may be necessary in SSF in order to convert the raw substrate into a form suitable for use. Besides size reduction, by either grinding, rasping or chopping, other pretreatments may include cooking, supplementation with nutrients or chemical or enzymatic hydrolysis of the polymeric substrate (Mitchell *et al.*, 1992). Particle size of substrate is extremely important since it affects the surface area to volume ratio of the particle and the packing density within the substrate mass. In SSF the microorganism is introduced onto the particle surface and therefore initially attacks the exposed substrate. The surface area to volume ratio determines the fraction of the substrate which is initially accessible to the microorganism. This fact becomes very important especially when the substrate is to be preserved by the fermentative action of the microorganism. The particle size will also affect how the substrate packs together, and therefore the void fraction. Loose packing is essential in aerobic SSFs to enable the interparticle diffusion of oxygen (Mitchell *et al.*, 1992). However, particle size reduction is costly, so the largest size which allows an acceptable fermentation is the probably the best and this will vary for different systems.

2.5.4 General types of bioreactors used in SSF.

2.5.4.1 Tray bioreactors.

Tray fermenters are the simplest of all types of fermenters and may be wooden, metallic or plastic (Pandey, 1991; Mitchell *et al.*, 1992). The bottom part is perforated in such a way that it holds substrate and allows aeration of the undersurface of the substrate. Usually, trays are arranged one above the other with a suitable gap between trays. The fermentation is carried out in a chamber where a controlled humidity atmosphere is created. The temperature of the fermenting substrate is controlled by circulating warm or cool air as necessary. A typical tray arrangement can be seen in Figure 2.8a.

Traditionally, most SSFs are conducted in shallow trays and the use of Soy Sauce Koji in the far East goes back possibly 3000 years (Pandey, 1991). Miso, a fermented food in Japan, China, Taiwan, the Philippines, Indonesia and in the Orient has also traditionally been prepared in shallow pans. Tempeh is yet another important solid-state fermented food used in Indonesia, New Guinea and Surinam and is prepared traditionally in shallow trays (Pandey, 1991).

Tray fermenters, however, need a large operational area, are labour intensive and this design does not lead readily to mechanical handling (Mitchell *et al.*, 1992). The substrate also requires separate sterilisation/cooking and these fermenters are therefore characterized as being of high cost (Pandey, 1991).

2.5.4.2 Drum bioreactors.

Drum bioreactors basically consist of a drum-shaped container mounted horizontally on a system of rollers which act both as support and as a rotating device (Figure 2.8b). The drum is rotated around its central axis to cause a tumbling motion of the substrate. Alternatively, a clock mechanism can be used for periodic rotation of the drum and a low rate of rotation (1 - 15 rpm) is usually applied (Lonsane, *et al.*, 1985). The kind of container used depends upon the volume of the fermentation that is to be carried out. These range from laboratory scale Fernbach flask (Pandey, 1991), to cement mixers for large scale work (Lonsane *et al.*, 1985). Improvements

have been made by providing internal baffles (Kargi and Curme, 1985) or by sectioning the fermenter into three or four parts (Hesseltine, 1977). Growth of the microorganisms in this type of bioreactor is considered to be rapid and more uniform than in tray fermenters (Pandey, 1991). One disadvantage, apart from handling difficulties with large-size drums, was the aggregation of substrate particles into balls.

2.5.4.3 Column or packed bed bioreactors.

This consists of a glass or plastic column enclosed at both ends (Figure 2.8c). Small columns can be placed in waterbaths for temperature control while larger columns can be water jacketed (Pandey, 1991). The column is filled with the substrate and supported on a perforated plate through which air is admitted as shown in Figure 2.8c (Mitchell, *et al.*, 1992). Column fermenters have most commonly been used in laboratory studies (Pandey, 1991; Castaneda *et al.*, 1992).

The advantage of packed bed bioreactors is that they remain relatively simple while allowing better process control than is possible with trays (Mitchell *et al.*, 1992). However, difficulties may arise when emptying the final product from the bioreactor (Lonsane *et al.*, 1985).

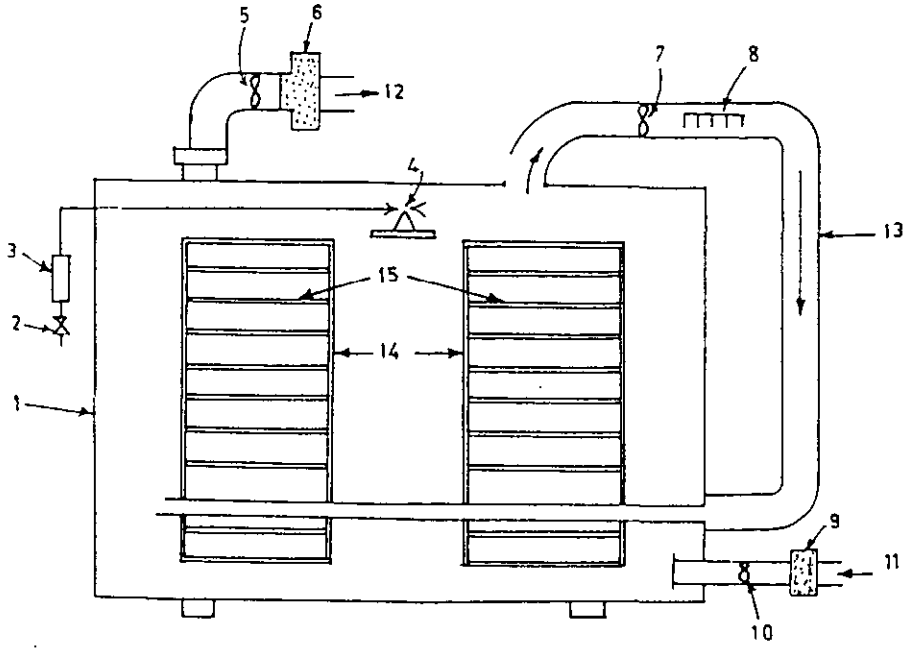
2.5.4.4 Stirred bioreactors.

Stirred bioreactors are of two main types depending on whether the axis of the bioreactor is horizontal or vertical. Horizontal stirred bioreactors (Figure 2.8d) are quite similar to rotating drum bioreactors except that the mixing is provided by an internal scraper or paddles, rather than by rotation of the body of the bioreactor (Mitchell *et al.*, 1992). Vertical stirred bioreactors are often subjected to forced aeration. They differ from packed bed bioreactors by the fact that they are agitated, and this may be either continous or intermittent (Mitchell *et al.*, 1992).

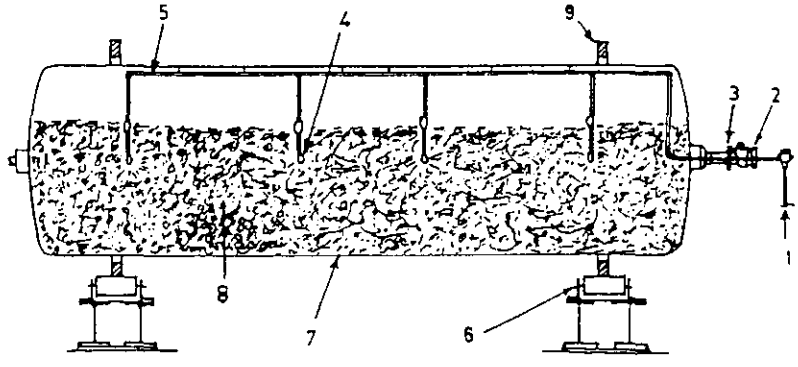
Viesturs *et al.* (1981) conducted a SSF of wheat straw by *Chaetomium cellulolyticum* and *Trichoderma lignorum*, using a horizontal Plexiglas cylinder with paddlelike stirrers. The fermenting mass was mixed initially with the stirrers for 1 minute,

thereafter, the stirrers were intermittently switched on for 1 second, for a full rotation. They compared SSF with SF using shake flask cultures, and noted that SSF is as efficient as SF but the added advantage of using SSF was that the solids content in SSF was ten times greater than in SF.

Durand and Chereau (1988) used a pilot-scale vertical stirred bioreactor for protein enrichment of sugar beet pulp. The reactor had a capacity of one ton and consisted of a reactangular box kiln, built of stainless steel. There were three vertical screws which moved the pulp forward and backward along the entire length.

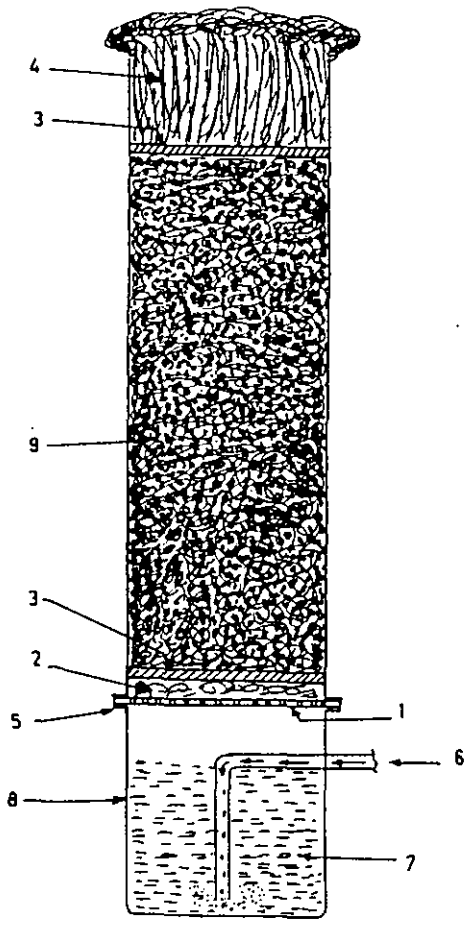


(a) Typical koji room for industrial scale operation with tray bioreactors. Key: 1. Koji room; 2. Water valve; 3. Germicidal UV tube; 4. Humidifier; 5. Air blower; 6. Filter; 7. Air blower; 8. Air heater; 9. Air filter; 10. Air blower; 11. Air inlet; 12. Air outlet; 13. Air circulation loop; 14. Tray holders; 15. Trays.

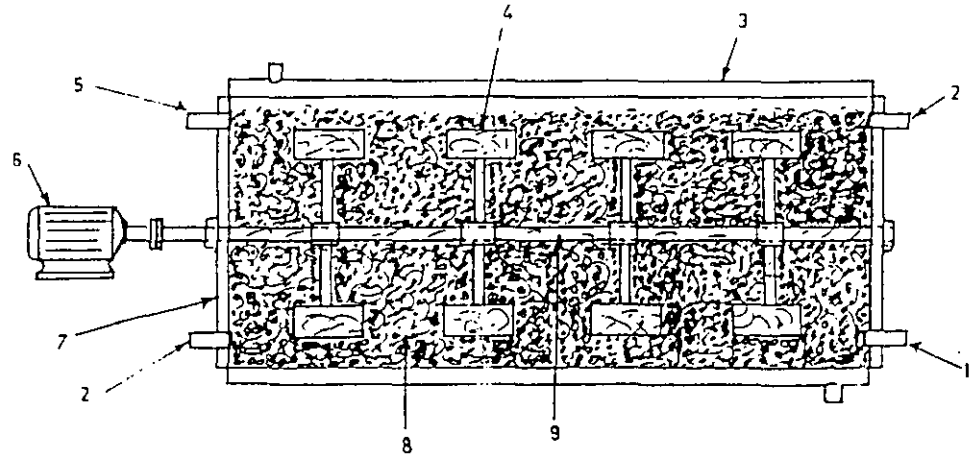


(b) Typical large scale design for a rotating drum bioreactor. Key: 1. Air inlet; 2. Rotating joint; 3. Coupling; 4. Air line nozzles; 5. Air line; 6. Rollers; 7. Rotating drum; 8. Moist solids; 9. Rim.

Figure 2.8: Bioreactor designs in solid state fermentation.



(c) Typical laboratory-scale packed bed reactor. Key: 1. Stainless steel mesh; 2. Glass wool; 3. Filter paper disc; 4. Cotton wool; 5. Flange; 6. Air inlet; 7. Water; 8. Humidifier; 9. Moist solids.



(d) Typical design for a stirred horizontal bioreactor. Key: 1. Air inlet; 2. Port for probes; 3. Water jacket; 4. Paddles; 5. Air outlet; 6. Motor; 7. Vessel body; 8. Moist solids; 9. Central shaft.

Figure 2.8: Bioreactor designs in solid state fermentation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganisms (lactic acid bacteria).

All lactic acid bacteria (LAB) used in the fermentation experiments were isolated in the Food and Bioprocessing laboratory (Loughborough University) from the various fermented shellfish waste sources mentioned below (3.2.1).

3.2 Materials.

3.2.1 Shellfish waste.

Whole frozen prawns from Thailand and India were purchased from a local market, and their heads, claws and shell were removed by hand. The waste (all components other than the meat) from these tropical prawns was used to isolate lactic acid bacteria.

For the chitin purification studies, scampi (*Nephrops norvegicus*) waste containing the heads and claws, obtained from Norfish Ltd., Sunderland, was used. Frozen waste was minced using an industrial mincer (model E 4522, Hobart, Southgate, London) with a mincer plate having holes of 6.0 mm diameter and was stored in approximately 1.0 kg amounts in plastic bags at - 20°C until required.

3.2.2 Media for lactic acid bacteria.

For the isolation of lactic acid bacteria, commercial MRS (Oxoid, Unipath Ltd., Basingstoke, Hants) and APT (Difco, Michigan, USA) broths were used (see Appendix 3.1 for preparation and content of broths and agar plates).

3.3 General methods.

3.3.1 Isolation and screening for potential LAB from shellfish waste.

LAB was isolated from fermented shellfish waste as described below. As the isolated strains were intended to be used as a starter culture in the fermentation of shellfish waste, isolation from a successfully fermented waste will ensure that well acclimatised strains are obtained (Jepperson, 1993).

Isolation. Slightly thawed waste (10.0 g) was minced with a domestic mincer (Model N705, Salter, Kent) and thoroughly mixed with 10 % (w/w) glucose. The mixture was incubated at 30°C in a lightly screw-capped Universal bottle to allow release of any gas that might be evolved during fermentation and so prevent the bottles from cracking. When the pH had dropped to a pH of 5.0 or less, samples of the silage were taken and a serial dilution made with sterilised PBS (Sigma). A rapid pH drop within 24 to 48 hours was taken as an indication of a good fermentation (Hall and De Silva, 1992). Aliquots of 0.1 ml of each dilution were spread onto APT and MRS agar plates. Both plates were incubated aerobically as well as under enriched carbon dioxide gas sealed in a plastic bag, at 30°C for between 24 and 48 h.

Small, isolated whitish colonies, typical of LAB (Collins *et al.*, 1989a) were randomly subcultured from plates containing less than 300 colonies, into APT and MRS broth (3.0 ml in bijou bottles) and the broths incubated at 30°C for between 24 and 48 hours. The bacteria were then purified by plating on APT or MRS agar depending on which of the two broths they grew best in. Isolates which were Gram-positive and catalase - negative rods or cocci (Sharpe, 1979) were taken as LAB and were further screened and identified. Several replicates of an 18 hour pure culture (MRS broth) were distributed in sterile Eppendoff tubes containing 15 % glycerol (Kandler and Weiss, 1986) and were kept as stock culture at - 20°C. Several working cultures on MRS slants were also prepared and kept at 4°C and were subcultured every two months from a newly opened stock culture in order to preserve activity.

Screening. This experiment was conducted to screen for potential strains on the basis of its ability to ferment extract of sterile scampi broth (Hall *et al.*, 1994). To prepare the inoculum, purified LAB strains from agar slants were grown in MRS broth (3.0 mL) and incubated at 30°C for 24 h. The bacteria was sub-cultured (2 % v/v, inoculation) into MRS broth (10.0 mL) and incubated for 18 - 24h and this culture served as the inoculum for the screening experiment. Sterilised aqueous extract (15.0 ml) of scampi waste (see Appendix 3.2 for preparation of scampi extract) containing 2 % glucose (v/v) was inoculated (2 % inoculation) with the LAB, lightly screw-capped and incubated at 30°C in a water bath for 48 hours.

Measurements of pH were taken regularly throughout the fermentation period and strains which showed the lowest pH within 48 hours were chosen and identified.

3.3.2 Identification of lactic acid bacteria.

Identification of LAB was done using a series of biochemical tests (Collins *et al.*, 1989a) and the determination of the carbohydrate fermentation pattern with API 50 Lactobacillus Identification System (API, Bio-Merieux, Basingstoke) was conducted according to the manufacturer's instructions.

3.3.2.1 Morphological and biochemical observations.

i) Preparation of inoculum.

Inoculum was prepared as in screening (page 71). The cells were harvested by centrifugation and resuspended in physiological saline and this served as the inoculum for all the biochemical tests requiring liquid cultures unless otherwise stated. All test broths were made with 2 % (v/v) inoculation (Mauguin and Novel, 1994).

ii) Hot loop test for CO_2 production.

Gas production from glucose using the hot loop test was determined as described by Sperber and Swan (1976). Cultures were grown in APT broth and incubated at 30°C. After 24 hours, a heated (red hot) inoculating loop was plunged immediately into the culture. Gas evolution in a positive culture is usually copious but occasionally only a small stream of bubbles observed. Negative cultures were retested after an additional 24 hours of incubation. The production of gas differentiates between homofermentative and heterofermentative LAB.

iii) Ammonia production from arginine broth.

Cultures were incubated in arginine broth (see Appendix 3.3 for preparation and content of media) at two glucose concentrations, 0.05 % and 2 %, w/v (Mauguin and Novel, 1994) for 24 to 48 hours. A brown or orange colouration produced when a few drops of Nesslers Reagent (1:1) were added indicated hydrolysis and a positive arginine degradation.

iv) Salt tolerance: growth in 4 % and 6.5 % NaCl.

Salt tolerance was used to differentiate among the cocci. The ability to grow in the presence of NaCl was tested in MRS broth containing 4 and 6.5 % NaCl (w/v). Growth was monitored during the incubation period of three to four days at 30°C.

v) pH tolerance: growth at pH 9.6 and 4.4.

Growth at pH 4.4 and 9.6 was determined in sterile MRS broth. The broth was prepared without phosphate and pH adjusted appropriately before autoclaving. Most pediococci are more acid tolerant than the morphologically similar aerococci and growth at pH 4.4 can differentiate these two genera.

vi) Growth at temperatures 10°C, 15°C and 45°C.

Growth at 10°C, 15°C and 45°C were observed using MRS broth (Collins *et al.*, 1989a). The temperatures of the broths were appropriately adjusted before inoculations. Results were read after 48 hours and for growth at 10°C, the results were read after an incubation period of up to 7 days.

vii) Morphology, Gram reaction and catalase test.

The morphology of the bacteria was observed with wet unstained preparations from an 18 hour culture in APT and MRS broth under a light microscope (Olympus, Tokyo). Rod-shaped cells were either that of *Lactobacillus* or *Carnobacterium* while all other genera have a spherical shape. Tetrad formation indicated cell division in two planes belonging to the genera *Aerococcus, Pediococcus* or *Tetragenococcus* and was distinguished from all other cocci. Gram stain was determined according to Collins *et al.* (1989a). For the catalase test, a few isolated colonies from MRS plate were smeared on a clean slide with a sterile inoculating loop. A few drops of hydrogen peroxide were added over the smear and covered with a cover slip. Effervescence indicated the presence of catalase (Collins *et al.*, 1989a).

3.3.2.2 API 50 CHL kit system.

The API 50 CHL (Bio-Merieux, Basingstoke) gallery allowed the simultaneous study of the fermentation of 49 sugars and sugar derivatives and was a quick way to

categorise and identify LAB. The gallery consisted of 50 microtubes containing a defined amount of dehydrated substrate (except the first one which acts as control) and was filled with the 50 CHL medium previously inoculated with the organism to be identified. The fermentation of the sugars were indicated by a colour change in the tube and was due to the anaerobic production of acid being detected by a pH indicator included in the 50 CHL medium. The results obtained made up a biochemical profile of the organism which was used in conjunction with a manual to identify the organism.

To prepare the inoculum and the API strips. Two to three well isolated colonies from MRS plate (18 hours) were transferred into 2.0 ml sterile water, mixed well and this served as the inoculum. The API 50 CHL medium was inoculated with 0.5 ml of this inoculum. When distributing the inoculated API 50 CHL medium into the tubes, the pipette tip was placed gently against the side of the tube to avoid the formation of bubbles. The tubes were then overlayed with mineral oil to ensure anaerobic conditions, placed in an incubation tray and incubated at 30°C. Observations were made after 6 to 48 hours and the results compared to a standard strip.

3.3.3 Determination of pH and total titratable acidity (TTA)

pH. The liquor sample (2.0 ml) was pipetted into a 5 ml glass sampling bottle and stirred with a small magnetic follower. The pH was measured using a small standard gelplas electrode (BDH Ltd., Poole, Dorset) of 3.0 mm diameter.

TTA. Using the same sample as above, TTA was measured by titrating the sample against 0.1 M NaOH to a final pH of 8.0 as the end point (Adams *et al.*, 1987). The normal phenolphthalein indicator was not used to mark the end point for the titration as the brownish colour of the liquor made observation of any colour change difficult. Assuming that all acid was lactic acid, then 1.0 ml of 0.1 M NaOH was equivalent to 0.009 g lactic acid (90 g/mol). Therefore, the percentage of acid is calculated as :

$$\% \text{ TTA (w/v)} = \frac{[\text{Titrant} \times 0.009]}{\text{Volume of sample}} \times 100$$

3.3.4 Determination of the moisture content (M)

Moisture content was determined by accurately weighing approximately 1 - 2 g of the sample (**W**) in a silica dish of known weight (**D**) and the sample dried in an air oven at 105°C for 16 hours (AOAC, 1980). After cooling in a dessicator for 1 hour, the weight of the dried sample and dish (**Y**) was taken and the moisture content calculated as :

$$\% \text{ M (w/w)} = \frac{[\text{W} - (\text{Y} - \text{D})]}{\text{W}} \times 100$$

The same symbol (W) was applied in section 3.3.5.

3.3.5 Determination of calcium content (Pearson, 1976).

To determine the calcium content the dried sample from the moisture content was used. The sample was charred over a bunsen burner in a fume cupboard to remove most of the fumes. After charring, the silica dish plus the contents, was placed in a muffle furnace at 550°C for 2 hours or until completely free from black carbon particles. After cooling, the ash was carefully moistened with distilled water before adding 5.0 ml of diluted hydrochloric acid (1:1 dilution) to avoid vigorous frothing during the addition of acid. The silica dish was then placed on a boiling water bath until the contents were completely dried. A further 5 ml acid and 5 ml distilled water were added and warmed before filtering the solution into a 50 ml volumetric flask and making up to the mark with distilled water. The sample was further diluted (**V**) if necessary, before reading the absorbance at 422 nm (for Calcium) using an atomic absorption spectrophotometer (Model 1100 B, Perkin Elmer, Norwalk, U.S.A.) with air/acetylene flame. The program was in the "run" mode which gives readings of concentration (**C**) in mg/L. A series of calcium standards (1, 2, 3, 4, and 5 mg/L) were used to prepare a standard curve. The percentage of calcium in the sample was then calculated as :

$$\% \text{ Ca (w/w)} = \frac{\text{C (g/ml)}}{1000 \times 1000} \times \frac{50\ (\text{V})}{\text{W}} \times 100$$

1 % Calcium = 2.5 % Calcium carbonate

3.3.6 Determination of Kjeldahl total nitrogen (TN) of the chitin sediment and the liquor.

The Kjeldahl method for determining TN involved first heating the sample with concentrated sulphuric acid (digestion). An added catalyst accelerated the reaction. The oxidation caused the nitrogen to be converted to ammonium sulphate. After making alkaline with concentrated sodium hydroxide solution, the ammonia was distilled into excess of boric acid and is estimated by titration.

Digestion. Estimation of total nitrogen content of the chitin sediment was made using the Büchi (323, 435, 412) Kjeldahl System (Büchi Laboratoriums-Technik, Switzerland). Dried sample (0.2 - 1.0 g) was accurately weighed and transferred to the digestion tube containing concentrated sulphuric acid (9.0 - 15.0 ml) and two to five tablets of catalysts (K_2SO_4 + $CuSO_4$) and was digested until the solution turned green or light green in colour (about 30 to 60 minutes). The digestion tube and the contents were cooled slightly before being transferred to the distillation unit.

Distillation. The program on the distillation unit was preset to allow automatic addition of distilled water for dilution and concentrated NaOH (32 %) to neutralise the mixture which turned black before distilling. If the solution did not turn black, more NaOH was added manually using a push button on the unit. The distillate was collected in 60.0 ml of Boric acid (2 %, w/v) containing methyl red and bromocresol indicator for 3 minutes (see Appendix 3.4 for the preparation of Boric acid). The distillate was titrated against 0.1 M HCl (standard solution) until a greyish end point was observed. The blank containing no sample (or filter paper where used) was determined and the titre deducted from the sample titre. The percentage nitrogen was then calculated as :

$$\text{\% Total Nitrogen (w/w)} = \frac{14.01 \times \{\text{sample titre - blank titre}\} \times \text{Molarity of acid}}{\text{weight of sample(g)} \times 10}$$

$$\text{or \% Total Nitrogen} = \frac{0.14 \times \{\text{sample titre - blank titre}\}}{\text{weight of sample(g)}}$$

To determine total nitrogen content of the liquor, 1.0 g sample of the liquor was used with 2 catalyst tablets and 9.0 ml sulphuric acid. Percentages of TN of liquor were calculated as above and expressed as either wet or dry weight basis. The percentage of protein of the liquor was then calculated as **TN × 6.25**.

3.3.7 Determination of chitin nitrogen (CN).

Chitin nitrogen was also estimated by the Kjeldahl method, but after the sample had been acid and alkali treated (Black and Schwartz, 1950). A 250 ml beaker containing 1.0 g (dry weight) or 2.0 g (wet weight) sample was placed on a boiling water bath with 100.0 ml of 5 % NaOH solution for 1.0 hour, with occasional stirring to remove the protein. After filtering through a Buchnel funnel with a filter paper (general purpose) and washing thoroughly with distilled water, the deproteinised sample was replaced in the water bath with 50.0 ml of 1.0 M HCl solution for 1.0 hour to remove calcium carbonate. The decalcified sample was again filtered and washed. During the filtration process, care had to be taken to avoid losses of material by using a slightly bigger-sized filter paper than the Buchnel funnel and gently pressed at the side to improve suction. In addition, in order to avoid losses of material, the filtered sample was transferred together with the filter paper into the Kjeldahl digestion tube. The filter paper was free from nitrogen and therefore did not affect the nitrogen determination. The rest of the procedure was the same as in the total nitrogen determination in 3.3.6. The percentage of chitin nitrogen was calculated as below and the percentage of chitin in the sample calculated by multiplying CN by 14.5, assuming that pure chitin has 6.89 % nitrogen (No *et al.*, 1989).

$$\% \text{ CN} = \frac{0.14 \times \text{Titre}}{\text{Weight of sample (g)}}$$

$$\% \text{ Chitin} = \% \text{ CN} \times 14.5$$

3.3.8 Determination of corrected protein.

Corrected protein was obtained by subtracting CN from TN and multiplying by 6.25, assuming that protein has 16 % nitrogen (Jay, 1992).

3.3.9 Determination of total soluble protein nitrogen (SPN).

During autolysis meat and visceral protein liquefy and total visceral nitrogen comprises both soluble and insoluble fractions. Soluble nitrogen is composed of both soluble protein and simpler nitrogen products such as free amino acids and volatile nitrogen bases like ammonia. To determine total soluble protein and non protein nitrogen (3.3.10), the liquor was first centrifuged at 3000*g* for 20 minutes to remove any insoluble fractions.

Total soluble protein (SP) was determined using the Bio-Rad reagent (Hempstead, Hertfordshire). Five protein standard solutions containing 0.2 to 1.0 mg/ml were prepared from a frozen bovine serum albumin (Sigma) standard stock solution (2.0 mg/ml). The liquor sample (0.5 ml, and weights taken, M_x) was appropriately diluted with distilled water (to contain about 0.1 - 1.0 mg/ml protein) to a final volume, V_x. In clean test tubes, 5.0 ml of Bio-rad reagent (previously diluted with 4 parts of distilled water) was added to each of 0.1 ml of standards, sample and a blank (distilled water). The mixtures were thoroughly mixed using a vortex mixer and left to settle for 15 minutes. The absorbance of standards and sample were read at 595 nm using a UV/VIS spectrophotometer against the blank. The concentration of the protein (mg/ml) was deduced from a protein standard curve (see Appendix 3.5). The percent soluble protein was then calculated as:

$$\% \text{ SP} = \frac{\text{conc (mg/ml)} \times V_x}{1000 \times M_x \text{ (g)}} \times 100$$

Also, $\% \text{ SPN} = \% \text{ SP} / 6.25$

3.3.10 Determination of non protein nitrogen (NPN).

Total soluble visceral nitrogen comprises protein and non-protein components. As a preliminary to the measurement of non protein nitrogen, residual undigested protein and soluble protein were removed from the samples by precipitation following the addition of 20 % (w/v) trichloroacetic acid (Adams *et al.*, 1987). The NPN was then determined by the Kjeldahl method of the supernatant liquid.

2.0 g of liquor (**N**) was mixed with 4.0 ml trichloroacetic acid (20 %) using a vortex mixer and centrifuged at 3000*g* for 15 minutes. The supernatant was pipetted and its volume measured (**v**). An aliquot of the supernatant (3.0 ml) was used and the nitrogen content determined by the Kjeldahl method. The percentage NPN was calculated as:

$$\% \text{ NPN} = \frac{0.14 \times \text{Titre} \times (v)}{3.0 \times N}$$

3.3.11 Determination of glucose content by Somogyi-Nelson Method.

Glucose content was determined according to the method of Somogyi and Nelson (Pearson, 1976). The method is based on the reaction of cuprous oxide with arsenomolybdate reagent, which is prepared by reacting ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) and sodium arsenate (Na_2HAsO_7) in sulphuric acid. Oxidation of cupric to cuprous by reducing sugars, with concomitant reduction of the arsenomolybdate complex, produces an intense blue-coloured solution (due to the reduced arsenomolybdate) that is very stable. The absorbance of this solution was determined at either 510 nm. Sodium sulphate was included in the reaction mixture to minimise the entry of atmospheric oxygen into the solution, which would cause reoxidation of cuprous oxide. The calibration curve depends to some extent on the sugar being estimated, so the method is not suitable for the determination of a complex mixture of reducing sugars. Protein must be removed before proceeding with the determination, and this was carried out using zinc hydroxide as the protein precipitant to give a neutral protein-free solution.

Precipitation. Any interfering material such as protein was first removed by adding 1.5 ml distilled water to 0.1 ml sample (diluted beforehand, if necessary), followed by 0.2 ml Barium hydroxide and 0.2 ml Zinc sulphate solution. The mixture was thoroughly mixed and centrifuged at 3000*g* for 20 minutes. The supernatant was used for the glucose analysis.

Colour development. Five glucose standard solutions containing 20 to 100 µg/ml were prepared from a glucose standard solution (Sigma) of 1 mg/ml. 1.0 ml of Copper reagent (see Appendix 3.6) was added to each of 1.0 ml of the supernatant, standard solutions and blank (distilled water), in a capped test tube, mixed well and heated in a boiling water bath for 15 minutes. The tubes were cooled in cold water. Arsenomolybdate reagent (1.0 ml) (see Appendix 3.7) was added to the solution with vigorously shaking. When effervescence has ceased, the blue colour (indication of the presence of reducing sugar) was diluted to a final volume of 10.0 ml. Absorbance was read at 510 nm against the blank. The concentration of glucose was obtained from a glucose standard curve (see Appendix 3.8), multiplying appropriately by the dilution factor. Normally at ($t = 0$), the sample required 30 - 50 times dilution, after which time no dilution was usually necessary.

$$\text{Thus, } \%\ \text{sugar left}_{(t=n)} = \frac{\text{conc. in liquor}_{(t=n)}}{\text{conc. in liquor}_{(t=0)}} \times 100$$

where t = time

3.3.12 Detection of lactate by Gas liquid chromatography.

Samples were centrifuged at 11,000 rpm for 20 minutes prior to methylation to remove microbial cells. Aqueous samples were prepared at concentrations within the range 0.5 % to 1.0 % of the acid volume.

Methylation procedure. The technique employed for the methylating the samples was that of Drummond and Shama (1982). 2 ml sample were combined with 2 ml methanol and 0.75 ml 50% (v/v) aqueous sulphuric acid in screw capped glass bottles that were subsequently incubated in a water bath at 50°C for 30 min. After cooling, 1 ml of deionised water and 0.5 ml chloroform were added to each tube for extraction by vigorously shaking for 5 min.

Chromatographic procedure. A gas chromatograph (series 304, PYE Unicam) equipped with flame ionisation detector was used. 1.0 µl quantities of the methylated acids in chloroform were injected onto a capillary column (S.G.E., 25QC3/BP1-0.5). The column temperature was programmed as follows: a delay of 1 min at 80°C

followed by an increase to 130°C at the rate of 12.5°C/min. Injector and detector temperatures were 225°C and 250°C respectively. The helium carrier gas flow was 25 ml/min.

3.3.13 Determination of particle size distribution in minced waste.

In order to evaluate the effect of particle size upon the fermentation processes, an experiment was conducted to determine whether there existed any significant differences in the composition of particles produced if scampi and tropical waste were minced with different sized mincer plates.

Batches of frozen scampi waste (about 500 g, wet weight) were minced using one of 3 different mincer plates having hole diameters of 3, 6 or 10 mm and each minced waste was designated as "3", "6" and "10" respectively. The waste was centrifuged at 17,000 rpm at room temperature for 30 min and the weight of liquid and solid fractions were taken. The dried solid fraction (105°C overnight) was sieved for 15 minutes using a sieve shaker (Fritsch) with 6 different sieve sizes. The weight of each particle size fraction obtained after sieving was taken and its percentage per total solid waste used calculated. Each fraction was analysed for chitin, total nitrogen and calcium content. Similar treatment was done with tropical waste and the product analysed and compared.

3.4 Fermentation parameters.

a) Bioreactor.

The horizontal rotating basket bioreactor is shown in Figure 3.1. It comprised a lightweight stainless steel cylindrical shell (23.0 cm long and 12.5 cm diameter) covered with a double layer of mesh to form a cylindrical basket. The outer, stainless steel, mesh had an aperture of 2 mm and the inner polyester mesh, an aperture of 0.9 mm. An axial shaft, supported in PTFE bearings, ran the length of the basket permitting it to rotate freely inside a glass outer casing (QVF Glass, 29.5 cm long and 15.5 cm diameter). The shaft was connected via a speed-reducing gear box to a fixed speed electric motor capable of delivering a torque of 52 foot-pounds. With this arrangement, the basket rotated at 20 rpm. Two circular stainless steel plates were used as end closures for the glass outer casing and these were bolted in place. Water

tight seals at the end plates were provided by PTFE end gaskets. The end plates were supplied with ports for sample withdrawal, liquid additions and gas removal. The maximum charge of scampi waste (wet weight) which could be loaded into the basket was approximately 2.0 kg. This design of bioreactor enabled the liquid released during fermentation to fill the annular gap between the basket and the glass casing and thereby keep the contents of the basket wetted. The electric motor was activated via a timer. For the batch fermentations (see section 3.4.1), a simple 24 hour electronic timer was used which had a fixed minimum "time on" of 10 minutes. In order to achieve a shorter level of agitation a purpose-built electronic timer with greater variations in the time "on" and "off " facilities (in seconds, minutes and hours), was used in all other experiments.

b) Preparation of inoculum.

Lactobacillus paracasei, strain A3 was used in all the fermentations described in this section. To prepare the starter culture, a loop of cells from a slope of MRS agar was transferred into 3.0 ml of sterile MRS broth (Unipath Ltd., Basingstoke, Hants) and incubated at 30°C for 24 hours. To prepare the inoculum for the fermentation, a 2 % inoculation (v/v) with the starter culture was made with sterile MRS broth and was incubated statically at 30°C for 24 hours. Inoculum prepared in this way had an approximate cell count of 10^8 cfu/ml.

c) Preparation of scampi waste.

All the scampi waste used was minced using a mincer plate with 6 mm diameter holes unless otherwise stated. The minced waste was partially thawed in a microwave oven (Deltawave III, Toshiba, defrosting mark 3) for 4 to 6 minutes. All preparations were performed without delay and while the waste was still slightly frozen to prevent spoilage taking place. The charge of waste used was determined in this slightly thawed condition in all the experiments. Preliminary investigation revealed that 1.0 kg charge of waste was found suitable to effect sufficient contact between sediment in the basket and the liquor and was used in all experiments.

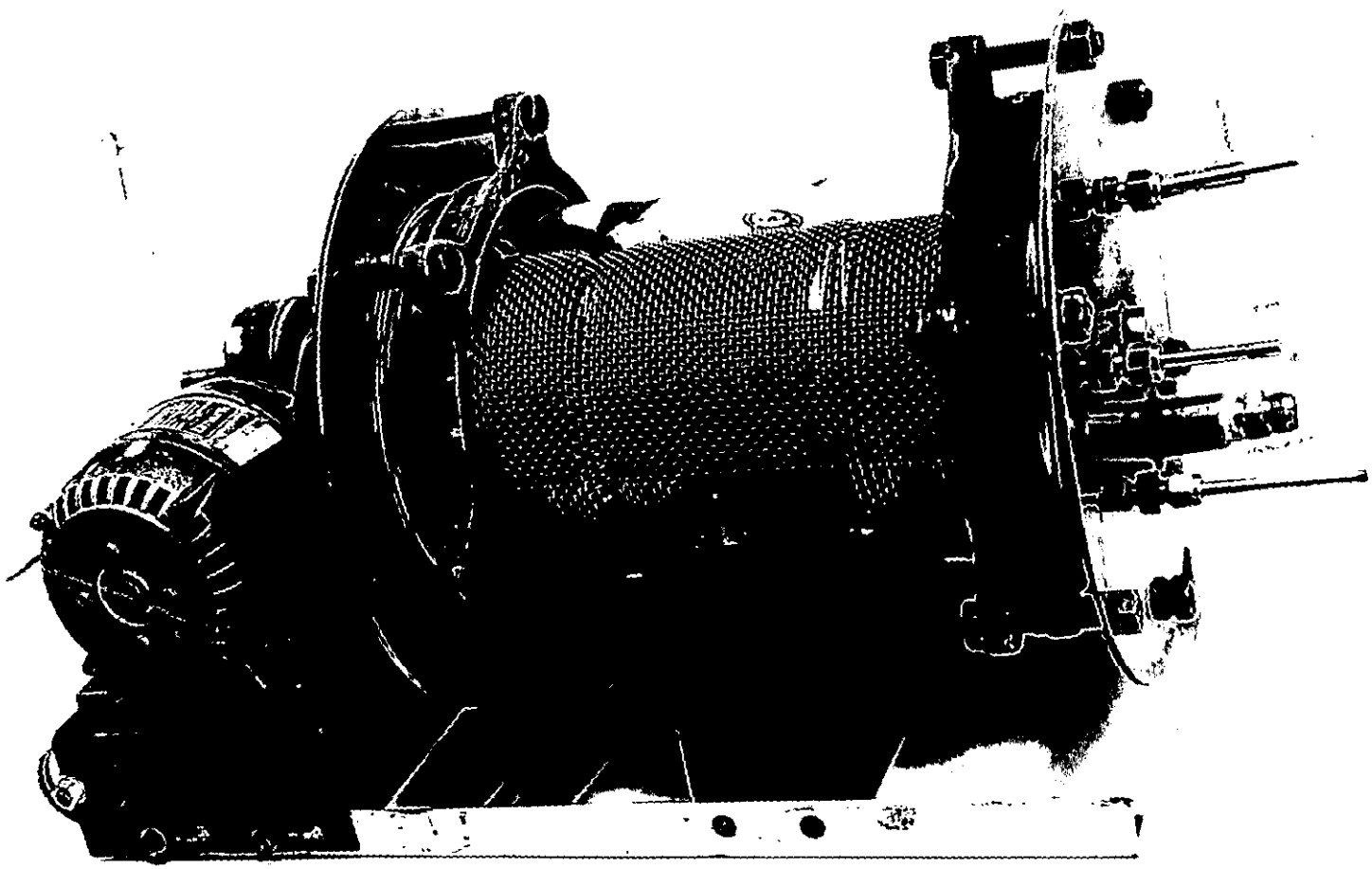


Figure 3.1: Horizontal rotating bioreactor

3.4.1 Batch fermentations.

Three similar runs were carried out according to the following procedures. Earlier studies by Hall *et al.* (1994) had revealed the importance of adding sufficient glucose and of using a large enough inoculum, therefore in this work the minced scampi waste (1.0 kg) was thoroughly mixed with 10 % (w/w) glucose and 10 % (v/w) of the prepared inoculum, to give a cell concentration of 10^8 cfu/g waste. The semi-solid mixture was transferred to the basket bioreactor and the entire bioreactor placed in a temperature controlled enclosure maintained at 30°C. Rotation of the basket was set to provide 10 minutes continuous operation every 6 hours. During the first experiment, agitation was set for 7 days but in the following two experiments, it was set for first 3 days only, to prevent the liquor from foaming. The gas vent was connected to a Dreschel bottle approximately half filled with water to provide a slight gas overpressure in the bioreactor relative to the surroundings.

Small aliquots of the liquor produced during fermentation were taken via the sampling port using a syringe, every 6 hours during the day. The pH and TTA of the liquor were determined immediately after sampling. Other liquid samples were kept frozen at - 20°C until required. These samples were analysed for TN, SPN, NPN, moisture and calcium content. Total weights of the liquor (W_{sam}) taken during the fermentation were noted. The fermentation was terminated when the pH started to rise to a value of 6.0, after which time, the protein-rich liquor was poured into a glass beaker and the weight taken (W_{tot}) and the chitin sediment was scraped out from the basket. The final liquor was analysed for TN, SPN, NPN, glucose, moisture and Ca content. It was noticed that the sediment still contained some liquor and to totally remove the adhering liquor, a small proportion of the wet sediment was placed in a circular muslin cloth, placed in a 20 ml screw-capped bottle and was centrifuged at 3000*g* for 30 minutes. During centrifugation, the muslin cloth facilitated the separation of liquor and chitin sediment. The total weight of the liquor (W_{cent}) and sediment obtained through centrifugation was noted and the sediment analysed for TN, CN and Ca content. The total weight of liquor obtained after centrifugation (W_{cent}) was added to the (W_{tot}) and (W_{sam}) to obtain total weight of liquor. From the moisture content determinations of liquor, sediment and silages, the total dried

weights of these fractions were obtained. These data were used to construct mass balances.

3.4.2 Variation in the glucose addition.

Theoretically (see Appendix 4.2), the addition of 10 percent glucose (w/w) in a batch fermentation would limit complete removal of calcium carbonate. However, during the actual fermentation process in which several interactions may have been occurring including growth of bacteria, hydrolysis of proteins and formation of calcium lactate, increasing the glucose concentration might significantly affect one or more of these processes and resulted in an overall deleterious effect on the fermentation. To investigate this, three experiments were conducted to investigate the effect of adding additional glucose during the course of fermentation in order to achieve a higher percentage of calcium removal. Three modes of glucose addition were investigated.

Mode A: Addition of anhydrous glucose.

Scampi waste was fermented with 10 % inoculum and 10 % glucose at 30°C as described above. A further 20 % glucose (200.0 g) was added at intervals between day 3 and day 4. This was done by withdrawing some liquor from the reactor, dissolving the glucose and then replacing it back into the reactor. The basket was rotated for 1 min after each addition of glucose and the basket rotated at 3 revolutions every hour throughout the fermentation. Liquor samples taken during fermentation were analysed for TN, TTA and Ca content. Also, in this experiment and in all subsequent experiments, the pHs' were monitored continously using a Gelplas electrode which was inserted into the bioreactor through the lowest port, so that the electrode was always immersed in the liquor. The pH meter was connected to a chart recorder (Pharmacia).

Mode B: Addition of dilute glucose solution.

Scampi waste was initially fermented as in mode A. After 72 hours, 15.0 g glucose and 100 ml water were added. A further 15.0 g glucose and 100 ml water were added at 120 hours. The basket was rotated for 1 min after each addition of glucose and the

basket rotated at 3 revolutions every hour throughout the fermentation. The liquor samples taken during fermentation were analysed for TN, TTA and Ca content.

Mode C: Substitution with dilute glucose solution.

Scampi waste was initially fermented as in mode A. After 54 hours, the liquor was totally withdrawn and replaced with an equal volume of distilled water and 50.0 g glucose. Two further additions were made when the pH started to rise, at 96 hours and 130 hours. The basket was rotated for 1 min after each addition of glucose and the basket rotated at 3 revolution every hour throughout the fermentation. The liquor samples taken during fermentation were analysed for TN, TTA and Ca content. In mode C, drainage of the bioreactor prior to making the glucose additions enabled samples of the material from inside the basket to be taken for analysis. These samples designated as F1, F2, F3 and F4, were washed, dried (105°C for 16 hours), and analysed for chitin nitrogen (CN), total nitrogen (TN) and calcium content.

3.4.3 Effect of waste particle size.

This experiment was conducted to evaluate the effect of particle size of scampi waste on the lactic acid fermentation. Earlier experiments conducted with the mincer fitted with plates having holes of 3, 6 and 10 mm revealed no significant differences in the particle size distribution of the minced product. Therefore a different approach was adopted to produce a material with relatively broad range of particle size. Initial trials with ball milling were not successful as the chitinous shells were pliable and did not break. In the end, a simple cutting and crushing procedure was used instead.

Method. Five fermentations were carried out in this series of experiments, each employing scampi waste of different particle size (Table 3.1). Where minced waste was required, the waste was minced with 6 mm hole mincer plate. Unminced waste was prepared by cutting the slightly thawed waste into lengths of either 0.5 or 0.25 inches. This was subsequently used as cut or was further crushed. Crushing was achieved by loosely packing the cut-waste into a thick double layered plastic bag and flattened it once with a hammer (Stanley, 1 lb weight). This had the effect of cracking the material and exposing the flesh present. However, some smaller sized material was also produced by this process. A fixed amount of waste (1.0 kg) was

used in all cases and was fermented at 30°C for 72 hours with 10 % inoculum and 10 % glucose. The basket was rotated at 3 revolutions every hour for 72 hours and the pH was monitored continuously. At the end of the fermentation, the sediment was separated from liquor and washed with 2 L of distilled water to remove any adhering protein liquor. The sediment was dried at 105°C for 16 hours and analysed for CN, TN and Ca content. For (D), the fermentation was stopped after 48 hours as it was beginning to spoil. No analysis was done on the liquor produced.

Table 3.1: Variation of particle sizes used in the fermentation:

Designation	Particle size
(A)	100 % minced
(B)	50 % minced and 50 % crushed (1/2" size)
(C)	100 % crushed (1/2" size)
(D)	100 % crushed (1/4" size)
(E)	100 % whole (1/2" size)

3.4.4 Effects of agitation.

When waste of a relatively large particle size was loaded into the basket it was observed that the proportion of solids in contact with the liquor generated during the fermentation was reduced. This reduced contact was viewed as undesirable as it might have affected the rate of fermentation. It was thought that contact time could be increased by increasing the rate of rotation of the basket. However, increasing the rotation rates had previously been shown to cause foaming of the liquor in some batch fermentations. Based on the results of the batch fermentations, three agitation regimes were investigated (Table 3.2).

Method. Fermentations were carried out using 1.0 kg of waste fermented with 10 % inoculum and 10 % glucose at 30°C for 72 hours. The following rotation rates and waste treatments were used:

Table 3.2: Variation of agitation rate used in the fermentation

Fermentations	Waste	Rotations
(i)	minced	3 revolutions per hour for 72 hours
(ii)	crushed (1/2")	3 revolutions per hour for 72 hours
(iii)	crushed (1/2")	15 revolutions per 15 min for first 36 hours, then at 10 revolutions per hour until 72 hour

At the end of the fermentation, the sediment was separated from liquor and washed with 2 L of distilled water to remove any adhering protein liquor. The sediment was dried at 105°C for 16 hours and analysed for CN, TN and Ca content.

3.4.5 Effect of temperature.

Temperature plays an important role in processes where enzymes and bacterial growth are involved. The A3 strain was able to grow at temperatures 15°C and 45°C but not at 10°C or 50°C. It can therefore be considered as a mesophilic bacterium (Banwart, 1989). Fermentations were carried out at three temperatures in order to evaluate the effect of temperatures on proteolytic activity and acidification of scampi waste.

Method. Minced waste was fermented with 10 % inoculum and 10 % glucose with a rotation rate of 3 revolutions per hour for 72 hours. Fermentations were conducted at four temperatures, 15°C, 20°C, 30°C and 45°C and pH was monitored continuously. Fermentations were stopped after 72 hours and the sediment was separated from the liquor, weights of the fractions were determined and the sediment washed with 2 L of distilled water, dried at 105°C for 16 hours and analysed as previously described.

3.4.6 Effect of loading.

Batch fermentations had revealed that the volume of liquor produced from a 1.0 kg minced waste was sufficient to keep the sediment well submerged in the liquor within the basket during the fermentation processes. A calculation was also made (see Appendix 3.9) to extrapolate the minimum amount of waste needed to effect such solid and liquid contact. In experiment 3.3.6, the effect of loading on the purification of chitin was evaluated using the lowest loading well within the suitable range.

Method. Minced waste (750 g, 1000 g and 1500 g) was fermented with 10 % inoculum and 10 % glucose at 30°C with a rotation rate of 3 revolutions per hour for 72 hours. pH was monitored continuously. Fermentations were stopped after 72 hours and the sediment was separated from liquor, weights of the fractions were

determined and the sediment washed with 2 L of distilled water, dried at 105°C for 16 hours and analysed as previously described.

3.4.7 Comparison of waste from tropical and temperate species.

Fermentations of scampi waste and tropical waste were compared. Minced waste (1.0 kg) was fermented with 10 % inoculum and 10 % glucose with a rotation rate of 3 revolutions per hour for 72 hours. Fermentations were conducted at 30°C and the pH was monitored continuosly. Fermentations were stopped after 72 hours. Sediment was separated from liquor, weights of the fractions were determined and the sediment washed with 2 L of distilled water, dried at 105°C for 16 hours and analysed as previously described.

3.5 Chemical purification of unfermented scampi waste.

In this experiment, unfermented scampi waste was purified by the conventional acid and alkali treatment according to the method of No *et al.* (1989). The acid and alkali purification of the 72 hour fermented scampi waste was conducted elsewhere (Baker and Milnes, 1995). In Chapter 4 (Results), comparison was made between the two methods in terms of chemical usage.

3.5.1 Acid treatment.

3.5.1.1 Effect of HCl concentration.

Dried waste was treated with HCl (0.2 - 1.5 M) at a 1:20 ratio(w/v), continuously stirred at room temperature for 1h. The treated waste was filtered, washed with distilled water and dried for 16h at 105°C prior to the determination of its calcium content by the method described in section 3.3.5.

3.5.1.2 Effect of HCl extraction time.

Dried waste was treated with 1.0M HCl (1:20 ratio, w/v) at room temperature for various extraction times ranging from 15 min to 2 h. A final calcium content of about 0.1% (dry weight basis) was taken as the optimum value. The treated waste was filtered, washed and dried before treating it with alkali to remove protein.

3.5.2 Alkali treatment.

3.5.2.1 Effect of NaOH concentration & temperature.

The partially purified chitin obtained by the optimum acid conditions above was treated with NaOH solutions of concentrations, 0.5, 0.75, 1.0 M at a ratio of 1:15 (w/v), stirred for 2 h at 3 different temperatures (21°C, 40°C, 65°C). The chitin was then filtered, washed, dried and its total nitrogen determined.

3.5.2.2 Effect of extraction time.

Similarly, partially purified chitin was treated with 1.0 M NaOH (1:15, w/v) at 65°C and at various extraction time ranging from 15 min to 2 h. The TN content of the dried chitin was determined. The final weight of the product was taken to determine the percentage yield. These results were then compared to the conditions obtained for fermentation-based purification and the savings in chemical usage was estimated.

CHAPTER 4

RESULTS

4.1 Isolation of lactic acid bacteria (LAB).

4.1.1 Isolation and screening.

Nine lactic acid bacterial strains were successfully isolated from the various fermented tropical wastes samples shown in Table 4.1. With 10 percent glucose (w/w) added and incubation at 30°C, the moist pinkish mixture soon became liquified, the pH dropped and bubbles of gas evolved, an indication of a good fermentation taking place. After 48 hours, the wastes were still pinkish in colour and had a fermented protein smell. However, one tropical sample from Thailand did not ferment at all and was spoilt and turned black within a short time, and no bacteria were isolated from this source. No further tests were done on the Thailand prawn waste to identify the reason behind this failure to ferment. The nine isolated colonies regarded as LAB showed positive reactions to Gram staining, were catalase negative and grew well in both APT and MRS broth. On MRS plates, these isolated colonies appeared very small (almost pin point) and whitish, typical of LAB. Besides the catalase negative isolates, no other types of bacteria seemed to thrive in the acidic conditions of the fermented waste except for Gram-negative bacillus species. The A1 - A5 isolates were all very short rod-shaped bacteria, some in chains whereas the B isolates contained both spherical (cocci) and rod-shaped bacteria (Table 4.2). The C1 strain (rod-shaped), referred to in Table 4.1 was supplied by Dr. Reid of Loughborough University and was used in the comparative experiments.

In order to choose a suitable bacterial strain which will serve as the inoculum or starter for scampi waste fermentations, a simple screening procedure was carried out in which pH drops were compared during fermentation in scampi extract with added glucose. The pH activity of the five A strains are shown in Figure 4.1a. Only one strain showed significantly different pH activity. Strain A1 displayed a lag phase of about 30 hours at pH of approximately 6.5, afterwhich a pH of 4.5 was attained at 48 hours. The other four strains were rather similar in activity with an immediate rapid drop of pH from 7.4 to approximately pH 4.0 in less than 10 hours.

Table 4.1: Isolation of lactic acid bacteria from various sources of prawn waste

Source of waste	No of isolates	Catalase test	growth in broth APT	MRS	Gram Stain
Tropical waste	5 [a] (A1-A5)	+	+	+	+
Indian shrimp	4 (B1-B4)	+	+	+	+
Thailand shrimp	none				
Scampi [b]	1 (C1)	+	+	+	+

[a] = The 5 isolates were very similar morphologically and only A3 was characterized.
[b] = temperate source

Table 4.2 : Morphological and biochemical observations of the various isolates

Characteristics	Isolates					
	B1	B2	B3	B4	A3	C1
Morphology in (APT) (MRS)	cocci cocci	rod rod	cocci cocci	rod rod	coccobacilli cocci	rod rod
Tetrad formation	+	-	-	-	-	-
CO_2 production	-	-	-	+	-	-
NH_3 production	+	-	+	-	-	-
Growth in 4 % NaCl	+	+	+	+	+	+
Growth in 6.5 % NaCl	+	+	-	+	+	+
Growth at pH 4.4	+	+	+	+	+	+
Growth at pH 9.6	-	-	-	-	-	-
Growth at 10°C	+	+	+	+	-	+
Growth at 45°C	+	+	-	-	+	-

- no growth; + good growth

These low pH values were maintained over 48 hours. The pH activity of the four B strains (Figure 4.1b) also showed similar trends but with a shorter lag phase of about 6 hours at pH 7.4 then rapidly dropped to pH 4.1 at 9 hours.

Since all the isolated strains showed similar pH activity, only those strains that were morphologically different were further identified using a series of biochemical tests and an API kit system i.e the A3 strain, all the B strains and also the C1 strain.

Figure 4.1a: Screening of potential LAB from tropical shrimp waste

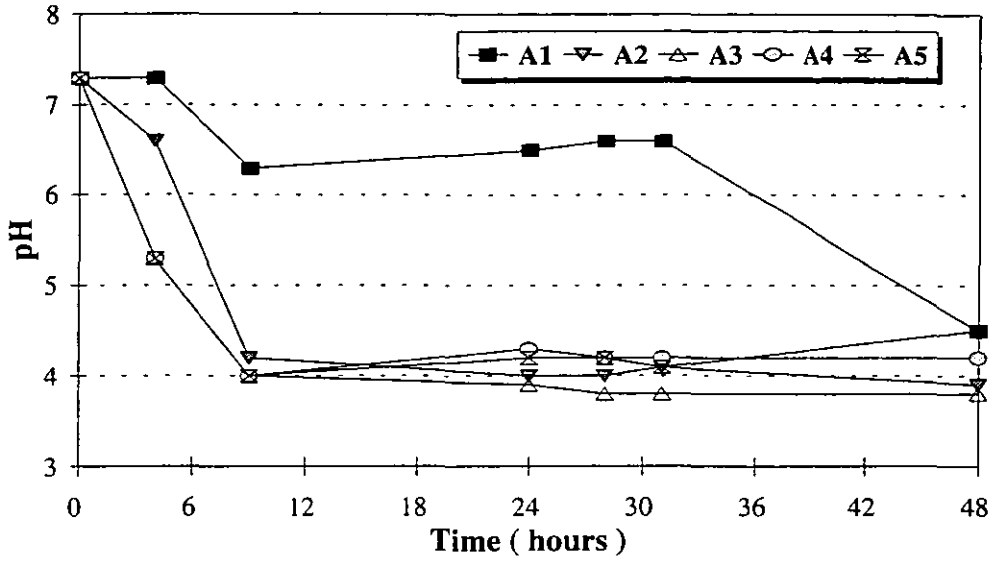
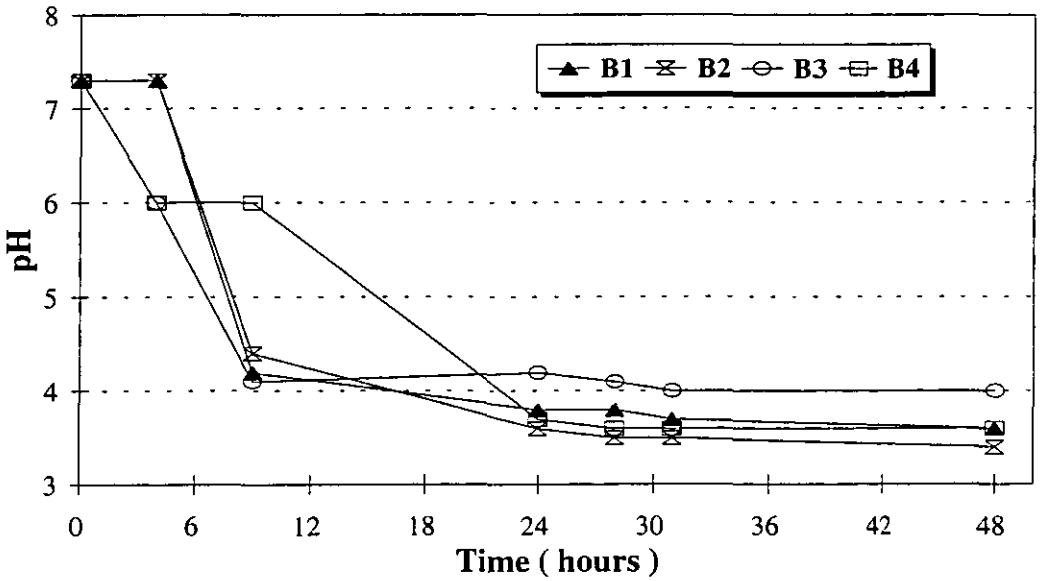


Figure 4.1b: Screening of potential LAB from Indian shrimp waste



4.1.2 Identification of isolated lactic acid bacteria.

The morphology of A3 strain was quite difficult to categorise as it appeared to be coccobacilli (very short rods) when grown in APT broth and as very short rods in MRS broth (Table 4.2). B2, B4 and C1 strains were typical lactobacillus species with characteristic slender rod forms which can be easily recognised. B1 strain had tetrad formation. All strains tested, with the exception of B4, did not produced carbon dioxide. All strains were able to grow in acidic conditions and in the presence of NaCl. In terms of growth temperature, A3 showed a slightly different growth pattern, able to grow at 45°C but not at 10°C. The A3 strain grew very well in MRS broth and was still active when grown on MRS slopes at 4°C even after 2 - 3 months. Other strains had to be subcultured within 1 - 2 months and became non viable after 2 months.

Table 4.3 shows differential characteristics of recent regroupings of the LAB genera (Axelsson, 1993). Comparing the results obtained in Table 4.2 with that of the reference Table 4.3, the results show that B1 is a *Pediococcus,* having cells with tetrad formation; B2, B3, A3 and C1 are either homofermentative *Lactobacillus* (i.e they do not produce carbon dioxide) or facultative heterofermentative and B3 is a heterofermentative *Lactobacillus* (producing carbon dioxide). From these isolates, three were further identified to the species level using the API kit i.e the A3, B1 and C1 strains, based on the criteria of ease of growth, non-producers of carbon dioxide and belonging to different genera.

Table 4.4 compares the carbohydrate fermentation profile of the isolated strains (A3, B1 and C1) to that of similar reference strains extracted from the Identification Table provided with the API Kit (see Appendix 4.1). Great similarities were observed in the fermentation profile of these isolated strains to that of the reference strains and thus strain A3 was identified as *Lactobacillus paracasei*, B1 as a *Pediococcus* spp. and C1 as *Lactobacillus plantarum.* The production of lactate by *Lactobacillus paracasei* was confirmed by gas chromatography from a 24 hours culture in MRS broth.

Table 4.3: Differential characteristics of lactic acid bacteria (after Axelsson, 1993).

Characteristics	Rods		Cocci or spherical						
	Carnob	*Lactob*	*Aeroc*	*Entero*	*Lactoc Vagoc*	*Leucon*	*Pedioc*	*Strep-to*	*Tetra-genoc*
Tetrad formation	-	-	+	-	-	-	+	-	+
CO_2 from glucose	-	±	-	-	-	+	-	-	-
Growth at 10°C	+	±	+	+	+	+	±	-	+
Growth at 45°C	-	±	-	+	-	-	±	±	-
Growth in 6.5 % NaCl	nd	±	+	+	-	±	±	-	+
Growth in 18 % NaCl	-	-	-	-	-	-	-	-	+
Growth at pH 4.4	nd	±	-	+	±	±	+	-	-
Growth at pH 9.6	-	-	+	+	-	-	-	-	+

nd = not determined; ± = some strains may show no growth (-).

Table 4.4 : API carbohydrate fermentation profile of isolated and reference strains

Sugar fermentation	Isolated Strains[a]			Reference Strain[b]		
	A3	B1	C1	*Lactobacillus paracasei*	*Lactobacillus plantarum*	*Pediococcus* spp
Control						
Glycerol	+			+		
L-Arabinose			+		+	
Ribose	+	+	+	++	++	+
Galactose	+	+	+	++	++	++
D-Glucose	+	+	+	++	++	++
D-Fructose	+	+	+	++	++	++
D-Mannose	+	+	+	++	++	++
Mannitol	+		+	++	++	
Sorbitol	+		+	++	+	
α Methyl-D-mannoside			+		+	
N Acetyl glucosamine	+	+	+	++	++	++
Amygdaline	+	+	+	++	++	+
Arbutine	+	+	+	++	++	+
Esculine	+	+	+	++	++	++
Salicine	+	+	+	++	++	++
Cellobiose	+	+	+	++	++	++
Maltose	+	+	+	++	++	++
Lactose	+	+	+	++	++	++
Melibiose			+		++	
Saccharose	+		+	++	++	++
Trehalose	+		+	++	++	
Melezitose	+		+	++	++	
D-Raffinose			+		+	
β Gentiobiose	+	+	+	++	++	++
D-Turanose			+	++	+	
D-Lylose						
D-Tagatose	+	+		++		+
D-Arabitol			+		+	
Gluconate	+	+	+	++	+	

Incubation temperature: 37°C
Inoculation medium : 50 CHL
[a] [+] = positive reaction
[b] [++] = 80 - 100 % positive reaction
[+] = few strains produce positive reaction

Note: Some of the sugars which appeared in the kit but did not produce any positive results with the above strains are omitted from Table 4.4

4.1.3 Comparison experiments between isolated strains to obtain potential starters for the fermentation of scampi waste.

The objective of these experiments was to find out whether the strains chosen for further investigation could perform better (in terms of achieving a rapid initial pH drop) either on their own or in combination when fermenting scampi waste. Some commercial starters contain combinations of LAB and are commonly used in various fermentations (Wood, 1985). The results obtained from these are considered in two sections; in the first, the pH profiles of the different species are compared, whereas in the second, the pH activity when using a combination of two strains was evaluated.

4.1.3.1 Comparison between strains A3, B1 & B2.

The pH profiles are shown in Figure 4.2a and their respective TTA in Figure 4.2b. The only difference observed between these strains is in the initial phase of pH decline (Fig 4.2a). Strain A3 reduced the pH immediately whereas B2 & B1 showed a lag phase of approximately 6 to 12 hours respectively then dropped rapidly to a similar low pH of 5.0 at 24 hours. The TTA were highest when the pH were lowest (Fig 4.2b). Analyses performed on the fermented products also showed very little difference between these (Tables 4.5, 4.6, 4.7 & 4.8). B1 and B2 achieved 89.6 and 89.2 percent conversion of glucose respectively, whilst A3 resulted in an 84.1 percent conversion of glucose. However, Table 4.7, shows that calcium carbonate removal using these three strains was comparable. The mass balances obtained were also not significantly different (Table 4.8).

Table 4.5: Wet weight[a] and moisture content[a] of the various components in the fermentation of scampi waste.

Bacterial strains		Silage	Sediment	liquor	Ratio of Liq:Sed
A3	Wt (g)	59.5	18.0	41.5	2.3 : 1
	% m.c..	68.3 ± 2.0	56.3 ± 1.6	79.6 ± 0.2	
B1	Wt (g)	60.5	15.5	45.0	2.9 : 1
	% m.c..	69.4 ± 0.9	56.0 ± 3.4	81.6 ± 0.5	
B2	Wt (g)	60.3	17.6	42.7	2.4 : 1
	% m.c.	69.0 ± 1.4	56.1 ± 0.7	82.0 ± 0.3	

[a] = Percentages of moisture expressed as mean of 3 replicates ± standard deviation
m.c. = moisture content

Figure 4.2a: Changes in pH during fermentation of scampi waste using different bacteria

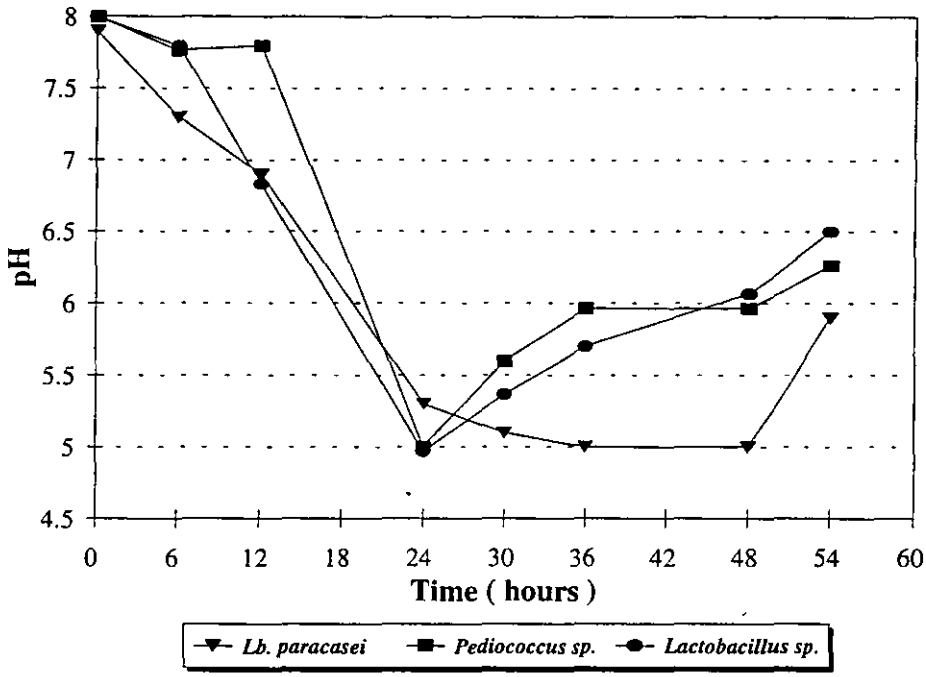


Figure 4.2b: Changes in TTA during fermentation of scampi waste using different bacteria.

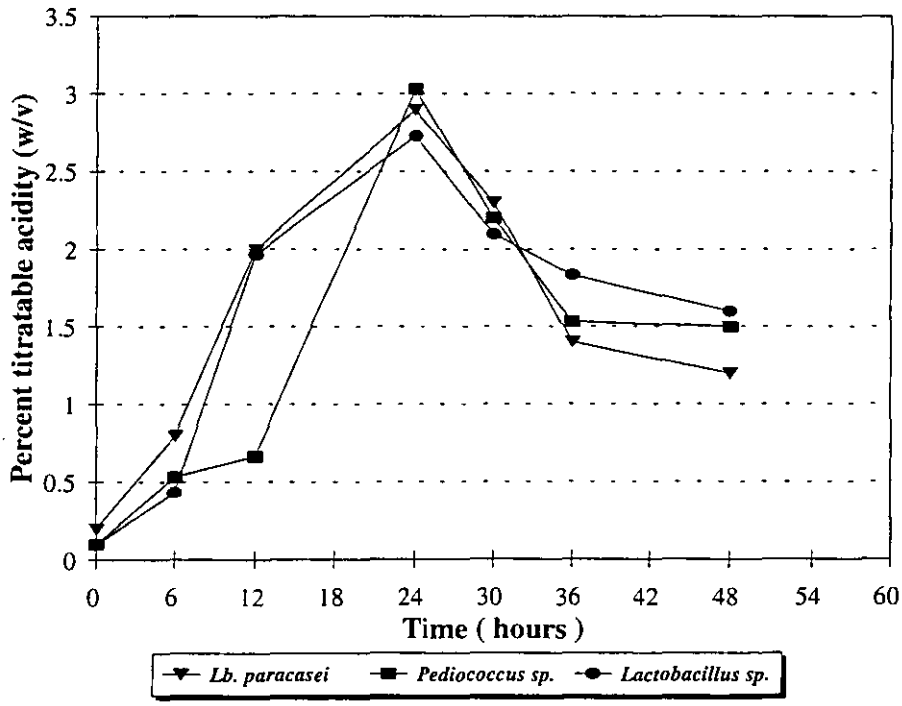


Table 4.6: Composition of the liquor at the end of the fermentation.

Bacteria strain	Conc. of glucose in the liquor (mg/ml)	% glucose left in liquor*	% NPN (w.w.b)	% PN (w.w.b)
A3	15.65 ± 0.85	15.9 ± 0.83	0.98 ± 0.06	0.13 ± 0.02
B1	10.24 ± 1.04	10.4 ± 1.07	0.80 ± 0.01	0.15 ± 0.02
B2	10.65 ± 0.84	10.8 ± 0.85	0.93 ± 0.04	0.17 ± 0.03

w.w.b.= wet weight basis

* % glucose left calculated as = glucose in liquor / glucose at t = 0 (i.e 98.3 mg/ml)

Table 4.7: Chitin, protein and calcium content[a] of the various components in the fermentation of scampi waste.

Bacteria strain	content of:	Silage	Sediment	Liquor
A3	CH (%)	8.7 ± 1.2	17.8 ± 2.5	nd
	(g)	1.6 ± 0.2	1.4 ± 0.2	
	PR (%)	27.6 ± 3.2	18.8 ± 2.3	40.4 ± 0.7
	(g)	5.2 ± 0.3	1.5 ± 1.2	3.4 ± 0.03
	Ca (%)	11.3 ± 1.6	14.8 ± 0.8	9.4 ± 0.3
	(g)	2.1 ± 0.4	1.2 ± 0.1	0.8 ± 0.03
B1	CH (%)	9.2 ± 1.2	15.1 ± 1.7	nd
	(g)	1.7 ± 0.3	1.04 ± 0.2	
	PR (%)	27.5 ± 5.0	18.8 ± 1.7	40.4 ± 0.4
	(g)	5.1 ± 0.7	1.3 ± 0.01	3.3 ± 0.1
	Ca (%)	11.6 ± 1.5	15.0 ± 0.8	7.1 ± 1.8
	(g)	2.1 ± 0.2	1.02 ± 0.1	0.6 ± 0.2
B2	CH (%)	9.8 ± 0.8	12.7 ± 1.3	nd
	(g)	1.8 ± 0.1	1.0 ± 0.1	
	PR (%)	26.7 ± 2.1	19.4 ± 0.3	44.2 ± 2.5
	(g)	5.0 ± 0.5	1.5 ± 0.02	3.4 ± 0.1
	Ca (%)	11.6 ± 0.6	14.4 ± 0.5	5.51 ± 0.3
	(g)	2.2 ± 0.2	1.1 ± 0.01	0.4 ± 0.02

nd = not determined [a] = dry wt basis, CH = chitin, PR = protein

Table 4.8: Overall balances[a] of the various components in the fermentation of scampi waste.

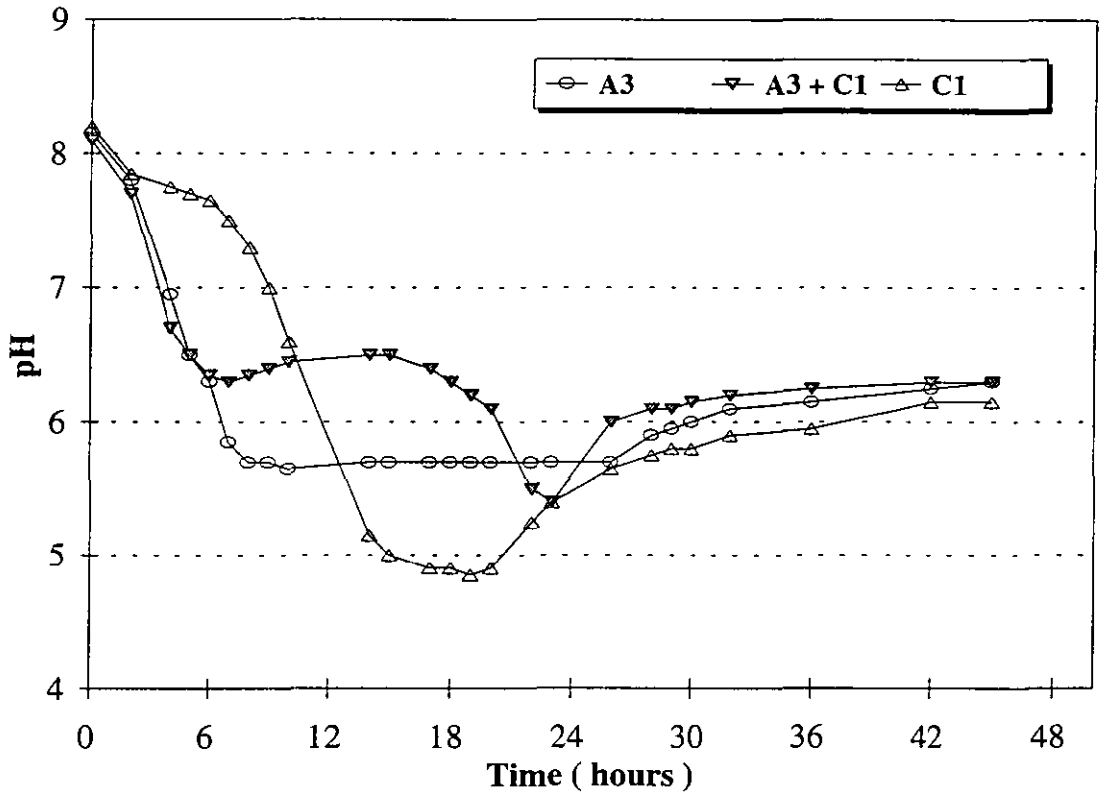
Bacterial strain		A Silage	B Sed+Liq	A/B[b]
A3	Chitin	1.6	1.4	1.14
	Protein	5.2	4.9	1.06
	Calcium	2.1	2.0	1.05
B1	Chitin	1.7	1.0	1.70
	Protein	5.1	4.6	1.11
	Calcium	2.1	1.6	1.31
B2	Chitin	1.8	1.0	1.80
	Protein	5.0	4.9	1.02
	Calcium	2.2	1.5	1.47

[a] = masses of chitin, protein and calcium content of the various components taken from Table 4.7
b = Since silage = sediment + liquor, theoretically A/B equals 1.0.

4.1.3.2 Comparison between mono and co-cultures.

The strains used in these experiments were C1 and A3 which had been identified as *Lactobacillus plantarum* and *Lactobacillus paracasei* respectively. C1 was chosen for study in this experiment besides *Lactobacillus paracasei* has been shown to be a good producer of lactic acid and has been used in many fermentations (Wood, 1985). Strain C1 was isolated from a temperate source and was able to grow at 10°C and not at 45°C, unlike the A3 strain. pH profiles during these fermentations are shown in Figure 4.3. The C1 strain showed a lag phase of approximately 6 hours when fermented alone, whereas when A3 strain was used on its own, or in combination with C1, the pH dropped almost immediately. However, C1 strain on its own resulted in a slightly greater pH drop than either A3 or A3 with C1. Although no further analysis was done to compare the products obtained, Figure 4.3 indicates that the strain A3 did not show any significant difference when fermented alone or in combination with strain C1. Although many commercial starters contain combinations of species (Wood, 1985) the results obtained here suggest that there appeared to be no overwhelming advantages to using co-cultures of *Lactobacillus paracasei* strain A3 and *Lactobacillus plantarum* strain C1.

Figure 4.3: Changes in pH during fermentation of scampi waste using mono and co-cultures.



A3 = *Lactobacillus paracasei* C1 = *Lactobacillus plantarum*

4.2 Analysis of particle size fractions obtained following mincing.

Before the shellfish waste underwent lactic acid fermentation, the waste was minced to produce a homogeneous material. Pretreatment such as particle size reduction has previously been found necessary in making fish silages (Beddows, 1985). However, it would be more attractive industrially, if fermentation of shellfish waste could be done with minimum pretreatment as this could reduce production costs. Therefore experiments were conducted to evaluate the products obtained when tropical and temperate or scampi waste are subjected to three different sized-mincer plates with diameter holes of 10, 6 and 3 mm and were designated as "10", "6", and "3" respectively. After centrifugation and drying, the solid products were sieved using a series of 6 sieves.

4.2.1 Composition of scampi (*Nephrops norvegicus*) waste.

When the minced waste products were centrifuged, three distinct layers of material were obtained. Besides the liquid and solid layers, there was also a layer of sludge. This sludge was incorporated with solids by thoroughly mixing it with the solids before drying. The sludge layer was most prevalent in the 3 product and when dried, the solids tended to clump together. The dried solids were separated as gently as possible before sieving to prevent disrupting the actual size of the materials. Table 4.9 shows the results obtained. Small differences occurred in terms of percentage of liquid or solid produced using the three different mincer plates. However, the number of particle size ranges obtained from 6 and 10 were seven, whereas there was only six size ranges from 3 which lacked the largest sized particles, which were present in both 10 and 6. The slightly higher moisture content in 3 was probably due to the moisture bound to the sludge fraction.

A plot of cumulative percentage by weight against particle size (Figure 4.4) revealed that when scampi waste was minced, it produced a distribution of particles. A similar pattern of particle size distribution were observed for 10, 6 and 3. However, the percentage of larger particles increased as the size of the mincer hole increased (Figure 4.4). Particles less than 0.10 mm appeared as sludge when wet and as a fine powder when dried.

Figure 4.4: Particle size distribution of scampi waste as a function of mincing plate hole diameter

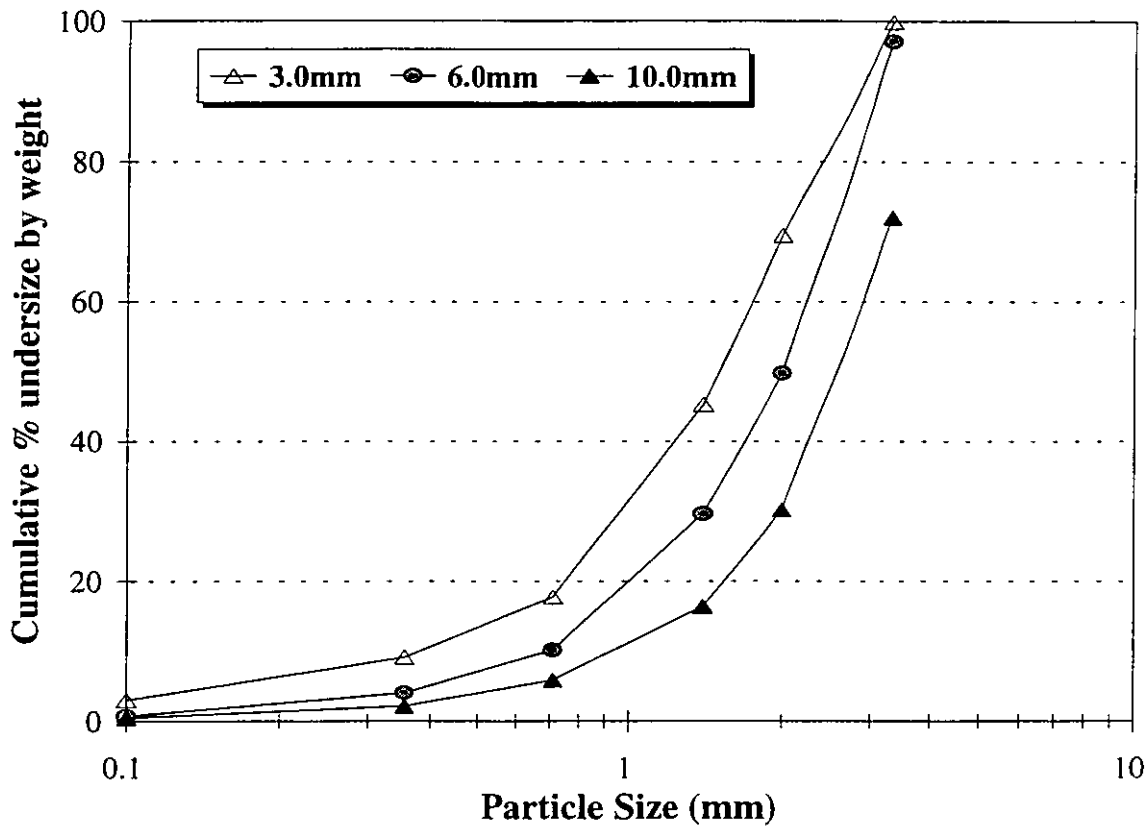


Table 4.9: Weights of various fractions obtained from scampi waste after mincing with different sized plates

Fractions	Mincer plate hole diameter (mm)		
	3	6	10
Wet weight of waste used (g)	600.6	599.4	456.8
Extracted liquid (ml)	265.0	283.0	204.5
Percentage of Liquid/ Waste (v/w)	44.1	47.2	44.8
Wet weight of Solid (g)	335.6	316.4	252.5
Percentage of Solid / Waste (w/w)	55.9	52.8	55.2
Percent moisture content of solid (w/w)	58.2	53.9	54.5
No. of particle sizes obtained	6	7	7

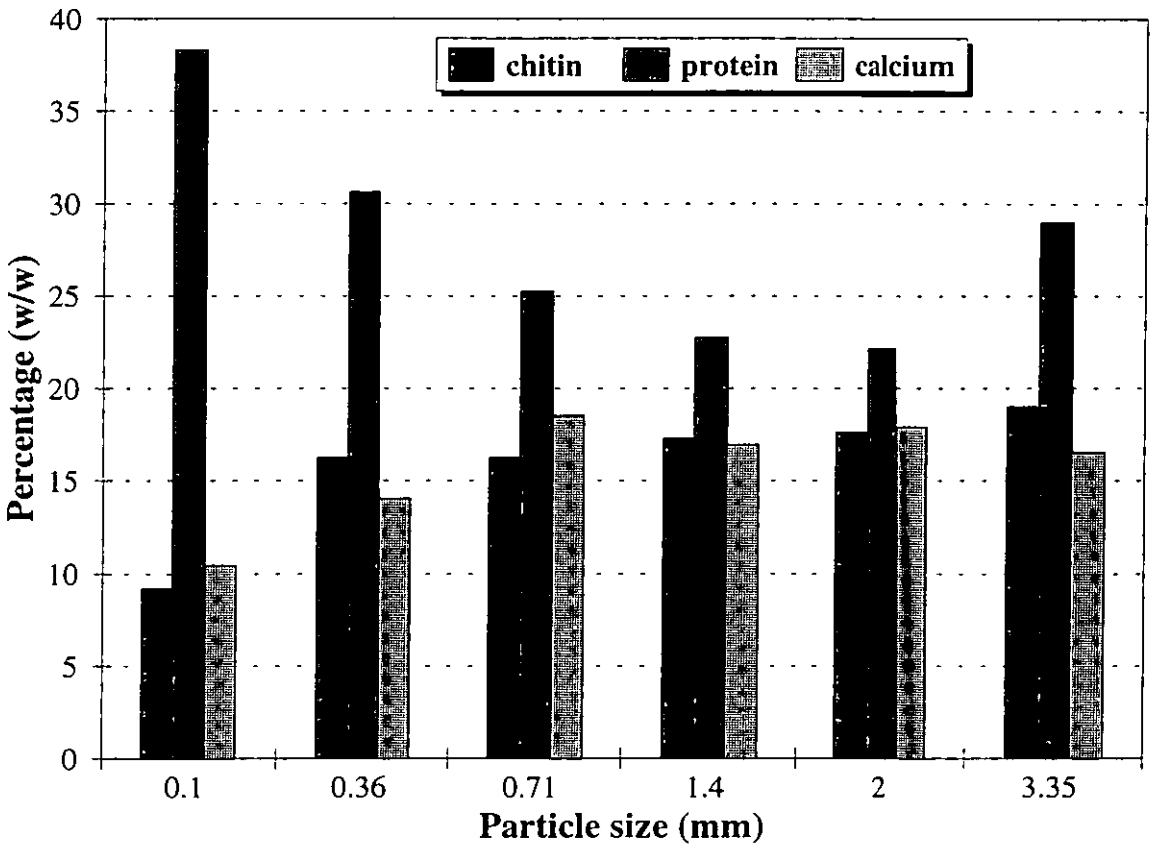
The percentage composition of chitin, protein and calcium of various particle sized materials obtained from 10, 6 and 3 are shown in Figures 4.5a, 4.5b and 4.5c respectively. Whilst chitin and calcium content remained quite constant in all the particle sizes, there was a significant difference in the protein content. The protein seemed to be concentrated in either the smallest or largest particles with the highest percentage being obtained in the smallest particle size (0.1 mm). Table 4.10 shows that more than 80 percent of the chitin, protein and calcium were found in the particle range above 0.71 mm size irrespective of the mincer plate used. This means that the plates used here did not significantly affect the particle size of the waste materials for fermentation.

Table 4.10: Chitin, Protein and Calcium content (g) of the fractionated solid waste

Particle Size(mm)	Mincer Plate	Whole solids	>3.35	2.00	1.40	0.71	0.36	0.10	<0.10	Total wt (g) [a]
Dried	10.0	114.8	32.0	48.0	16.0	12.0	4.2	2.0	0.6	114.8
Wt(g)	6.0	131.8	2.8	63.4	26.5	25.7	8.0	4.4	1.0	131.8
	3.0	140.0	none	42.5	34.0	38.5	12.0	8.5	4.5	140.0
Chitin	10.0	18.78	6.08	8.46	2.77	1.95	0.68	0.18	nd	20.12
	6.0	20.26	0.45	11.03	4.76	4.36	1.22	0.56	nd	22.94
	3.0	23.55	none	6.75	6.07	6.89	1.71	1.03	nd	22.45
Crude	10.0	31.06	9.28	10.62	3.64	3.03	1.29	0.77	nd	28.63
Protein	6.0	36.00	0.85	14.70	5.62	5.32	2.12	1.46	nd	31.07
	3.0	34.83	none	10.65	7.20	8.11	3.28	2.82	nd	32.06
Ca	10.0	19.93	5.30	8.60	2.71	2.22	0.59	0.21	0.05	19.68
	6.0	25.31	0.45	13.03	5.33	4.96	1.51	0.59	0.13	26.00
	3.0	28.59	none	8.49	6.70	7.23	2.28	1.08	0.60	26.38

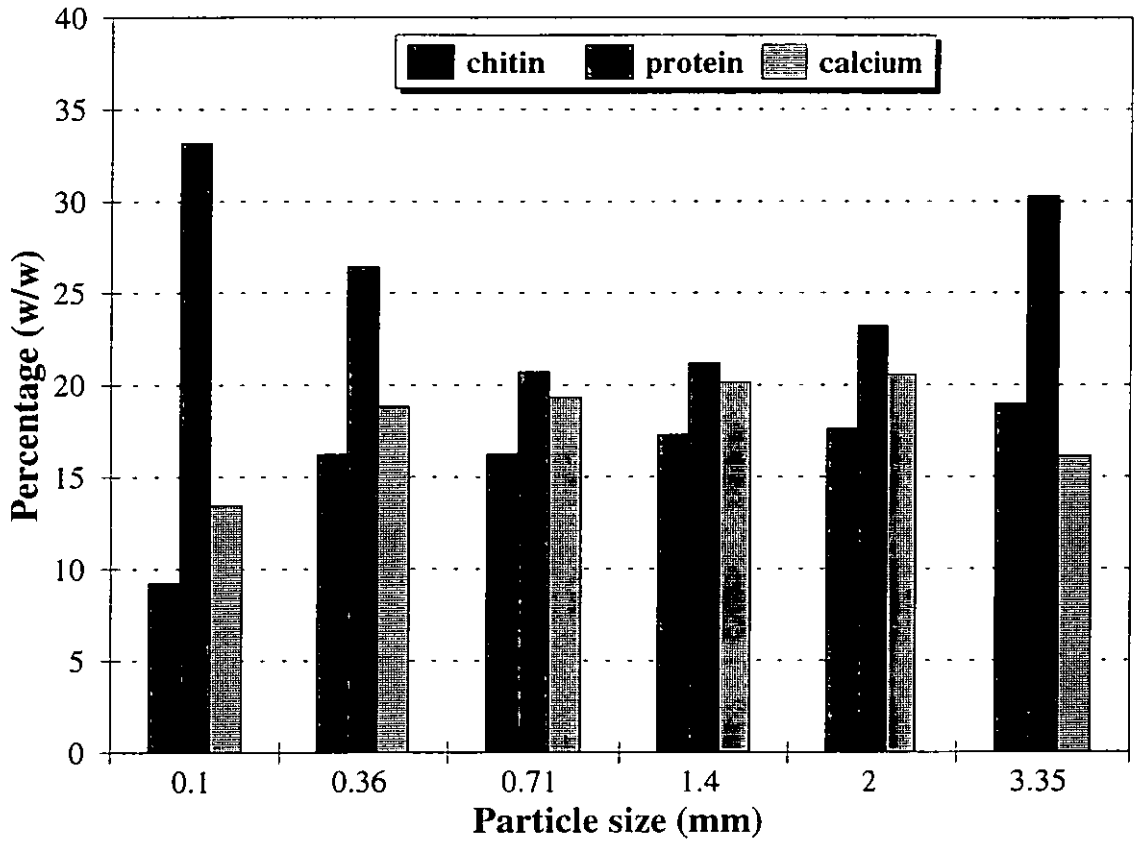
a= total weights obtained by adding the weights in each fractionated solids.

Figure 4.5a: Distribution of chitin, protein and calcium of scampi waste* minced using plate with 10 mm holes.



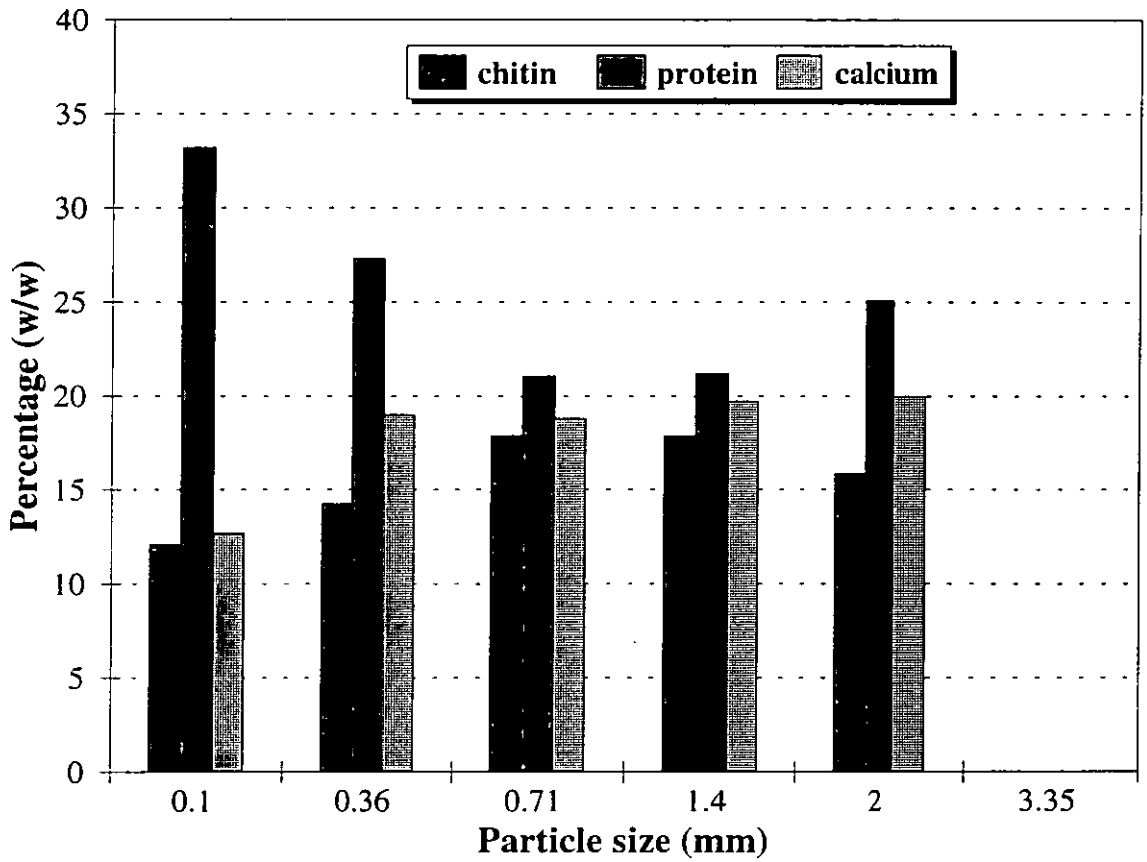
* Chitin and protein content of particles less than 0.1 mm size were not determined.

Figure 4.5b: Distribution of chitin, protein and calcium of scampi waste* minced using plate with 6 mm holes.



* Chitin and protein content of particles less than 0.1 mm were not determined.

Figure 4.5c: Distribution of chitin, protein and calcium of scampi waste* minced using plate with 3 mm holes.



* Chitin and protein content of particles less than 0.1 mm were not determined and particles bigger than 3.35 mm was absent.

4.2.2 Composition of tropical waste (*Penaeus monodon*).

The waste was of a darker brownish colour than the scampi waste and the carapace covering the head portion was much softer. Table 4.11 shows the increase in the percentage of liquid obtained while that of solids decreased as the plate size was increased. Similar particle size distributions as for the scampi waste were obtained (Figure 4.6) except for the presence of a much higher amount of 0.1 mm particles in the tropical waste (~ 38 percent) compared to ~ 4 percent, (Figure 4.4) in the scampi waste. Similarly, the number of particle size fractions obtained from plates 10 and 6 were seven, whereas there was only six from the 3 plate.

The chemical composition of the various particle sizes are shown in Figures 4.7a, b and c for 10. 6 and 3 respectively. In contrast to the scampi waste, the tropical waste contained a higher percentage of protein but a lower calcium content. More than 77 percent of the chitin, protein and calcium was contained in the particles of sizes 0.71 mm and above (Table 4.12).

The results obtained from both studies showed that the tropical waste had a lower calcium content but slightly higher protein and chitin content than the scampi waste. It also showed that mincing the waste (in both cases) with the three mincer plates with holes of diameter 3, 6 or 10 mm did not produce significantly different products.

Table 4.11: Weights of various fractions obtained from tropical waste after mincing with different mincer plates

Fractions	Mincer Plate Size (mm)		
	3	6	10
Wet weight of waste used (g)	231.90	336.00	293.50
Extracted liquid (ml)	106.82	165.05	159.9
Percentage of Liquid/ Waste (v/w)	46.06	49.11	54.48
Weight of Solids (g)	125.08	170.95	133.6
Percentage of Solid / Waste (w/w)	53.94	50.88	45.52
Percent moisture content of Solid (w/w)	61.38	66.16	55.58
No. of particle sizes obtained	6	7	7

Table 4.12: Chitin, Protein and Calcium content (g) of the Fractionated Solid Waste

Particle Size (mm)	Mincer Plate	Whole solids[a]	>3.35	2.00	1.40	0.71	0.36	0.10	<0.10	Total wt.[a] (g)
Dried Wt(g)	10.0	55.64	7.3	15.74	10.16	10.09	3.38	1.08	7.74	55.64
	6.0	54.45	4.17	14.07	9.88	9.53	3.09	0.98	12.35	54.45
	3.0	44.70	none	4.00	5.91	11.55	4.8	1.60	16.60	44.70
Chitin	10.0	12.67	1.46	3.57	2.27	2.18	0.68	0.18	nd	10.34
	6.0	12.08	1.01	3.33	2.32	2.06	0.61	0.19	nd	9.52
	3.0	11.16	none	0.96	1.54	2.72	1.13	0.31	nd	6.66
Crude Protein	10.0	16.86	2.85	5.70	3.33	3.01	1.03	0.38	nd	16.30
	6.0	16.10	1.63	4.68	3.04	2.98	1.02	0.32	nd	13.67
	3.0	13.66	none	1.51	1.87	3.05	1.41	0.48	nd	8.32
Ca	10.0	6.37	0.69	1.72	1.22	1.15	0.36	0.11	0.27	5.52
	6.0	6.05	0.35	1.29	1.19	1.52	0.45	0.13	0.33	5.26
	3.0	5.69	none	0.50	0.71	1.34	0.72	0.24	0.37	3.88

a = total weights obtained by adding the weights in each fractionated solids.

nd = not determined

Figure 4.6: Particle size distribution of tropical waste as a function of mincing plate hole diameter

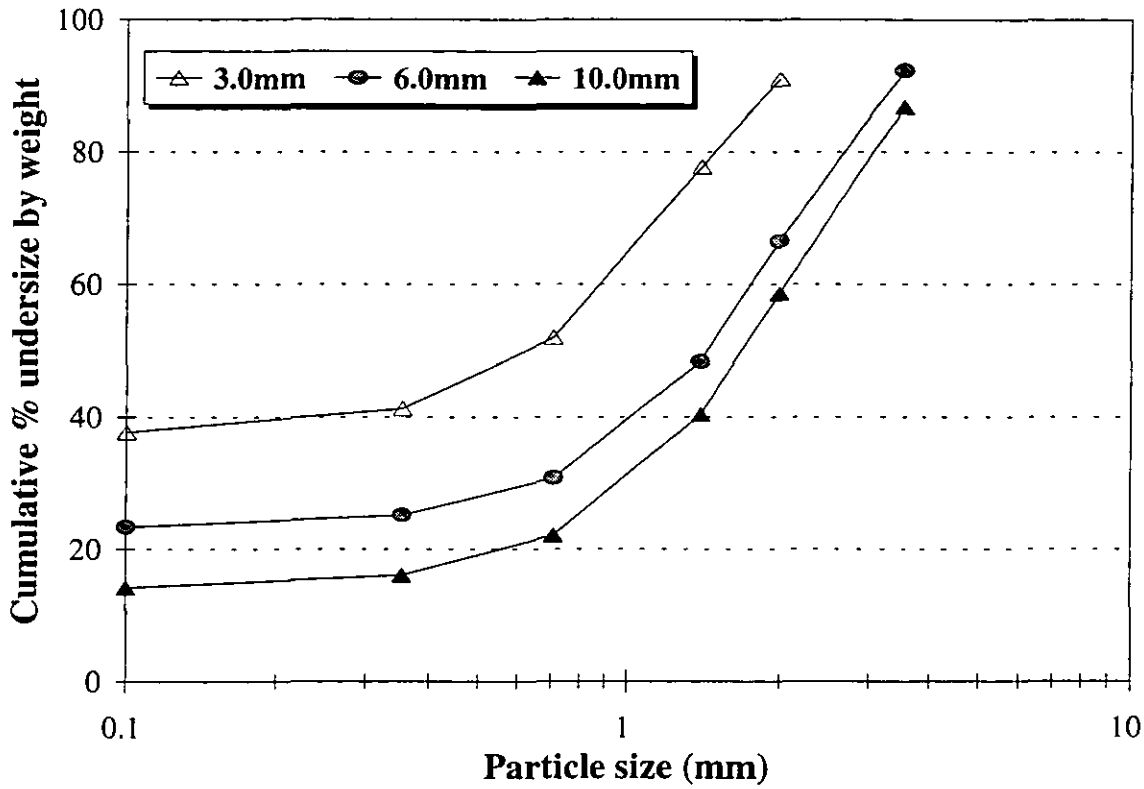
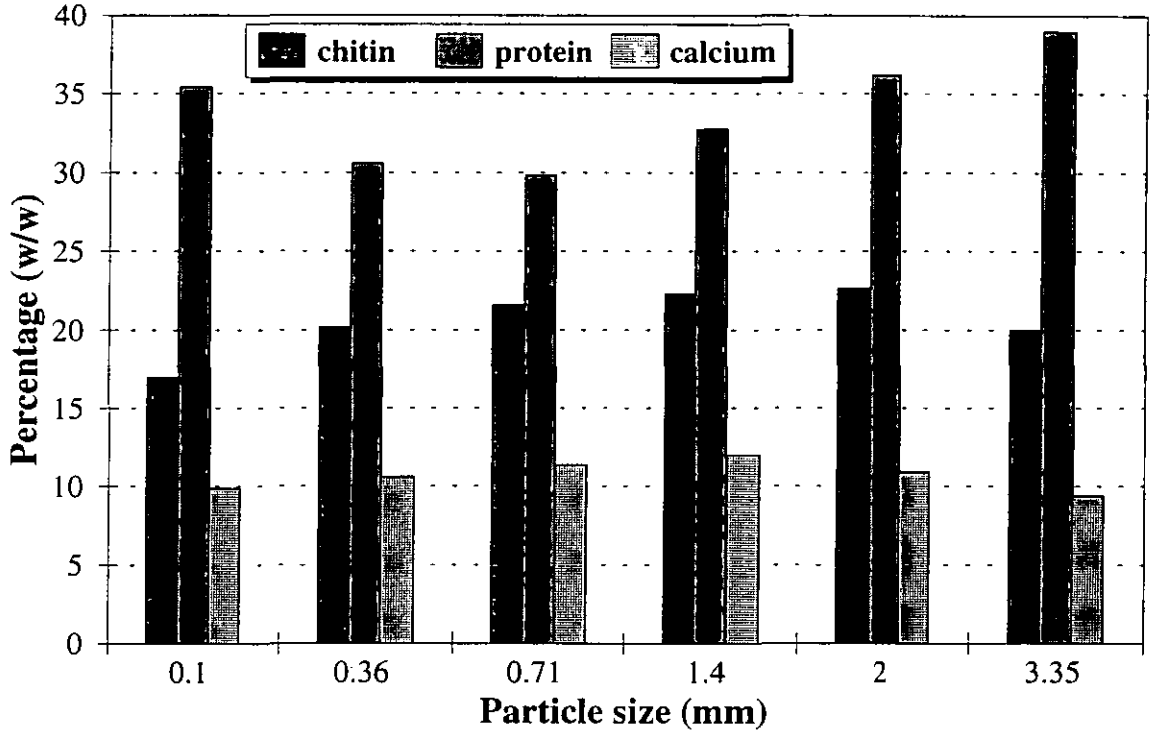
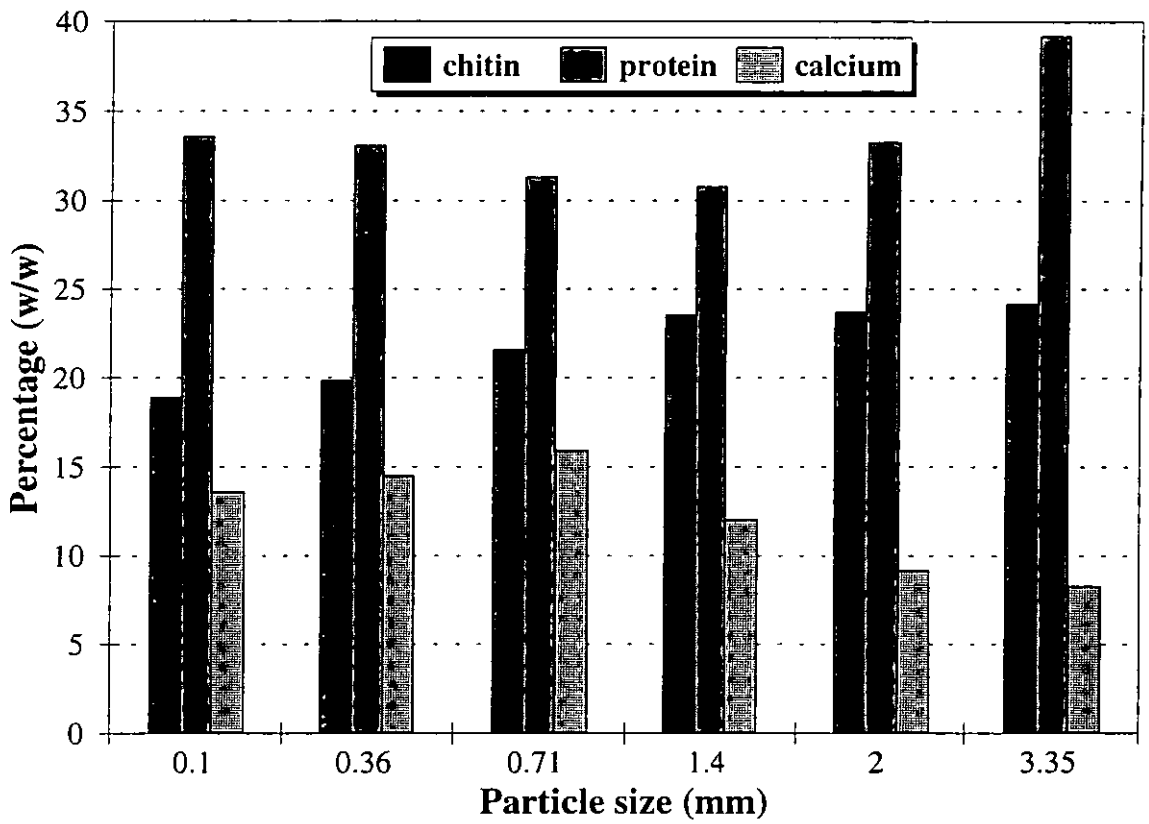


Figure 4.7a: Distribution of chitin, protein and calcium of tropical waste* minced using plate with 10 mm holes.



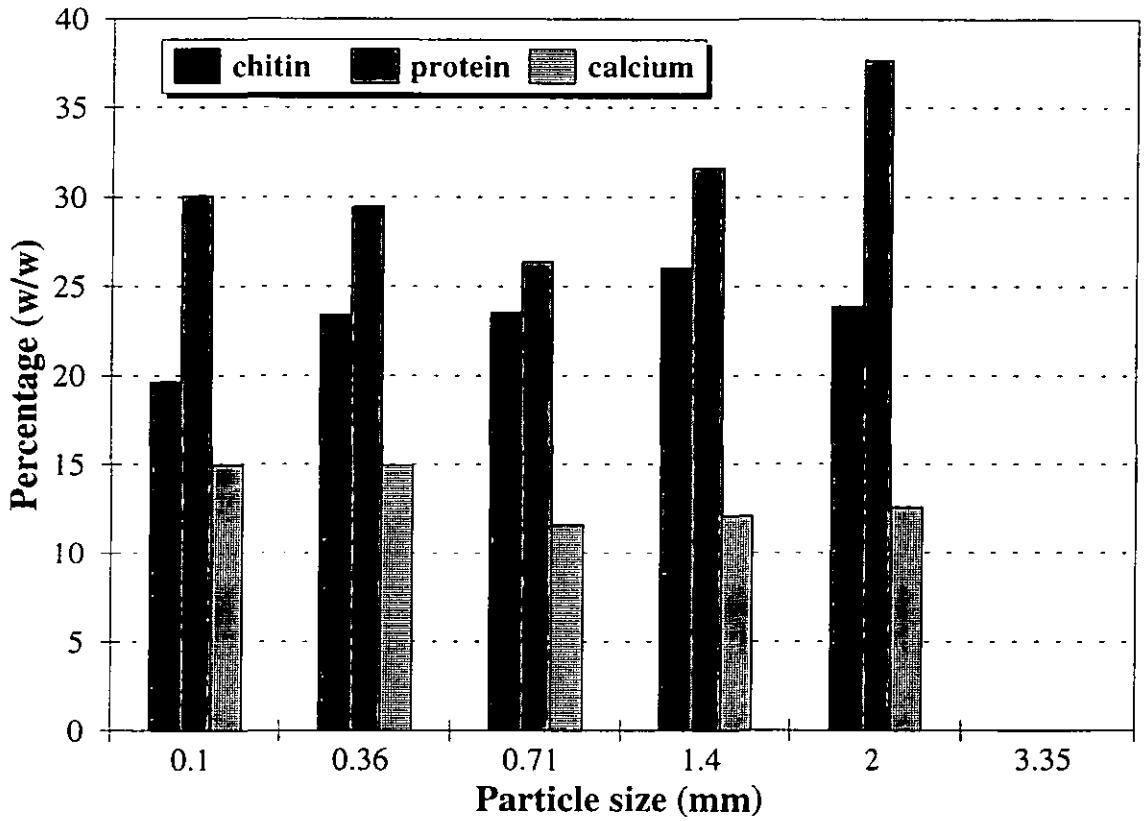
* Chitin & protein content of particles less than 0.1 mm were not determined.

Figure 4.7b: Distribution of chitin, protein and calcium of tropical waste* minced using plate with 6 mm holes.



* Chitin & protein content of particles less than 0.1 mm were not determined.

Figure 4.7c: Distribution of chitin, protein and calcium of tropical waste* minced using plate with 3 mm holes.



*** Chitin & protein content of particles less than 0.1 mm were not determined and particles bigger than 3.35 mm was absent.**

4.3 Lactic acid fermentation of scampi waste using a horizontal rotating bioreactor (HRB).

This section is divided into seven parts (4.3.1 - 4.3.7). The first part (4.3.1) deals with the results from three batch fermentations while the remainder of this section, show the results of six fermentation parameters being studied. The batch fermentation experiments were conducted to provide data which could give direction to the subsequent optimisation studies.

4.3.1 Batch fermentations.

The results of the three replicates of batch fermentation designated as fermentations (I), (II) and (III), were compared. Where appropriate the results are jointly presented, otherwise they are shown separately as subsection Fermentation (I) and the other two fermentations as subsection Fermentation (II) & (III).

4.3.1.1 Fermentation (I).

Table 4.13 shows the various weights of the materials involved and their respective moisture contents for all the three fermentations. The initial scampi waste had a high moisture content of 68.2 percent (w/w).

At the start of fermentation (I), the mixture was in a condition best described as a moist solid state. During fermentation the state of the waste changed to that of a dilute slurry as production of a brownish liquor occurred, particularly during the first 24 hours. Liquid samples withdrawn during the course of the fermentation had a fermented protein smell. The pH of the liquor dropped from 7.8 to 5.0 within 48 hours (Figure 4.8). The rapid pH drop was almost certainly the result of acid produced from glucose by the culture and this is reflected in the rapid increase in the titratable acidity of the liquor (Figure 4.8). However, the low pH was not maintained and the pH started to increase again after 72 hours. After 108 hours it was found that the liquor began to thicken slightly and the fermentation was terminated after 120 hours when the final pH was 6.4.

Table 4.13: The weights and moisture content[a] of the various components.

Batch & Duration of fermentation(h)	%Moisture & Weights (g)	Starting material	Silage	Liquor	Sediment
(I)	%Moisture	68.2	67.1	70.0	62.7
	Wet wt.	1100	840.0	430.0	410.0
120	Dry wt.	350.2	276.4	129.0	152.9
(II)	%Moisture	68.2	69.8	75.5	52.1
	Wet wt.	1100	1065.6	759.2	306.5
132	Dry wt.	350.2	321.8	185.9	146.9
(III)	%Moisture	68.2	70.6	79.3	55.0
	Wet wt.	974.0	953.0	715.0	220.0
84	Dry wt.	310.1	274.6	148.2	99.1

[a] = Mean of duplicate readings; for the various weights taken, refer to Materials and Methods (section 3.3) on how weights were derived.

An increase was observed in the calcium content of the liquor samples as the fermentation progresses (Figure 4.9). Gas formation occurred during the fermentation particularly in the early stages and could be seen bubbling through the Dreschel bottle attached to the gas exit line. Figure 4.9 also shows that the calcium solubilisation followed closely behind acid production.

Figure 4.10 reveals a gradual increase in the total nitrogen (TN) content of the liquor. A small amount of soluble protein was detected at the start of the fermentation reaching a maximum at approximately 12 hours after which its concentration fell. In contrast, there was a large increase in non protein nitrogen (NPN) compounds in the liquor, being highest at the end of the fermentation period. Figure 4.10 also shows that the sum of the soluble protein nitrogen (SPN) and NPN is almost equal to the TN.

A slight modification was done to the second and third fermentations in order to avoid the thickening of the liquor by changing the mode of rotation. Although the frequency of rotation of the basket was relatively low, i.e it was rotated for 10 minutes every six hours, it was sufficient to cause the formation of a stable foam. This was presumably the result of "whipping up" the proteins present in the liquor. In order to avoid this happening in the subsequent fermentations, agitation was

Figure 4.8: Changes in pH & TTA in the liquor during batch fermentation (I)

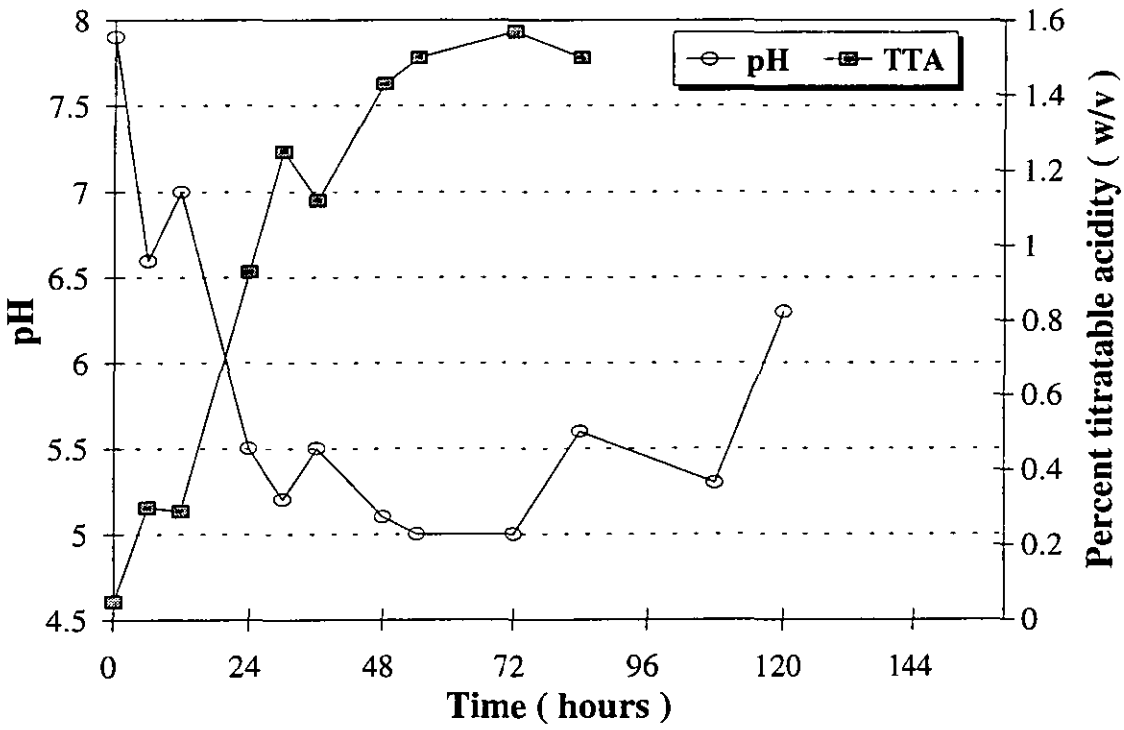


Figure 4.9: Changes in TTA & calcium content in the liquor during fermentation (I)

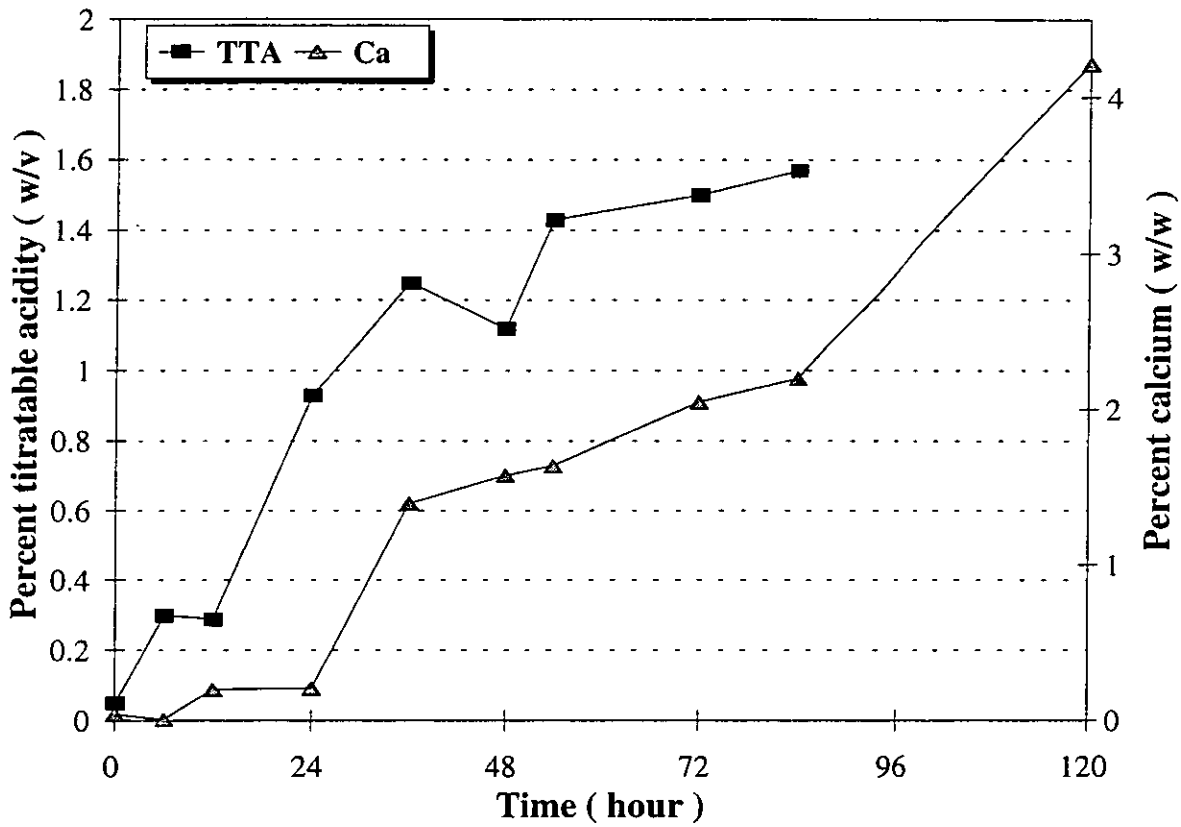
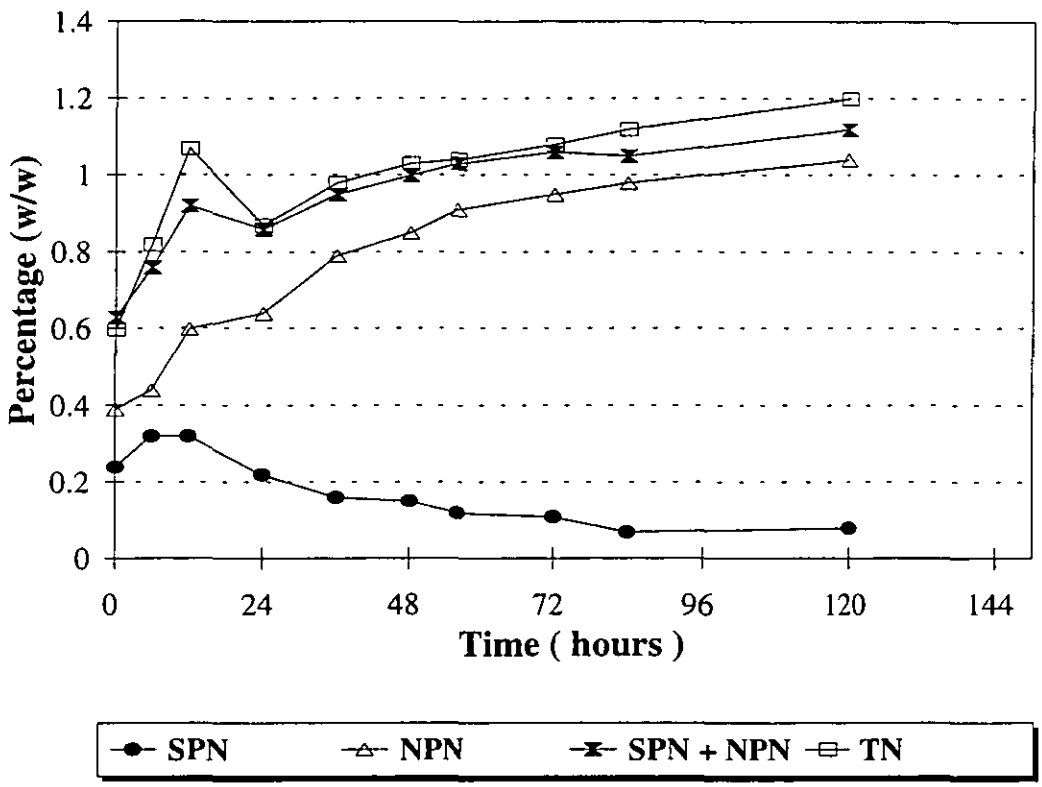


Fig 4.10: Changes in SPN, NPN & TN in the liquor during fermentation (I)



stopped at day three and the fermentation continued under static conditions until the end of the fermentation period. With this arrangement no thickening of the liquor occurred and the insoluble chitin sediment was easily separated from the brownish, viscous liquor.

At the end of the fermentation period, the concentration of glucose in the liquor as determined and the results of all three fermentations are shown in Table 4.14. The results show that the amount of glucose left varied between 11.7 to 7.2 percent which represents a conversion of between 88.3 percent and 92.8 percent respectively.

Table 4.14: Glucose content in the liquor at the end of fermentation.

Fermentation	Duration of fermentation (h)	Conc. of glucose in the liquor (mg/ml)	% glucose left in the liquor*
(I)	120	11.46 ± 0.31	11.7
(II)	132	11.10 ± 0.28	11.3
(III)	84	7.05 ± 0.32	7.2

* % glucose left calculated as = glucose in liquor / glucose at t = 0 (i.e 98.3 mg/ml)

In order to establish a mass balance, analysis of the sediment, liquor and silages was done and the results are shown in Table 4.15. In this case, the whole product of fermentation is designated as "silage", and if the silage is centrifuged, two fractions are obtained i.e. the insoluble chitin solids which is the "sediment" and the liquid fraction which is referred to as "liquor". Proximate analysis on the sediment and the liquor in fermentation (I) revealed that with 27.0 g of protein and 33.1 g calcium left on the sediment, the removal of protein and calcium from the initial waste (114.9 g and 72.7 g) were 76.5 percent and 54.5 percent respectively. The liquor contained 28.13 percent protein and 11.46 percent calcium, indicating a substantial amount of calcium and protein removal from the waste into the liquor.

To complete the mass balance, it was assumed that the weight of silage (A) should equal the sum of the weights of sediment and liquor (B) and as such the ratio of A/B should be one. Table 4.16 shows the overall mass balances for protein, chitin and calcium content during fermentation (I) and slightly higher or lower values of the ratio A/B (1.02, 0.97 and 0.83 in the protein, calcium and chitin content respectively)

were obtained. These differences may be due to the masses lost during dismantling of the bioreactor. Extracting fermented waste sediment which remained in between the basket and the plastic mesh was quite difficult, resulting in small amount of material loss. Overall, there is a 98.3 percent recovery of protein, 102.6 percent of calcium and 121.1 percent of chitin (calculated as masses in B over masses in A from Table 4.16).

Table 4.15: Distributions of calcium, protein and chitin content* in the various fractions at the end of fermentation (I)

Fractions		Protein	Calcium **	Chitin
Silage	(%)	23.19	16.86	10.30
	(g)	64.10	46.7	28.51
Sediment	(%)	17.56	21.65	17.70
	(g)	26.85	33.10	26.98
Liquor	(%)	28.06	11.46	5.80
	(g)	36.20	14.80	7.48
Waste	(%)	32.83	20.77	12.05
	(g)	114.90	72.70	42.20

* = % expressed on a dry weight basis;
** 1.0 percent calcium = 2.5 percent calcium carbonate

Table 4.16: Mass[a] balance for protein, calcium and chitin during fermentation (I)

Components	(A) Silage	(B) Sed + Liq	A/B[b]
Protein	64.1	63.1	1.02
Calcium	46.7	47.9	0.97
Chitin	28.5	34.5	0.83

[a] masses in this table were obtained from Table 4.15.
[b] Since silage = sediment + liquor, theoretically, A/B should equal 1.0.

4.3.1.2 Fermentations (II) and (III).

Similar fermentation patterns were observed during fermentation (II) and (III) (Figures 4.11 - 4.16) as was shown in fermentation (I). However, in both cases, the pH started to rise again after 48 hours (Figure 4.11 and 4.12 respectively). The liquor at the end of fermentation (II) produced a slightly off smell and the final pH was 6.5. Fermentation (III) was stopped as soon as the pH reached approximately pH 6.0.

Figure 4.11: Changes in pH & TTA in the liquor during fermentation (II)

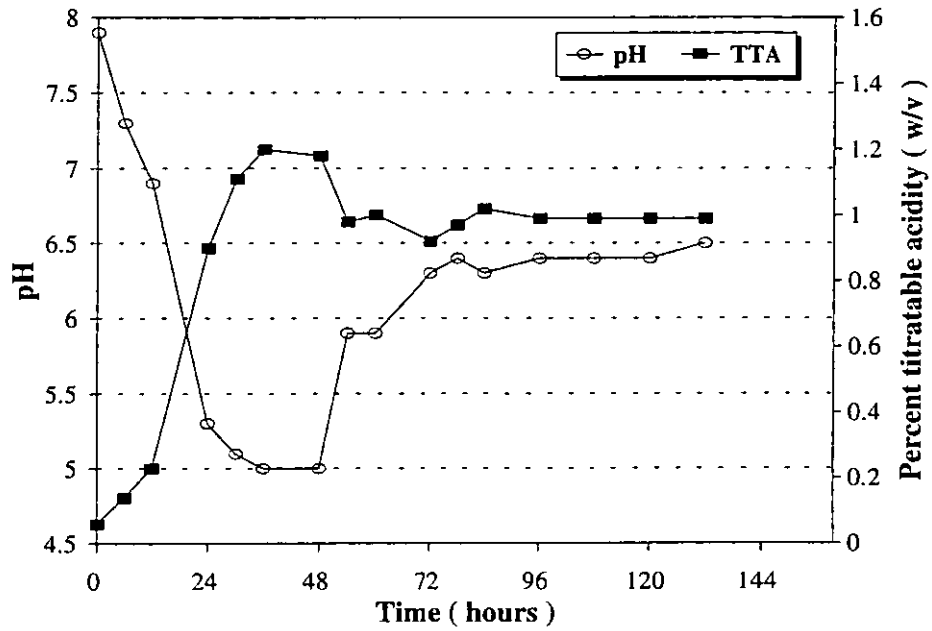


Figure 4.12: Changes in pH & TTA in the liquor during fermentation (III)

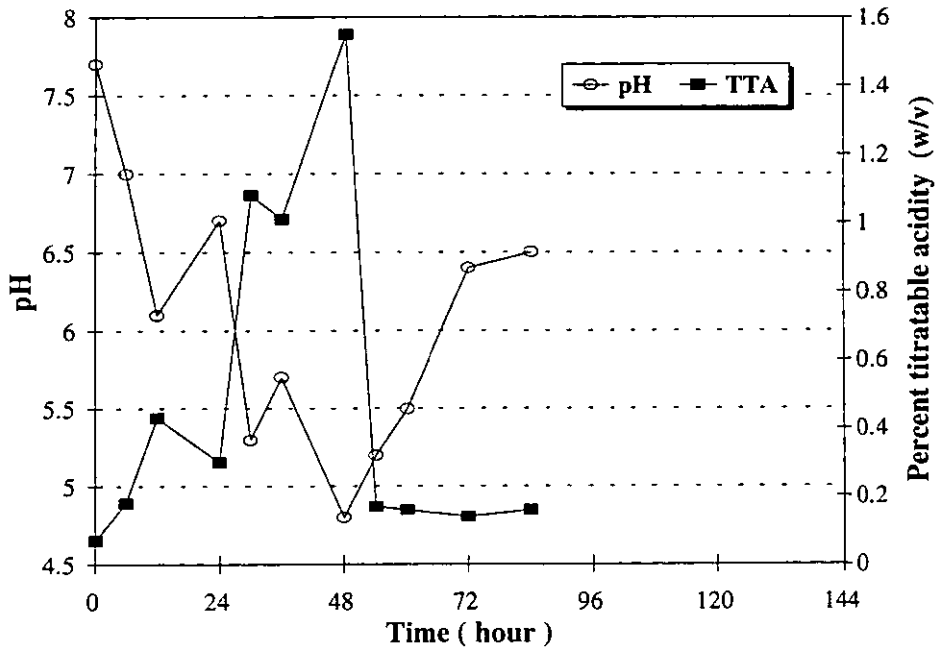


Figure 4.13: Changes in TTA and calcium content in the liquor during fermentation (II)

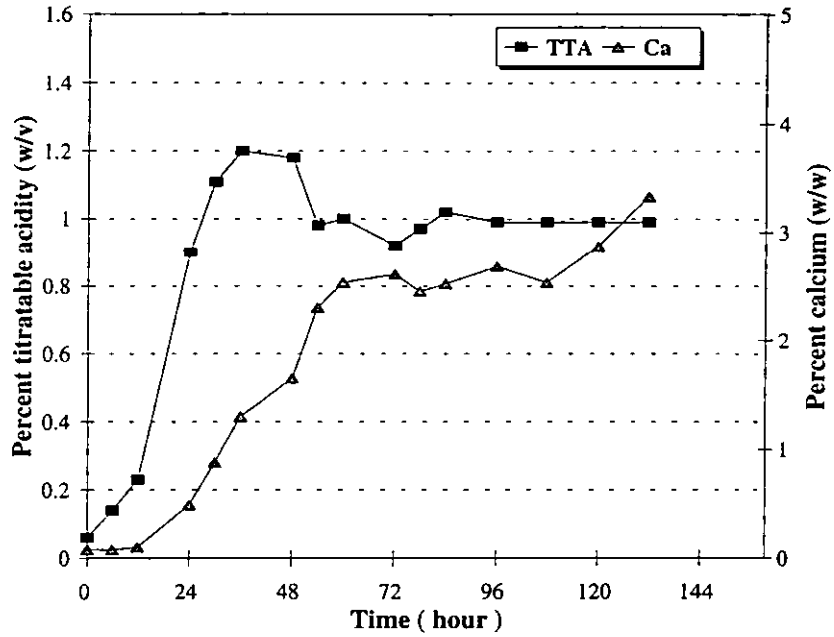


Figure 4.14: Changes in TTA and calcium content in the liquor during fermentation (III)

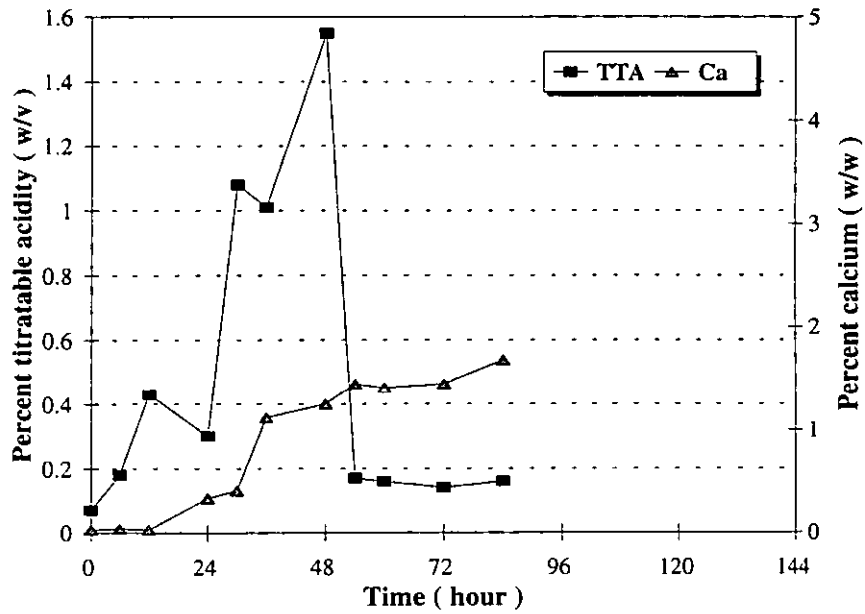


Figure 4.15: Changes in SPN, NPN & TN in the liquor during fermentation (II)

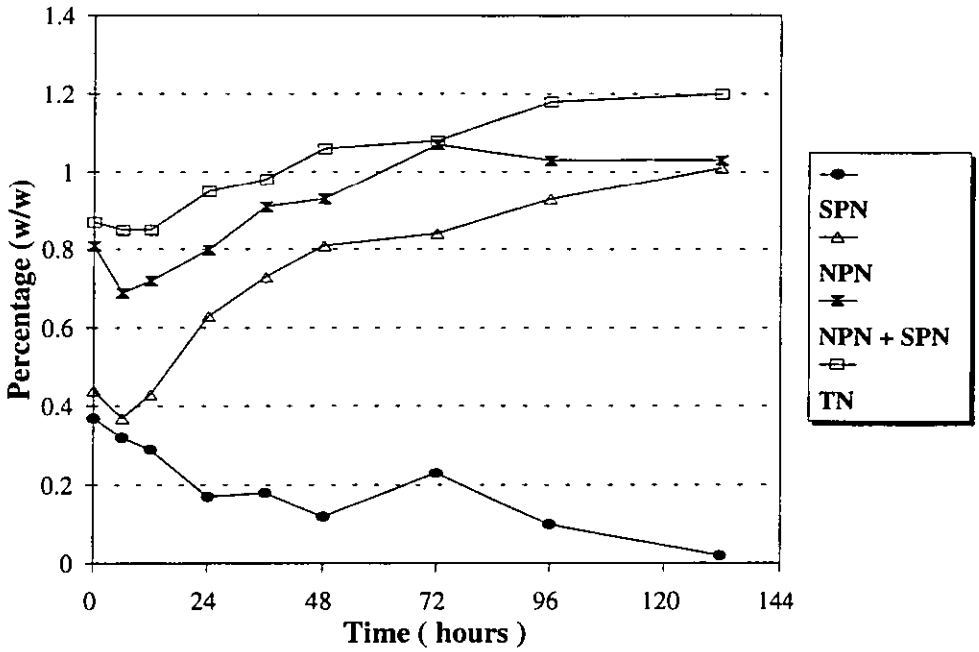
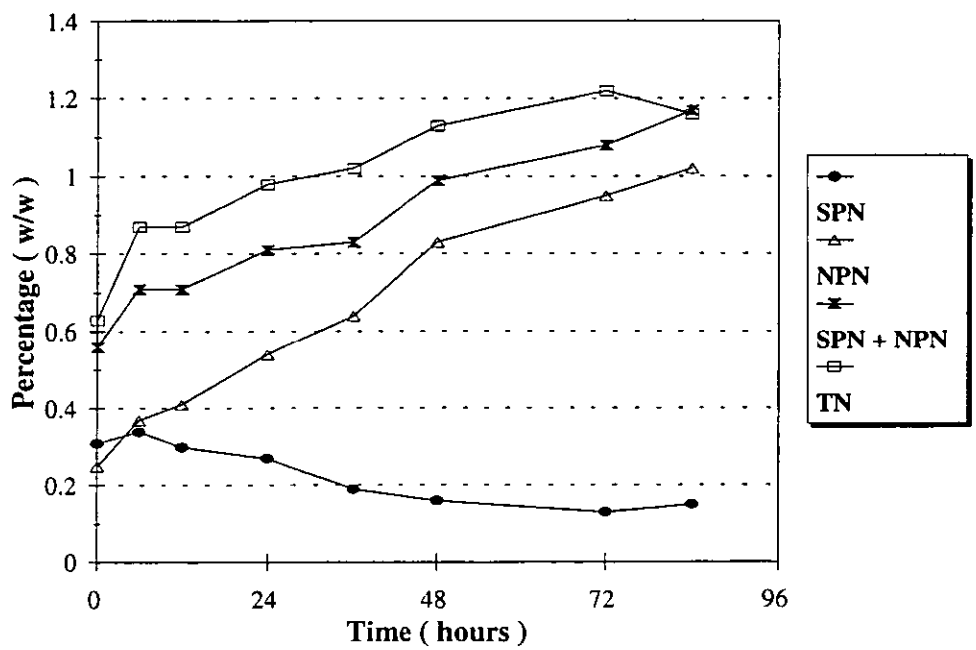


Figure 4.16: Changes in SPN, NPN & TN in the liquor during fermentation (III)



The results of proximate analysis on the fermentation products in (II) and (III) are shown in Table 4.17 and 4.19 and their corresponding mass balances shown in Table 4.18 and 4.20 respectively. Proximate analysis on the sediment and the liquor in fermentation (II) (Table 4.17) revealed that with 25.9 g of protein and 28.4 g calcium left on the sediment, the removal of protein and calcium from the initial waste was 77.5 percent and 61 percent respectively. The liquor contained 44.38 percent protein and 9.2 percent calcium. The recovery of protein, calcium and chitin was 120.6, 111.0 and 92.4 percent respectively (calculated as in (I), and the results from Table 4.18).

Table 4.17: Distributions of Ca, Protein and chitin content* in the various fractions at the end of fermentation (II).

Fractions		Protein	Calcium**	Chitin
Silage	(%)	28.00	12.70	11.82
	(g)	90.10	40.90	38.00
Sediment	(%)	17.63	19.3	17.55
	(g)	25.90	28.40	25.80
Liquor	(%)	44.38	9.20	5.08
	(g)	82.50	17.00	9.40
Waste	(%)	32.83	20.80	12.05
	(g)	114.90	72.80	42.20

* = % expressed on dry weight basis;
** 1.0 percent calcium = 2.5 percent calcium carbonate

Table 4.18: Mass[a] balance for protein, calcium and chitin during fermentation (II).

Components	(A) Silage	(B) Sed + Liq	A/B[b]
Protein	90.1	108.4	0.83
Calcium	40.9	45.4	0.90
Chitin	38.0	35.2	1.08

[a] masses in this table were obtained from Table 4.17.
[b] Since silage = sediment + liquor, theoretically, A/B should equal 1.0.

Proximate analysis on the sediment and the liquor in fermentation (III) (Table 4.19) revealed that with 19.6 g of protein and 20.7 g calcium left on the sediment, the removal of protein and calcium from the initial waste was 80.7 percent and 67.9

percent respectively. The liquor contained 36.69 percent protein and 9.29 percent calcium. The percentage recoveries of protein, calcium and chitin are 112.6, 82.5 and 82.8 respectively (calculated as in (I), results from Table 4.20).

Table 4.19: Distributions of Ca, protein and chitin content* in the various fractions at the end of fermentations (III).

Fractions		Protein	Calcium**	Chitin
Silage	(%)	23.94	15.2	11.01
	(g)	65.7	41.8	30.23
Sediment	(%)	18,00	20.91	18.85
	(g)	17.84	20.7	18.68
Liquor	(%)	36.68	9.29	4.88
	(g)	54.4	13.8	7.23
Waste	(%)	32.83	20.8	12.05
	(g)	101.8	64.5	37.4

* = % expressed on dry weight basis;
** 1.0 percent calcium = 2.5 percent calcium carbonate

Table 4.20: Mass[a] balance for protein, calcium and chitin during fermentation (III)

Components	(A) Silage	(B) Sed + Liq	A/B[b]
Protein	65.7	74.0	0.89
Calcium	41.8	34.5	1.21
Chitin	30.2	25.9	1.17

[a] masses in this table were obtained from Table 4.17.
[b] Since silage = sediment + liquor, theoretically, A/B should equal 1.0.

Overall, the three batch fermentations showed a very similar pattern. The HRB proved to be particularly efficient in separating the insoluble chitin solids from the liquor. The system also made sampling of the liquor straightforward and at the same time allowed the solids to be in frequent contact with the liquor. This made purification of chitin from protein and calcium carbonate possible with a high percentage removal being obtained. The data obtained has also made other information available such as, i) the glucose consumption requirement (see Appendix 4.2) which is further discussed in section 4.3.2. and ii) the agitation regimes which did not result in thickening of the proteinaceous liquor (see Appendix 4.3).

4.3.2 Variation in glucose additions.

The addition of 10 percent glucose during the batch fermentations (section 4.3.1) resulted in a a significant amount of protein and calcium removal from the scampi waste. It was reasoned that improved purification might follow if additional glucose were added during the course of fermentation. According to the theoretical glucose conversion to lactic acid if it is assumed that lactic acid is produced stoichiometrically only from glucose and that the lactic acid subsequently reacts with calcium carbonate, the initial charge of glucose might be the limiting factor in increased purification. Since glucose has been shown to be inhibitory to the growth of lactic acid bacteria (Jay, 1992), it was added in small amounts at various time intervals. In all, three modes (A, B and C) of glucose addition were evaluated.

Mode A: Addition of anhydrous glucose.

In mode A, 50 g glucose was added each at 48, 57, 72 and 80 hours. Figure 4.17 show changes in the pH and TTA over 144 hours. The pH dropped rapidly during the first 48 hours and a low pH (between pH 5.3 to 5.6) was maintained until the end of the fermentation.

After 144 hours, the fermentation was stopped. Surprisingly, the liquor was very viscous and sticky and the sediment had formed into hard balls of various sizes. The balls consisted of chitin sediment and whitish solids, presumably solid calcium lactate. As it was quite difficult to clean the chitin sediments, no analysis was done on the sediments.

Analysis of the liquor samples revealed that following each glucose addition a relatively rapid decrease recurred in the concentration of glucose (Figure 4.18). However, after 80 hours, when the last batch of glucose was added, the glucose concentration reduced slightly, then it remained constant until the end of the fermentation period, indicating that no further utilisation of glucose was taking place. However, the final glucose concentration (approximately 17 mg/ml) was much higher than the initial starting concentration (approximately 7 mg/ml). The apparently inactive period at the end of the fermentation was also reflected in the changes of

Figure 4.17: Changes in pH of the liquor during fermentation (mode A)*

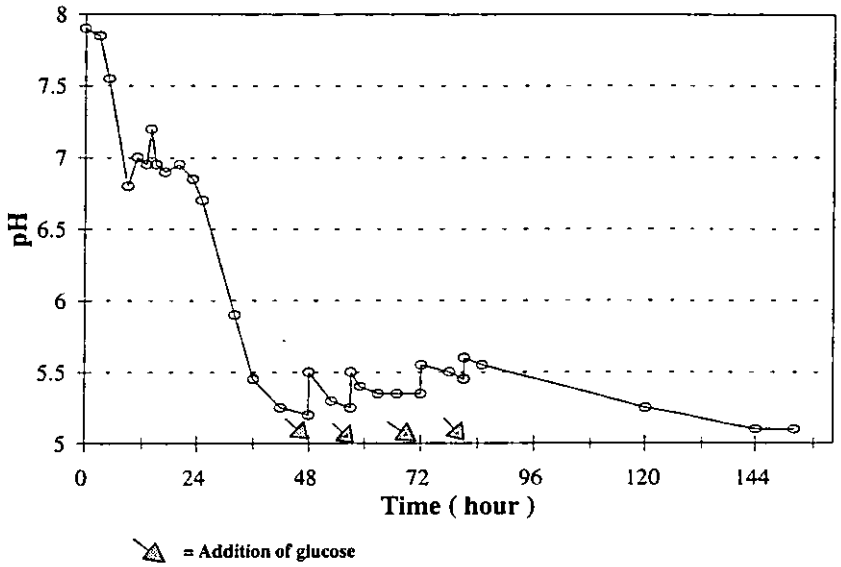
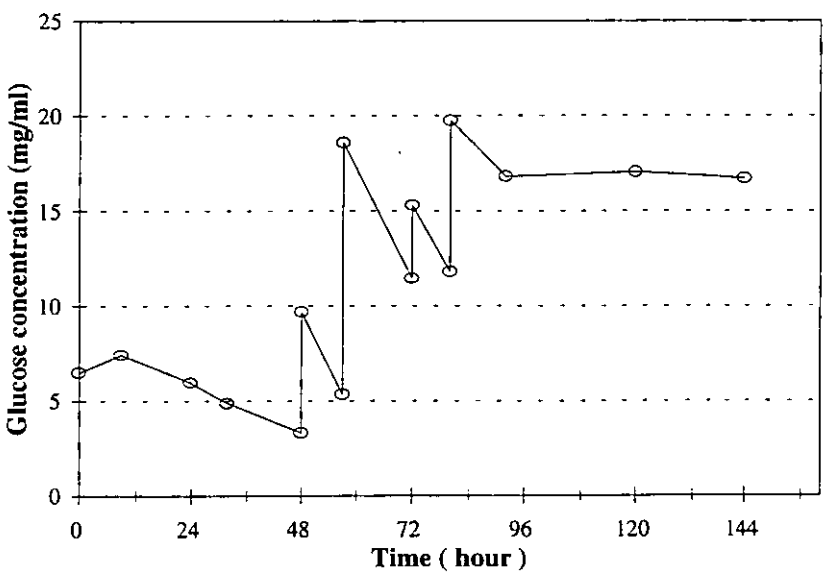


Figure 4.18: Changes in glucose concentration in the liquor during fermentation (mode A)



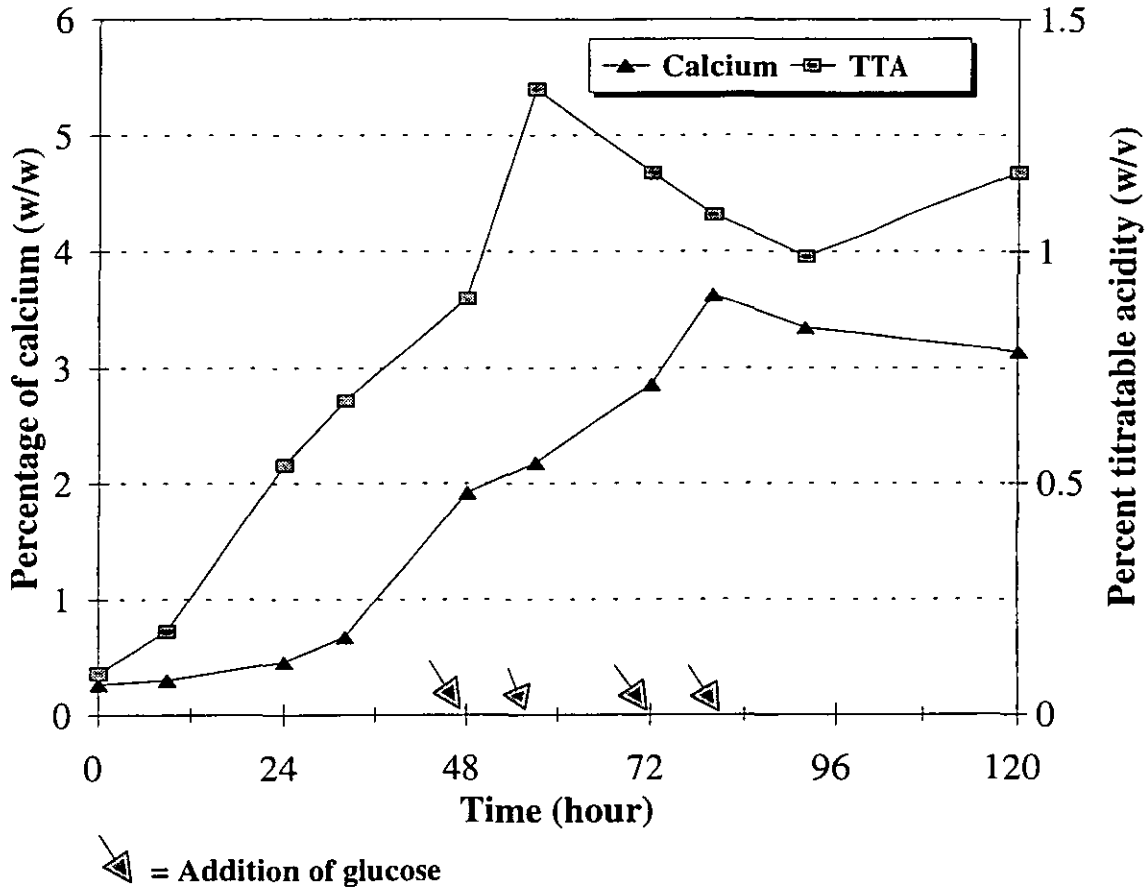
*** Direct addition of anhydrous glucose at the times shown**

calcium concentration of the liquor (Figure 4.19) where the concentration continuously increased until 82 hours then dropped slightly until the end of the fermentation period. This is in contrast to the batch fermentation described in section 4.3.1 where the calcium concentration of the liquor increased until the end of the fermentation. This slight reduction of calcium in the liquor after 82 hours may have been caused by the crystallization of calcium lactate. This substance together with the high glucose concentration in the liquor might have caused the chitin sediment to form balls which was observed during fermentation (I). Therefore to summarise, adding glucose at various times during the fermentation did maintain a low pH but the subsequent formation of putative calcium lactate solids made purification of chitin more difficult to achieve.

Mode B: Addition of glucose solution.

In this fermentation, dilute glucose solution was added rather than solid glucose powder. Glucose solution was added as soon as the pH was observed to rise. On this occasion the chitin sediment was not covered with the whitish substance at the end of fermentation period but instead the whitish substance precipitated out from the liquor and started covering the steel basket after the 120th hour and became thicker towards the end. The fermentation was stopped after 144 hours. The pH dropped each time glucose was added (Figure 4.20) and again the same observation occurred in the calcium content of the liquor whereby the percentage of calcium in the liquor decreased slightly after the 96th hour i.e. when it started to precipitate out (Figure 4.21). The chitin sediment was extracted from the basket and analysed. The analysis of the chitin sediment revealed a high calcium content (27.6 %) and 8.68 percent of protein. Chitin nitrogen was low, at only 2.36 percent, dry weight basis which corresponds to 34.72 percent chitin. Therefore it seems that the strategy of adding glucose during fermentation does result in enhanced removal of calcium from the waste. However, the increase in the calcium content of the liquor resulted in the formation of whitish solids which hindered further purification.

Figure 4.19: Changes in TTA and calcium content during fermentation (mode A)*



* Direct addition of anhydrous glucose at the times shown

Figure 4.20: Changes in pH of the liquor during fermentation (mode B)*

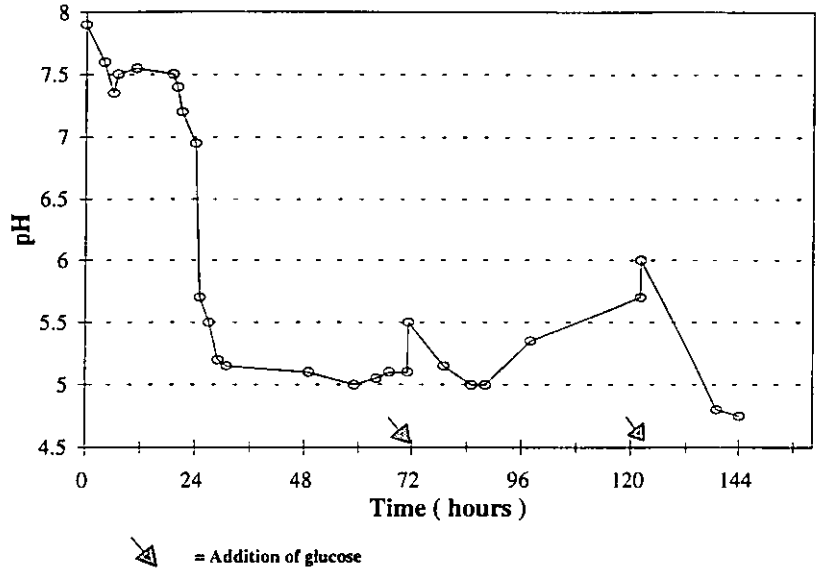
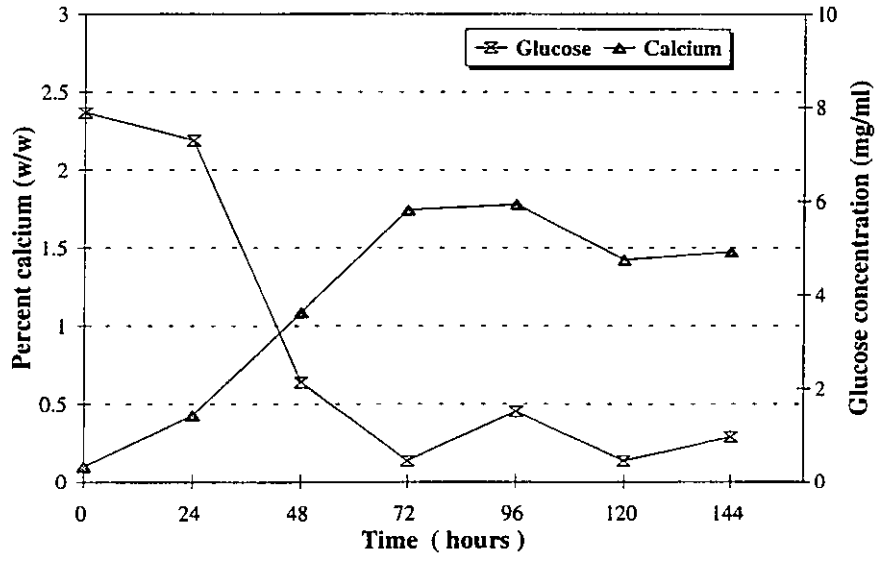


Figure 4.21: Changes in glucose & calcium in the liquor during fermentation (mode B)



*** Addition of glucose solution at the times shown**

Mode C: Substitution of fermentation liquor with glucose solution.

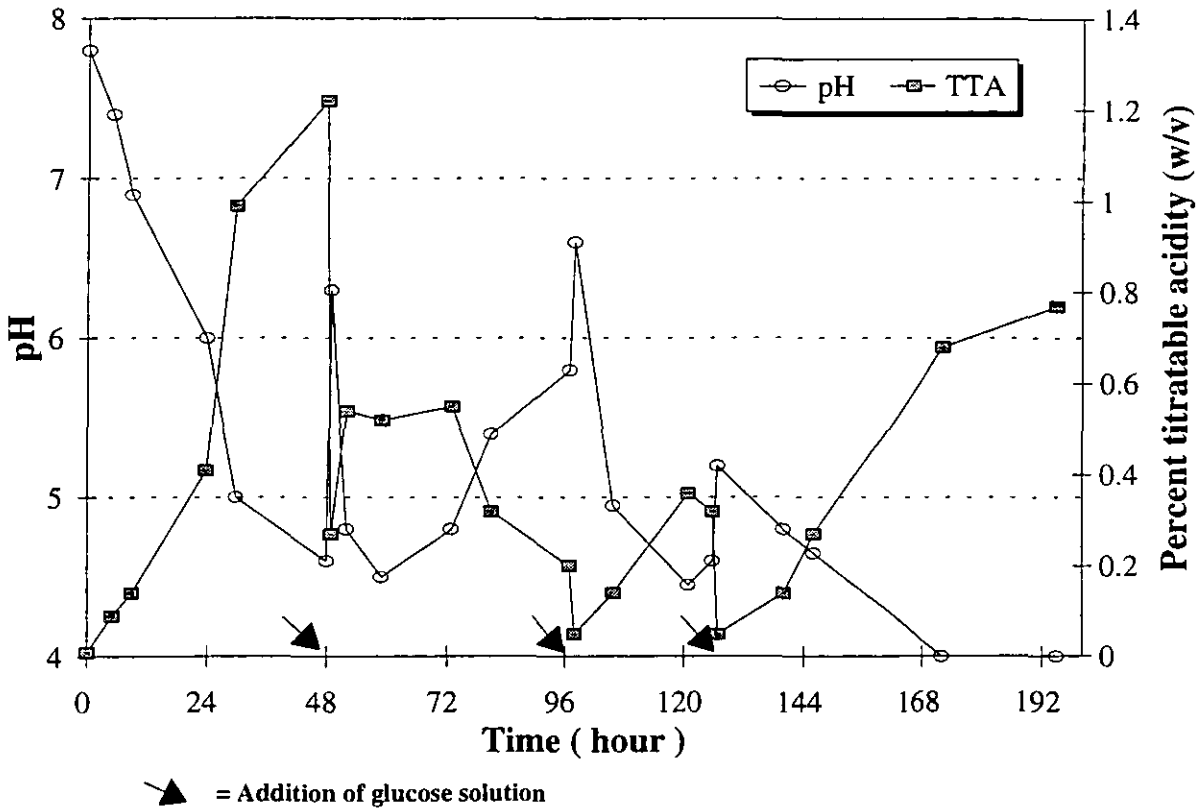
In the first two variations described above, where glucose was added either in solid form or in dilute solution, additional calcium was removed from the waste but the corresponding increase in calcium concentration in the liquor caused difficulties as described above. It was also observed that when the 10 % glucose fermented liquor obtained after 72 hours was stored in the refrigerator, similar white solid material was precipitated.

Therefore in mode C, the liquor produced during the first period of fermentation i.e. up to 48 hours was totally removed from the bioreactor before adding new glucose solution. Two more similar additions were added at approximately 96 hours and 130 hours (Figure 4.22). It was hoped that this arrangement would minimise the precipitation of solids.

During the initial glucose addition at 48 hours, the liquor produced was very dark in colour but the intensity of the colour became reduced as further additions of glucose were made until a pale yellow liquor was finally produced. Changes were also perceived to the odour of the liquor which changed from that of characteristic "fermented protein" smell to that of a "fermented starch" one. Although smells are subjective impressions, what is important is that changes were taking place after each liquor substitution. The changes from a characteristic fermented protein smell to a fermented starch suggested that protein extraction from the waste into the liquor was being reduced progressively during this fermentation and that the final liquor had contained almost negligible amounts of protein. The sediment also became lighter in colour after each fermentation. A small sample taken out from the basket after each fermentation (designated as F1 to F4) enabled analysis to be done on the sediment and the results are shown in Table 4.21.

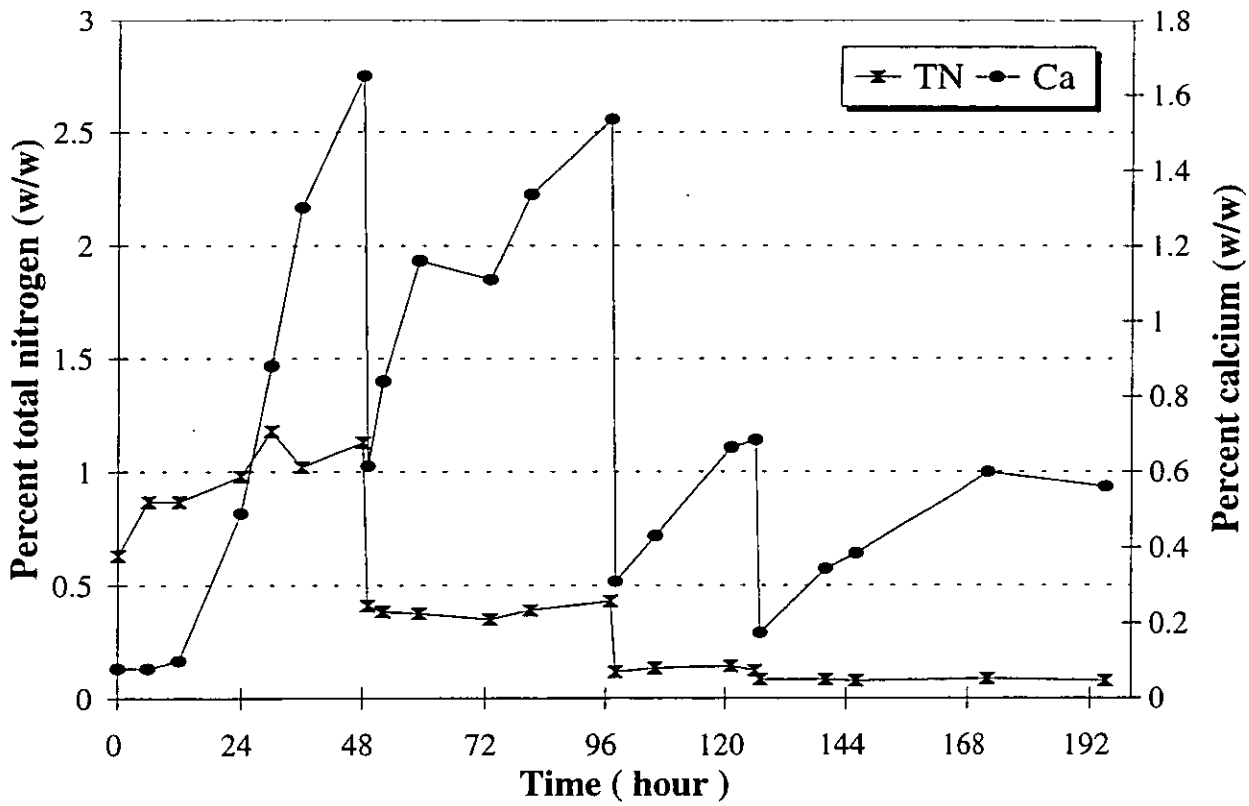
The pH drop and the subsequent changes in TTA during the fermentation are shown in Figure 4.22. A low pH was maintained throughout the fermentation period. Figure 4.23 shows that there was a substantial removal of calcium from the waste into the liquor at each fermentation period continuing until the termination of fermentation.

Figure 4.22: Changes in pH and TTA of the liquor during fed batch fermentation (mode C)*



*** Replacement of fermentation liquor with glucose solution at times shown**

Figure 4.23: Changes in TN and calcium content of the liquor during fed batch fermentation (mode C)



This progressive removal of calcium from the waste is reflected in the progressive reduction of the calcium content in the sediment from 22.51 percent in F1 to 8.25 percent in F4 (Table 4.21). This corresponds to 92.94 percent removal of calcium from the waste in 192 hours (Table 4.22).

However, the total nitrogen content of the liquor decreased after 48 hours (Figure 4.23) and only small amounts (approximately 0.1 percent TN which corresponds to 0.6 percent protein) was detected after 96 hours. This data suggested that protein was mostly removed by 48 hours and that almost complete removal was achieved within 96 hours. It is interesting to note that the sediment at each period (F1-F4) contained a constant protein concentration of 6 to 7 percent (Table 4.21). This result suggests that total removal of protein may be difficult to achieve by fermentation.

The economic implications of making glucose additions to remove additional calcium when compared to the 'base case' of a 72 hour fermentation with a single glucose charge is further evaluated in section 4.4.

The amount of glucose detected in the liquor at the end of each fermentation period was approximately fixed i.e. 8.00 to 12.35 mg/ml or 8.14 to 12.56 percent of glucose left (Table 4.23). This shows that the glucose was being utilised following each addition and that the addition of a 22 percent glucose (in total) resulted in the removal of 92.94 percent calcium from the scampi waste (Table 4.22) in 8 days. The theoretical amount of glucose required to remove all of the calcium carbonate from the waste is about 30 percent (see Appendix 4.2).

Table 4.21: Proximate analysis of sediments (F1-F4)[a] collected at each fermentation period

Percentage (dwb)	F1	F2	F3	F4
Total N	2.91	4.19	4.43	5.34
Chitin N	1.86	3.03	3.29	4.27
Chitin	27.00	43.90	47.70	61.89
Corr. Protein	6.56	7.25	7.13	7.17
Calcium	22.51	15.93	11.17	8.25

Corrected Protein = (TN - CN) x 6.25; (dwb) = dry weight basis

[a] = sediments were taken at 48, 96, 130 and 192 hours, washed and dried before analysis

Table 4.22: Percentage removal of protein and calcium from the waste during fed batch fermentation.

Components	Dry weight	Calcium*		Protein		Chitin	
	(g)	(%)	(g)	(%)	(g)	(%)	(g)
Waste used	318.0	20.80	66.14	32.83	104.40	12.05	38.32
Final Sediment (F4)	56.57	8.25	4.67	7.17	4.06	61.89	35.01
Percentage Removal			92.94		96.11		

* To convert Ca to $CaCO_3$, multiply by 2.5

Table 4.23: Glucose content in the liquor at the end of each fermentation period.

Fermentation period	Conc. of glucose in the liquor (mg/ml)	% glucose left in the liquor*
F1	12.35	12.56
F2	8.00	8.14
F3	9.20	9.36
F4	11.20	11.39

* glucose left calculated as = glucose in liquor / glucose at t = 0 (i.e. 98.3 mg/ml)

4.3.3 Effect of particle size.

In section 4.2, detailed studies on the effect of mincing (using three mincer plates with holes of diameter 3, 6 and 10 mm) on the particle size of temperate and tropical waste were evaluated. The results of these experiments showed that changing the aperture of the holes of the plates did not significantly affect product size distributions. In the work present below a different strategy was adopted. Size reduction was done by cutting and then lightly crushing the waste. Waste material (as received) was cut into either 1/2 or 1/4 inch pieces. This material was either crushed or used directly. In addition fermentations were conducted with both cut and minced scampi waste (refer Table 3.1, section 3.4.3 for details).

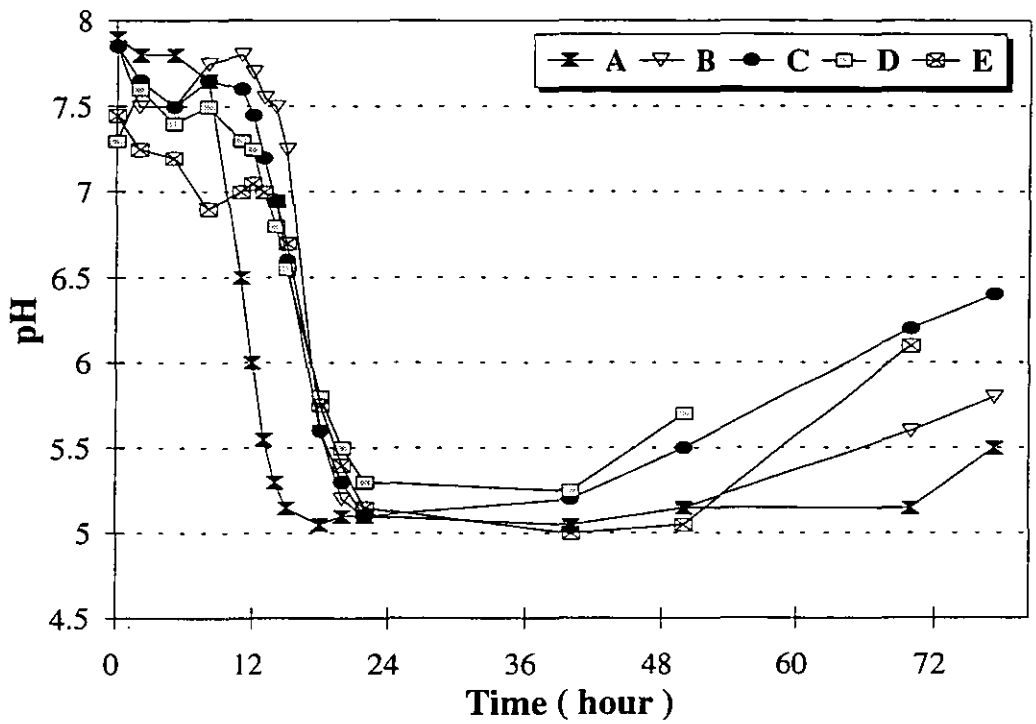
All previously described fermentations were conducted with minced scampi waste and the fermentation was successful resulting in a substantial purification of the chitin. Pretreatment by mincing produces relatively homogeneously small scampi waste particles. However, such pretreatment would normally add to the cost of production and as such it is best avoided if possible.

In all the fermentations, observations were made on the extent to which the sediment in the basket was submerged in the liquor. These observations were made purely by sight and as such are somewhat subjective, nonetheless they provide some useful information. The results obtained were as follows:

(A), all sediment was fully submerged
(B), ~ 1/2 was submerged, most of the bigger pieces stayed out of the liquid.
(C), ~ only 30% was submerged.
(D), ~ a degree of submergence was obtained which was intermediate to B and C
(E), only a very small amount of waste was in contact with the liquor

The odour given off during fermentation (C) was indicative of spoilage whereas all the other fermentations had a fermented protein smell. Figure 4.24, shows that with the 100 % minced waste (A) rapid acidification was achieved within 12 hours whereas the rest of the fermentations lagged behind by approximately 6 hours. Significant differences between the fermentation became manifest after approximately 40 hours. It appears that the lower the content of minced waste, the

Figure 4.24: Effect of particle size of scampi waste on the pH profile of the liquor during fermentation



A = 100 % minced
B = 50 % minced + 50 % crushed (1/2" size)
C = 100 % crushed (1/2" size)
D = 100 % crushed (1/4" size)
E = 100 % whole (1/2" size)

faster the pH started to rise. It was also noticed that an off smell was generated during fermentation (C) which implies that spoilage organisms may have become active as the pH increased. Fermentations (D) and (E) were stopped when it became clear that the pH was rising.

Table 4.24 shows that the volume of liquor produced decreases with increasing particle size. The percentage of protein in the liquor remains comparable for all ferments but the percentage of protein in the sediment increases slightly with increasing particle size. A drastic increase in the protein left on the sediment was observed when whole waste was used in fermentation (E) (Table 4.24). These obervations were also similar in the calcium content of the sediment (Table 4.24).

Table 4.25, clearly shows that the best purification of chitin was achieved when a 100 percent minced waste was used with 93.8 percent and 78.3 percent removal of protein and calcium respectively being achieved. Very little difference in the protein and calcium removal were observed between fermentations (A) - (D) but the results suggest that purification is better when the waste contains higher proportion of minced waste. In fermentation (E) when whole waste was used, the removal of both protein and calcium dropped significantly. Although the pH drop in (E) did reach a value of 5.0, the relatively low amount of liquor being produced and the higher proportion of solids not in contact with the liquor, suggest that good contact between the acidified liquor is a necessity for calcium removal from the waste. These results show that using unminced waste did not affect the protein removal as much as calcium removal.

Table 4.24: Proximate analysis of sediment and liquor

Fractions	Moisture content (%)	Volume (ml)	Wet wt (g)	Dried wt(g)*	Chitin (%)	Protein (%)	Calcium (%)
Waste		-					
(%)	68.20				12.05	32.83	20.80
(g)			1000	318.0	38.32	104.40	66.14
Sediment *		-					
(A) (%)	nd				28.28	7.51	16.50
(g)			377.0	87.0	24.60	6.53	14.36
(B) (%)	nd	-			25.32	10.18	16.93
(g)			415.0	121.0	30.64	12.32	20.49
(C) (%)	nd	-			24.23	11.73	15.05
(g)			510.0	159.2	38.57	18.67	23.96
(D) (%)	nd	-			25.61	9.41	17.34
(g)			525.0	115.0	29.45	10.82	19.94
(E) (%)	nd	-			18.75	18.34	34.60
(g)			585.0	175.0	32.81	32.10	60.55
Liquor							
(A) (%)	79.30				nd	40.40	9.40
(g)		620	625	-			
(B) (%)	79.43				nd	42.48	10.45
(g)		660	670	-			
(C) (%)	78.24				nd	42.92	7.30
(g)		550	560	-			
(D) (%)	76.68				nd	41.44	nd
(g)		506	503	-			
(E) (%)	78.36				nd	41.31	nd
(g)		457	451	-			

* Sediment washed and dried before being analysed

Table 4.25: Percentage removal* of calcium and protein from scampi waste

Fractions	Protein	Calcium
(A)	93.8	78.3
(B)	88.2	69.0
(C)	82.1	63.7
(D)	89.6	69.9
(E)	69.3	8.5

*Percentage removal calculated from the weights in Table 4.24.

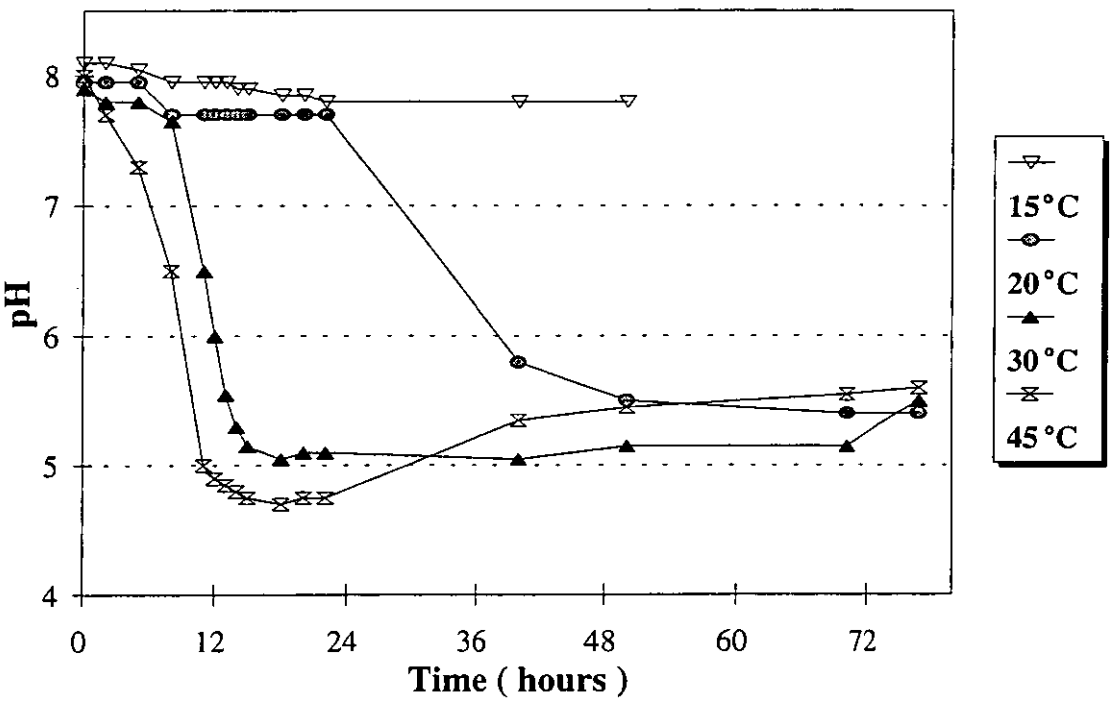
4.3.4. Effect of temperature.

This study was conducted to evaluate the effect of temperature upon the fermentation of scampi waste and on its subsequent purification. Therefore four temperatures were studied i.e. 15°C, 20°C, 30°C and 45°C. Analyses were performed on the solid sediments obtained at the end of the fermentation.

Figure 4.25 shows the pH profile of each fermentation. At 15°C, there was no fermentation activity taking place and the pH remaining high at 7.8 until the 48th hour, after which time the fermentation was stopped. Although the waste was not fermented, neither did it spoil. The low temperature had preserved the waste material but could not initiate growth of the inoculum, hence no production of acid occurred. However, at 20°C, there was a lag phase of 24 hours before the pH suddenly dropped to a value of 5.5. Increases in the rate of pH decline were observed for the fermentations conducted at 30°C and 45°C. The lowest pH achieved was at 5.1 at 45°C.

Table 4.26 shows that there was an increase in the weight of sediment remaining at the end of fermentation as the temperature decreased whereas in Table 4.27, it is seen that the volume of liquor decreases with decreasing temperature. Analysis of the liquors revealed no significant differences with respect to protein content. However, there was a slight increase in the protein content of the sediment fermented at 20°C. For ease of reference the data has been summarised in Table 4.28, which shows the percentage removal of calcium and protein from scampi waste. The percentage

Figure 4.25: Effect of temperature on the fermentation of scampi waste



removal of these two components is greatest (81.9 percent calcium and 96.5 percent of protein) when fermentation was conducted at 45°C and the percentage removal gradually decreased with temperature.

Table 4.26: Proximate analysis[a] of the chitin sediment after 72 hours fermentation

Temperature (°C)		Calcium	Protein	Chitin	Total wet wt (g)	Total dried wt (g)
45	(%)	22.62	6.73	30.26		
	(g)	11.99	3.57	16.04	195.0	53.0
30	(%)	16.50	7.51	28.28		
	(g)	14.36	6.53	24.60	377.0	87.0
20	(%)	21.92	12.87	29.44		
	(g)	19.73	11.58	26.50	440.0	90.0
waste	(%)	20.80	32.83	12.05		
	(g)	66.14	104.40	38.32	1000	318.0

[a] = Results expressed as percentages on dry weight basis

Table 4.27: Proximate analysis of the liquor produced after 72 hours fermentation

Temperature (°C)	Calcium (%)	Protein (%)	Moisture content (%)	Total wet weight (g)	Volume (ml)
45	11.35	36.88	78.03	747.0	726.5
30	9.40	40.40	79.30	625.0	620.0
20	12.94	35.60	77.90	557.5	525.0

(%) on dry wt basis

Table 4.28: Percentage removal of calcium and protein from scampi waste

Temperature	Calcium	Protein
45°C	81.9	96.5
30°C	78.3	93.8
20°C	70.1	88.9

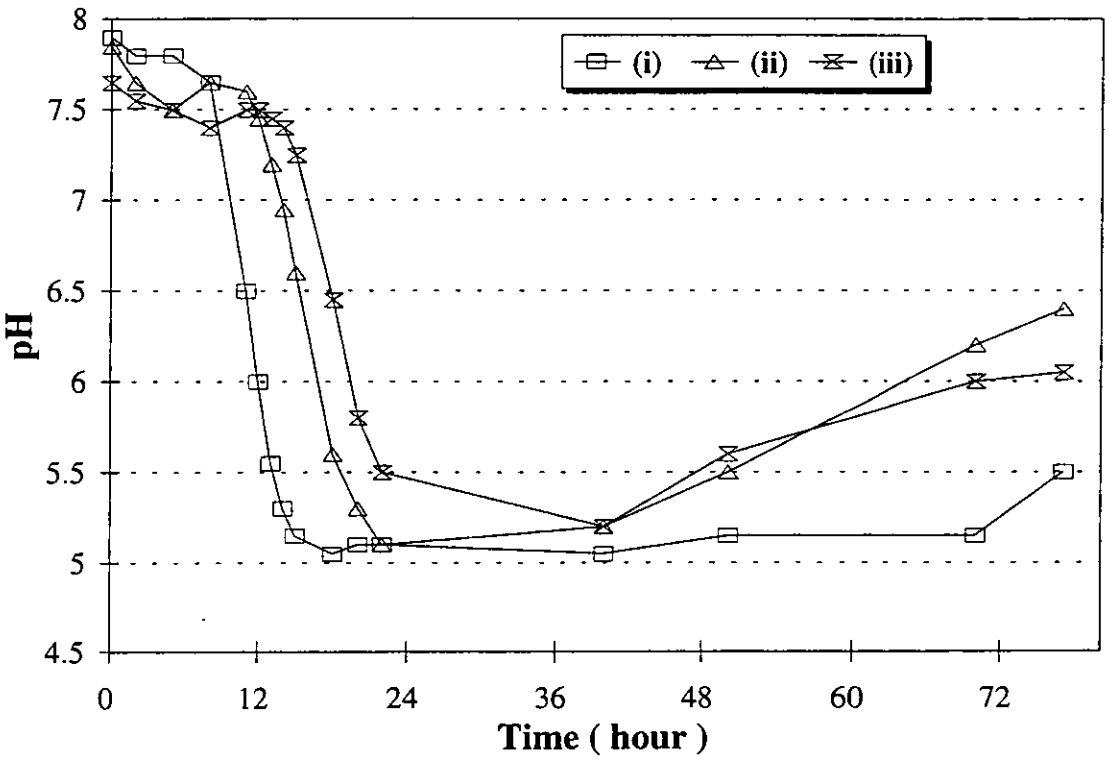
4.3.5 Effect of agitation.

The results from a previous experiment (section 4.3.3), show that when unminced waste was fermented, the volume of the liquor produced was much less than the minced waste. The unminced waste also occupied a much greater volume than the minced waste and thus most of the sediment in the basket was above the liquor level. In this condition, contact between the calcium carbonate of the solids and the acidified liquor can only occur during the relatively short time when the basket was rotated (i.e. 3 turns every 1 hours). If however, this contact time could be increased by increasing the number of rotations, calcium removal could also be increased further. Therefore a series of experiments were conducted to evaluate the effect of agitation on the fermentation of unminced scampi waste. These are designated as:

(i) fermentation with minced waste, at 3 revs per hour.
(ii) fermentation with unminced waste (1/2 inch), at 3 revs per hour.
(iii) fermentations with unminced waste (1/2 inch) with increased rotation rate of 15 revs per15 min for first 36 hours, then at 10 revs per hour until 72 hours.

The result of the pH activity is shown in Figure 4.26. The results show that although there was very little difference in liquor pHs between (ii) and (iii), the pH drop in (iii) lagged behind that obtained in (ii). The lowest concentration of calcium in the liquor was obtained in (iii) (Table 4.30) signifying a relatively lower calcium removal. This result is reflected in the higher amount of calcium left on the chitin sediment (Table 4.29). The protein content of the sediment is also slightly higher in (iii) than in (ii). Calculation of the percentage removal of calcium and protein from the waste (Table 4.31), shows that providing additional agitation did not improve the purification of chitin as expected but actually resulted in a low level of purification. Interestingly, the protein removal was also affected with only 77.9 percent removal from the waste in (iii).

Figure 4.26: Effect of rotation on the pH of the liquor during fermentation of scampi waste



(i) = Minced waste; standard agotation rate
(ii) = Cut waste; standard agitation rate
(iii) = Cut waste; high agitation rate
(See text for fuller description)

Table 4.29: Proximate analysis on the chitin sediment after 72 hour fermentation.

Type of Rotation		Calcium (%)	Protein (%)	Chitin (%)	Moisture content(%)	Total wet wt (g)	Total dried wt(g)
(i)	(%)	16.50	7.51	28.28			
	(g)	14.36	6.53	24.60		377	87.0
(ii)	(%)	15.05	11.73	24.23			
	(g)	23.96	18.67	38.57		510.0	159.2
(iii)	(%)	21.18	14.88	19.98			
	(g)	32.83	23.06	30.97		544.0	155.0
Waste	(%)	20.80	32.83	12.05	68.20		
	(g)	66.14	104.40	38.32		1000	318.0

Table 4.30: Proximate analysis on the liquor produced after 72 hour fermentation

Rotation	Calcium (%)	Protein (%)	Moisture content (%)	Total wet wt (g)	Volume (ml)
(i)	9.40	40.40	79.30	625	620
(ii)	7.30	42.92	78.24	550	560
(iii)	6.80	39.80	75.65	454	440

Table 4.31: Percentage removal of calcium and protein from scampi waste

Rotation	Protein	Calcium
(I)	93.8	78.3
(ii)	82.1	63.7
(iii)	77.9	50.4

4.3.6 Effect of charge size.

The objective of this series of experiments was to determine the treatment capacity of the HRB. A particular characteristic of this bioreactor is the necessity of maintaining good solid liquid contact in order to achieve acceptable purification. Although it was anticipated that increasing the initial charge of scampi waste would result in increased liberation of liquor, it was not known whether the increase would be directly proportional. In previously described experiments, a charge of 1 kg of scampi waste was used and resulted in satisfactory operation. However, the basket has a maximum capacity between 1500 and 2000 g waste depending on the physical condition of the waste. In order to maximise the productivity of batch fermentations, it would be useful to determine the maximum charge of scampi waste which could be treated without adversely affecting product quality. Loadings are designated as L1 (750 g), L2 (1000 g), and L3 (1500 g) and the results of the production of liquor and sediment are shown in Table 4.32.

Table 4.32 shows that, as expected the volume of the liquor and the amount of chitin sediment produced at the end of the 72 hour fermentation increased as the initial amount of waste used increased. No significant difference occurred in the pH profiles (Figure 4.27). A linear relationship with high correlation coefficient (r^2 = 0.9) was obtained when mass of liquor generated was plotted against mass of charge (Figure 4.28). Similarly, a graph of volume of liquor generated versus mass of charge (Figure 4.29) was linear with a correlation coefficient of 0.9968. These results provides a good basis for establishing the relationship between the mass of waste used and the volume of liquor produced. From Figure 4.29, the relationship between the amount of waste used and the volume of liquor produced after 72 hour fermentation is $y = 0.67x - 16.7$.

Table 4.33 shows no difference among the chitin, calcium and protein content of the various fractions. This implies that, over the range studied, the level of purification was maintained.

Table 4.32: Weights of liquor and chitin sediment after 72 hour fermentation.

Loading (g)	Weight of liquor (g)	Liquor volume (ml)	Sediment wet weight (g)	Sediment dry weight (g)
L1	471.0	450.0	166.0	45.5
L2	673.0	650.0	290.0	91.0
L3	1027.0	1000.0	481.0	126.0

Table 4.33: Proximate analysis on the chitin sediment and liquor after 72 hour fermentation.

Product components	Moisture content (%)	Calcium	Protein	Chitin
Scampi waste*	nd	18.45 ± 0.15	26.87	14.48 ± 0.39
Liquor				
(L1)	75.17 ± 0.08	7.77 ± 1.07	37.37 ± 0.16	nd
(L2)	76.20 ± 0.07	7.25 ± 1.24	38.50 ± 0.18	nd
(L3)	75.94 ± 0.01	7.16 ± 0.29	37.65 ± 0.50	nd
Sediment				
(L1)	nd	22.87 ± 0.02	7.06	29.44 ± 0.58
(L2)	nd	23.52 ± 1.56	6.02	29.68 ± 0.81
(L3)	nd	23.89 ± 1.23	6.48	29.21 ± 0.62

* new batch of scampi waste was used, nd = not determined
All percentages of chitin, protein and calcium expressed as dry weight basis

Figure 4.27: Changes in pH of the liquor at different loading capacities

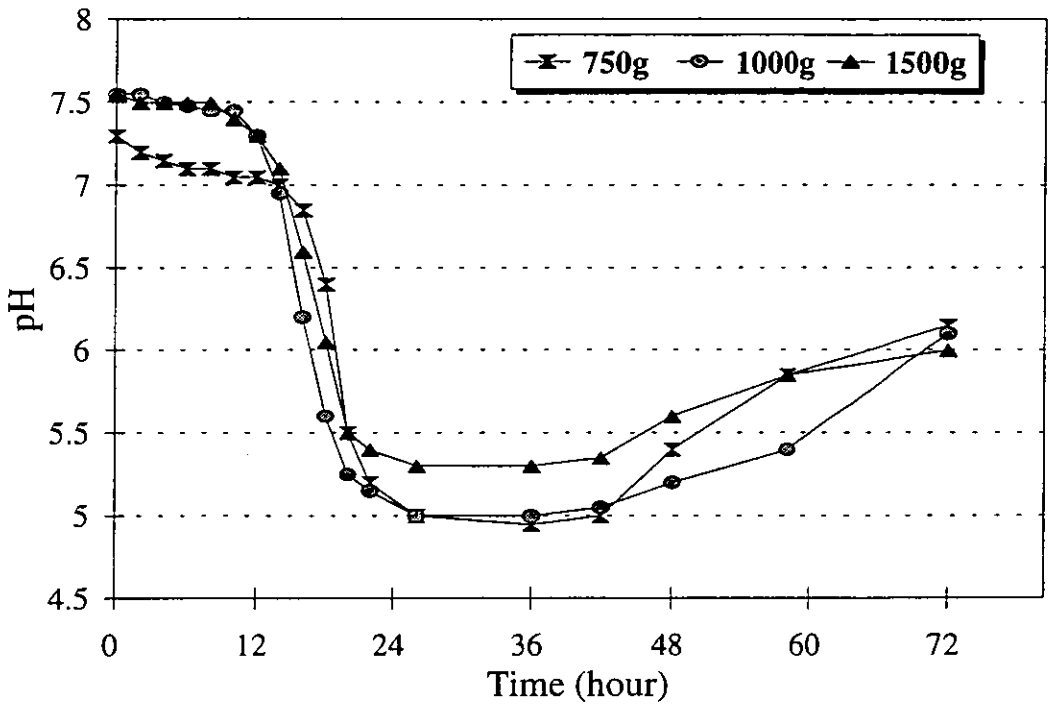
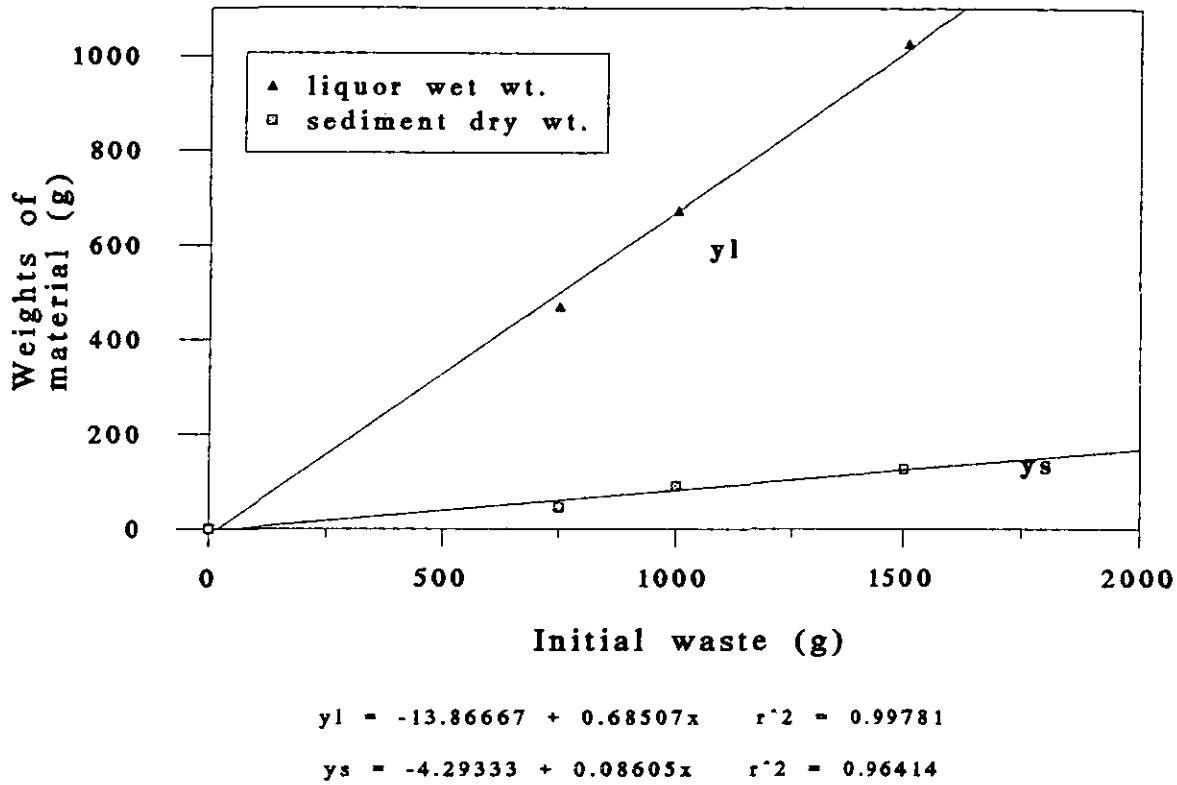


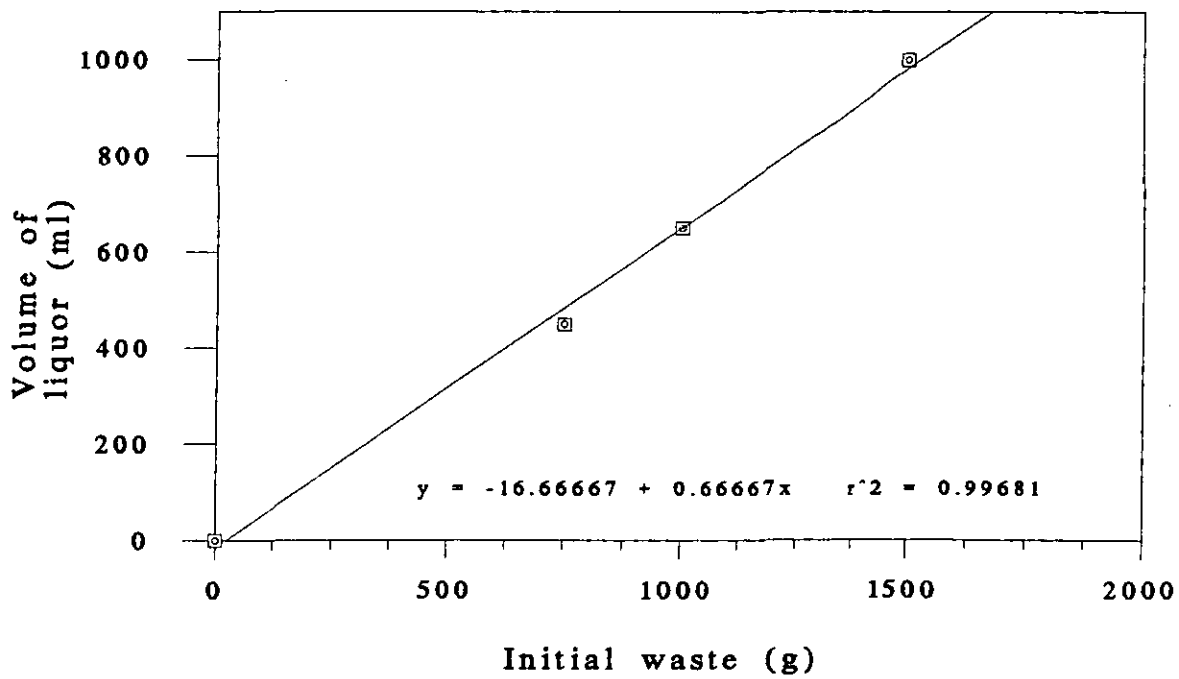
Figure 4.28: Relationship between weights of liquor and sediment produced and the initial waste



yl = -13.86667 + 0.68507x r^2 = 0.99781

ys = -4.29333 + 0.08605x r^2 = 0.96414

Figure 4.29: Relationship between production of liquor and initial waste



4.3.7 Effect of waste type.

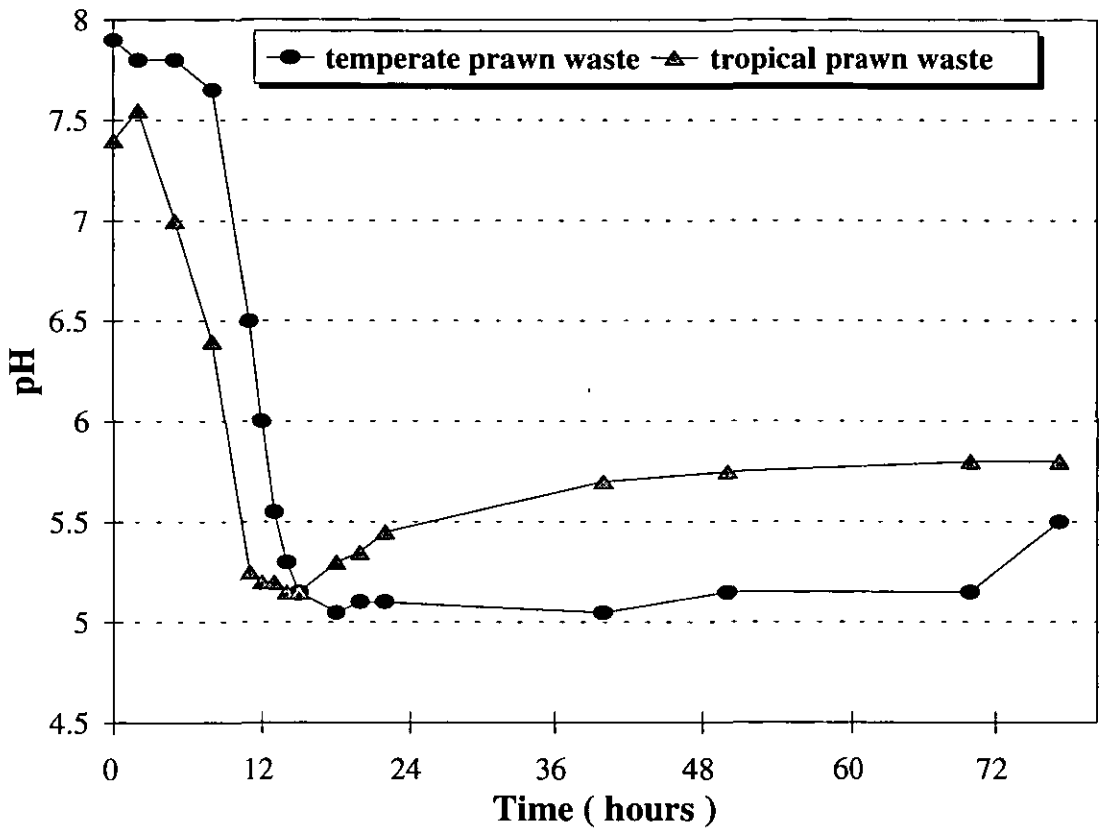
In all the previous experiments, fermentations were conducted using waste from temperate prawn or scampi (*Nephrops norvegicus).* In this section, the fermentation of temperate waste was compared to a waste from the tropical prawn (*Penaeus monodon*). Both waste containing heads and claws, were minced with a mincer plate with 6 mm diameter holes and fermented with A3 strain of *Lactobacillus paracasei.*

The tropical waste was slightly darker in colour compared to the temperate waste which had a pinkish colour and the shells of the tropical species were softer. During fermentation, a very dark brown, viscous liquor was collected from the tropical waste while the liquor from the temperate waste was slightly less viscous and lighter in colour. The tropical waste had a slightly lower initial pH than the temperate waste, however their pH profile were quite similar (Figure 4.30). The volume of liquor produced from the tropical waste (720 ml) was slightly higher than that from the temperate waste (620 ml) (Table 4.34). The protein content of the tropical liquor (Table 4.35) was slightly higher (44.13 percent) than the temperate liquor (40.40 percent).

Table 4.35 shows, the protein content of tropical unfermented waste to be higher than that of unfermented temperate waste. In contrast, the calcium and chitin contents of tropical waste were lower. The compositions of the produced liquor in both fermentations were quite similar and both had a similar fermented smell.

The percentage removal of calcium and protein from temperate waste was 78.3 percent and 93.7 percent respectively (Table 4.36). In the tropical waste there was a 79.9 percent removal of calcium and 89.2 percent removal of protein (Table 4.36). The results show that for a 72 hour fermentation with 10 percent glucose substantial amounts of calcium and protein were removed from the waste. However, in both cases, further treatment may still be necessary to provide chitin of acceptable purity.

Figure 4.30: Changes in pH of the liquor during fermentation of fermentation of temperate and tropical waste



Temperate prawn = ***Nephrops norvegicus***
Tropical prawn = ***Penaeus monodon***

Table 4.34: Weights of the various components of temperate and tropical waste

Components	Temperate		Tropical	
	Dry wt	Wet wt	Dry wt	Wet wt
Initial waste used	318.0	1000.0	271.5	1000
Sediment	87.0	377.0	67.3	248.0
Liquor	129.4	625.0	196.2	752.0

Table 4.35: Proximate analysis[a] on the unfermented waste and sediment and liquor fraction of fermented waste

Components		Temperate Waste			Tropical Waste		
		Calcium [b]	Protein	Chitin	Calcium	Protein	Chitin
Waste	(%)	20.80	32.83	12.05	11.12	45.00	22.18
	(g)	66.14	104.40	38.32	30.19	122.18	60.22
Sediment	(%)	16.50	7.51	28.28	9.01	19.60	37.57
	(g)	14.36	6.53	24.60	6.07	13.20	25.30
Liquor	(%)	9.40	40.40	nd	5.94	44.13	nd

[a] = on dry wt.basis; [b] To convert % Ca to $CaCO_3$, multiply by 2.5,
nd = not determined.

Table 4.36: Percentage removal of calcium and protein from scampi waste

Waste type	Calcium	Protein
Temperate	78.3	93.7
Tropical	79.9	89.2

4.4 Acid & alkali purification of unfermented shellfish waste.

At present, chitin is purified from shellfish waste by chemical means. Chitin is usually treated with acid (usually HCl) and an alkali (usually NaOH) to remove calcium and protein respectively as exemplified in Route (A) below. In this project, the scampi waste underwent an alternative method of purification i.e. through a lactic acid fermentation for 72 hours (Route B), during which time a considerable amount of calcium and protein removal took place, producing a partially purified chitin.

Scampi waste→ mincing → acid / alkali treatment → chitin **(Route A)**

Scampi waste→ mincing → fermentation → acid / alkali treatment → chitin **(Route B)**

Unlike the chemical method, the results so far suggested that the chitin purified by the lactic fermentation still contained small amounts of protein (7 %) and calcium (16.5 %) on a dry weight basis. To obtain a highly purified chitin, the chitin sediment may be further treated with acid and alkali as in Route A. Pure chitin normally contains less than 1.0 percent calcium and 6.89 percent nitrogen (No *et al.*, 1989).

Thus in this section, a study was made to compare treatment by Route A and Route B in terms of acid and alkali consumption. In Section 4.4.1. are described the results of chemical treatments for Route A and these results were compared to the treatments in Route B (section 4.4.2). The chemical treatment of a 72 hour fermented waste of the same source had been carried out elsewhere (Baker and Milnes, 1995) and their results shown in Table 4.38.

In section 4.4.3, another comparison was made between the overall performance of a 72 hour batch fermentation and a fed batch fermentation (192 hour) in terms of overall chitin purification.

4.4.1 Acid and alkali treatment.

When dried unfermented minced waste was added to the acid solution, the mixture became frothy due to rapid production of carbon dioxide gas which lasted between 15 and 20 minutes. Figure 4.31 shows the effect of HCl concentration on the calcium content of the chitin sediment. An almost total calcium removal can only be obtained when a concentration higher than 1.0 M is used. In Figure 4.32, the minimum time taken to remove all the calcium carbonate using a 1.0 M solution was 1 hour. For treatment with NaOH solution, Figure 4.33 shows that the optimum concentration was 1.0 M at a reaction temperature of 65°C under which conditions total nitrogen residual became constant at 6.4 percent. This figure was taken as the percent nitrogen content for purified chitin in this study. This value was a slightly lower than theoretical value of 6.89 percent obtained by No *et al.* (1989). In Figure 4.34, the optimum extraction time to remove protein using the above conditions was found to be 1 hour. The purified product was cream coloured. A summary of the optimum acid and alkali treatments of the unfermented waste is shown in Table 4.37 and that of the fermented waste in Table 4.38. From the results of these two tables, a comparison was made in terms of chemical usage, and is described below.

4.4.2 Comparison between fermented and unfermented waste in terms of acid and alkali utilisation.

(a) Purification of unfermented scampi waste.

If a 100 g (wet weight) of minced scampi waste having a moisture content of 68.20 percent was treated with 1.0 M HCl (36.5 g/mol) @ ratio of 1: 20 (dry wt / v), and 1.0 M NaOH (40 g/mol) @ ratio of 1 : 50 (dry wt / v) (i.e. under optimal conditions, see Table 4.37), then the amount of acid and alkali required to purify the chitin would be:

100g waste = 31.8g dry weight

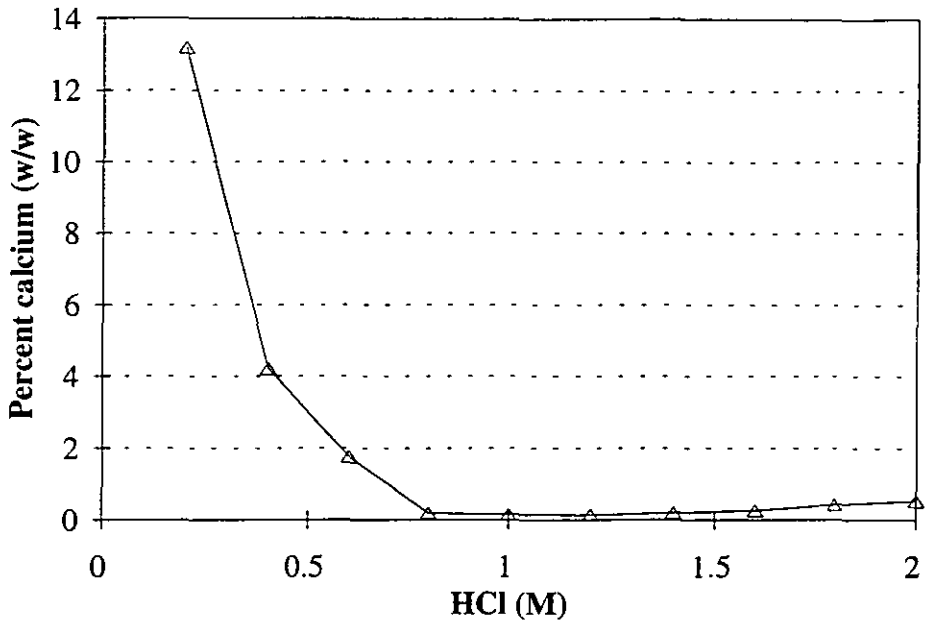
Amount of HCl required = [(20 x 31.8)/1000] x 36.5

= **23.21 g HCl**

Amount of NaOH required = [(50 x 31.8) / 1000] x 40

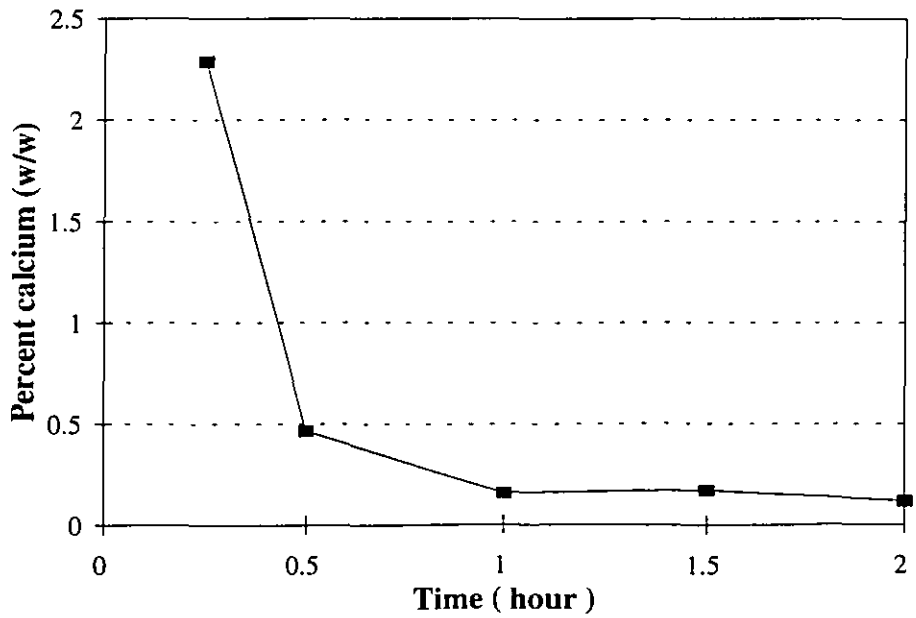
= **63.60g NaOH**

Fig. 4.31: Effect of HCl concentration on the calcium content of chitin*



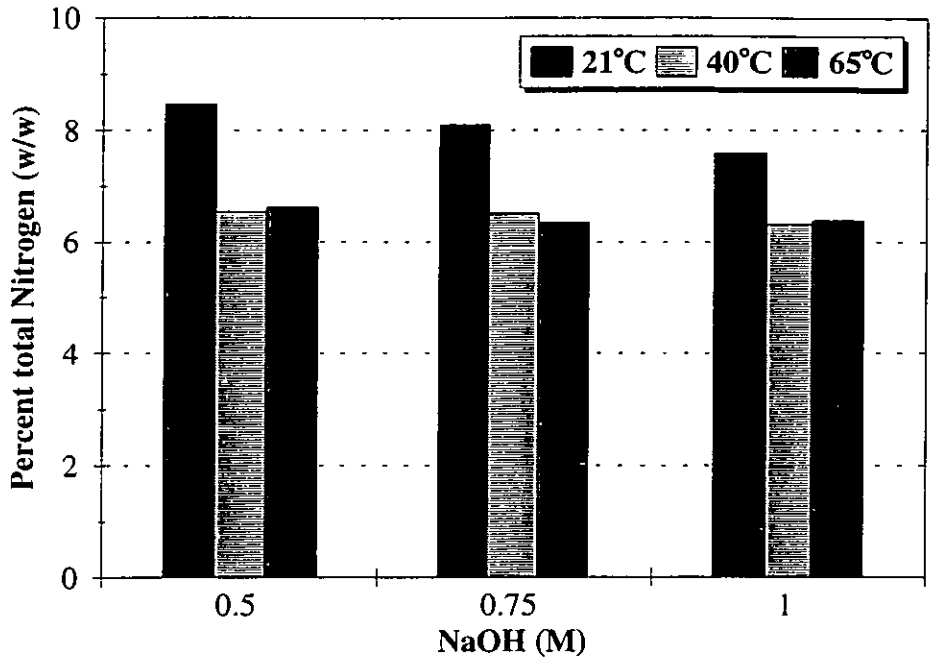
* Reaction time = 1.0 hour

Fig. 4.32: Effect of acid extraction time on calcium content of chitin *



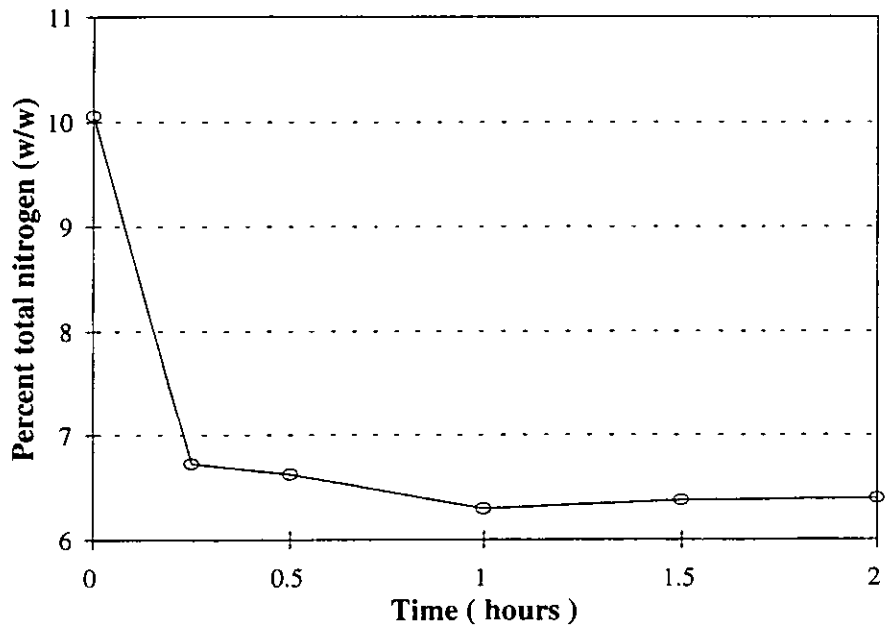
* Concentration of HCl used = 1.0 M

Figure 4.33: Effect of NaOH concentration and temperature on the TN content of chitin *



* Reaction time = 2.0 hours

Figure 4.34: Effect of alkali extraction time on the nitrogen content of chitin *



* Concentration of NaOH = 1.0 M

(b) Purification of fermented waste.

Similarly, fermenting 100 g of minced scampi waste (wet weight) will produce 8.7 g dried sediment (obtain from previous result in section 4.3.3). Applying the optimal conditions for acid treatment for fermented waste (Table 4.38) i.e. 1.2 M HCl @ ratio of 1 :15 (dry wt / v), and 1.0 M NaOH @ ratio of 1 : 15 (dry wt / v), then the amount of acid and alkali required to purify the chitin sediment would be:

Amount of HCl required = [(15 x 8.7) x 1.2] /1000
= 0.1566 mol HCl
= **5.72 g HCl**

Amount of NaOH required = [(15 x 8.7)/1000] x 40
= **5.22 g NaOH**

Table 4.39 shows that when Route (B) was used prior to acid and alkali treatment, a saving of 91.8 percent NaOH and 75.4 percent HCl were obtained. This result is very encouraging especially in terms of environmental preservation. Besides these large savings in chemicals, protein could also be recovered safely and cheaply in the form of a fermented protein liquor.

Table 4.37: Optimum conditions for chemical treatment of **unfermented** scampi waste

Parameters Alkali	Acid	Alkali
Concentration(M)	1.0	1.0
Weight to volume ratio	1 : 20	1 : 50
Extraction time(h)	2.0	1.0
Temperature (°C)	21	65

Table 4.38: Optimum conditions for chemical treatment of **fermented** scampi waste[a]

Parameters Alkali	Acid	Alkali
Concentration(M)	1.2	1.0
Weight to volume ratio	1 : 15	1 : 15
Extraction time(h)	2.0	2.0
Temperature (°C)	21	linear

a = Results were obtained elsewhere (Baker and Milnes, 1995).

Table 4.39: Summary of the amount of chemicals used as presented in the above calculations and the respective savings obtained.

Chemicals	NaOH (g)	HCl (g)
Fermented waste	5.22	5.72
Unfermented waste	63.60	23.21
Savings (%)	**91.79**	**75.36**

4.4.3 Comparison between a batch fermentation (72 hour) and a fed batch fermentation (192 hours).

During a 72 hour batch fermentation, the percentage removals of protein and calcium were 78.29 and 93.74 percent (dry weight basis) respectively, whilst in the fed batch fermentation with additional glucose added, the percentage removals of protein and calcium were 92.94 and 96.11 percent respectively (Table 4.40). This result shows that the effect of adding more glucose improved the calcium removal by 17.82 percent but only increased the protein removal by 3.17 percent.

Table 4.40: Comparison between batch and fed batch fermentations

		Batch (72 h)	Fed Batch (192 h)
Waste used (g)		1000.0	1000.0
Glucose added (g)		100	220
Sediment (%)	Ca	16.5	8.25
	Pr	7.51	7.17
	CH	28.28	61.89
% Removal of protein		93.74	92.94
% Removal of calcium		78.29	96.11

Ca = calcium, Pr = protein, CH = chitin.

CHAPTER 5

DISCUSSION

5.1 Isolation of lactic acid bacteria (LAB) from fermented prawn waste.

5.1.1 Isolation of LAB.

In the past LAB have been isolated from a wide variety of sources such as fermented products, food and dairy products, in the mouth and intestinal tract of humans and animals and in vegetable materials (Sharpe *et al.*, 1966; Sneath, 1986; Axelsson, 1993). The variety of habitats from which they can be isolated suggests that they are highly competitive bacteria. As such their isolation should not be too difficult. Their presence in these various habitats proves to be beneficial to the host or their surroundings. For example, their presence in the intestinal tract provides protection against undesired bacteria (Vandevoorde *et al.*, 1992). Fermented samples normally contain bacterial species which produce large amount of acids which preserve the food and feed products. Since their energy gain is through the metabolism of fermentable carbohydrates (Axelsson, 1993), it is not surprising that fermentations are usually performed by high carbohydrate content food materials such as rice, cereals, milks, vegetables and grass (Platt, 1987).

In the present work, LAB were isolated from minced prawn waste. The prawn waste which constitutes the heads and claws were first fermented with 10 percent glucose to form a silage. From these silages, nine strains of LAB were successfully isolated. The reason for using shellfish waste for isolation of LAB was because bacteria isolated from a particular substrate generally produce satisfactory performance when used to ferment that substrate (Jepperson, 1993).

The fermentation of shellfish waste was successful possibly because of the presence of natural microflora and also the addition of a fermentable carbohydrate (Lindgren and Pleje, 1983). Shellfish like other animals have populations of bacteria associated with them and referred to as "resident flora". In a normal living shellfish there are a variety of types of bacteria present that cause no harm to the shellfish. Once the shellfish is caught and dies, changes occur in the environment in which the bacteria

live which provide conditions conducive to bacterial growth. Refrigeration or freezing of the waste effectively controls the growth of these bacteria which may have originated from the waters from which it was harvested (Jay, 1992). If however, conditions become conducive to growth, such as raising the temperature, these bacteria can increase in number and if allowed to continue, can cause spoilage of the food. It appears that the spoilage organisms first utilise the simpler compounds present in the food and in the process release various volatile "off" odour components such ammonia, hydrogen sulphide, and amines (Jay, 1992). It is the dominance of these bacteria which renders the shellfish waste unsuitable for any form of further utilisation. Shrimp has been reported to have a higher content of free amino acids than fish and to contain cathepsin-like enzymes that rapidly break down proteins (Jay, 1992). The presence of higher quantities of free amino acids in particular and of higher quantities of nitrogenous extractives in crustacean meats in general makes them susceptible to rapid attack by the spoilage flora. When shrimp was allowed to spoil at 0°C for 13 days, *Pseudomonas* spp. were found to be the dominant spoilers (Matches, 1982). Because of the very low sugar content in the shellfish (Table 5.1) it is unlikely that the LAB will dominate.

If however, a substantial amount of glucose is present, then it is expected that the LAB will dominate (provided the number of other bacteria was originally low). When LAB proliferate, they produce lactic acid from glucose. The acid conditions create the right environment for the native enzymes to work, halts the growth of spoilage bacteria (Lindgren and Pleje, 1983; Booth and Kroll, 1989) and helps to break down protein. If isolation is carried out during the course of active fermentation, it is highly likely that a good acid producer will be obtained. Similar proliferation of LAB has been reported from fish and fish/vegetable silages (Woolford, 1985). In this work, the LAB strains were isolated from minced prawn waste fermented with a 10 percent sugar where the production of acid was rapid and immediate as reflected by the pH drop. A low pH of 5.0 was obtained within 48 hours. By comparison, it has been reported that an addition of a 10 percent glucose (w/w) gave the highest level of acid with the lowest pH drop of 4.9 within 48 hours (Hall and Reid, 1995). With no added glucose, the scampi waste was soon spoil and

fermentation will not occur. Therefore it seems that glucose must be present to promote rapid fermentation. This will ensure that LAB will dominate and produce high levels of acid essential to preserve the prawn waste. However, increasing the concentration of glucose may not result in a better fermentation but may actually inhibit the growth of bacteria (Jay, 1992). During fermentation of the various tropical wastes with 10 percent glucose (w/w) at 30°C, the mixture stays fresh pinkish in colour, indicating no spoilage at all. Other types of bacteria may have been fully suppressed at this stage by the low pH (Booth and Kroll, 1989). Furthermore the fermentation was done in a lightly capped bottle, creating a partially anaerobic environment, thus reducing the possibility of aerobic microorganisms being present.

Table 5.1: Fish and shellfish : Approximate percentage chemical compositions (after Jay, 1992)

	Water	Carbohy-drates	Proteins	Fat	Ash
Bony Fish					
Cod	82.6	0	16.5	0.4	1.2
Haddock	80.7	0	18.2	0.1	1.4
Herring	67.2	0	18.3	12.5	2.7
Mackerel	68.1	0	18.7	12.0	1.2
Salmon	63.4	0	17.4	16.5	1.0
Crustaceans					
Crab	80.0	0.6	16.1	1.6	1.7
Lobster	79.2	0.5	16.2	1.9	2.2
Molluscs					
Oysters	80.5	5.6	9.8	2.1	2.0

One tropical sample did not ferment at all and was spoilt and turned black within a short time, thus no bacteria were isolated from this source. As no further tests were done on this sample, the reason behind this failure could not be identified. If the presence of indigenous microorganisms is essential for the fermentation to take place, then it seems possible that the reason for its failure is probably the absence of these natural microorganisms. There have been concerns over the years of large doses of antibiotics being found in cultured prawns especially during the harvesting season.

One of the most commonly used antimicrobial agents in food production is the tetracycline group. Antibiotics have been used extensively in the shrimp aquaculture. However, it is important to withdraw the drugs well in advance of harvesting to enable the animals to clear themselves of any residual antibiotics. In spite of the fact that most countries have strategies to prevent the occurrence of antibiotic residues in their exports, failures of these measures are occasionally reported (Srisomboon and Poomchatra, 1995). In the making of grass silages, where the presence of indigenous LAB is of the utmost important, it has been found that freshly cut herbage does sometimes contain very minimal amount of LAB (less than 10^2 per g material). This relatively low number has been suggested to be the consequence of either antibiotic or antagonistic activity on the part of other components of the microflora (Woolford, 1985).

The nine isolated colonies regarded as LAB showed positive reactions to Gram stain, are catalase negative and grew well in both APT and MRS broth. On MRS agar plate, these isolates were easily recognised by their prominantly small, almost pin-point shape and whitish, typical to that of LAB (Kitchell and Shaw, 1975; Sharpe, 1979, Collins *et al.*, 1989a). Besides the catalase negatives isolates, no other types of bacteria seemed to thrive on the acid medium of MRS agar (Collins *et al.*, 1989a). However, Gram-negative bacillus species were able to grow on a slightly less selective medium of APT agar plate, appearing as big, whitish colony. From these isolates only a few were chosen for identification. It was rather difficult to pick up colonies which were markedly different from each other as revealed by the results obtained.

In conclusion, to ensure successful fermentation as a prerequisite to the isolation of LAB, it is important to obtain fresh waste, as it is less likely to contain high numbers of spoilage bacteria. A sufficient amount of fermentable sugar must be added and the preparation done as quickly as possible, moreover, the optimum fermentation temperature must quickly be attained. It should be appreciated that when doing a lactic acid fermentation using the indigenous microflora, the risk of spoilage is relatively high as spoilage microorganisms may also be present. It is also important

to intermittently mix the fermenting waste so that all the waste comes into contact with acid because pockets of untreated material can putrefy rapidly.

5.1.2 Identification of isolated strains and their potential as starter cultures for the fermentation of scampi waste.

LAB are a large group of bacteria containing ten different genera (Axelsson, 1993; Schleifer and Ludwig, 1995). Each genera has quite prominant biochemical and physiological characteristics which can easily be used to differentiate them such as the production of gas and morphological characteristics. The results of the biochemical tests in section 4.1.2 showed that the isolated colonies were those of homofermentative *Lactobacillus* (i.e they do not produce carbon dioxide) and heterofermentative *Lactobacillus* (producing carbon dioxide). Also identified were those of a tetrad-forming colonies typical to that of *Pediococci* and an unidentified cocci species. It is not surprising to find these genera in the fermented prawn waste as *Pediococci* and *Lactobacillus* are typically dominant in many fermented food products (Hammes and Vogel, 1995; Sharpe, 1979).

From the fermentation profile using the API Kit, strain A3 was identified as *Lactobacillus paracasei*, formerly known as *Lactobacillus casei* (Collins *et al.*, 1989b), B1 as a *Pediococcus* sp. and C1 as a strain of *Lactobacillus plantarum*. B2 was a *Lactobacillus* species. From these species, A3 was finally chosen for use as a starter culture for the fermentation of scampi waste for chitin recovery. A3 had a higher maximum growth temperature (it grew well at 45°C) and could be maintained at a longer period on MRS agar slant at 4°C than B1 and C1. Nevertheless, the other two strains were equally good producers of acid and could warrant further investigation as starters for fermentation purposes though they suffered from the disadvantage of displaying a lag phase of between 6 to 12 hours. This is reflected in the comparison experiments between A3, B1 and B2 and C1. Analysis on the fermented products also showed very little differences among these strains.

The difference in the conversion of glucose during fermentation using strains B1, B2 and A3 were small and no difference was also observed in the calcium content of the chitin sediment in each case (refer Table 4.7). These results suggest that these three

strains were acceptable as potential starters for the fermentation of shellfish waste. Also strain A3 did not show any significant difference when fermented alone or in combination with strain C1.

During the isolation of LAB, the fermentation of prawn waste was initiated by the presence of the indigenous LAB. Once isolated and purified, these LAB can themselves be used as starters for fermentation purposes. Although many commercial starters are available such as Lactostart (*Lactobacillus plantarum*), Pediostart (*Pediococcus pentosaceus*), and Stabisil (combination of *Streptoccus faecium*, *Lactobacillus plantarum* and *Pediococcus acidilactici*), they have been isolated for different purposes, thus may not be suitable for the formation of prawn silage. Indeed, a study conducted by Guerrero-Lagarreta *et al.* (1996) comparing the performance of these commercial starters and the A3 strain isolated in this work shows that some of the commercial vegetable starters are not suitable for the fermentation of prawn waste. They found that the isolated strain, A3 was as good as some of the commercial meat starter cultures. Jepperson (1993) also stresses the importance of using starters which have been isolated from the same conditions in which they will ultimately be used.

5.2 Lactic acid fermentation (LAF) of scampi waste using a horizontal rotating bioreactor (HRB).

5.2.1 Batch fermentations.

The LAF of scampi waste is similar in principle to the ensilation method used to preserve crops and grass for preparation of animal feed (Woolford, 1985). During ensilation, the material is preserved from pathogenic and spoilage organisms by creating acid conditions either by adding acids or the acid can be produced *in situ* by LAB from a carbohydrate source. The LAB may be naturally-occurring in the substrate but best results are achieved with starter cultures. The term "silage" has also been used to describe the product of anaerobically-stored mixtures of animal by-products and plant materials (usually cereals) and the liquid digest resulting from the treatment of fish and fish offal with acid (Woolford, 1985).

As mentioned in the Materials and Method section, the LAF of scampi waste was carried out using a ten percent (v/w) inoculum of *Lactobacillus paracasei* (A3 strain) and ten percent glucose (w/w). Under these conditions fermentations carried out in the HRB was shown to be successful in stabilising the waste and to yield reproducible results. During the process of LAF of scampi waste, the results obtained suggested that a unique combination of synergistic processes had occurred to bring about the purification of chitin. These processes were (a) production of acid from glucose by the bacteria and, (b) autolysis or self digestion of protein. In this section the discussion on the batch fermentations of scampi waste is divided into subheadings.

(a) Production of acid and its role in preserving the waste and removing calcium carbonate.

One important aspect of the lactic acid fermentation (LAF) of scampi waste is the acidification process. As presented in the results, rapid acidification of scampi waste occurred during the first 48 hours (refer Figure 4.8). Similar results of rapid acidification was also obtained by Hall and De Silva (1992) in their study of LAF of tropical prawn waste (*Penaeus monodon*). The production of acid occurred as a result of glucose metabolism by the starter cultures. A conversion of about 85

percent of the initial glucose into lactic acid has been being reported in the literature (Jay, 1992). In the present study, a conversion of between 88.3 and 92.8 percent of glucose were obtained and this result shows that total conversion of glucose is difficult to achieve.

In common with all fermentation processes, the nutritional and environmental requirements of LAB have to be met if successful operation is to be achieved. The nutritional requirements of LAB include fermentable carbohydrates, amino acids, peptides, nucleotides and vitamins (Vandevoorde *et al.*, 1992). Initially the LAB can obtain these requirements from the scampi waste as seafood products has been reported to contain small amount of amino acids and short peptides (Early and Stroud, 1982). These smaller components are easily absorbed by microorganisms and seafood products are notorious for their perishability due to the ready availability of the former (Jay, 1992). However, as these nutrients become depleted during fermentation the LAB will have to utilise the short chain peptides generated as a result of autolysis. The fermentation temperature employed here for the majority of experiments was 30°C, this is well within the temperature range (20°C - 37°C) for growth of spoilage and pathogenic organisms (Hobbs, 1982). As the scampi waste used was not sterile, there will be competition between the natural scampi flora and the starter inoculum for nutrients at the initial stages of the fermentation. However, the addition of a large inoculum (10^8 cfu/g waste) of A3 strain was successful in enabling the LAB to gain a position of dominance over spoilage organisms. Jepperson (1993) also reported similar success by this strategy. Spoilage organisms such as *Pseudomonas* sp. have been reported in spoiled shrimp and prawns (Banwart, 1989). These organisms, which also utilise small peptides and amino acids in the substrate, break down these substrates into foul smelling volatile bases such as ammonia and amines (Early and Stroud, 1982). It is the production of these foul smelling products (which are markedly different from the fermented smell) which is actually responsible for making food products unacceptable for human consumption. Scampi waste must be in a fresh condition otherwise it cannot be used for fermentation as the essential enzymes, vital for the autolytic processes become denatured (Arason, 1994).

The precise mechanisms by which LAB inhibits other organisms is not yet clear (Jay, 1992). However, several potential factors have been identified such as low pH, organic acids (lactic, acetic), hydrogen peroxide, diacetyl, nutrient depletion and bacteriocins (Daeschel, 1989). Bacteriocins are typically plasmid-borne and heat resistant, and inhibit or kill other closely related species or different strains of the same species but do not affect the producing strains; many are peptides (Earnshaw, 1992).

It is well known that most microorganisms grow best at pH values around 7.0 (Jay, 1992), whereas few grow below 4.0. Bacteria tend to be more fastidious in their relationships to pH than are moulds and yeasts, with the pathogenic bacteria being the most fastidious. Lactic and acetic acids produced by certain LAB have anti-microbial activity towards many microorganisms including those that cause food spoilage and food borne diseases (Earnshaw, 1992; Mayra-Makinen and Bigret, 1993). Low pH, dissociation constant (pKa) and the acid concentration determine inhibition by both lactic (pKa = 3.08) and acetic acid (pKa = 4.73) (Adams and Hall, 1988). At a given pH, the acid with the highest pKa value will have the most undissociated acid present, resulting in the strongest antimicrobial activity (Vuyst and Vandamme, 1994b). However, the minimal inhibitory level of acid at different pH is different for different species (Woolford, 1985). Table 5.2 shows the minimum inhibitory concentration of lactic acid and acetic acid against various groups of microorganisms. The antimicrobial effect markedly increases as the pH declines.

Table 5.2: Minimum inhibitory concentrations (mmol/L) of lactic acid and acetic acid against various groups of organisms at different pH values.

Tested microorganisms	Lactic acid		Acetic acid	
	pH		pH	
	5	4	5	4
LAB (heterofermentative strains)	31	4	379	94
Clostridium sp	8	nd	47	nd
Bacillus sp.	6	nd	< 12	nd
Other Gram-positive bacteria	31	nd	47	nd
Other Gram-negative bactetia	16	nd	23	nd
Yeasts	> 250	> 250	281	94
Fungi	>250	> 250	188	94

Adopted from Woolford (1985).

Table 5.2, shows that lactic acid is inhibitory to *Clostridium* at a level of 8 mmol/L at pH 5. From the results of the present study (see Figure 4.11), the highest level of acid detected was about 1.2 percent TTA (w/v) which is equivalent to 132 mmol/L of lactic acid at pH 5.0. It can therefore be assumed that during the fermentation of scampi waste, pathogenic organisms such as *Clostridium* sp. were being effectively inhibited. Therefore if the protein-rich liquor is to be incorporated into animal feed it is vitally important to terminate the fermentation during the stage when the pH is low i.e. after 48 - 72 hours after inoculation. In the food industry, acetic and lactic acid are among the most widely employed preservatives (Jay, 1992). These compounds are employed as sprays at levels of 1 to 3 percent, with 1 percent solutions being used most often (Jay, 1992). Clearly, when the pH rises, the inhibitory effect of organic acids produced by LAB can hardly be expected (Vandevoorde *et al.*, 1992). At pH values higher than 5.6, the possibility that spoilage will occur is increased as will be the growth of pathogens (Booth and Kroll, 1989). Therefore pH monitoring can be used as a good indicator of a successful fermentation as well as providing a safety measure. This further emphasizes the importance of obtaining a rapid lowering of pH in order to ensure good preservation. With reference to the problem of spoilage, it must be stressed that the fermentation needs to be done quickly, as soon as the waste is received, this is even more critical where ambient temperatures are high such as in hotter countries. Although this process does not require sterilisation, every measure must be taken to keep processing equipment and the working surroundings as clean as possible.

It has been noted that adverse pH affects at least two aspects of a respiring microbial cell: namely the functioning of its enzymes and the transport of nutrients into the cell (Ray and Sandine, 1992), with regard to the latter, the bacterial cell tends to have a residual negative charge. Microbial cells are bounded by a membrane which is relatively impermeable to ions, and protons in particular. Non-ionised compounds therefore can enter cells, while ionised ones can not. At neutral or alkaline pHs, organic acids do not enter the cell, whilst at acid pH values, these compounds are non-ionised and are able to enter the negatively charged cells. The accumulation of acid can denature the membrane and transport enzymes leading to growth inhibition (Booth and Kroll, 1989; Jay, 1992). Eklund (1984) predicted that the dissociated

form of a weak acid is approximately 10 - fold less effective as a growth inhibitor. Lipophilic acids such as lactic and acetic acids can penetrate the microbial cell in their undissociated form, and at higher intracellular pH, dissociate to produce hydrogen ions, and interfere with essential metabolic functions such as substrate translocations and oxidative phosphorylation, thus reducing the intracellular pH (Baird-Parker, 1980).

The amount of acid produced is largely dependent upon the microorganism used, the availability of glucose and the buffering capacity of the substrate or the waste material. As in other lactic fermentation processes the main role of the acid is to preserve the substrate. However, in the fermentation of scampi waste, the acid has an added role, that of calcium carbonate removal. As scampi waste contains high amounts of calcium carbonate, any acid produced will react with calcium carbonate forming carbon dioxide and calcium lactate. The removal of calcium carbonate from the waste is reflected in the gradual increase in the calcium content of the liquor samples as the fermentation progresses (see Figure 4.9). This reaction explains the formation of copious amounts of gas seen bubbling through the Dreschel bottle. Whitish crystals formed in the liquor when the latter was cooled in the refrigerator were likely to be calcium lactate. It has also been shown (Appendix 4.2) that in the case of scampi waste, the ten percent glucose used is a limiting factor in the further production of acids.

In the formation of fish silage, the fish is preserved by lactic acid, produced *in situ* by LAB from a carbohydrate source such as malt, cereals, molasses or tapioca (Wyk and Heydenrych, 1985). Under these conditions, the production of lactic acid reduces the pH of the mixture to about 4.5, a pH lower than that achieved with scampi waste, this is because fish waste is not as well buffered as scampi waste which contains calcium carbonate. Another example is the fermentation of fructans in a grass ensilation study using *Lactobacillus paracasei* (Muller and Lier, 1994) which reduced the pH to less than 4.0. This shows that the buffering capacity of the substrate affects the overall pH drop during a fermentation process and determines the lowest pH reached. It has also been shown that the extent of the pH drop will in turn have an impact on the efficacy of the lactic acid as a preservative.

Regarding the bacteriocins produced by certain LAB, Silva *et al.* (1987) found a low molecular mass inhibitory substance produced by a *Lactobacillus casei* strain isolated from human faeces. It was found active against a broad spectrum of both Gram-negative and Gram-positive bacteria, including LAB, but excluding other lactobacilli. It was resistant to various proteases such as proteinase, chymotrypsin, bromelain, trypsin and carboxypeptidases and had a narrow optimal pH range, i.e. pH 3·0 - 5·0. However, no experiments were conducted here to ascertain whether bacteriocin were produced by *Lactobacillus paracasei* (A3 strain).

Although heterofermentative bacteria are capable of producing greater levels of antimicrobial agents such as carbon dioxide, hydrogen peroxide and acetic acid, which is a strong inhibitory acid, maximal conversion of glucose into acids is the more important criterion for effective removal of calcium carbonate.

The production of carbon dioxide gas is also an added advantage to the overall preservation of the waste as carbon dioxide has long been known for its antagonistic properties towards spoilage microorganisms (Jay, 1992). Gram negative bacteria are reported to be more sensitive than Gram positive, with pseudomonads being among the most sensitive and LAB and anaerobes being among the most resistant (Jay, 1992). Carbon dioxide has been used on its own or in combination with other gases in vacuum-packed meat as a preservative (Wood, 1985). Its inhibitory activities are due to the creation of an anaerobic environment by replacing molecular oxygen, its extra- and intracellular pH-decreasing effect and to its destructive effect on cell membranes (Eklund, 1984)

(b) Protein solubilisation (autolysis).

During the course of the scampi waste fermentations, the liquid level in the bioreactor rose as a result of protein solubilisation, forming a protein-rich liquor. Protein solubilisation was evidenced by the increase in the total nitrogen (TN) content of the liquor (see Figure 4.10). Rapid production of the liquor occurred during the first 24 hours then slowed down towards the end of the fermentation period. This demonstrates the typical pattern of nitrogen release during autolysis which is rapid during the first two days then slower thereafter (Lindgren and Pleje,

1983; Beddows, 1985). It was also shown that the sum of soluble protein nitrogen (SPN) and non-protein nitrogen (NPN) was almost equal to the TN content in the liquor but was always slightly lower. These results suggest that autolysis or self-digestion of protein was taking place and that proteolytic enzymes were active in the silage. During autolysis, protein is being broken down into smaller components such as smaller molecular weight soluble proteins and NPN compounds (free amino acids and polypeptides). Similar observations have been reported during ensilation of fish and shrimp products using added acids (Backhoff, 1976; Barratt and Montano, 1986) and also during LAF of tropical shrimp waste (Hall and De Silva, 1992). Several studies have shown that the autolysis occurring during ensilation of fish products was caused by the proteolytic activity derived from the head and viscera and its enzymic nature is shown by the fact that boiled minced fish preserved in acid does not liquefy (Backhoff, 1976; Adams *et al.*, 1987). It was also suggested that mincing promotes the autolytic process by releasing the enzymes responsible for autolysis and brought into contact with the substrate proteins (Arason, 1994). Protein solubilisation process probably occurred as a result of proteases released from the gastrointestinal tract of the scampi and also of cathepsins from muscle tissue both of which are active at acid conditions (DeVillez and Buschlen, 1967; Lindgren and Pleje, 1983). Studies by Jiang *et al.* (1992) on the proteolytic enzyme in grass shrimp (*Penaeus monodon*) revealed the presence of trypsin-like and chymotrypsin-like enzymes. More evidence on the effect of mincing is discussed in section 5.2.3. The slightly lower value of the sum of SPN + NPN compared to the TN was probably due to measurement errors.

Autolysis is the term used to describe a variety of processes controlled by the enzymes present in the flesh and various organs of fishes and marine organisms at death. These processes result in the breakdown of many components of the tissues. Autolysis in certain shellfish such as lobster and shrimp can occur extremely rapidly, the flesh being attacked by gut enzymes within a few hours after death (Early and Stroud, 1982). The enzymes in the muscle and especially in the gut can break down proteins of the flesh to smaller compounds or even to free amino acids. Mincing the fish can increase the rate of enzymic reactions.

In unfermented scampi waste, the protein of the waste originates from two main sources. Firstly, the waste contains large amounts of meat in the claws and heads and secondly the exoskeleton also contains protein. Most protein in the exoskeleton is readily extractable by native proteolytic enzymes in the epidermal cells except for the outermost protein layer in the epicuticle which is tightly bound (see 2.1.1.4), (O'Brien *et al.*, 1993). It is therefore assumed that most of the proteins in the liquor comes from these two sources. Further discussions on this matter are covered in 5.2.2.

(c) Bioreactor.

During their study on the fermentation of scampi waste in a simple unstirred reactor, Hall and Reid (1995) observed that rapid production of gas caused solids to float to the top and if the material was not frequently mixed (manually) spoilage quickly ensued. This problem was not encountered during the present study as the horizontal basket is slightly above the liquor and was rotated intermittently. Moreover, gas generated during fermentation was vented through the Dreschel bottle. Bioreactors of horizontal configuration have been widely used in many solid state fermentations (Kargi and Curme, 1985; Lonsane *et al.*, 1985; Shama and Berwick, 1991). For larger scale work, improvements may be made by providing baffles in the horizontal drum (Kargi and Curme, 1985). One shortcoming relating to the use of drum bioreactors is the formation of aggregated substrates. This is perhaps not unexpected when the substrates are in the form of moist particles. However, in the LAF of scampi waste, the solid state condition was not maintained for long periods as liquid was released during the course of fermentation and aggregation did not occur.

5.2.2 Variation in glucose additions.

Attempts were made to improve calcium removal from scampi waste by operating the HRB in fed batch mode. Three modes of glucose addition were investigated. During the first two modes (A & B) where glucose or glucose solution were added at intervals, the low pH of the liquor was maintained and subsequently resulted in the removal of more calcium carbonate from the waste, resulting in a higher concentration of calcium in the liquor. However, the increase in the calcium content

of the liquor posed a new problem, namely that of the formation of whitish crystals especially towards the end of the fermentation periods (i.e. after the 96th hour). During mode A (direct addition of anhydrous glucose), as the whitish crystal precipitated out of the liquor, it caused aggregation of the chitin sediment into the form of balls. The clumping of the whitish crystals to the chitin sediment may well have been initiated by the stickiness of the high glucose-content liquor. In such conditions, further removal of calcium carbonate from the chitin sediment would be difficult. The precipitation of the crystals caused a slight decrease in calcium content in the liquor after the 96th hour which remained steady thereafter.

However, in mode C where the existing liquor was removed before adding new glucose solution, this problem was not observed and the removal of calcium continued until only a small percentage was left in the chitin sediment. Besides the continued removal of calcium carbonate, following the replacement of liquor by glucose solution, protein solubilisation was also found to be taking place although at a much lesser extent than during the first 48 hours of the fermentation. After the 96th hour, i.e. in between the third and the fourth addition of glucose, the protein content of the liquor was very low showing that almost complete protein removal occurred within 3 to 4 days and that high levels of protein were being removed during the first stage of the fed batch fermentation. This was confirmed by comparing the protein content of the chitin sediments collected at the end of each fermentation period (designated as F1, F2, F3 and F4) which remained similar i.e. six to seven percent protein (see Table 4.21).

One possible explanation for the almost constant protein values is that, since protein removal was enzymic in nature, there may be a limit to the extent of protein solubilisation with indigenous enzymes. The reason for this may be due to the establishment of conditions not conducive for the enzymes or alternatively the residual protein may simply not be completely digested by the indigenous enzymes. The protein in scampi waste originates from two sources, from the meat and organs and the exoskeleton (Skinner *et al.*, 1992). The meat protein can be easily solubilised by indigenous enzymes (Guillou *et al.*, 1995), however the proteins in the exoskeleton vary in their bonding structures (O'Brien *et al.*, 1993). While most of

the proteins in the inner part of the exoskeleton are either soluble in water or can be easily removed by enzymes, the outermost layer, the epicuticle protein is strongly bonded (O'Brien *et al.*, 1993). The epicuticular protein is usually discarded as exuviates during the moulting period (ecdysis) and are not recycled during the premoulting period (Figure 5.1) as are the protein, chitin and calcium in the lower parts of the exoskeleton (O'Brien *et al.*, 1993). It seems possible that the constant six to seven percent protein detected in the chitin sediment during the fed batch fermentation is that of the epicuticle type. Several studies have been done in which chitin was purified from associated proteins by enzymic methods. Takeda and Abe (1962) utilised proteolytic enzymes such as papain and proteinase to remove protein from shell material and resulted in chitin containing 5 percent protein. Roberts (1992) reasoned that the residual protein ranging from 1 - 7 % approximately is inaccessible to the proteinase involved. However, this residual bound protein may not prove problematic as most chitin is currently converted to chitosan as applications for the former are limited. This conversion involves treatment with strong alkali at high temperature and any residual protein will be removed by this process (Coughlin *et al.*, 1990). In this way, the alkaline step to purify the fermented chitin can be omitted.

The production of acid by LAB is not just confined to simple sugars such as glucose. Other cheaper sources could also be used. This includes whey powder and molasses (Wyk and Heydenrych, 1985), cereals (Lindgren and Pleje, 1983) and cassava (Hall and De Silva, 1992). The need to minimise costs in developing countries indicates that the carbohydrate source should be based on indigenous staples such as rice,

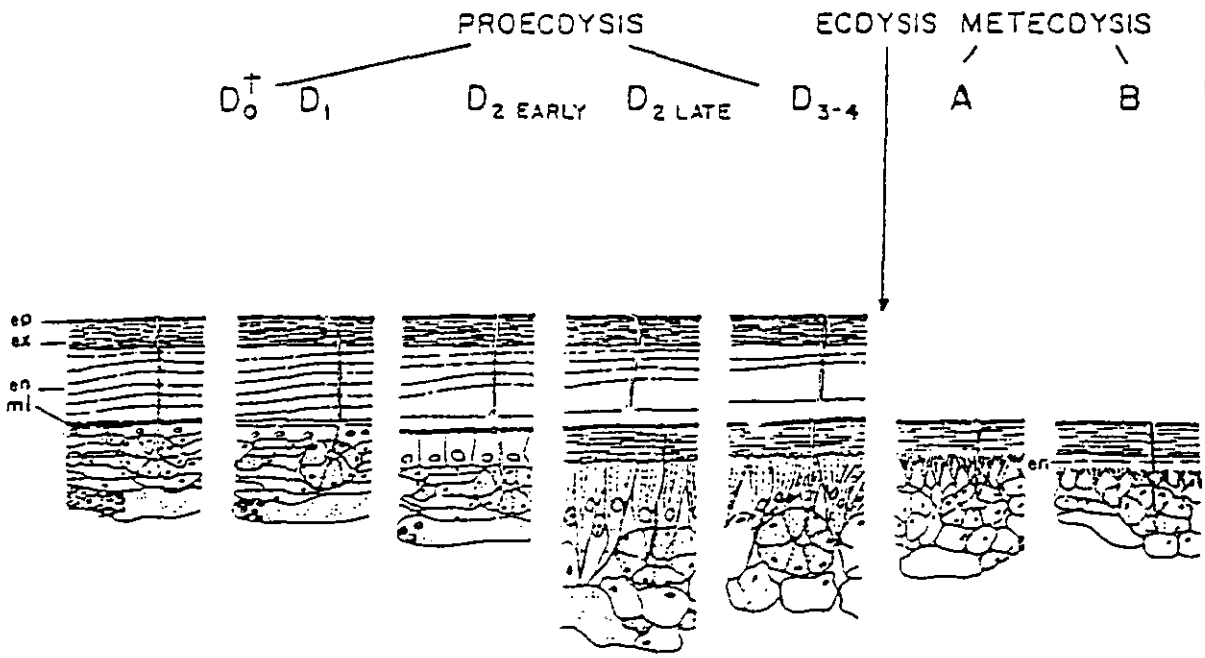


Figure 5.1: Diagram of cross sections of the exoskeleton and underlying integumentary tissues of *G. lateralis* during an intermolt cycle. During proecdysis, inner layers of the old exoskeleton are degraded and new epicuticle and exocuticle are synthesised. At ecdysis, the animal separates from the old exoskeleton. ep, epicuticle; ex, exocuticle; en, endocuticle; ml, membranous layer.

cassava or sago (Twiddy *et al.*, 1987). Many of these studies are related to making fish silages. Wyk and Heydenrych (1985) found that molasses is better than whey powder in terms of rapid acidification, however, molasses on its own did not give adequate preservation of silage for long term storage. The use of 3.8 percent whey powder caused rapid putrefaction. Their results also suggested that a ten percent inclusion (whether a single type of carbohydrate or combinations) was able to provide the required acidity to produce a stabilised product for long term storage. Clearly not only the type of carbohydrate used, but also the total level of carbohydrate source is important in determining successful lactic acid fermentations.

In conclusion, the removal of calcium carbonate from scampi waste is dependent upon generating sufficient acid. However, successful removal of calcium during subsequent fermentations in a fed batch fermentation, can only be obtained if the liquor produced is being removed before replacing new glucose solution. This mode of operation avoided the complications caused by the aggregation of scampi waste brought about by excess glucose and sticky white precipitates of putative calcium lactate during subsequent fermentations.

The discovery of a constant protein content in the chitin sediment during subsequent fermentations during the fed batch fermentations exposes the limits to which protein could be removed by the fed batch approach which would not be apparent during a normal single batch fermentation. The fed batch fermentations succeed in removing more calcium carbonate while the protein solubilisation mostly occurred during the first 48 to 72 hours.

5.2.3 Effect of particle size on the fermentation of scampi waste.

The ultimate objective here was to establish whether the pre-fermentation step of particle size reduction could be omitted. Obviating the need for this step would be attractive, especially when applied at large scales as it would result in reduced costs of production. Previous experiments had shown that the use of minced scampi waste resulted in a stabilised product with reasonably high levels of purification. Moreover, the production of a brown protein-rich liquor was rapid reflecting the rapid breakdown of the proteinaceous materials. Much of the production of fish and

shrimp silages by ensilation employs minced substrates (Backhoff, 1976; Wyk and Heydenrych, 1985; Giullou *et al.*, 1995). All of these workers reported rapid protein solubilisation or autolysis. The process of mincing is thought to promote autolytic processes by rupturing the guts and distributing the hydrolytic enzymes (Arason, 1994). Mincing also produced a homogeneous mixture thus ensuring good preservation by removing any pockets of high pH which can initiate spoilage (Arason, 1994). However, the results of the present experiment reveals some interesting observations regarding LAF of scampi waste in the bioreactor.

In the fermentations (A) - (C), the effect of using respectively: 100 percent minced, a mixture of minced and crushed (1/2 inch size), and 100 percent crushed (1/2 inch size) scampi waste were evaluated. In fermentation (D) the effect of using crushed but with smaller particles (1/4 inch size) than (C) was evaluated whilst (E) shows the effect of using whole scampi waste (1/2 inch size).

The pH profiles of all the above fermentations, revealed very few differences between them. Although, the fastest rate of pH decline was achieved with the 100 percent minced waste, the other four fermentations also showed that acid production was occurring at a relatively high rate, dropping to about pH 5.0 within 24 hours. However, slight differences became apparent from the pH profiles after 48 hours when pHs of the fermentations of crushed and whole waste rose rapidly. The 100 percent crushed waste in (C) produced an off-smelling product at 72 hours. With the 100 percent minced waste, the rise in pH after 48 to 72 hours was related to the neutralising effect of calcium carbonate on the acid, thus reducing the acidity of the liquor. However, the rapid rise in pH with the crushed waste is more compatible with spoilage. Comparisons of the percentage removal of calcium carbonate and protein in each case, revealed that mincing the waste did not affect the protein solubilisation as much as it did the removal of calcium. This effect was even more prominent when whole waste was used (E). Therefore in (C) where a 100 percent crushed waste was used, the calcium removal was not as efficient as when 100 percent minced waste (A) was used, yet the rise in pH was faster than (A). It is therefore reasonable to suggest that besides calcium neutralisation of the acid, spoilage processes may have also contributed to the rapid rise in pH in crushed waste

as these processes normally produce basic compounds which can increase the pH. At 72 hours, the pH of 100 percent minced waste was slightly above pH 5.0 but the 100 percent crushed waste had already attained a pH of about 6.3. It seems likely that the crushed waste which was not in contact with the acidified liquor contained pockets of high pH which could initiate spoilage. Since rotation rates were low, contact between these bigger particles and the liquor was infrequent. A pH higher than 5.6 was also previously reported to greatly increased the risk of spoilage (Jay, 1992).

To summarise, at this scale of operation mincing did produce the highest level of waste purification. However, at larger scales of operation mincing might not be required. The results obtained here suggest that it is important to ensure full or substantial submergence of the waste in the liquor. At higher scales of operation this will be dependent on drum diameter and loading of the drum, despite the increase in the volume of liquor as the particle size decrease. However, studies need to be conducted at intermediate scales of operation.

5.2.4 Effect of rotation rate.

In the foregoing it was concluded that minced waste resulted in a higher level of purification of chitin whilst the use of unminced waste resulted in a less well purified product. The bulkiness of the unminced waste resulted in a situation where most of the scampi waste remained above the liquor level in the basket. Without proper contact between the acidified liquor and the waste material, calcium solubilisation can not take place. One way to increase contact is to increase the rotation rates of the bioreactor. A relatively intensive mixing regime was evaluated as it was reasoned that small variations from what had become the accepted regime would not yield significantly different results.

The application of more intensive agitation resulted in the disruption of the growth of bacteria. This effect can be seen from the increased lag phase and a lower pH drop. The pH also increased faster after the lowest pH had been reached. This effect was even more noticeable in the degree of purification of chitin. Both processes (autolysis and calcium solubilisation) were adversely affected.

The reason for the impaired bacterial activities when rotated at higher rates was probably due to the disruption of the microaerophilic growth environment. Kandler and Weiss (1986) reported that although most strains of lactobacilli are fairly aerotolerant, optimal growth is achieved under microaerophilic or anaerobic conditions. They also reported that increased carbon dioxide concentration (~ 5 %) may stimulate growth. The increased tumbling nature of the rotation of the basket might have resulted in a more efficient stripping out of carbon dioxide from the system which would reduced the growth rate of the bacteria. Reduction in growth of the bacteria will affect the production of acid and subsequent calcium carbonate removal.

Similar oxygen toxicity is encountered in the dairy industry (Mayra-Makinen and Bigret, 1993) where in order to maintain constant pH a continuous agitation is applied which induces aeration. Oxygen can also cause the production of hydrogen peroxide by some starter strains, which can be autoinhibitory (Mayra-Makinen and Bigret, 1993).

5.2.5 Effect of temperature.

Temperature is one of the most important environmental factors influencing the growth and activities of microorganisms. During a lactic acid fermentation, beside the availability of a fermentable carbohydrate, the population levels and activity of the lactic acid bacteria are of importance for rapid acid production (Deschamps, 1993). Whilst very low temperatures will inhibit or reduce microbial growth, high temperatures may kill the cells.

During the lactic acid fermentation of scampi waste, there are two main aspects of fermentation that are affected by temperatures, i.e. the growth of bacteria and the activity of the autolytic enzymes in the waste. The results obtained here showed that virtually no activity was observed when attempts were made to operate at 15°C. Increasing the temperature to 20°C resulted in some activity with the pH decreasing after 24 hours. Fermentation at 30°C and at 45°C proceeded vigorously indicating good growth of the bacteria and a concomitant rapid decrease in pH. Regarding the removal of calcium and protein from the waste, increasing the temperature from 20 to

30°C resulted in increased levels of purification. Whilst further improvements were obtained by increasing the fermentation temperature to 45°C, these were not as great as those obtained by increasing the temperature from 20 to 30°C. Since the emphasis of this work was on developing a process with low investment costs, fermentations of shellfish waste conducted in the tropical regions would seem attractive as high ambient temperature will eliminate the need for heating. Furthermore the protein-rich liquor produced during the fermentation could be incorporated into animal feed, and could be easily sun-dried. Wood (1985) noted that many of the food and feed fermentations are conducted at temperatures between 28°C to 30°C and Beddows (1985) reported that the optimum temperatures for visceral enzymes in fish are in the range 35 to 45°C. With the A3 strain used in these experiments, fermentation at 20°C seems to be very slow with longer lag period. It may require a slightly longer fermentation period if a similar effect is to be obtained as at 30 or 45°C.

In general, microorganisms are usually divided into three arbitary classes: psychrophilic (low temperature), mesophilic (medium temperature), and thermophilic (high temperature). The temperature ranges for the various types are listed in Table 5.3. Insufficient data was obtained on *Lactobacillus paracasei* (strain A3) to enable it to be categorised in these terms with certainty. However, it can be regarded more as mesophilic in nature as it did not grow at 10°C but grew well at 45°C but not at 50°C in MRS medium. However, since the A3 strain is relatively insensitive to the effects of temperatures, no adverse effects would follow if the temperature were to increase as high as 40°C.

Table 5.3: Approximate temperature ranges of growth for arbitary classes of microorganisms (after Banwart, 1989).

	Temperature (°C)		
	Minimum	Optimum	Maximum
Psychrophilic	-15 - 5	10 - 30	20 - 40
Mesophilic	5 - 25	25 - 40	40 - 50
Thermophilic	35 - 45	45 - 65	60 - 90

5.2.6. Effect of loading

The result of this experiment has very important implications for large scale fermentations. It is obviously desirable to maximise loading of the bioreactor as long as purification of the waste is not compromised. LAF of scampi waste is a type of solid state fermentations and the starter used is microaerophilic in nature (Axelsson, 1993). As such it does not require vigorous agitation or oxygen rich-atmosphere. Furthermore in a solid state conditions, large volumes of water was not necessary.

Investigations conducted into the effects of increasing the loading of the bioreactor from 750 g to 1500 g proved encouraging. The increase in loading did not adversely affect the quality of the end product obtained. Moreover, useful additional data was obtained showing the relationship between mass of waste treated and volume of liquor generated (see Figures 4.28 & 4.29). What has been experienced using the present bioreactor is that it requires a certain amount of liquid in order to keep the chitin sediment and the liquor in contact. Such contact was important for preservation as well as calcium removal.

5.2.7 Effect of waste type.

The objective of this experiment was to evaluate the feasibility of applying the fermentation method using the HRB and the isolated strain, A3 to other waste type, in particular a tropical species. Hall and De Silva (1992) have shown some encouraging results using commercial starters and the waste of a tropical species (*Penaeus monodon*). Currently, the production of the seafood industry in particular prawns and shrimp had risen tremendously in the Asian countries (Ferdouse, 1996). Furthermore cultured shrimps had also made an impact on the global crustacean production (Csavas, 1993). With this rise in production, the availability of shellfish waste is also expected to rise.

The results obtained here may not be representative of the entire tropical species as shrimps are biological species and as such its characteristics are subjected to various environmental factors, age and species (Nicol, 1967). Nevertheless, the results in this experiment could give implications as to the expected results when using this type of waste. The results showed that the fermentation process was also successful

using the tropical waste and that the pH profiles were almost similar. The results also showed that the tropical waste had higher protein content and that the liquor produced was darker in colour compared to the scampi waste (a temperate species), whereas the calcium content in the former was lower. These differences in the initial composition of waste resulted in a different chitin product. The tropical chitin sediment had a higher protein but lower calcium content and vice versa for the temperate chitin sediment (see Table 4.35). The lower calcium content in the tropical waste (11.12 percent) compared to the temperate waste (20.80 percent) was thought to be an attractive characteristics for chitin recovery by the fermentation method as it would require less glucose.

5.3 Comparison of chitin purification by the acid/alkali treatment and fermentation.

5.3.1 The consumption of chemicals.

The chemical method of extracting chitin from shellfish waste is simple but requires large volumes of acid and alkali which results in the generation of large volumes of aqueous effluents. Alternative approaches have been suggested to reduce or eliminate the use of chemicals (Hall and De Silva, 1992; Healy *et al.*, 1994).

In the present work, by conducting a lactic acid fermentation, partially purified chitin was obtained. The purification level of the fermented waste was high, with approximately 94 percent removal of protein and 78 percent removal of calcium carbonate having being achieved (see Table 4.28). Further purification would require treatment with acid and alkali, but the quantities of these two reagents required would be relatively low in comparison to those needed to purify chitin solely using acid and alkali. In sub-section 4.4, comparison was made in terms of chemicals used to purify waste that had been fermented for 72 hours and unfermented waste. The fermented waste required 5.22 g NaOH/100 g waste compared to 63.6 g NaOH/100 g waste for unfermented waste and this is a saving of 92 percent of NaOH. Similarly, fermented waste required 5.72 g HCl/100 g waste whereas unfermented waste required 23.2 g HCL/100 g waste which is equivalent to a 75 percent saving of HCl. In terms of chemicals, this is a substantial saving and coupled with the protein recovery, the fermentation method may prove to be a worthwhile method of chitin purification, either on its own or as a pretreatment prior to production of chitin or chitosan. However, further work needs to be done to enable realistic estimates of fermentation costs to be achieved. Further saving may also be achieved if cheaper, more locally abundant carbohydrate sources were used instead of glucose.

The optimum conditions obtained by other workers (see Tables 2.2 and 2.3) to purify chitin by the acid and alkali treatment varied with species. The Tables show that optimal acid treatment was achieved at room temperature whilst protein hydrolysis required temperatures in the range of 65°C to 100°C. This supports the result obtained here. Thus to the large savings in terms of quantity of alkali used for

purifying fermented waste must be added energy savings. In the case of using HCl for the demineralisation step, it had been reported that using HCl at concentration above 1.25 N adversely affects the viscosity of the final product (Muzzarelli, 1977). Subsequent to the demineralisation and deproteinisation steps, the product may be decolourised with acetone and/or hydrogen peroxide (Simpson *et al.*, 1994). All these treatments would require special corrosion resistant equipment and the reagents would have to be handled with care.

5.3.2 Comparison between a 72 hour batch fermentation and a fed-batch fermentation (192 hour).

Comparing the 72 hour batch fermentation with a fed batch fermentation, an increase in the overall calcium removal was observed i.e. a 17.8 percent increase whereas no significant change occurred in the protein removal. This means that in terms of acid and alkali used (if further purification is required), the prolonged fermentation with increased addition of glucose, would only increase savings on the acid used. Thus the cost of overall production must be balanced between the cost of the additional glucose and the increased fermentation period with the incremental increase in purification achieved. From an economic point of view, the 48 to 72 hour batch fermentation is likely to be more attractive, though further work need to be undertaken to estimate accurate costings. Furthermore, this approach of chitin purification has only been conducted on a small scale and would need to be repeated at higher scales.

However, as had been mentioned in section 5.2.2., the fed batch fermentation experiment provided important information leading to a better understanding of the overall process. These include operating strategies for reducing foaming, estimates of a suitable rotation rates and considerations of maximum loading whilst maintaining purification.

CHAPTER 6

OVERALL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK.

The present trend towards using natural-based polymers in many fields, has increased the demand for chitin and chitosan (its simplest derivative). Chitin, an important structural component of the exoskeleton of crustaceans is produced from shellfish waste material from the seafood industry. Its attractive characteristics such as biodegradability, ease of conversion into various forms and its chelating properties have led to major applications such as waste water treatment, medical applications, biotechnology and cosmetics. The conventional process of producing chitin requires the use of corrosive chemicals (acid and alkali) and produces large amounts of effluent. Realising the disadvantages of using such chemicals, several recent studies have examined the possibilities of applying alternative milder methods such as the use of enzymes to remove protein. Alternatively, as in the present work, chitin is purified from the waste by using a solid-state lactic acid fermentation similar to the ensilation technique and the results obtained have shown it to be promising and meriting further studies with reference to scale-up. In this process, chitin is being purified from calcium carbonate and protein by the acid produced and the indigenous enzymes from the waste respectively. The following paragraphs conclude the findings of this study and at the end of the chapter, some suggestions for further work are provided.

The initial part of the work involving the isolation of lactic acid bacteria was found to be crucial to the fermentation process. As the purification of chitin from shellfish waste was dependent upon acid production by the bacteria, fast growing strains with rapid production of acid were important selection criteria which would bring about rapid preservation of waste and allowing the purification process to proceed. In this study, nine strains of lactic acid bacteria were successfully isolated from various sources of fermented shellfish waste. Identification of these strains showed that they belonged of the genera *Pediococcus* and *Lactobacillus*. One strain A3, which was identified as *Lactobacillus paracasei*, showed a rapid lowering of pH and had a

broader temperature growth profile (growth between 15 to 45°C) than the rest of the isolated strains and was used as starters for the optimisation studies using the horizontal bioreactor. This characteristic may prove useful when applied to large scale fermentation operations as close temperature control is often difficult to exercise in solid state fermentations. The upper growth temperature displayed by this strain, represented an added advantage if the procees were to be applied in tropical regions where the higher ambient temperatures would not be detrimental to the growth of the starters. The effect of temperature upon the lactic acid fermentation was clearly shown in the fermentations conducted in the bioreactor at various temperatures. Fermentation conducted at 20°C showed very slow activity, whilst higher temperatures of 30°C and 45°C showed rapid fermentation rates. In terms of the level of purification of chitin, the differences was greater when fermenting between 20°C and 30°C as compared to between 30°C and 45°C. Taken together, this result suggest that temperatures of around 30°C would be adequate to obtain good levels of purification. It is also important to bring up the desired fermentation temperature as quickly as possible to ensure rapid dominance of the starter cultures (LAB) over other undesired microorganisms.

One of the factors determining the success of the lactic acid purification of chitin was the use of the horizontal rotating bioreactor. The bioreactor was found to be suitable for the fermentations of scampi (*Nephrops norvegicus*) waste and fulfilled the criteria of providing easy separation of the fermented products, namely the protein-rich liquor and the chitin sediment. The problem of flotation of chitin sediment (which may initiate spoilage) caused by the production of carbon dioxide gas as experienced by Hall and Reid (1995) was not observed in this bioreactor. This is due to the horizontal position of the basket which is slightly above the liquor and the tumbling nature of the agitation. The horizontal arrangement also provided ease of intermittent mixing by the motor-controlled rotation of the inner basket. In large scale operations, the liquor could easily be emptied via an outlet or pipe at the bottom of the reactor and once the liquor had been removed, washing of chitin sediment or further treatment could be done *in-situ*.

Batch fermentations conducted in the bioreactor revealed that successful fermentation was dependent upon the quality of the waste, providing a high enough inoculum level and adding sufficient glucose to ensure rapid production of acid by the bacteria. Perhaps one of the principal advantages of this method is that it does not require the addition of water. After mixing the waste with the inoculum and glucose, rapid production of acidified liquor was achieved which dissolved the calcium carbonate of the waste. Charging of the waste was found to be critical in effecting contact between waste and the produced liquor. Since the LAB are generally microaerophilic, only low intermittent mixing was necessary for good growth whilst high rotation rates led to the formation of mousse-like product which hindered the purification of chitin. High rotation rates were also found to reduce bacterial growth resulting in a much reduced production of acid during fermentation of scampi waste. The gentle mixing provided by the tumbling action of the reactor also helped to prevent pockets of high pHs from persisting which might initiate spoilage. Fermentation products were best recovered at fermentation times in the range of 48 to 72 hours when the pH was still low because longer periods tended to favour the growth of spoilage organisms as the pH increased due to the neutralisation of the acid by the calcium carbonate.

Mincing the shellfish waste was found to promote good fermentation resulting in substantial level of purification. Fermentation of minced scampi waste with 10 percent glucose at 30°C for 72 hours led to the removal of 78 percent calcium carbonate and 94 percent protein. In order to maximise the batch productivity at the same condition as above, experiments conducted with increased charges of waste revealed that the increased loading did not affect the level of purification of chitin. However, evaluating the effect of particle size upon fermentation revealed that larger particles (cut to 1/2 inch followed by crushing) occupied a greater volume of the basket, thereby greatly reducing the contact between waste and the liquor produced. Crushing the waste was found particularly effective in exposing meat fractions to the acid conditions and it also helped to reduce the bulkiness of the waste material.

Since purification of chitin during a lactic acid fermentation involved the removal of calcium carbonate and the solubilisation of protein, the presence of acid was of great

importance to both processes. If it is assumed that the reaction between calcium carbonate and the acid proceeded stoichiometrically, then it appeared that the addition of a 10 percent glucose in a batch fermentation was the limiting factor towards further removal of calcium carbonate. It was found that further removal of calcium carbonate could be achieved in a fed batch fermentation with increased addition of glucose. The fed batch fermentation revealed that whilst calcium removal improved by 15.5 percent when more glucose was added, the protein content of the chitin sediment remained almost constant. This result suggested that the autolytic process which resulted in protein solubilisation, was achieved within 48 to 96 hours whilst calcium removal was dependent upon the availability of acid. The production of acid by LAB is generally not just confined to simple sugars such as glucose. Other cheaper sources could also be used such as whey powder, cereals, molasses or cassava. In developing countries, the need to minimise costs indicates that carbohydrate source should be based on indigenous staples such as rice, cassava or sago.

The lactic acid fermentation was also found applicable to waste from tropical prawns (*Penaeus monodon*) and it is not inconceivable that it could be applied to waste from a wider range of crustaceans. Krill for instance, is a high protein content material and contains 25 percent chitin (on a dry weight basis) and is usually used whole for aquaculture. The minute size of krill hinders peeling processes to separate the meat and the shells thus reducing the availability of chitin. The simplicity of the lactic acid fermentation and its rapid separation of the fermentation products into protein-rich liquor and the chitinous material may prove useful for separating chitin from krill. It is anticipated that crab waste which contains a higher amount of hardened protein-chitin composite in the exoskeleton might prove more difficult to treat. However, further investigations would need to be conducted to optimise fermentation conditions for wastes from different shellfish species.

Complete purification of the fermented (72 hours) and unfermented chitin product by the acid and alkali method showed that by pre-fermenting the waste to partially remove calcium carbonate and protein resulted in savings of 75.4 and 91.8 percent of acid (HCl) and alkali (NaOH) respectively. This indicates that the operation of lactic

acid fermentation method of recovering chitin from shellfish waste on a large scale either on its own or as a pretreatment prior to further acid and alkali treatments, is very much less polluting to the environment as compared to the traditional acid and alkali method. Furthermore, protein is recovered and the purification level of chitin after 72 hours fermentation is high. In addition, if chitin is to be eventually converted to chitosan, the small amount of protein left during fermentation would be eliminated in the course of the conversion process, as the conversion process employs boiling concentrated NaOH solution which would easily remove any residual protein.

The recovery of the protein-rich liquor after fermentation is an added advantage of this process. Further investigation into the protein rich liquor could improve the profitability of the process either as nutritious animal feed or as a source of food flavourings. The liquor could be mixed with other carbohydrate fillers such as malt wastes, rice husk, corn, bananas and other carbohydrate-rich substances. Once dried, it could be included in formulations in poultry, cattle as well as shrimp-feeds. Transporting dried product is also simpler and cheaper. The liquor product from this work has also been tried as an "attractant" by researchers at Hull University. Although experimentation is at an early stage, initial results are promising. Continuing studies on the enzymes present in the protein liquor is also underway at the Food and Biotechnology Laboratory at Loughborough University.

It seems appropriate to suggest that the immediate work should be conducted to evaluate the process on a larger scale. This could easily be conducted on site, as freshly processed waste material would be available. The relatively higher growth temperature of *Lactobacillus paracasei*, strain A3 means that the process is best conducted at tropical regions as heating could be omitted, thus reducing production costs. Further studies need to be conducted to permit economic evaluation of both the fermentation method and the traditional purification method. This should be extended to examine whether the fed batch process shows real advantage over the simple batch process. The results obtained here suggest that futher investigations into the effect of particle size when applied to larger scale horizontal rotating bioreactor need to be undertaken. The possibility of omitting the size reduction

pretreatment stage or reducing the amount of communition required would enhanced the attractiveness of the process. At the present scale of operation, whole untreated waste would spoil rapidly but crushing the waste helped to reduce the problem of spoilage and bulkiness of material. However, it is difficult to forecast what would happen at higher scales as the extent of submergence depends upon the volume of liquor produced and the drum diameter. Finding a direct use of the partially purified chitin (from a 72 hour fermentation) would be highly rewarding as it would obviate the need for further processing. The application of partially purified chitin by Coughlin *et al.* (1990) in purifying electroplating wastewater is one example of such an application.

On the whole the results obtained in this work suggest that lactic acid fermentation of shellfish waste merits serious consideration by the chitin production industry. With expected future increases in the production of shellfish waste, a process based on that developed here could have a significant impact on the continued viability of the shellfish industry.

APPENDICES

Appendix 3.1: APT and MRS Broths

APT Broth (Difco, Michigan, U.S.A.)

Contents	g / L
Yeast extract	7.5
Tryptone	12.5
Dextrose	10.0
Sodium citrate	5.0
Thiamine hydrochloride	0.001
Sodium chloride	5.0
Dipotassium phosphate	5.0
Manganese chloride	0.14
Magnesium sulphate	0.8
Ferrous sulphate	0.04
Sorbitan monoleate complex	0.2

To prepare broth: dissolve 46.2 g of APT Broth powder in 1.0 L of distilled water, autoclave at 121°C for 15 min. Avoid excessive heating. To make APT agar plates, add 1.5 % (w/v) of bacteriological agar (Oxoid) to the broth and after autoclaving, cool the agar broth to 45°C in a water bath before plating out.

MRS Broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire)

Contents	g / L
Peptone	10.0
Lab Lemco powder	8.0
Yeast extract	4.0
Glucose	20.0
Tween 80	1.0 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
Magnesium sulphate	0.2
Manganese sulphate	0.05

To prepare: dissolve 52.0 g in 1.0 L of distilled water, autoclave at 121°C for 15 min. To make MRS agar plates, add 1.5 % (w/v) of bacteriological agar (Oxoid) to the broth and after autoclaving, cool to 45°C in a water bath before pouring.

Appendix 3.2: Scampi Broth

A broth made from scampi waste was used for the screening procedures. Scampi broth is rich in essential amino acids will provide a good medium for growth of lactic acid bacteria.

Method: Minced scampi waste (250 g) was mixed with 1.0 L of distilled water, stirred and left to stand for 10 min at room temperature. The mixture was filtered and the filtrate boiled for 30 min to coagulate the proteins and was left to stand for another 30 min to settle down the solids. The clear solution was decanted and centrifuged at 3000*g* for 15 min before autoclaving at 121°C for 15 min. The scampi broth was kept at 4°C until further use. Sterilised glucose solution was added to the scampi broth prior to fermentation (10 ml of 20 % (w/v) glucose solution with every 90 ml of scampi broth).

Appendix 3.3: **Arginine solution** (Collins *et al.*, 1989a).

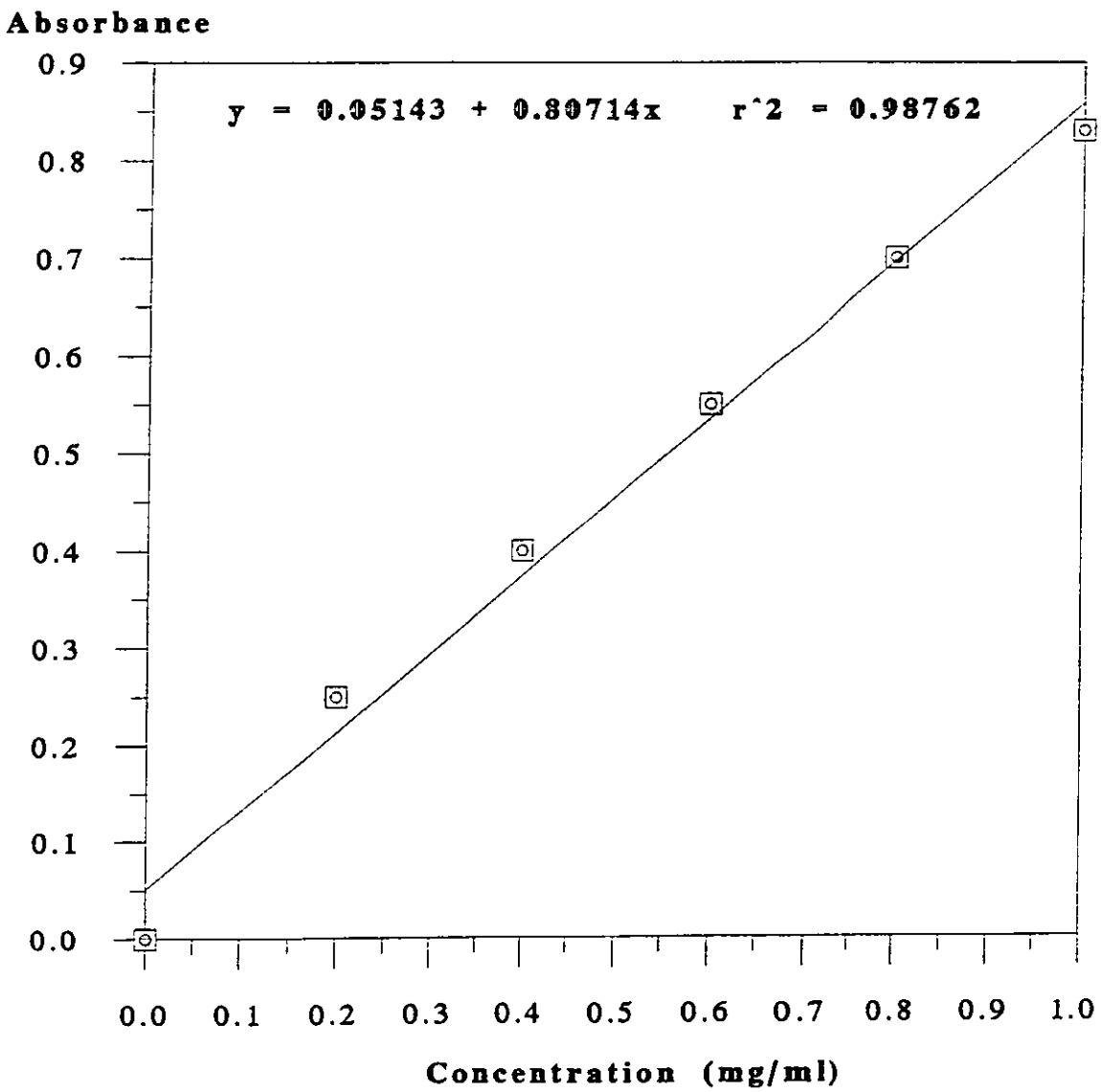
To prepare arginine broth, dissolve the following by heating, adjusting the pH to 7.0 and autoclaving at 115°C for 10 min. This broth was used to identify streptococci but for arginine breakdown by lactobacillus, MRS broth in which ammonium citrate is replaced with 0.3 % (w/v) arginine hyfrochloride was used.

Contents	g / L
Tryptone	5.0
Yeast extract	5.0
Dipotassium hydrogen phosphate	2.0
L - arginine monohydrochloride	3.0
Glucose	0.5
Water	1.0 L

Appendix 3.4: Boric acid solution

To prepare a 2 % Boric acid solution, dissolve 50 g of boric acid in 2.5 L of boiled distilled water. Allow to cool. Add to the cooled boric acid solution, 25 ml of bromocresol green (1.0 mg / ml in ethanol) and 17.5 ml of screened methyl red (1.0 mg / ml in ethanol) indicator. Transfer 25 ml of the boric acid solution to a flask and titrate with 0.1 M NaOH until a neutral grey colour is obtained. Calculate the amount of NaOH solution required to adjust the 2.5 L of boric acid solution to neutrality (approximately 30 drops).

Appendix 3.5: Protein Standard Curve



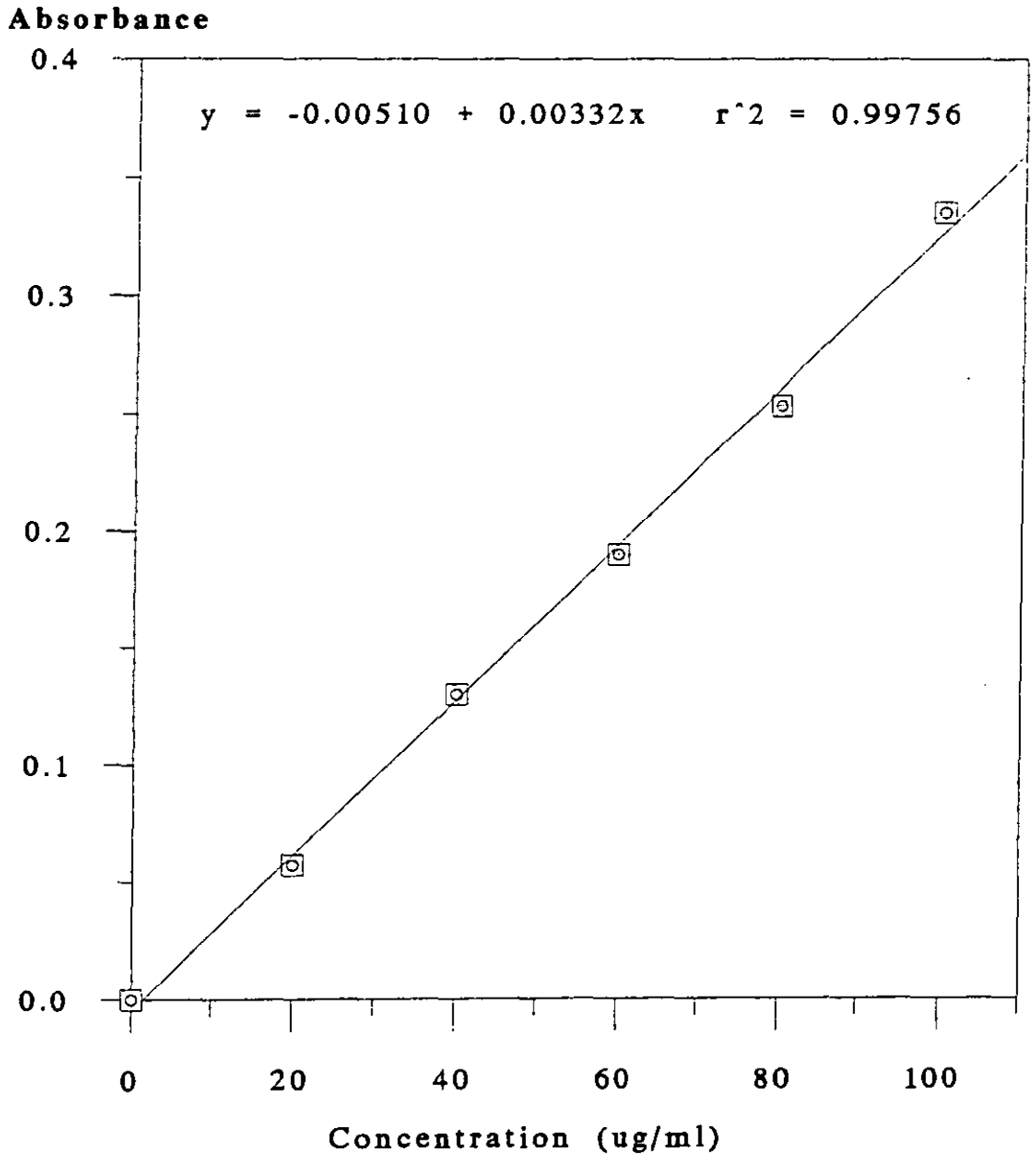
Appendix 3.6: Copper reagent (Pearson, 1976).

To prepare Somogyi's modified alkaline copper tartrate stock reagent, dissolve 28 g anhydrous disodium phosphate and 40 g sodium potassium tartrate in 700 ml distilled water. Add 100 ml of 1.0 M of sodium hydroxide (aq) and 80 ml of a 10 % (w/v) solution of crystalline copper sulphate. Lastly, add 180 g of anhudrous sodium sulphate and make up to a litre. Allow to stand for a few days before decantibg and filtering.

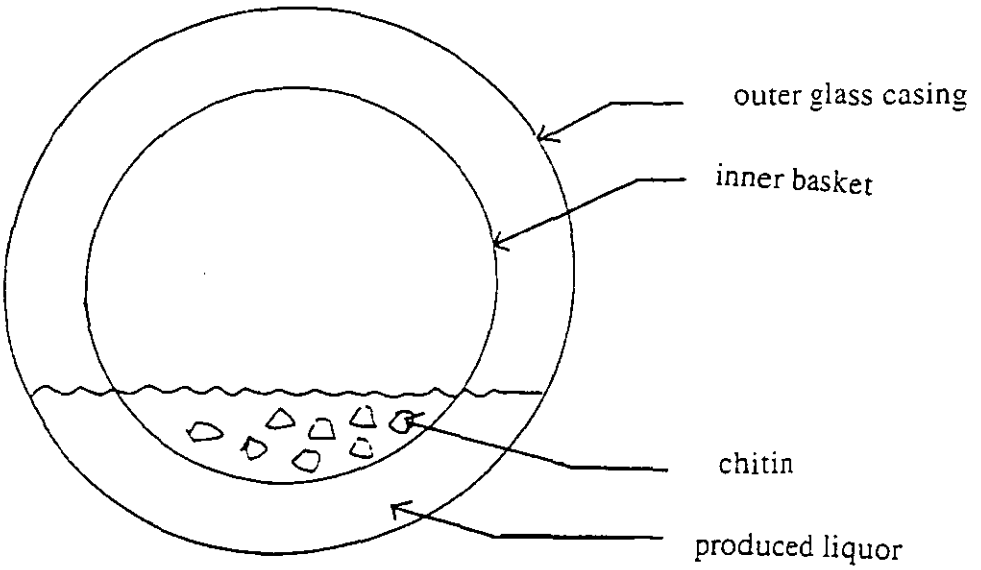
Appendix 3.7: Arsenomolybdate reagent (Pearson, 1976).

Dissolve 25 g of ammonium molybdate in 450 ml distilled water. Add 21 ml of concentrated sulphuric acid and 3.0 g $Na_2HAsO_4.H_2O$ which has been previously dissolved in 25 ml water. Incubate at 37°C for 2 days.

Appendix 3.8: Glucose Standard Curve



Appendix 3.9: Estimation of the minimum amount of waste needed to produce substantial submergence of waste and liquor.

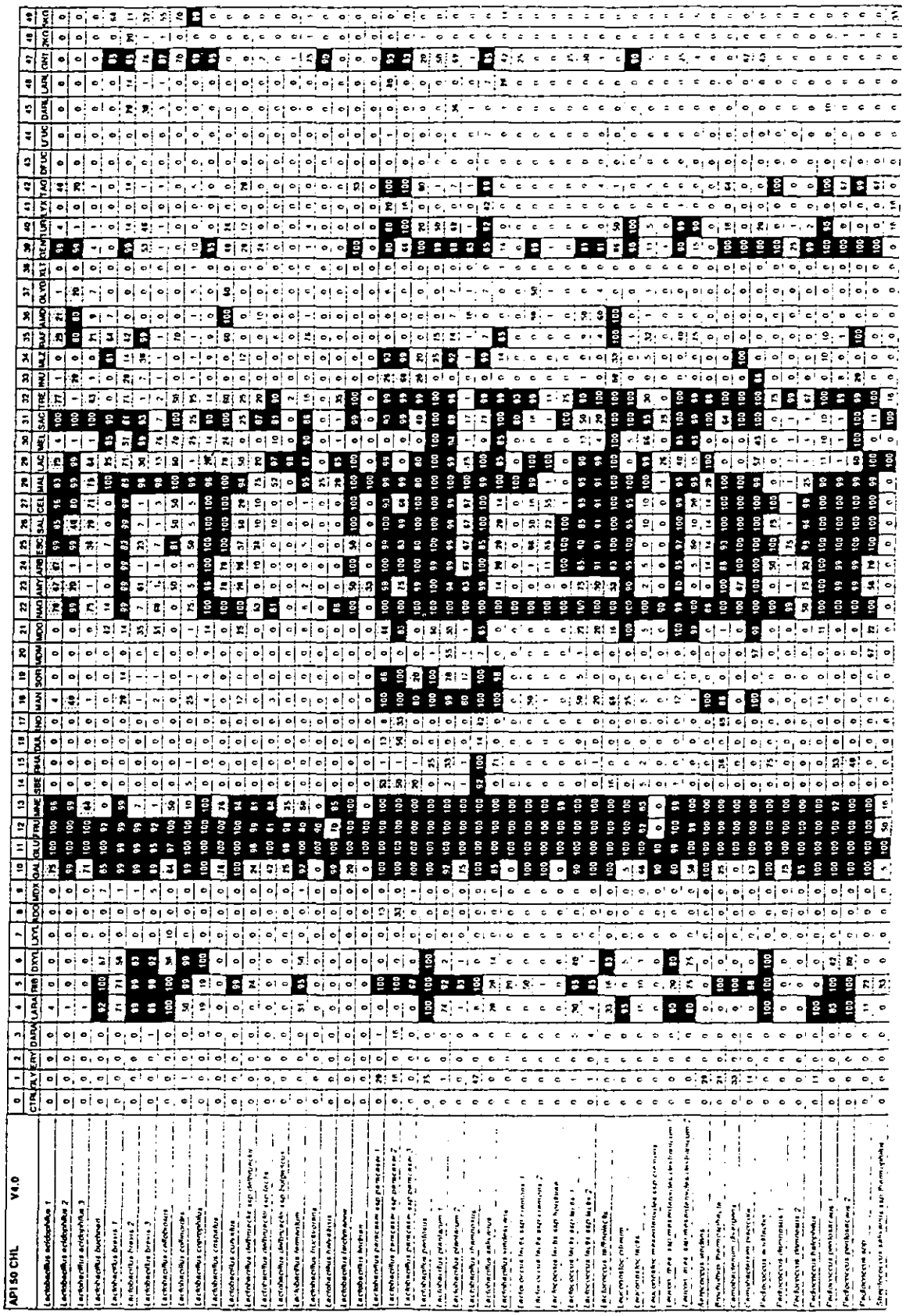


During fermentation, shellfish waste is placed in the inner basket. Subsequently, acidified protein liquor is being produced as a result of protein digestion and production of lactic acid by the bacteria, which accumulates in the outer glass casing. To produce successful fermentation and purification of chitin, substantial amount of the waste material must be well submerged in the liquor. Therefore, to obtain an estimate of the minimum waste required to produce such effect, firstly, submergence of the inner basket was tested with the volume of water added. Then based on the results of the batch fermentations, an average percentage of liquor production was noted (which is 62 %, v/w) and was related to the amount of waste needed to produce it. The results are as follows;

Volume of water (ml)	Approximate submergence of basket (inch)	Amount of waste required (g)
375	touching	605
750	0.5	1210
1500	1.5	2419

Therefore approximately 750 g waste was considered the minimum amount of waste required to produce sufficient submergence of waste in the liquor.

Appendix 4.1: Identification Table



Appendix 4.2: Theoretical Calculation of the Calcium Solubilisation from Scampi waste.

Homofermentative lactic acid bacteria produces lactic acid (90 g/mol) as their major product from glucose (180 g/mol). In reality more than 85 % glucose is converted to lactic acid (Jay, 1992). The production of acid is important in removing the calcium carbonate (100 g/mol) from scampi waste. However, from batch fermentations results (see section 4.3.1), it was shown that when scampi waste was fermented with 10 percent (w/v) glucose, only 54 to 68 percent of the calcium carbonate was removed. As the scampi waste contains large amounts of calcium carbonate, it might be possible that more glucose is required. Since prawns and shrimps contain only minute amount of carbohydrate, it is assumed that the lactic acid produced during fermentation of scampi waste mainly originated from the conversion of glucose. Also with the assumption that intimate contact between acid and calcium carbonate occured, then the theoretical glucose consumption necessary to effect complete removal of calcium carbonate from scampi waste can be calculated using Eqn 1 and 2 as shown below;

1 mol glucose = 2 mols pyruvate = 2 mols lactic acid -----> Eqn 1 (100 % conversion)
1 mol glucose = 2 mols pyruvate = 1.7 mols lactic acid ---> Eqn 1 (85 % conversion)

2 mols acid + calcium carbonate--> carbon dioxide + water + calcium lactate---> Eqn 2

For every 1.0 kg waste, 100 g glucose was added, which is equals to (100/180) or 0.56 mols of glucose. Assuming an 85 % conversion of glucose to lactic acid, this will produce (0.56 x 1.7) mols of acid. The acid produced reacts with calcium carbonate according to Eqn. 2 to produce calcium lactate. Assuming all acid produced readily reacted with calcium carbonate, then (0.56 x 1.7) mols acid will dissolve [(0.56 x 1.7) / 2] mols calcium carbonate or 47.6 g calcium carbonate. Since 1.0 kg (318.0 g dry weight) of scampi waste contains 20.8 percent calcium (Ca^{2+}, 40 g/mol) on dry weight basis, then it follows that

318 g drywaste contains = 66.14 g calcium
= (66.14 / 40) mols calcium
= 1.65 mols calcium carbonate
= 165 g calcium carbonate.

Therefore if 100 g glucose are required to remove 47.6 g of calcium carbonate, the amount of calcium carbonate left in the sediment should be;

$$165 - 47.6 = 117.4 \text{ g calcium carbonate}$$
$$= 1.17 \text{ mol calcium}$$
$$= 47.0 \text{ g calcium}$$

From the result of fermentation (a) in section 4.3.1, the average calcium content of the sediment was 27.4 g calcium which is lower than the theoretical value. This result seems to suggest that removal of calcium carbonate had also been initiated by some other mechanism other than acid reaction. However, it remains possible that further addition of glucose may provide the essential acid required to remove more calcium carbonate, then the theoretical amount of glucose required to remove 165 g calcium carbonate would be 294.6 g glucose, i.e. approximately 30 percent glucose.

Appendix 4.3: Optimisation of agitation to avoid foaming.

Initially a simple 24 hour timer was used during the batch fermentations (section 4.3.1) which switches on at least for ten min. Since the bioreactor has a motor which operates at 20 revs per min, then switching on the timer for 10 min will rotate the basket for 200 revs. From the results of the batch fermentations, it was observed that during fermentation (a), the liquor started to solidify after the 4th day of the fermentation. During this fermentation, the timer was on every 6 hours which means that it will be on for 4 times a day. This is equivalent to (4 x 200) revs a day or to 3200 revs in 4 days. Previously, another batch fermentations (result not shown) started to solidify after 3 days when rotated at 4 hourly intervals. This is equivalent to 6 X rotations a day or 3600 revs in total. Therefore during fermentations (b) and (c), the timer was switched on every 6 hours but only for the first 3 days. With this arrangement, the problem of solidification was overcome.

However, in other experiments, a purpose-built timer was used instead. With this timer, shorter durations of rotation period were possible. From the above observations, a general rule can be postulated regarding the minimum rotations possible to avoid solidification of the liquor from taking place and the new arrangements could be easily set with the new timer.

For a 3-day fermentation, taking an average of 1000 revs a day as the maximum rotations possible to avoid foaming or solidification of liquor, then the following variations of rotation rates may be used;

No of revs	Time on(min)	Intervals(h)	No of frequency a day	Total rev/day
250	12.5	6	4	1000
166	8.3	4	6	1000
83	4.1	2	12	1000
41	2.1	1	24	1000
20	0.5	0.5	48	1000
10	0.25	0.25	96	1000

Therefore the rate in the last row (i.e. 0.25 h interval) represents the upper limit in which the timer can be set based on the problem of the solidification of the liquor. However, it must be remembered that the lactic acid bacteria is a microaerophilic bacteria, meaning it only requires minimal amount of oxygen for growth. The rotation of the inner basket is a type of stirring mechanism and as such it can also be considered an aeration mechanism. Although no data is available to provide the maximum rates at which the bacteria could withstand without affecting its growth rate, it is reasoned that a low rotation rate is only necessary.

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