BIOTECHNOLOGICAL UTILISATION OF *NEPHROPS* SHELL WASTE

BY

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For my mum and dad.

DECLARATION

I hereby declare that this thesis, and the experiments described therein, are entirely my own work, except where attributed otherwise. No part of this thesis has been submitted for a degree at this or any other university. All sources of information have been acknowledged by means of reference.

Julia C. Morrow

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SUMMARY

Shellfish processing results in the disposal of large quantities of waste to landfill sites. This project sought to deal with the issue of the processing of marine crustacean waste, specifically that of the prawn, *Nephrops norvegicus*.

The exoskeleton of *Nephrops norvegicus* contains chitin, β-(1→4)-N-acetyl-D-glucosamine. Chitin is a polymer with widespread applications in many different industries. Traditionally it has been isolated from crustacean shell waste via chemical demineralisation using 1-2M HCl and deproteinisation using 4% (w/v) NaOH.

In this study shell waste was demineralised, via lactic acid fermentation, using a commercial silage inoculant. The product contained 11.2% (w/w) ash, 31.1% (w/w) protein and 53.5% (w/w) chitin. Addition of the proteolytic enzyme, bromelain and a nitrogen source to the fermentation system led to deproteinisation and further demineralisation of the shell. The final product contained 4.2% (w/w) ash, 5.3% (w/w) protein and 79.6% (w/w) chitin.

Chitin is relatively inert but can be converted to its more reactive derivative, chitosan, by deacetylation. Chitosan, prepared from bioprocessed shell waste, was investigated as a potential coating for urinary catheters. Attempts are currently being made to prevent blockage of these biomedical devices due to mineral ions, via the use of different manufacturing materials. Shell waste that had been demineralised using lactic acid fermentation was deacetylated using 50% (w/v) NaOH at 90°C. Membranes were prepared from the resultant chitosan. After immersion in a bath containing artificial urine, adherence of calcium and magnesium ions to the membranes was measured and compared with adherence of the same ions to polyurethane membranes. Adherence of calcium to the chitosan membranes was no higher than adherence to the polyurethane control membranes. Adherence of magnesium ions was significantly less than to the polyurethane control. The chitosan was hence shown to have potential for use as a coating for urinary catheters.

LIST OF ABBREVIATIONS

AS	Autoclaved Shell
BMW	Biodegradable Municipal Waste
EDTA	Ethylene-diamine-tetra-acetic acid
EN	Endocuticle
EP	Epicuticle
EX	Exocuticle
ICES	International Council for the Exploration of the Sea
LAF	Lactic Acid Fermentation
ML	Membranous Layer
MRS	De Man Rogosa Sharpe
N:C	Nitrogen : Carbon
TAC	Tri-Ammonium Citrate
TTA	Total Titratable Acid
US	Untreated Shell

CHAPTER ONE

LITERATURE REVIEW

1.1 ENVIRONMENTAL POLLUTION

During the mid-1700s the effects of the 'Industrial Revolution' became noticeable in Britain. Daily life, which had been based on farming for thousands of years, shifted to crowded cities. In 1769 the first efficient steam engine was developed. It could run anything from a pump or factory machine, to a train or ship. The steam engines were grouped together with machines in large workshops and by 1800 these workshops had become the world's first factories. New factories were often set up near sources of coal and water. Towns grew up around them where the workers lived. Very soon these towns had expanded into cities. At the same time new methods of transport developed. The first steam locomotive was built in 1803 and the first steam powered public railway opened in 1825. The first petrol cars were built in 1885/1886 and the first sizeable powered aeroplane was built in 1842/1843.

The advent of industry has not occurred without hazard. The dumping of waste products from industry, into the water supply and the air, has created an enormous threat to our environment and currently the environmental hazards caused by pollution are on such a scale that government agencies are addressing issues involving environmental protection, at a national and an international level. Thus, globally, politicians have debated changes in climate caused by emission of 'greenhouse gases', from factories, transportation and households. In 1992 the United Nations Framework Convention on Climate Change was agreed at the Earth Summit in Rio de Janeiro. All developed countries involved aimed to reduce their greenhouse gas emissions to 1990 levels by the year 2000. Following this, the Kyoto Protocol, which was agreed in 1997, was designed to produce deeper cuts in the emissions.

1.2 WASTE MANAGEMENT

We live in a throwaway society where the policy of the majority of people is to discard rather than re-use. However, with calls for a 'greener' environment now being made law it is essential that recycling is carried out wherever possible. In Northern Ireland 'The Waste Management and Contaminated Land' branch of the Environment and Heritage Service is responsible for implementing the Government's environmental policy on waste management.

The main legislation governing wastes, prior to 1997, was the Pollution Control and Local Government Order (N.I.) 1978 and the Waste Collection and Disposal Regulations (N.I.) 1992. This legislation came under the control of individual district councils. The Waste and Contaminated Land (N.I.) Order 1997 made provision for the transferral of the major responsibility for waste matters from the district councils to the Department of the Environment's Environment and Heritage Service. In March 2000, a waste strategy was launched. The strategy, which was based on principles designed to implement objectives of the 1997 Order, advocated the following options: reduction of waste, re-use of materials, recycling of waste into new raw materials, recovery of energy from waste products and disposal of waste to a high standard (Environment and Heritage Service, Northern Ireland, 2001). To do this, local authorities have been encouraged to set up 'municipal waste management strategies' showing how they can meet the objectives and targets in the Waste Strategy 2000. In particular, the strategies must contain ways of increasing recycling and composting.

European countries rely heavily on landfill as a waste management option. More than 60 percent of municipal waste is currently landfilled (Environmental Protection Agency, Ireland, 2001). Badly operated sites can, however, cause annoyance due to offensive odours, insects and litter, as well as having the potential to pollute air, water and land. In an effort to reduce the amount of biodegradable municipal waste (BMW) going to

landfill sites the EC Landfill Directive (99/31/EC) that came into force on 16 July 2001 set targets relating to landfill waste. The U.K. national targets are:

BMW to landfill is to be reduced to 75% of 1995 levels by 2010.

BMW to landfill is to be reduced to 50% of 1995 levels by 2013.

BMW to landfill is to be reduced to 35% of 1995 levels by 2020.

Approximately 10 million tonnes of packaging waste are produced annually in the U.K. (Environment and Heritage Service, Northern Ireland, 2001). Most of this also ends up in landfill sites. The EC Directive on Packaging and Packaging Waste (94/62 EC) set targets for recycling and recovering packaging waste. At least 50% of the U.K.'s packaging waste must be re-utilised by the year 2001. The Producer Responsibility Obligations (Packaging Waste) (Northern Ireland) Regulations 1999 implements this directive.

In the short-term, landfill remains the only way to meet the need for the disposal of rising levels of waste in Northern Ireland. However, it is envisaged that with the implementation of new strategies, Northern Ireland will see a reduction in the number of landfill sites and the development of alternative facilities for waste disposal/recycling.

1.3 FISH AND SHELL FISH WASTES

As island communities the British Isles have a heritage of exploiting marine resources, the result of which is the generation of large amounts of waste. Since the majority of this waste ends up in landfill sites this area of environmental concern demands attention in order that the fish and shellfish industries may develop the capabilities to fall in line with any governmental waste minimisation strategies which may be imposed upon them. The amount of waste generated has been monitored and it is estimated that less than half of the fish and shellfish resources end up as products for human consumption (SeaFish Industry

Authority, 2001). The remainder is classified as waste and much of it, in particular the shellfish waste, goes to landfill sites.

The U.K. fish and shellfish trade can be broken down into three main categories: demersal (deep water fish), pelagic (open sea fish) and shellfish. The landings for 1999 are summarised in Table 1.1.

	Official landings[1] (tonnes)	Imports[2] (tonnes)	Exports[2] (tonnes)	Overall trade[2] (tonnes)
Demersal	236,398	249,404	72,640	413,162
Pelagic	107,277	147,363	114,970	139,670
Shellfish	147,891	87,652	81,866	153,677
Total	491,566	484,419	269,176	706,509

Table 1.1 *The U.K. fish trade for 1999* (SeaFish Industry Authority, 2001).
1 - liveweight, 2 - actual weight

Most demersal fish are processed, to some extent, before landing. The waste, which consists of guts, liver and other viscera, is currently discarded at sea. Pelagic fish and shellfish are not normally processed before landing, however small-sized or damaged shellfish, in particular *Nephrops norvegicus*, will have their head and claws removed and discarded at sea. These can make up 67% of the liveweight of the animal (SeaFish Industry Authority, 2001). Table 1.2 shows an estimate of total processing waste produced at sea in one year.

Sector	Off-Shore Processing Waste (Tonnes)
Demersal	36,966
Pelagic	-
Shellfish	
- *Nephrops*	8,796
Total	45,762

Table 1.2 *Estimated quantity of processing waste produced at sea* (SeaFish Industry Authority, 2001).

On shore the viscera, frame, skin, fins and head of demersal fish are discarded. Only 50% of the whole fish are edible (SeaFish Industry Authority, 2001). In the pelagic sector the waste material includes heads, viscera, frames, flaps and sometimes skin, with the edible portion accounting for approximately 53% (SeaFish Industry Authority, 2001). The waste products from shellfish processing include shell and viscera. The edible portion of shellfish varies from 14 - 58% (w/w) depending on species and final product (Table 1.3).

Species	Edible Portions % (w/w)
Crab	32
Lobster	44
Nephrops (whole)	24
(unshelled tails)	58
Shrimp (brown)	35
Prawn	40
Crustacea (Average).	39
Oyster	14
Cockle	12
Winkle	23
Scallop	14
Mussel	14
Whelk	42
Mollusc (Average)	20

Table 1.3 *Edible portions of some shellfish species* (SeaFish Industry Authority, 2001).

The estimated total annual on-shore processing waste is given in Table 1.4.

Sector	On-Shore Processing Waste (Tonnes)
Demersal	154,142
Pelagic	50,269
Shellfish	
- **molluscs**	67,166
- **crustacea**	29,459
Total	301,037

Table 1.4 *Estimated quantity of on-shore processing waste* (SeaFish Industry Authority, 2001).

Fish processing waste is of high nutritive value and can be widely utilized via incorporation into animal feed. The waste is dried and ground up, or preserved as a proteinaceous liquid via addition of acid/the anaerobic production of lactic acid by bacteria (Raa and Gildberg, 1982). Both treatments result in a product that can be incorporated into animal feeds. In contrast to fish, shellfish waste is not as nutritious for animals because of its high ash content and is therefore disposed of via landfill sites or by direct return to the sea (Healy and Bustos, 1993). Due to the implementation of waste minimization strategies and environmental concerns new methods of disposal for shellfish waste are being sought and this project seeks to deal with the issue of the processing of marine crustacean waste, specifically that of the prawn, *Nephrops norvegicus*.

1.4 *Nephrops norvegicus* - THE DUBLIN BAY PRAWN

Shellfish processing is a global industry. Species of decapod crustacean such as shrimps, prawns, lobsters, crayfish and crabs are a favourite delicacy. Of the 30 or so types of existing lobster three species inhabit the north eastern Atlantic Ocean - the European clawed lobster, *Homarus gammarus*, the scarlet clawed lobster, *Nephropsis atlantica* and the Norway clawed lobster, *Nephrops norvegicus*. Of these three, only the European clawed lobster and the Norway clawed lobster are common and fished commercially. However, the numbers of European clawed lobsters landed have declined since 1975 (Ingle, 1997).

The Norway lobster (*Nephrops norvegicus*) is also known as the Dublin Bay Prawn, Scampi, Cigala or Langoustine depending on the country of use. It was not fished to a great extent until the 1950's. However, since then, the landings in Europe have increased considerably. France lands the largest quantity, followed by Scotland, Spain, Ireland, Iceland and Italy (Ingle, 1997). France lands *Nephrops norvegicus* chiefly from the Bay of Biscay, Scandinavian countries from the Skagerrak and Kattegat regions, Iceland from

waters off the south and southwest coasts of Iceland and Mediterranean countries from the Adriatic and Tyrrhenian Seas (Ingle, 1997).

Nephrops norvegicus represents the major shellfish industry in the United Kingdom and the Republic of Ireland. The major U.K. *Nephrops* fisheries occur in the Farne Deep region of the North Sea, all around the Scottish coast, particularly off north east Scotland, off the Northern Irish east coast and off the north west coast of England. Landings in Northern Ireland come from stocks in the Irish Sea to the west of the Isle of Man, International Council for the Exploration of the Sea (I.C.E.S.), area VIIa (Fig.1.1) (Briggs, 1985).

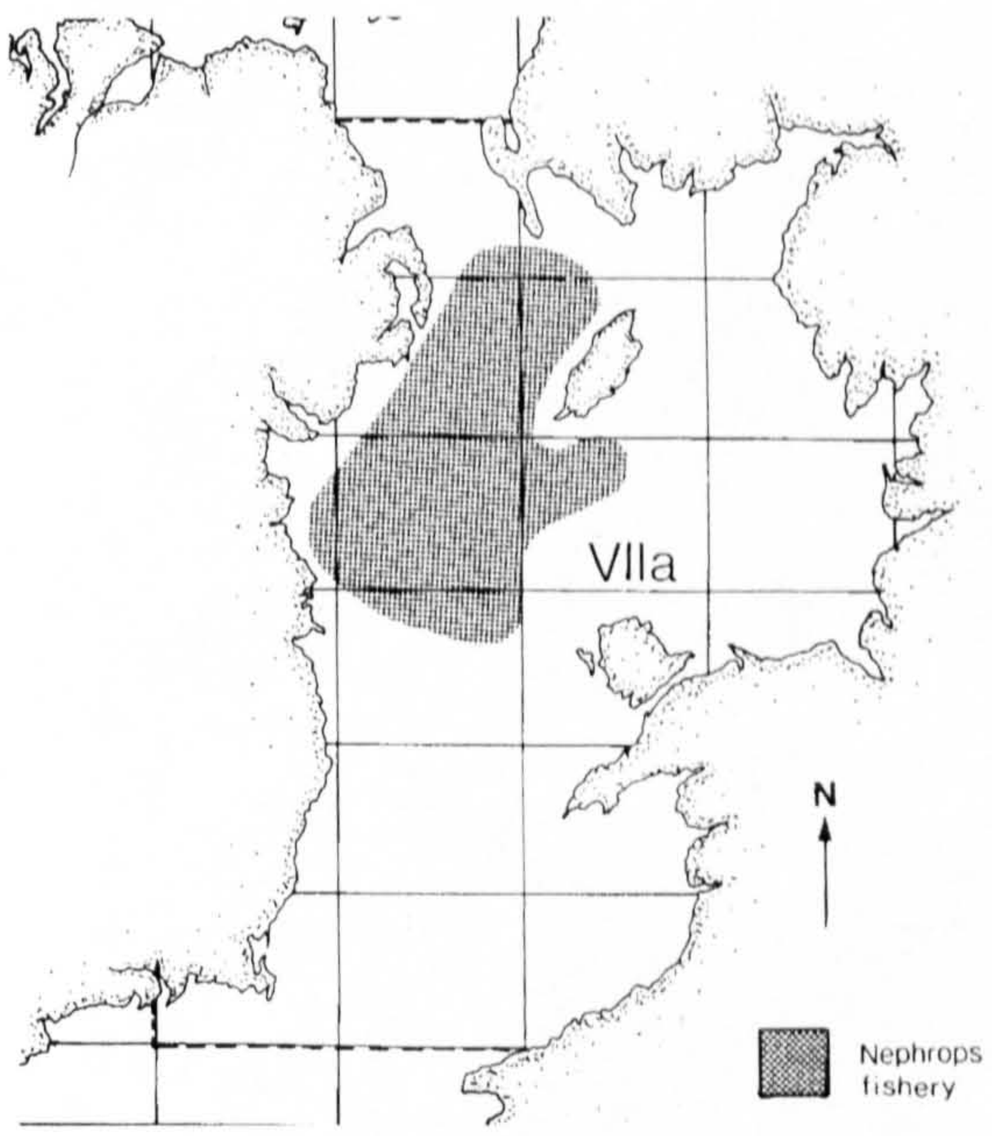


Fig. 1.1 *Map of Irish Sea (I.C.E.S. area VIIa) showing Nephrops grounds* (Briggs, 1985).

In the mid-1950's the annual landings of *Nephrops* in Northern Ireland were less than five hundred tonnes. By the early 1980's this figure had risen to more than four thousand tonnes (Briggs, 1995). Over the last decade approximately six to seven thousand tonnes of *Nephrops norvegicus* were landed annually in the U.K. (mainly in N. Ireland) and two to three thousand tonnes in the Republic of Ireland from the I.C.E.S. area VIIa (Table 1.5).

Year	Belgium	France	Republic of Ireland	Isle of Man	U.K.	Total
1989	0	19	2484	8	6011	**8522**
1990	0	8	2724	25	6165	**8922**
1991	1	12	3390	62	6864	**10327**
1992	1	6	2381	14	5596	**7997**
1993	0	8	2750	32	5939	**8729**
1994	0	17	1797	16	6312	**8142**
1995	2	7	3269	23	6020	**9321**
1996	1	2	1614	10	6127	**7756**
1997	1	0	3320	7	7180	**10509**
1998*	1	0	3008	25	6388	**9422**

*provisional

Table 1.5 *Total Nephrops landings (tonnes) by country, 1989 - 1998, in management area J (VIIa, North of 53°N)* (International Council for the Exploration of the Sea (I.C.E.S.) *Nephrops* working group report (1999 Meeting).

In 1982 *Nephrops* landings in Northern Ireland were worth over £3.4 million at first sale value (Briggs, 1985). By 1998 landings of *Nephrops* represented 46.1% of the first sale value of Northern Ireland landings of all species (£20.2 million). The value per tonne was £1506 in 1998 (Briggs, 1999).

In Northern Ireland there are three main fisheries. These are situated at Ardglass, Kilkeel and Portavogie in the east of the province. Around 20% of the Dublin Bay Prawns are landed 'whole' at the ports for export to France and Spain. The other 80% have been decapitated at sea and only the tail portion is brought to land (Briggs, 1997). Shrimp flesh spoils at a much faster rate than fish such as cod (Walker *et al.*, 1970) but bacterial spoilage can be prevented by temperatures less than 10°C (Shewan, 1971). The headless prawns are therefore stored frozen to prevent deterioration. When required, the meat is removed from the tail shell by water jet and frozen as it is or coated in breadcrumbs to be sold as scampi (Briggs, 1997).

The waste shells from all crustaceans pose a highly polluting environmental hazard if not disposed of correctly. The presence of meat fragments still attached to the shell leads to colonisation by spoilage organisms and rapid decomposition occurs. The presence of

foul odours, which accompanies the decomposition, necessitates rapid action. At present, the shells are dumped promptly at sea or in landfill sites, however new methods of disposal are being sought.

Although the shells pose a pollution problem and occupy large volumes within a landfill site, they also contain potentially valuable products - proteins, pigments and chitin, a structural polysaccharide. These commodities currently have widespread applications and potential applications in many different fields. Therefore, the emphasis of research in this area lies on the utilisation rather than the disposal of the shell. This project focuses on the isolation of one of these products - chitin. The raw material investigated was the tail shell of *Nephrops norvegicus* because of its wide availability in Northern Ireland and Scotland. The methods used could however be adapted for use with many shellfish species such as crab, krill and shrimp.

1.5 THE MORPHOLOGY OF *Nephrops norvegicus*

Nephrops norvegicus (Fig.1.2) belongs to the phylum Arthropoda, the order Decapoda, the class Crustacea and the family Nephropidae. Arthropods, in general, possess a hard exoskeleton. They are bilaterally symmetrical and consist of a longitudinal series of segments. Flexibility in the exoskeleton at intervals allows movement at joints. The exoskeleton of arthropods can be divided into two regions, the head or cephalothorax and the abdomen. The cephalothorax consists of the first twelve segments but, except for one joint, the joints are not present on the dorsal surface. Two kidney shaped eyes on the cephalothorax give the animal its name. The generic name *Nephrops* may be translated as 'kidney eye'. The abdomen contains six segments and the telson. The telson is an extension of the body, which possesses the anal opening. Each segment is made up of a convex dorsal plate, the tergum and a ventral transverse bar, the sternum. All the

abdominal segments except the first one also possess pleura, plates projecting down at either side.

Every segment has two jointed appendages. The appendages consist of the protopodite (the basal region), which bears two branches, an inner endopodite and an outer exopodite. The appendages vary in name and size depending on the segment to which they are attached. The segments of the abdomen bear five pairs of 'swimmerets'. The sixth segment bears 'uropods' that are flattened appendages. The swimmerets are involved in the transferance of sperm from male to female, and for attachment of eggs. The uropods are used for swimming (Hegner and Stiles, 1963).

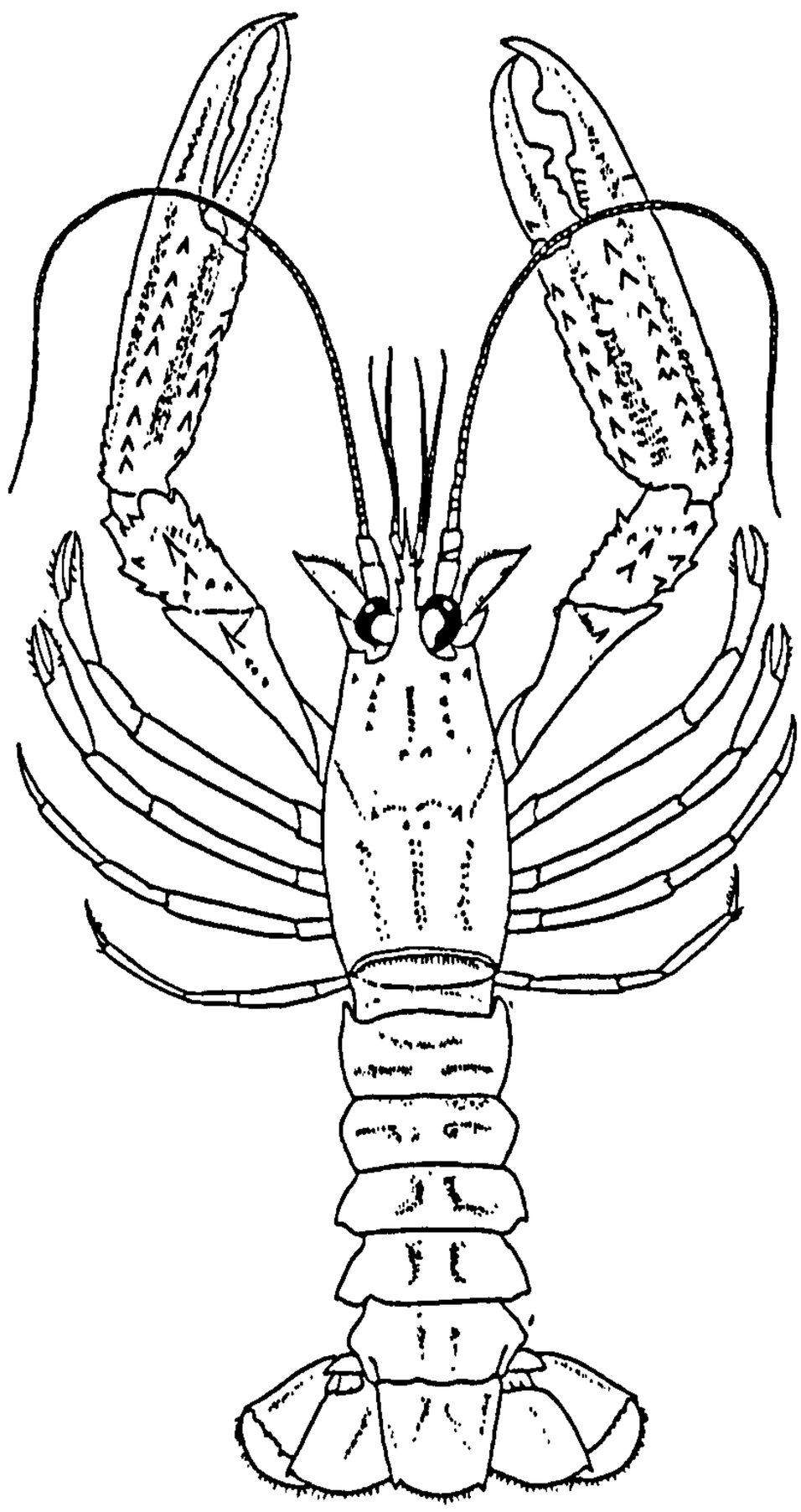


Fig.1.2 *Nephrops norvegicus, Norway Clawed Lobster.*

The cuticle of decapod crustaceans consists of four layers: the epicuticle, the exocuticle, the endocuticle and the membranous layer (reviewed by Roer and Dillaman, 1984). The epicuticle is the outermost layer. It is the thinnest layer and consists of tanned lipoprotein impregnated with calcium salts. The exocuticle, endocuticle and membranous layer contain horizontal, parallel microfibrillar sheets of protein covalently linked to chitin.

Throughout the procuticle (cuticle minus the epicuticle) the chitin-protein microfibrils have been laid down in layers. The fibrous direction rotates continuously as new levels are added so that each lamina corresponds to a 180° rotation leading to a lamellate appearance in cross section. Many extracellular fibrous composites are secreted layer by layer in this manner e.g. plant cell walls, insect cuticle, vertebrate cornea, moth eggshell, and fish eggshell (Neville, 1993). The endocuticle of the fiddler crab, *Uca tangeri*, contains larger and less-tightly stacked lamellae than the exocuticle (Green and Neff, 1972). The horizontal lamellae in the shells of the European clawed lobster and the shore crab, *Carcinus maenas*, are also more closely spaced together in the exocuticle than in the endocuticle (Mutvei, 1974).

The exocuticle is hardened by sclerotization (crosslinkage of structural proteins by quinones), and the presence of mineral crystals between the fibres. The endocuticle possesses the thickest lamellae and the most calcium although it is not sclerotised. The membranous layer is situated next to the cuticle-secreting epidermal cells. It is composed of just chitin and protein.

Fine helical tubules, or pore canals, extend throughout the procuticle as seen in the shore crab (Compère and Goffinet, 1987). Vertical fibres sheath these canals. They terminate at the epicuticle or are extended by epicuticular pores. The pore canals make up a three-dimensional branched system. The pro-cuticular pores, but not the epicuticular pores, have a helical or twisted ribbon morphology so that the pitch of the helix equals the lamellar period. During the moulting period the pore canals contain cytoplasmic

extensions from the epithelial cells. The very large number of these pore canals means that the whole cuticle is in close contact with the hypodermis and is therefore not a static entity but rather can be considered as living tissue.

A second canal system seen in the shore crab is the fibre canal system. It is composed of unbranched, vertical canals containing fibres (invaginations of the plasma membrane), in close association with myoepidermal junctions (Compère and Goffinet, 1987). These canals are not associated with the epithelial microvilli but may be associated with a second type of epidermal cell that acts as a mechanical attachment site. Like pore canals they show a twisted ribbon structure.

1.6 UTILIZATION OF CRUSTACEAN CUTICLE

The protein content of the cuticle makes crustacean shell a viable alternative to other protein sources. Protein present in shell waste can be classified either as adventitious - remnants of flesh and connective tissue, or firmly bound protein - complexed with chitin and minerals as an integral part of the shell (Bustos, 1996). The amino acid composition of *Nephrops* shell waste protein has been analysed. The protein contains a good balance of essential amino acids considered to be suitable for inclusion in salmon or trout diets (Bustos, 1996).

Shrimp by-products have been processed into meals and used successfully as a protein source for shrimp feed (Cruz-Suárez *et al.*, 1993; Fox *et al.*, 1994). Feed conversion rates improved when the shrimp by-product meal was included in the diet. In Thailand shrimp biowaste (head + tail) is sold at very low prices for animal feed production (Kungsuwan *et al.*, 1996). However, due to the high ash content of the shellfish waste, it has not been incorporated into animal meals as widely as fish waste.

The carotenoid pigments in the shell can also be put to use. The shells contain astaxanthin and are a natural source of pigmentation for wild salmonids. In a farmed

situation the pigments are not naturally available and an artificial supply is required. If the pigments from the shell could be isolated they would provide an alternative to artificial sources. Several studies have examined the possibility of releasing these carotenoids from the shell using proteolytic enzymes (Simpson and Haard, 1985; Cano-Lopez *et al.*, 1987) or a soy-oil extraction process (Chen and Meyers, 1982 and 1983).

Crustacean shell waste can be used to produce single cell protein. Carroad and Tom (1978) proposed the conversion of shellfish waste to yeast single cell protein. Wang *et al.*, (1998) grew a strain of *Pseudomonas aeruginosa* K-187 on powder made from shrimp and crab shell. Rhishipal and Philip (1998) grew marine yeasts on a mixture of waste from the prawns *Penaeus indicus*, *Penaeus monodon* and *Metapenaeus dobsoni*.

The most valuable component of the shell is potentially the polymer chitin, due to the widespread potential applications for both chitin and its main derivative chitosan. Chitin was first described in 1811 by Braconnot when he isolated it from mushrooms and named it fungine. It was given the name chitine (derived from the Greek for a tunic) in 1823 by Odier who found it in insect cuticles. It is present in vast quantities in nature joining the ranks of other skeletal macromolecules such as cellulose and collagen. These macromolecules form fibrous strengthening structures important to the architecture or design of the animal or plant.

Chitin is found naturally in the exoskeletons of arthropods, the cell walls of fungi, algae and protozoa, and the shells of bivalves. It has never been reported as a component of bacteria (Gooday, 1983). In 1991 it was estimated that the total production of chitin in the marine biocycle was at least 2.3 million tonnes per year (Jeuniaux and Voss-Foucart, 1991). The annual production of chitin in zooplankton alone is estimated to be several billion tons (Peter, 1995). Shigemasa *et al.*, (1998) stated that 150×10^3 tonnes of chitin was produced annually and in 1997 Stankiewicz *et al.*, estimated that the annual production in the biosphere was in the order of 10^{11} tons.

1.7 BIOSYNTHESIS OF CHITIN

Chitin in crustaceans is manufactured from blood glucose in the epidermal cells beneath the cuticle. The glucose is first phosphorylated, then aminated and N-acetylated to form N-acetylglucosamine. Uridine triphosphate reacts with the phosphorylated N-acetylglucosamine to form uridine diphosphate-N-acetylglucosamine, the chitin precursor. The UDP-glucosamine bond is a high-energy bond that provides the energy for the polymerisation reaction. The polymerisation occurs under the catalytic action of the enzyme chitin synthase but it is unclear whether the chitin is synthesised inside or outside the epidermal cells. Once the polymer has been formed and is outside the cell the polymeric chains have to be arranged into the chitin-protein microfibrils. It is thought that this occurs under the influence of the synthase enzymes taking up a particular spatial orientation (Salmon and Hudson, 1997).

This synthesis of chitin takes place several times throughout the life cycle of the prawn. *Nephrops norvegicus*, like all crustaceans, sheds its integument in order to increase in size. A new, larger cuticle is formed with room left inside for expansion. Each spiny larva formed moults through several stages before becoming a juvenile and then an adult Norway lobster (Ingle, 1997).

In the Irish Sea, sexually mature females moult between egg hatching and egg laying. Eggs are carried between September and April/May and they hatch between April and June. Moulting, followed by pheromone-encouraged copulation, takes place from May to August. The eggs are laid in August and September (Farmer, 1974).

This reproductive cycle may be temperature dependent. Spawn produced further north appear to have a longer incubation period than that produced in warmer water. This period varies from 7 months in the High Adriatic to 12-13 months in Iceland. This in turn has an effect on the moulting period. Both male and female Norway lobsters from

Galician waters have been shown to moult throughout the year in the laboratory and no particular seasonality has been noted (González-Gurriarán *et al.*, 1998).

Moulting, or ecdysis, occurs in various stages - pre-ecdysis, ecdysis and post ecdysis. It occurs under both hormonal and environmental control (reviewed by Chang, 1995). Methyl farnesoate (M.F.) produced by the mandibular organ increases the synthesis and/or secretion of moulting hormone (ecdysteroids) by the Y organ in the thorax. Moult inhibiting hormone (M.I.H.) produced by the X-organ in the sinus gland of the decapod eyestalk inhibits the process. External factors such as temperature, food supply, photoperiod and space all bear influence on the moult interval, the moult increment, or both.

1.8 THE MOULT CYCLE IN DECAPOD CRUSTACEANS

Changes in the crustacean integument due to stage of moult have been studied in several decapod crustaceans, for example the fiddler crab (Green and Neff, 1972), the European clawed lobster (Arsenault *et al.*, 1984) and the shore crab (Giraud-Guille, 1984; Compère and Goffinet, 1987; Compère *et al.*, 1998). The changes are also the subject of comprehensive reviews by Roer and Dillaman (1984 and 1993). The four main stages of the moult are pre-ecdysis, ecdysis, post-ecdysis and intermoult.

1.8.1 Pre-ecdysis

Moulting hormone (β-ecdysone) from the Y-organ in the thorax causes the secretory epidermal cells below the cuticle to become elongated and more structurally complex as shown in the fiddler crab. This complexity is caused by an increase in the levels of endoplasmic reticulum, Golgi apparatus, mitochondria and microtubules in the cells. The intraepidermal connective tissue cells also change in complexity (Green and Neff, 1972). An ecdysial cleft appears between the cuticle and epidermis. This separation of the cuticle

from the underlying cellular layer is known as apolysis. Moulting fluid is released from the epidermal secretory cells into the ecdysial space. This fluid contains enzymes that begin to solvate the membranous layer of the cuticle. Large vacuoles are seen at the apices of the epidermal secretory cells in the shore crab (Compère *et al.*, 1998). These are thought to contain the raw materials for the moulting fluid. At this stage the cytoplasmic extensions in the pore canals are lost but not before a partial resorption of the protein, chitin and calcium in the old cuticle takes place, initiated by the moulting fluid.

Precursor material, for example lipid, protein, chitoprotein and calcium, for the two outermost cuticular layers of the new cuticle are produced and stored by the intraepidermal connective tissue cells. The epicuticular secretory cells are responsible for the distribution of this material. Microvilli on the cuticle-secreting cells release these precursors, as shown in the fiddler crab (Green and Neff, 1972). Once outside the cell the precursors fuse to form the respective layers. The epicuticle and the exocuticle are formed completely before the old cuticle is shed. Hornung and Stevenson (1971) have shown that chitin synthesis starts to increase during pre-moult. This increase therefore correlates with the formation of the pre-exuvial cuticular layers.

1.8.1.1 Shell Resorption

During pre-ecdysis the moulting fluid aids in the resorption of the cuticular layers so that some of the components of the old cuticle can be recycled. Resorption involves the dissolution of the mineral component of the shell and the breakdown of the organic portion. Nicol *et al.*, (1992) have shown variations in the levels of chemical components of the Antarctic krill (*Euphasia superba*) at different stages of the moult cycle. For example, the levels of ash, lipid, chitin and calcium are at their lowest immediately after ecdysis, suggesting that dissolution of these substances has occurred.

Ecdysial droplets containing proenzymes are released from the epidermal cells as seen in the shore crab. The pore canals appear to be the route for these hydrolytic enzymes in some layers (Compère *et al.*, 1998). An increase in the activity of chitinase and N-acetyl-β-D glucosaminidase has been noted at pre-moult (Roer and Dillaman, 1993), as has the production of acid proteases and alkaline cysteine proteinases (O'Brien and Skinner, 1987 and 1988). These enzymes provide the means to break down the chitin and proteins in the shell. In the shore crab digestion of the chitin-protein complex appears to occur before the calcium carbonate portion of the shell is dissolved (Compère *et al.*, 1998). For the dissolution of the mineral elements H^+ ions are secreted into the moulting fluid. This causes the breakdown of $CaCO_3$ to Ca^{2+} and CO_3^{2-} or $H^+CO_3^{2-}$ (Roer and Dillaman, 1993). The calcium and bicarbonate ions are resorbed into the hypodermis. The concentrations of these ions increase in the hemolymph. It should be noted however that the majority of the calcium in marine crustaceans is lost rather than resorbed (Roer and Dillaman, 1984). *Carcinus maenas*, for example, loses 92% of its body calcium at ecdysis. This deficit is restored by exogenous sources, for example calcium from food and water.

1.8.2 Post-ecdysis

After moult, the remaining layers of the shell, the endocuticle and membranous layer, are formed again from precursors secreted by the epidermal cells (Green and Neff, 1972). Calcification and tanning of the appropriate layers also occur post moult as discussed below. The epidermal secretory cells and the connective tissue cells shorten and lose their structural complexity (Green and Neff, 1972) and the animal enters intermoult - the fully formed shell stage.

1.8.2.1 Calcification and Tanning.

Cuticular hardening in crustaceans is caused mainly by calcification of the integument. This cannot be allowed to occur until ecdysis has taken place and the animal has expanded by the intake of water. For this reason the pre-exuvial layers, i.e. the layers formed post-moult, are not calcified as they are formed but instead calcium is added to them post-moult. The calcium used comes from two different sources - (1) exogenous calcium i.e. calcium taken up from food and water and (2) endogenous calcium i.e. calcium resorbed during pre-moult into storage sites (Roer and Dillaman, 1984). Calcium flux across the epithelial layer in crustaceans does differ depending on species and the habitat i.e. marine or freshwater, however some form of active transport plays a part (Neufeld and Cameron, 1993).

Calcification occurs under the action of the enzymes alkaline phosphatase, phosphorylase and carbonic anhydrase. All three are found in the epidermis and the calcified layers but decrease in amount as calcification increases (Chockalingam, 1971). It is thought that phosphorylase produces phosphate esters from glycogen. The alkaline phosphatase uses these esters to produce a localised high concentration of phosphate ions. These react with the calcium salts released from the epidermis to produce calcium phosphate. Likewise carbonic anhydrase produces calcium carbonate from calcium salts and CO_2 (Chockalingam, 1971). The underlying reaction taking place is $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$. The bicarbonate comes from the external solution or hydration of metabolic CO_2 (Neufeld and Cameron, 1993).

Mineralisation of the epicuticle and exocuticle begins at the start of the post moult period. Calcium carbonate is first deposited in the most external regions of the shell. The calcium salts are transported to the epicuticle and exocuticle, from the epidermis, through the pore canals as strings of granules (Chockalingam, 1971). Pore canals have not been found in the cuticle of larval crustaceans. The larval cuticle is very thin and has little

tanning and calcification. Therefore it has been suggested that pore canals are not present because little material has to be transported to the outer layers of the cuticle (Freeman, 1993).

The endocuticle is calcified directly from the epidermis at the same time as the lamellar sheets are laid down (Chockalingam, 1971). This is possible because the endocuticle is formed post-moult rather than pre-moult. The mineral eventually invades the walls of the pore canals and the lumina of the canals. The cell extensions are replaced with mineral. Calcification deposition stops at the end of the post moult period when the non-calcified membranous layer is laid down (Roer and Dillaman, 1984).

Biomineralisation usually takes place by nucleation of an organic matrix, containing acidic proteins and glycoproteins, with crystals of $CaCO_3$. The proteins and glycoproteins act as a template for nucleation. Compositional changes, which allow mineralisation to occur, have been shown to take place at ecdysis. For instance, changes in proteins and glycoproteins present in the cuticle have been shown to differ most between late pre-ecdysis (stage D_4) and early post-ecdysis (stage A_1) (Roer and Dillaman, 1993). However, Manoli *et al.*, (1997) have shown that a chitin substrate favours the deposition of calcite crystals so perhaps it is the chitin surface that allows mineralisation to occur.

Another post moult occurrence is the tanning of the shell. It also contributes to the hardening of the cuticle (reviewed by Roer and Dillaman, 1993). The enzyme polyphenol oxidase is transported to the cuticle from the epithelial cells. Under the action of polyphenol oxidase, dihydroxyphenols are converted to quinones. The quinones act to cross-link proteins.

1.9 CHITIN

Chitin is considered to be a linear, unbranched polymer made up of N-acetyl D-glucosamine monomers. In reality up to one in six glucose residues may be unacetylated

(Giles *et al.*, 1958). The subunits are joined by β-(1→4) glycosidic bonds. Every sugar residue is flipped 180° with respect to its neighbour so that the repeat segment is a chitobiose dimer (Fig. 1.3).

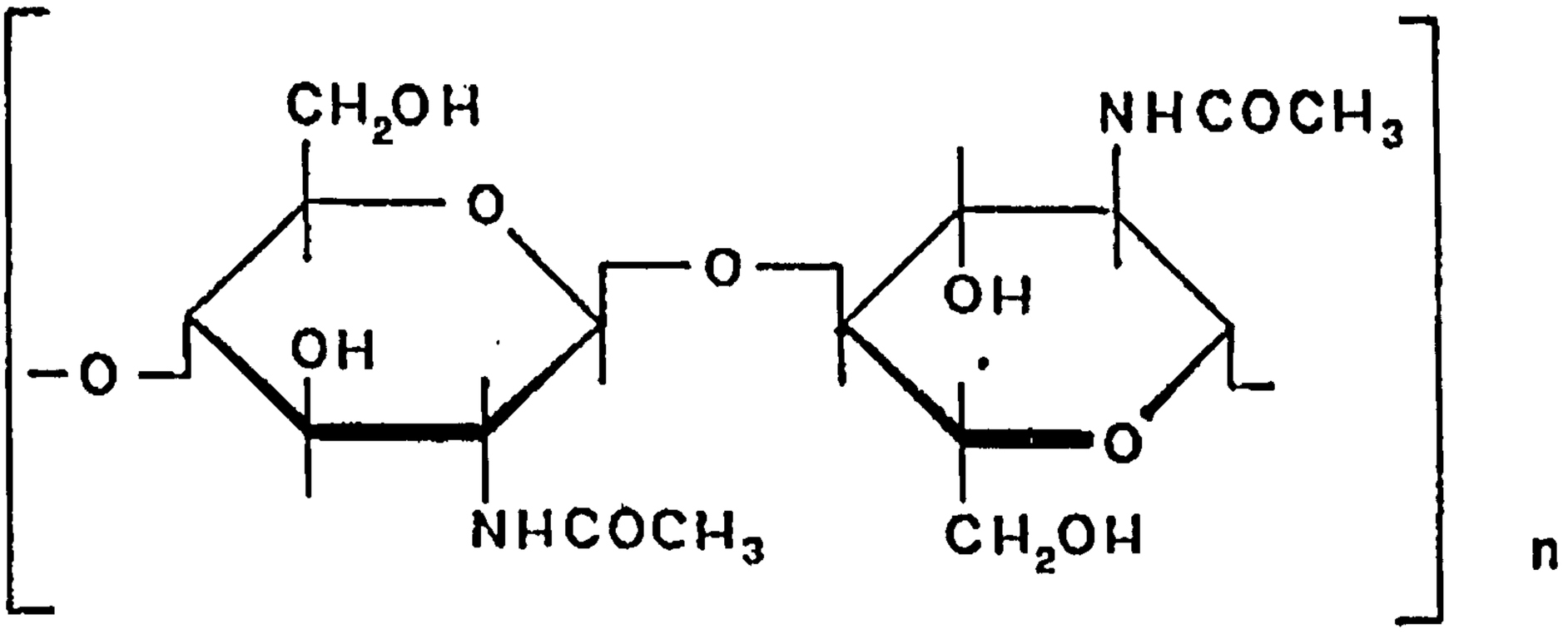


Fig.1.3 *The repeating subunit of chitin.*

The chitin residues are restricted to the low energy chair conformation i.e. the hydrogens occupy axial positions and all other groups equatorial positions (Fig. 1.4).

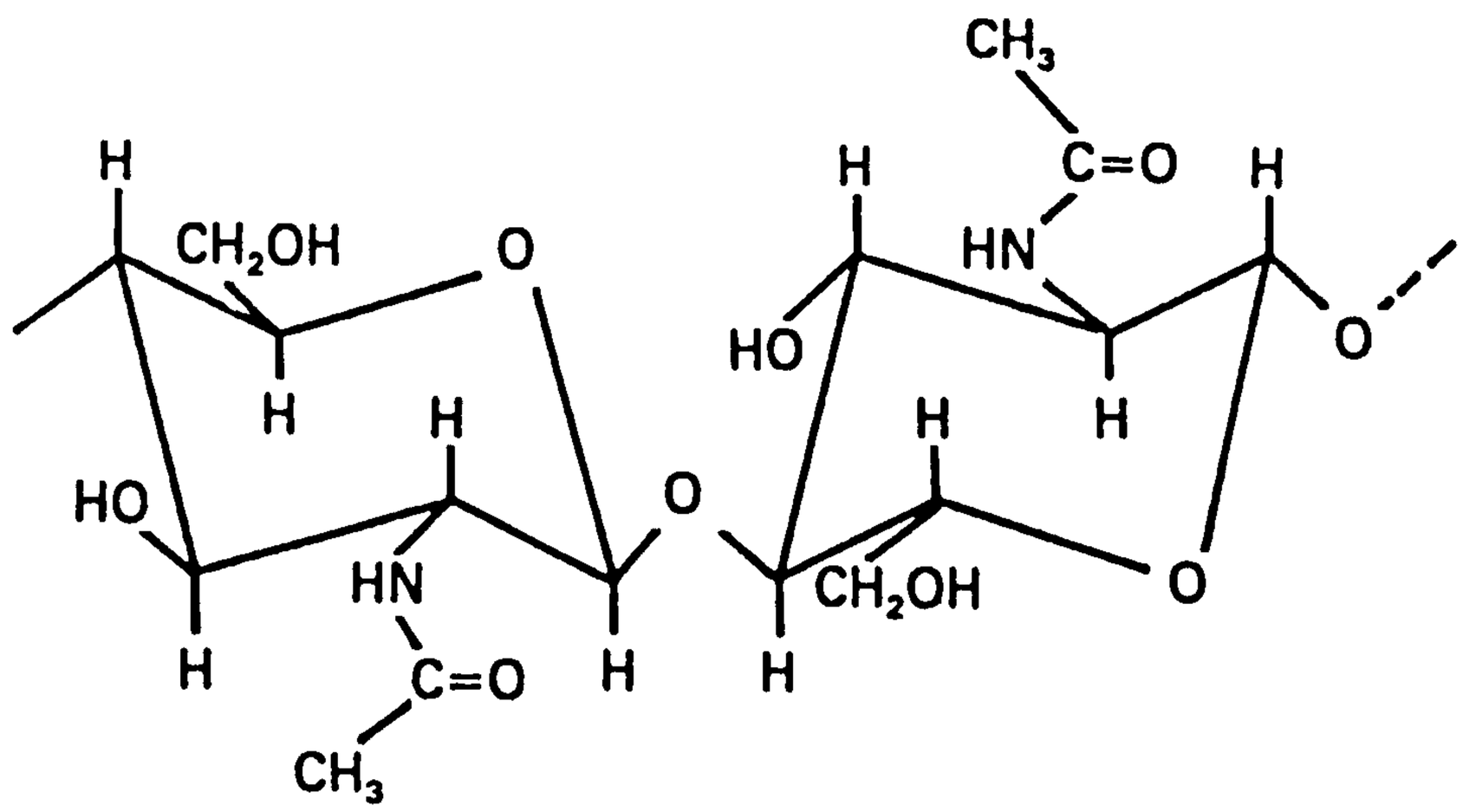


Fig. 1.4 *The low energy chair conformation of chitin.*

A hydrogen bond extends from the O(3')-H hydroxyl to the O(5) ring oxygen of the next unit across each β-1→4 -glycosidic linkage so that the chair is held in rigid position. The

backbone of the polymer is therefore also rigid and linear (Salmon and Hudson, 1997). The N-acetyl groups at the C(2) position produce hydrogen bonds in 3 dimensions so that chitin has a stack like morphology. Cellulose, the most abundant naturally occurring polysaccharide possesses a very similar structure to chitin, differing only at the C(2) carbon atom. At this position cellulose has a hydroxy group whereas chitin has an acetamido group (Fig. 1.5).

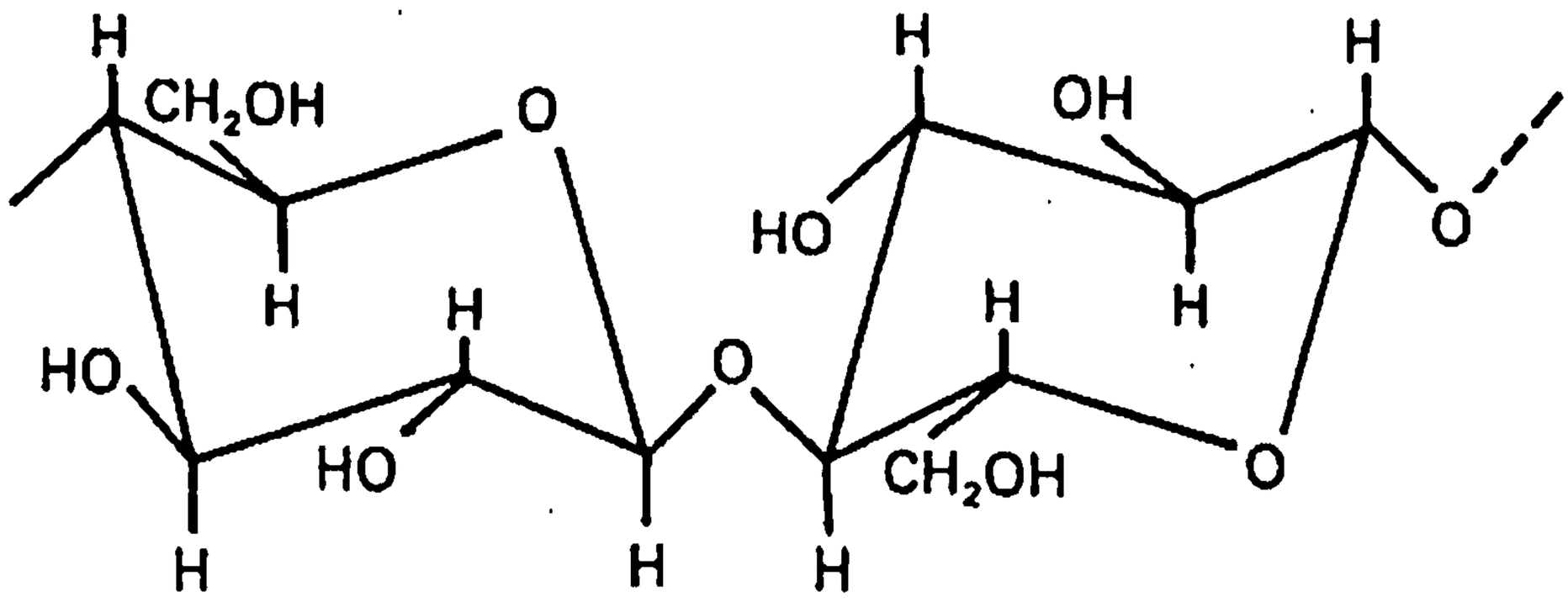
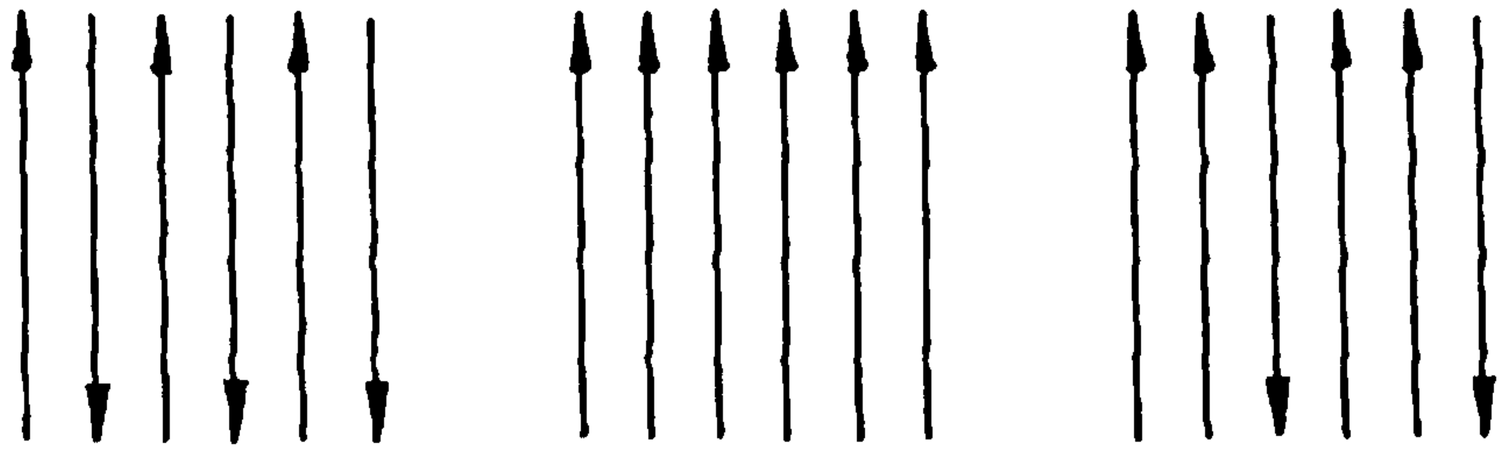


Fig. 1.5 *The repeating subunit of cellulose.*

X-ray diffraction studies have shown that chitin is a very ordered, crystalline structure. Three different polymorphic forms, as shown in Fig. 1.6, have been distinguished by differences in their crystal diffraction patterns (Rudall and Kenchington, 1973). They differ in the arrangement of the chains in the crystalline regions. α-Chitin contains anti-parallel chains, β-chitin contains parallel chains and in γ-chitin two chains up alternate with one chain down (Rudall, 1963).



α-chitin **β-chitin** **γ-chitin**

Fig. 1.6 *Arrangement of the polymer chains in the three forms of chitin* (Roberts, 1992).

All three polymorphs have been found in a single organism, for example the squid *Loligo* (Rudall, 1963). α-Chitin, the type present in decapods such as *Nephrops norvegicus*, is the most abundant form and also seems to be the most stable. The other two can be converted irreversibly to the α form by treatment with 6M HCl in the case of β (Rudall, 1963) and treatment with LiSCN in the case of γ (Rudall and Kenchington, 1973). β- and γ- chitin are found where flexibility and toughness are required whereas α-chitin is found where extreme hardness is required e.g. arthropod cuticle (Roberts, 1992). Chitin is generally found in a complex with other substances. Fungal chitin is found in association with other polysaccharides while exoskeletal chitin in insects and crustaceans is usually located in a complex with protein. Blackwell and Weih (1980) have suggested a model for the chitin-protein microfibril structure based upon x-ray diffraction studies of the Ichneumon fly *Megarhyssa*. An α-chitin core consisting of 18-20 chitin molecules running antiparallel to each other is surrounded by a helix of protein subunits (Fig. 1.7).

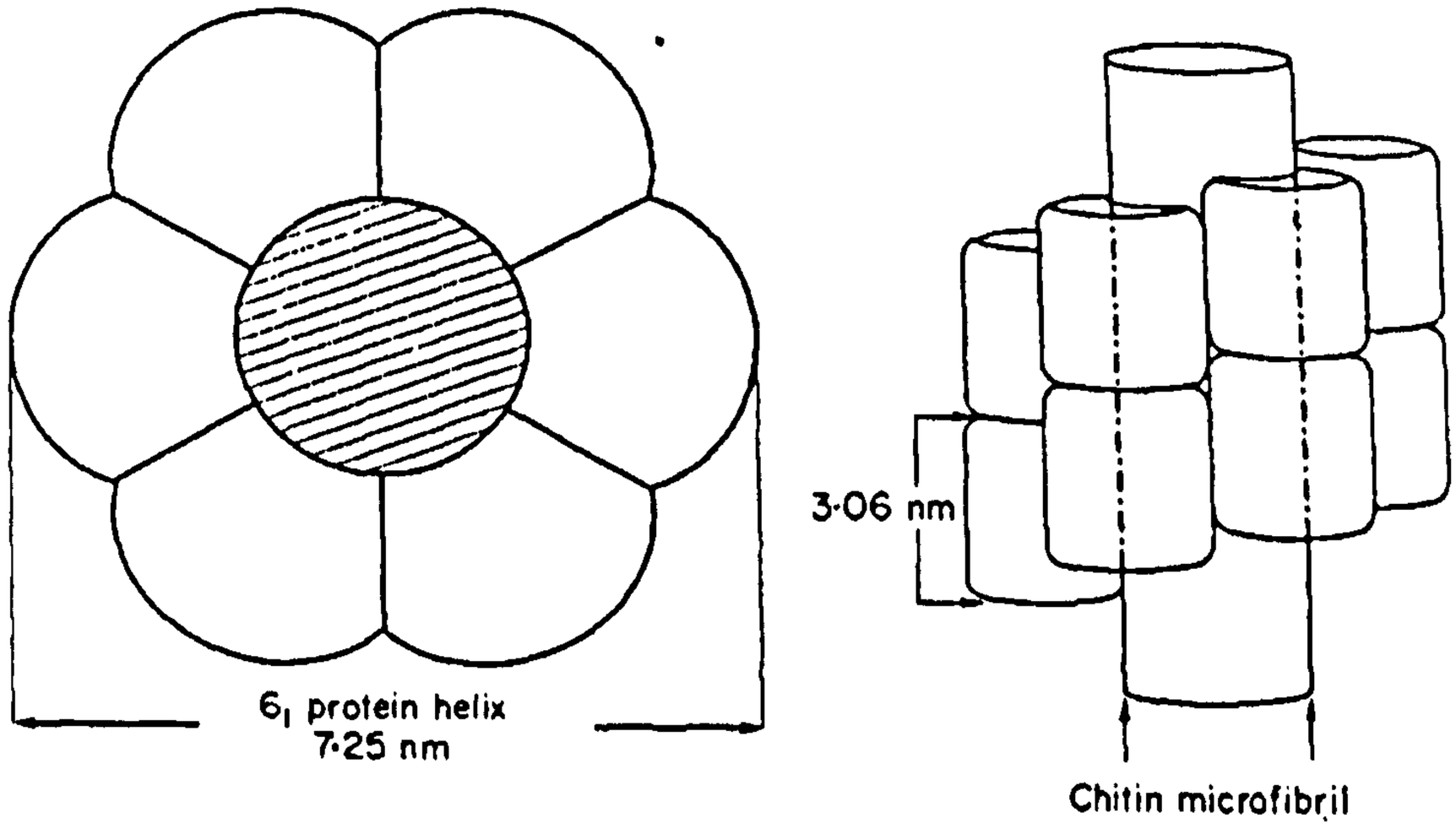


Fig. 1.7 *Arrangement of the protein subunits around the chitin core in the microfibril viewed (a) perpendicular to the fibre axis, and (b) along the fibre axis* (Blackwell and Weih, 1980).

The protein matrix is believed to protect the chitin against the action of chitinases. Some of the protein is attached by weak forces and some by covalent bonds (Hackman and Goldberg, 1958). The chitin-protein link does not appear to be universal. Hackman (1960) hydrolysed shell, previously deproteinised by treatment with 1M NaOH at 100°C, in 5.7M HCl at 105°C. Two amino acids remained - aspartic acid and histidine. These residual amino acids were thought to be involved in the covalent linkages between chitin and protein. Other studies have however shown alternative residual amino acids. For instance Brine and Austin (1981b) found aspartic acid, glycine and serine to be the main amino acids remaining after studying chitin from five different sources.

1.10 CHITOSAN - A DERIVATIVE OF CHITIN

α-Chitin is a very inert substance. It generally shows poor solubility in dilute acids, cold alkalis and in most organic solvents (Muzzarelli, 1973) and is very rigid and brittle. Some of the solvents available are hot concentrated solutions of neutral salts, for example LiCNS; acid solvents such as concentrated HCl, H_2SO_4, H_3PO_4 but not HNO_3 and some

organic solvents, for example N,N-dimethylacetamide-lithium chloride (DMAc)-LiCl and N-methyl-2-pyrrolidone (NMP)-LiCl (Rutherford and Austin, 1978). This limited solubility of chitin makes it difficult to work with and cuts down on the number of potential applications that otherwise would have been available.

The inertness of chitin can be overcome by the conversion of chitin to its main derivative - chitosan. Chitosan (Fig. 1.8) possesses the same structure as chitin (Fig. 1.9) except that the acetyl group at the C(2) carbon atom has been removed leaving a free amine group. This amine group accounts for the higher reactivity of chitosan compared with chitin.

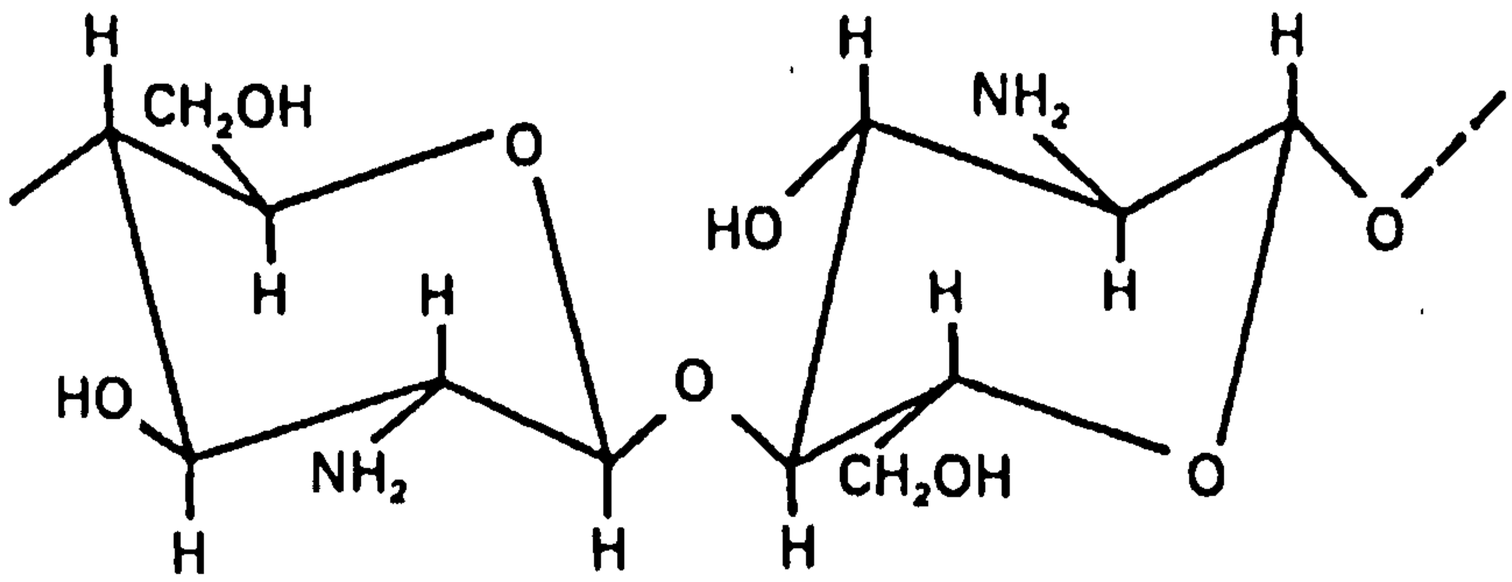


Fig. 1.8 *The structure of chitosan.*

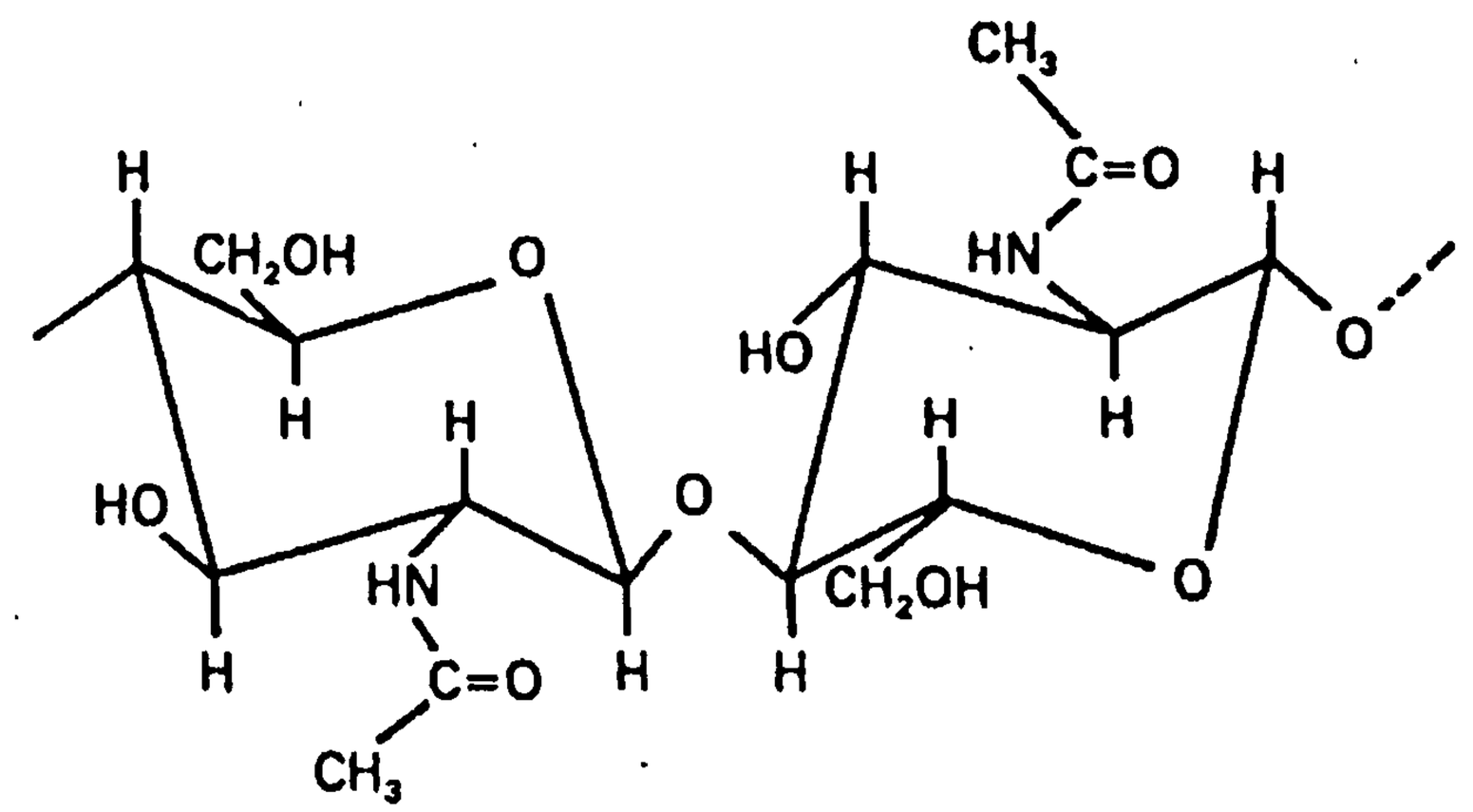


Fig. 1.9 *The structure of chitin.*

Chitosan occurs naturally only in the hyphal walls of zygomycete fungi such as *Mucor* species (Bartnicki-Garcia, 1968) but is obtained commercially by partial, chemical N-deacetylation of chitin. Neither chitin nor chitosan can be thought of as single materials with a defined composition. Chitin is not normally 100% acetylated, nor is chitosan usually 100% deacetylated. Instead there is a gradient of change somewhere between these two extremes and the terms chitin and chitosan describe co-polymers of N-acetyl D-glucosamine and D-glucosamine. For this reason chitin and chitosan are not normally distinguished by the percentage of deacetylated groups but instead by their solubility in dilute, aqueous acids (Muzzarelli, 1977). Chitosan dissolves as a polycation in dilute acids whereas chitin is insoluble.

1.11 APPLICATIONS OF CHITIN AND CHITOSAN

Chitin and chitosan perform several chemical reactions that make these substances appropriate for use in many different industries. Hackman (1955) was the first to show that chitin could adsorb proteins from aqueous solution. Many studies since then have applied this ability. Skaugrud and Sargent (1990) stated that chitosan could be used as a flocculent to recover proteins generated in the food processing industry. Hayashi and Ikada (1991) successfully immobilised papain, ficin and bromelain onto the surface of porous chitosan beads by covalent fixation. Ohashi and Koriyama (1992) immobilised glucose oxidase on a chitin membrane and used this in conjunction with an oxygen electrode to measure glucose in a fermentation system.

Chitin and chitosan also form complexes with metal ions, particularly transition metals and post-transition metals (reviewed by Muzzarelli, 1973 and 1977). The metal ions complex with free amine groups so that the greater the level of deacetylation the more metal ions are taken up (Roberts, 1992). Hence, chitosan is more active than chitin in this capacity. In one study making use of this ability, chitosan, graft-co-polymerised with

alkenedioic acids to give it an amphoteric flocculent character, was used in the purification of wastewater contaminated with metal ions (Kim *et al.*, 1998).

Another useful property of chitin and chitosan is the ability to adsorb dyes. This was first shown by Giles *et al.*, (1958b). It was found that as the pH decreased more dye was adsorbed. It is therefore assumed that ion exchange is the principal mechanism of dye uptake. At lower pH values more amine groups are protonated and therefore more sites are available at which ion exchange can take place. Since then studies have been carried out using synthetic dyes to dye natural fibres treated with chitosan and chitin (Rippon, 1984; Davidson and Xue, 1994). Chitin has also been proposed as an adsorbent to treat textile effluent (McKay *et al.*, 1987 and Kumar, 2000).

Chitosan has been used in the food industry to coagulate cheese whey solids (Wu *et al.*, 1978) and to clarify beverages (Imeri and Knorr, 1988). In Japan, where food and drug regulations are not so strict, chitin and chitosan have been approved for use as food additives (Skaugrud and Sargent, 1990).

Chitin and chitosan are both reported to be biodegradable, biocompatible and non-toxic (Agboh and Qin, 1997). Research into applications in medical fields is therefore widespread. In Japan both polymers appear to have been used in many biomedical fields to treat ailments such as high blood pressure, high blood cholesterol, angina, diabetes, constipation, cancer, baldness and senile dementia to name but a few (Asaoka, 1996). Other applications reported include wound dressings, topical treatments for dermatitis, artificial skin for burns and filling agents at the site of tumour removal (reviewed by Shigemasa and Minami, 1995).

Chitin and chitosan have been converted into suitable forms for use as biomaterials - flakes, powders, suspensions, cottons, sponges, membranes, sticks, tablets, papers, composites and gels (Shigemasa and Minami, 1995). Bioadhesive tablets for intraoral drug delivery have been prepared by compressing the drug with a mixture of sodium alginate

and chitosan (Miyazaki *et al.*, 1994). A sustained release of drug three hours after administration was obtained for some combinations of alginate and chitosan. Carboxymethylchitin discs have been tested as a bone substitute material to encourage calcification and therefore biointegration (Wan *et al.*, 1996). Chitosan membranes and chitosan-ascorbic gels in contact with the dura mater in the brain stimulated connective tissue rebuilding, mimicking the action of glycosaminoglycans (Muzzarelli *et al.*, 1988).

The reactive nature of chitosan, compared to chitin, due to the presence of free amide groups, makes it possible to crosslink chitosan with other molecules such as polyamides (Ratto *et al.*, 1996), proteases (Hayashi and Ikada, 1991) and poly(vinyl alcohol) (Blair *et al.*, 1987). Chitosan with wide ranging properties can thus be produced. Amiji (1995) produced chitosan-poly(ethylene oxide) blend membranes for haemodialysis. The poly(ethylene oxide) increased the permeability of the chitosan to urea and reduced platelet adhesion and activation which are common problems in membranes for haemodialysis. N-carboxybutyl chitosan forms a gel in contact with wound fluids. It led to the formation of regularly organised cutaneous tissue and reduced anomalous healing (Biagini *et al.*, 1991). A co-matrix of chitosan/polyethylene vinylacetate has been used to release aspirin-heparin to prevent cardiovascular thrombosis (Vasudev *et al.*, 1997).

The antibacterial properties and film/gel forming capabilities of chitosan make it a potential candidate for use in prosthetic devices. Use of current devices results in complications due to bacterial adherence (Mawhinney *et al.*, 1992; Keane *et al.*, 1994; Tunney *et al.*, 1996a) and encrustation due to build up of salts (Keane *et al.*, 1994; Tunney *et al.*, 1996b and 1996c; Winn, 1998). New biomaterials with which these implants can be manufactured or coated are being sought and chitosan could provide the answer.

Whilst widespread research continues in biomedical applications caution should be exercised. Chitosan can bind dietary lipids and is sold as a food supplement. However, reductions in bone mineral content and growth retardation have been shown in rats after

administration of chitosan supplements (Koide, 1998). It is also possible that prolonged ingestion may alter the normal flora of the intestine (Koide, 1998). Peluso *et al.*, (1994) reported that chitosan stimulates macrophages to produce nitric oxide. This could amplify the inflammatory process and lead to the destruction of an implant and surrounding tissue. Carreño-Gómez and Duncan (1997) showed that varying the chitosan salt used and the molecular weight of the chitosan employed can enhance cytotoxic properties towards a cell line. However, in contrast to these *in vitro* results, *in vivo* experiments on systemic toxicity, animal pyrogen testing, haemolysis, sterility tests and intramuscular implantation studies indicate that chitosan is non-toxic (Rao and Sharma, 1997). Landes and Bough (1976) showed that whilst chitosan included in rat diets at a level of 15% caused enlargement of the liver and kidney, no adverse effects were seen at levels of 5% or less. Further study is needed to ensure that chitosan does indeed meet regulatory requirements for use as a biomaterial.

1.12 TRADITIONAL ISOLATION OF CHITIN FROM SHELL

Chitin is produced commercially in India, Japan, Poland, Norway and Australia (Kumar, 2000). The primary sources of chitin in industry are waste crab and krill shells from the fishing industry (Peter, 1995). The isolation of chitin from shell waste involves two major steps, demineralisation and deproteinisation, to remove the other main components of the shell. The quality of the chitin obtained depends both on the source of the shell and on the variables employed within each step. No and Meyers (1995) have written a comprehensive review on the traditional techniques used.

Demineralisation of the shell involves removal of calcium carbonate and calcium phosphate. Hydrochloric acid at a concentration of approximately 1 - 2M and at room temperature is usually the reagent chosen (No and Myers, 1995). However, other reagents have been successfully employed. Foster and Hackman (1957), and Brine and Austin

(1981a and b) used ethylene-diamine tetra-acetic acid (EDTA) on blue crab (*Callinectes sapidus*), horseshoe crab (*Limulus polyphemus*), stone crab (*Menippe mercenaria*) and red crab (*Geryon quirquendons*). BeMiller and Whistler (1962) used 37% HCl at -20°C to demineralise lobster shell. Lobster shell has also been treated with 90% formic acid and 22% HCl at room temperature by Horowitz *et al.*, (1957).

For deproteinisation NaOH or KOH are usually the reagents of choice. Hackman in 1954 used repeated treatments of 1N NaOH at 100°C. Horowitz *et al.*, (1957) used 10% NaOH over a steam bath for 2.5h. BeMiller and Whistler (1962) used 10% NaOH at room temperature for 3 days. Other reagents have also been employed. Giles *et al.*, (1958a) deproteinised *N. norvegicus*, the common crab, *Cancer pagurus*, and the edible lobster by boiling in 1% aqueous sodium carbonate, and compared this with an enzymatic removal of protein using 0.5% pepsin. Takeda and Abe (1962) also used an enzymatic method to deproteinise king crab (*Paralithodes camtschatica*). Shimahara and Takiguchi (1988) employed proteolytic bacteria.

After removal of the minerals and proteins the shell lipids and pigments are removed by extraction with chemicals such as acetone, ether, ethanol or hypochlorite to give a chitin, almost white in colour.

The reagent of choice and its concentration are not the only variables in the extraction procedure. Various workers have employed different temperatures, lengths of time and solid:liquid ratios in an attempt to find the optimum conditions (Chang and Tsai, 1997; Madhavan and Ramachandran Nair, 1974; Shahidi and Synowiecki, 1991 and No *et al.*, 1989). The alteration of each of the variables can contribute to the degradative nature of the processes. For instance, the use of EDTA to demineralise the shell leads to the production of a less degraded product than 1M HCl as measured by the average molecular weight (Brine and Austin, 1981a). The amount of acid present in the demineralisation step must be enough to react with all the minerals in the shell to cause complete

demineralisation, so the solid:liquid ratio must be taken into account (Shahidi and Synowiecki, 1991). Increasing the temperature in either step may also affect the product formed, especially if the length of time of reaction is prolonged (No and Meyers, 1995).

Brine and Austin (1981a) have shown, in their studies with blue crab, stone crab and red crab that the chitin produced varies with species as well as with method of isolation. For instance, when 1N HCl at room temperature for 3 hours was used to demineralise blue crab, the chitin produced had an average molecular weight of 0.56 x 10^6. It was 40% soluble in lithium chloride-dimethylacetamide (LiCl-DMAc). On the other hand, treatment of red crab by the same method resulted in chitin with an average molecular weight of 0.92 x 10^6. It was 71% soluble in LiCl-DMAc.

Chitin has been extracted from many different crustaceans, for example crabs (Brine and Austin, 1981a and b), pink shrimp (*Solenocera melantho*) (Chang and Tsai, 1997), prawn shell (*Metapenaeus dobsoni*) (Madhavan and Ramachandran Nair, 1974), Louisiana crawfish (No *et al.*, 1989) and freshwater crayfish (Lovell *et al.*, 1968) using a wide range of chemical treatments. Each of these methods produces a large volume of aqueous waste. This waste is difficult to dispose of and due to the harsh nature of the chemicals involved, proteins removed from the shell are degraded and difficult to recover. Novel biotechnological fermentation methods, however, produce less waste and allow the majority of the shell by-products, for example proteins, pigments and chitin, to be recovered.

1.13 FERMENTATION

Fermentation is strictly defined as the catabolism of organic compounds in the absence of oxygen. To the industrial biotechnologist, however, fermentation involves the application of anaerobic or aerobic microorganisms in the manufacture of valuable products. The earliest record of fermentation is the conversion of sugar to alcohol under the action of

yeasts, particularly *Saccharomyces cerevisiae*. This method is still used in the brewing industry. Another example is the production of acetone and butanol, using *Clostridium acetobutylicum*, used by the British during the 2nd World War. This method survived until these products became available as by-products from the petroleum industry. Another example is the production of lactate by lactic acid bacteria. This fermentative path is in widespread use in many fields today, especially in the human and animal food industries. It has also been used as an alternative to the traditional chemical demineralisation treatments in the extraction of chitin from shell waste (Hall and De Silva, 1992; Healy *et al.*, 1992).

1.13.1 Lactic Acid Fermentation

In all the applications for lactic acid the same essential principle applies, that of food preservation. Lactic acid producing bacteria are provided with a suitable source of carbohydrate e.g. lactose in milk, cellulose in grass, or any available sugars such as molasses, cassava (Fagbenro and Bello-Olusoji, 1997) or even rejected candy bars (Deshmukh and Patterson, 1997b) if the substrate does not possess its own source of carbohydrate. The cruder forms of carbohydrate may carry the inoculant required. The bacteria convert the carbohydrate to lactic acid that in turn reduces the pH of the system to less than pH 4.5. This leads to a reduction in the number of spoilage and pathogenic organisms such as coliforms, salmonella and staphylococci, clostridium species and lysteria species and the material in question is effectively preserved. The lactic acid bacteria themselves can tolerate a high level of acidity but are inhibited at pH levels below 4.5. The bacteria may also produce antimicrobial agents such as bacteriocins, which are antibacterial peptides (Jeppesen, 1993), hydrogen peroxide and acetoin (Zakaria *et al.*, 1998; Hugas and Monfort, 1997). These can aid in the preservation process by helping to reduce the numbers of spoilage/pathogenic organisms present.

In the dairy industry lactic acid production was originally brought about by the action of indigenous lactic acid bacteria in milk. However, since milk is now normally pasteurised before consumption, a starter culture of lactic acid bacteria is added to the milk for the production of cheese, yoghurt and sour cream. These bacteria act on the milk sugar, lactose, to produce lactic acid and lower the pH. At the same time proteolytic enzymes of the bacteria hydrolyse milk proteins, e.g. alpha and beta casein to small peptides and amino acids (El Soda *et al.*, 1986). Free amino acids contribute to the flavour development of some products, for example, cheese (Puchades *et al.*, 1989).

Meat starter cultures have evolved in the last forty years (Hugas and Monfort, 1997) and meat fermented products such as salami are treated in a similar manner to dairy produce. The inoculants contain a mixture of lactobacillus, pediococcus, staphylococcus and micrococcus species. The lactobacilli/pediococci act to produce lactic acid and therefore aid in the preservation of the meat by lowering pH (Smith and Palumbo, 1983). The staphylococci/micrococci reduce nitrate to nitrite. Nitric oxide, produced from nitrite, converts myoglobin to nitrosomyoglobin. This is required for the development of red colour (Johansson *et al.*, 1994). Proteolysis, lipolysis and formation of volatile components also occur during the ripening of some meats (Johansson *et al.*, 1994). This is due in part to the tissue enzymes and partly to the bacteria (Hugas and Monfort, 1997; Fadda *et al.*, 1998).

Another similar process is that of silage manufacture (reviewed by Muck, 1988). The crop is kept in an anaerobic environment in a silo. Plant enzymes and acid hydrolysis break down plant structural carbohydrates e.g. hemicelluloses to water-soluble carbohydrates (WSC). Any indigenous lactic acid bacteria convert the WSC to organic acids. The number of indigenous lactic acid bacteria present may be low so the crop is often sprayed with an inoculant containing lactic acid producing microorganisms in an attempt to increase the inoculant concentration of the silage. New inoculants are

continuously being developed. In some studies an organic acid, such as formic acid, has been used to lower the pH at the start of fermentation (Muck, 1988). The acid prevents proliferation of unwanted indigenous bacteria but may be detrimental to the fermentation.

Other potential foods for livestock such as poultry waste (Deshmukh and Patterson, 1997a and b) have been ensiled with lactic acid bacteria or an inorganic/organic acid. Poultry waste is highly perishable. Therefore, if it is to be recycled for use in feeds, it needs to be preserved. Deshmukh and Patterson (1997a) have ensiled poultry waste using a mixed inoculant of *Lactobacillus plantarum*, *Streptococcus faecium* (now known and referred to hereafter as *Enterococcus faecium*) and *Pediococcus acidilactici*. The fermented product was dried and successfully used in feeding trials for broiler chicks (Deshmukh and Patterson, 1997b). In a similar trial Sander *et al.*, (1995) showed that protein, fat, ash and moisture levels changed little during the lactic acid fermentation and so gave a product suitable for use in animal feed.

1.13.2 Fermentation of Fish Waste

Fish waste is ensiled in a similar manner (Raa and Gildberg, 1982; Adams *et al.*, 1987; Samuels *et al.*, 1991; Faid *et al.*, 1997; Hassan and Heath, 1986; Jackson *et al.*, 1984; Lindgren and Pleje, 1983; Tatterson and Windsor, 1974). Its high protein content makes it a useful supplement for animal feeds. Ensiling has been proposed as an alternative to the production of fishmeal for animal food where high drying costs are incurred (Samuels *et al.*, 1991; Faid *et al.*, 1997; Martin, 1996). Fish waste is highly perishable but if the pH can be lowered quickly to less than 4.5 the action of spoilage organisms is inhibited. Lactic acid bacteria are present in fish waste but in very low numbers. Many studies show the benefits of reducing the pH by using an additive of lactic acid bacteria (Hassan and Heath, 1986; Lindgren and Pleje, 1983), lactic acid bacteria plus yeast species (Faid *et al.*, 1994; Faid *et al.*, 1997) or inorganic/organic acid, for example formic acid or sulphuric

acid (Samuels *et al.*, 1991; Jackson *et al.*, 1984). The advantage of using an organic acid rather than an inorganic one is that the silage does not have to be neutralised before use in animal feed (Tatterson and Windsor, 1974). However, addition of lactic acid producing microorganisms instead leads to the added benefits of an end product with a higher nutritive value and the possible production of biopreservatives. Some studies use an acid, at the same time as an inoculant, to reduce the pH at the beginning of the fermentation. This helps to prevent the growth of indigenous pathogenic or spoilage organisms before the pH begins to fall, however the acid can inhibit the fermentation (Samuels *et al.*, 1991).

Fish waste contains a low free sugar content. Therefore, for lactic acid bacteria to cause fermentation, a carbohydrate must be added to the waste. A range of different carbohydrate sources have been added to different fermentation systems e.g. glucose (Adams *et al.*, 1987), lactose (Hassan and Heath, 1986), cane molasses (Faid *et al.*, 1997), whey (Van Wyk and Heydenrych, 1985), refined sugar (Van Wyk and Heydenrych, 1985) and dextrose (Lassén, 1995). An inexpensive carbohydrate source will reduce the overall cost of the system. The inoculant used needs to be matched to the chosen carbohydrate and the raw material. For instance Giurca and Levin (1993) found that only one species of bacteria out of a commercial mixed culture was able to efficiently utilise the carbohydrate they had chosen.

1.13.3 Fermentation of Shell Fish Waste

Fish processing wastes from the shellfish industry have also been ensiled to form an alternative to fish meal. In some cases, the crustacean waste, for example crab shell, is mixed with low quality roughage before ensilation. Disposal of these roughages can be expensive and they can boost the nutritive value of the fish waste (Samuels *et al.*, 1992; Abazinge *et al.*, 1993; Evers and Carroll, 1996). Fagbenro and Bello-Olusoji (1997)

ensiled river prawn (*Macrobrachium* vollenhovenii) with *Lactobacillus plantarum* and cassava. The resulting liquid silage was used as a protein supplement for catfish.

Hall and De Silva (1992) adopted the process of lactic acid fermentation to isolate chitin from tropical prawn waste, *Penaeus monodon* and cold-water prawn waste, *Nephrops norvegicus* (Hall *et al.*, 1994). Lactic acid bacteria were added to minced prawn shell and prawn head. Glucose was added to supply the bacteria with carbohydrate. The added inoculant used the carbohydrate to produce lactic acid. The lactic acid dissolved the calcium carbonate in the shell, mimicking the action of 1M HCl in the chemical demineralisation of shell. Healy *et al.*, (1994) considered a similar strategy advocating the recovery of protein and astaxanthin as by-products of chitin isolation.

During ensilation of fish/fish waste any proteins present are hydrolysed by tissue proteases such as cathepsins, gut proteases (Lindgren and Pleje, 1983) and by proteolytic microorgansisms (Faid *et al.*, 1997) to short peptides etc. This results in a liquid silage, rich in protein/amino acids. When whole shrimp waste is ensiled, i.e. shell and head, enzymes from the digestive system are present in the head. They are released and can act on any protein present in the fermentation system. Zakaria *et al.*, (1996) have achieved a 77% (w/w) solubilisation of protein in whole prawn waste using lactic acid fermentation and the action of indigenous enzymes.

Bacteria, other than lactic acid bacteria, for example *Bacillus*, *Proteus* and *Pseudomonas* species, are more proteolytic than lactic acid bacteria. Some of these species may be indigenous to the shell and will therefore act alongside proteolytic enzymes at the start of fermentation. However, once the pH drops they will no longer be active. Lactic acid bacteria do have proteolytic equipment (Law and Kolstad, 1983) but this may need to be induced. In dairy products the lactic acid bacteria have to degrade the milk proteins, alpha and beta casein, proteolytically, to get enough amino acids and peptides to support their growth in milk. Different strains and species of lactic acid bacteria used in milk

production show different proteolytic abilities (Farkye *et al.*, 1995; Exterkate and Alting, 1995; Frey *et al.*, 1986). Various proteolytic enzymes, for example cell wall bound proteinases, endopeptidases and dipeptidases (Bockelmann, 1995) are situated in different parts of the bacterial cell. Proteinases are found outside or near the cell wall surface making them accessible to large molecules such as proteins. Peptidases are situated inside the cell or are membrane bound making them accessible to peptides that can diffuse into the cell. Some of these enzymes may also be released into the cell media (Thomas and Mills, 1981). Law and Kolstad (1983) have suggested that outside influences, such as media components, can lead to the differential stimulation of the proteolytic enzymes. Bockelmann (1995) also stated that the growth medium used probably determines the proteolytic activity obtained.

Most of the lactic acid bacteria characterised so far on a proteolytic basis are those used in the dairy industry, for example strains of *Lactococcus lactis* and *Lactobacillus delbrueckii*. Nevertheless other strains of lactic acid bacteria may be similarly affected by their growth medium and contain comparable proteolytic systems. Starter cultures used in meat often contain *Lactobacillus* and *Pediococcus* species (Johansson *et al.*, 1994). The meat proteins are broken down by the proteolytic systems of these bacteria as well as by meat enzymes (Fadda *et al.*, 1998). Therefore, based on this evidence, it is feasible that lactic acid bacteria could act to deproteinise crustacean shell if induced properly. However, most studies have used proteolytic bacteria or proteolytic enzymes to fulfil this function.

Shimahara *et al.*, (1984) used *Pseudomonas maltophilia* LC102 (now known and referred to hereafter as *Stenotrophomonas maltophilia*) to deproteinise chemically demineralised shell. This gave a chitin comparable with chemically deproteinised shell. A comparison of the action of various proteolytic inoculants/mixed inoculants on chemically demineralised *Nephrops* shell waste led to the extraction of 58 - 82% of the shell protein

(Bustos, 1996). The 82% extraction rate was achieved by BAFP202, a mixed grass silage inoculant containing *Bacillus subtilis*, *Pediococcus pentosaseus* and *Enterococcus faecium.* The waste did not contain prawn head so digestive enzymes were not present and lactic acid fermentation alone did not lead to an adequate reduction in protein.

Several other studies have used proteolytic enzymes to fulfil the same process. Broussignac (1968) used pepsin and trypsin to produce a chitin with little deacetylation. Takeda and Katsuura (1964) used the bacterial protease (Pronase-P), papain and an enzyme from tuna fish to deproteinise crustacean shell. Pronase-P was the most effective, reducing the protein level to 1%. Gagné and Simpson (1993) optimised the conditions for the use of chymotrypsin and papain to deproteinise chemically demineralised waste. The percentage protein extracted varied from 22 - 48% depending on the starting material used. Synowiecki and Al-Khateeb (2000) chemically demineralised *Crangon crangon* shells and followed this by treatment with the enzyme alcalase. The final chitin contained 4.45% protein.

A few studies have combined lactic acid fermentation and proteolytic enzyme treatment. Rao *et al.*, (1998) used lactic acid fermentation and a protease to demineralise and deproteinise shrimp biowaste. Ninety percent deproteinisation was achieved. Shirai *et al.*, (1998) compared chemical, lactic acid and enzymic treatments. Lactic acid treatment followed by enzymic treatment removed 77% of the waste protein.

As mentioned previously the applications of chitin/chitosan are many and further research into possible uses, in particular in the biomedical field, is widespread. However, no chitin/chitosan is extracted commercially in an environmentally friendly manner and until this occurs the full potential of these naturally occurring polymers will never be reached. In the light of the above evidence this project was devised to further the research already carried out on the isolation of chitin from *Nephrops* shell waste and to examine the role the extracted polymers could play in the biomedical device industry.

1.14 AIMS AND OBJECTIVES

The aims of the project were:

1.14.1 To isolate chitin from *Nephrops norvegicus* shell waste using the following biotechnological methods:

(a) Lactic acid fermentation

(b) Enzymatic proteolysis

1.14.2 To convert the isolated chitin to chitosan and test the suitability of the chitosan for use in biomedical devices, in particular in urinary catheters.

CHAPTER TWO

THE MORPHOLOGY OF NEPHROPS SHELL WASTE

2.1 INTRODUCTION

Arthropod cuticle consists of a headpiece or cephalothorax and an abdomen. The cephalothorax is made up of twelve segments but joints between the segments are not apparent from the dorsal side. The abdomen possesses six jointed segments. Each segment is made up of a convex dorsal plate, the tergum and a ventral transverse bar, the sternum. All the abdominal segments except the first one also possess pleura, plates projecting down at either side.

Every segment has two jointed appendages. The appendages consist of the protopodite (the basal region), which bears two branches, an inner endopodite and an outer exopodite. The segments of the abdomen bear five pairs of 'swimmerets' - small, paired appendages. The sixth segment bears flattened appendages known as 'uropods'.

The ultrastructure of the decapod crustacean has been studied extensively in various species such as the fiddler crab (Green and Neff, 1972), the European clawed lobster (Mutvei, 1974), the common crab (Hegdahl *et al.*, 1977a, b and c), the American clawed lobster, *Homarus americanus* (Arsenault *et al.*, 1984) and the shore crab, (Mutvei, 1974; Giraud-Guille, 1984; Compère and Goffinet, 1987 and Compère *et al.*, 1998), but not to the same extent in *Nephrops norvegicus*.

The cuticle of decapod crustaceans generally consists of four layers: the epicuticle, the exocuticle, the endocuticle and the membranous layer (reviewed by Roer and Dillaman, 1984). Scanning electron microscopy has shown the same four layers in *Nephrops norvegicus* (Bustos, 1996). The thickness of the layers differs depending on the decapod studied. Table 2.1 shows a comparison between the thickness of the layers in *Nephrops norvegicus* and in *Carcinus maenas*.

Crustacean	Layer Thickness (μm)				
	Epicuticle	Exocuticle	Endocuticle	Membranous Layer	
C. maenas	5	65	195	35	Roer & Dillaman, 1984
N. norvegicus	8	25	50	21	Bustos, 1996

Table 2.1 *Dimensions of the carapace of decapod crustaceans.*

Chitin is found in the three innermost layers (the exocuticle, the endocuticle and the membranous layer) where it is covalently linked to protein. These chitin-protein microfibrils are laid down in layers. The fibrous direction rotates continuously as new levels are added. The endocuticle of the fiddler crab contains larger and less-tightly stacked lamellae than the exocuticle (Green and Neff, 1972). The horizontal lamellae in the shells of the European clawed lobster are also more closely spaced together in the exocuticle than in the endocuticle (Mutvei, 1974).

The epicuticle is the outermost layer. This protective layer is the thinnest layer and consists of tanned lipoprotein impregnated with calcium salts (Roer and Dillaman, 1984). The exocuticle and the endocuticle are also hardened by the presence of mineral crystals between the fibres. No calcium is found in the membranous layer.

Eighty percent of the *Nephrops* landed in Northern Ireland have been decapitated and declawed and the waste discarded at sea. Only fifty eight percent of the unshelled tail is edible leaving forty two percent of the landed product as waste that has to be disposed of (Seafish Industry Authority, 2001). The waste material received for this project therefore consisted only of abdominal segments. A study of the gross appearance and ultra structure of the shell waste was carried out. The aim was not to definitively decipher the structure of the shell but to gain insight into the nature of the raw material to be used for the extraction of the chitin and to assess the homogeneity of the material used.

2.2 MATERIALS AND METHODS

2.2.1 Prawn Shell Waste

Prawn shell waste was obtained from Euroshell Ltd., Portavogie, County Down, Northern Ireland and from Scotprime Seafoods Ltd., Troon, Scotland. Fresh shell was collected after removal of meat by water jet. The shell was placed in a wire basket and washed in cold water, using a power hose, before drying in an incubator (Western 2000) for 5 days at 40°C. The dried shell was stored in black polythene bags at room temperature.

2.2.2 Morphological Studies

2.2.2.1 Gross Morphology

Whole wet shell was examined by visible inspection and the gross morphology recorded.

2.2.2.2 Ultrastructural Studies

Dried samples were sectioned to give particles of approximately 0.5cm^2 in size. These particles were attached to aluminium stubs using double-sided tape. The samples were coated with gold/palladium (AuPd) in an Emscope SC500. Coated samples were transferred to a Philips 501B scanning electron microscope where electron micrographs were taken at various magnifications (x40 - x5000).

2.3 RESULTS

2.3.1 Gross Morphology of the Shell Waste

Fig. 2.1 shows a diagram of the gross structure of a whole decapod crustacean.

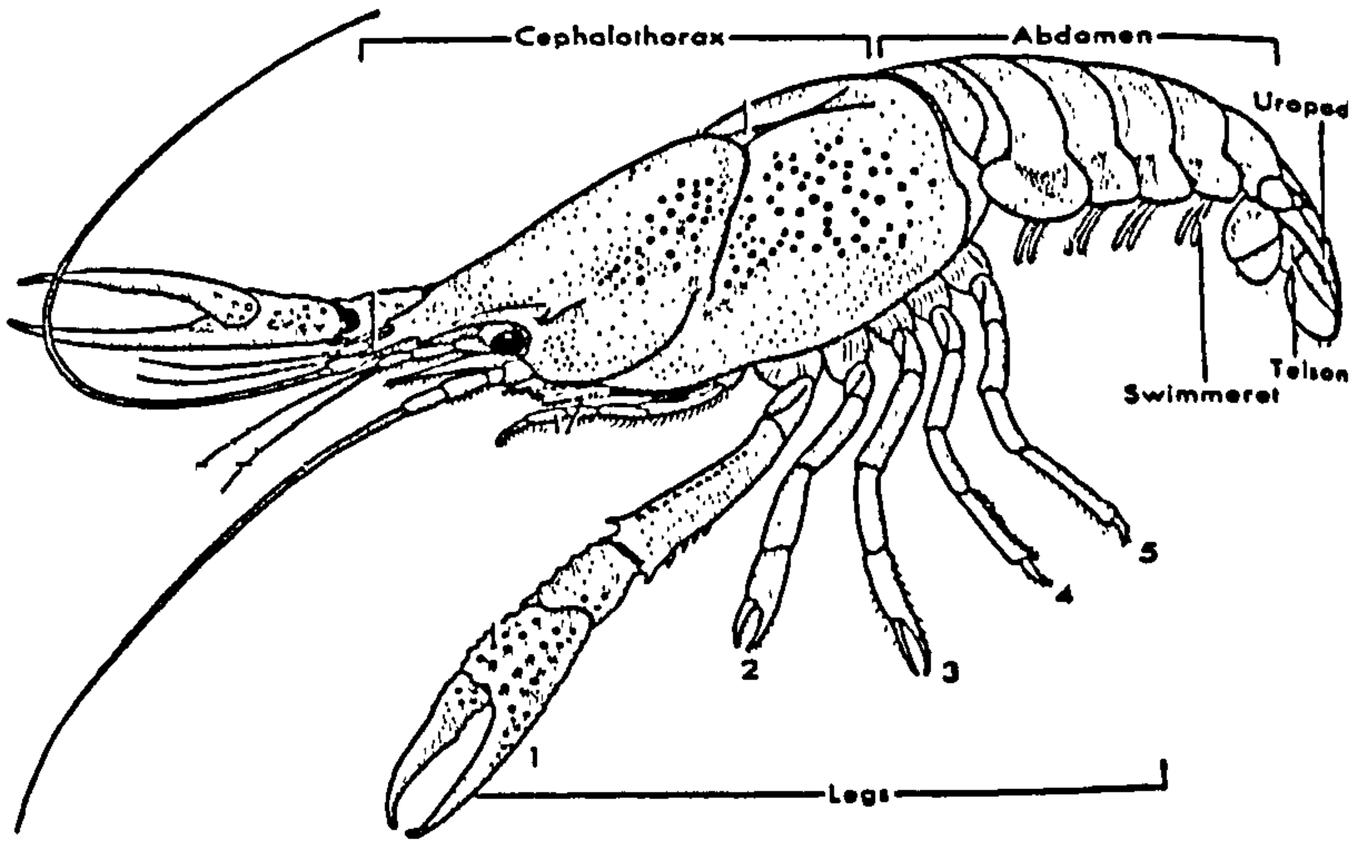


Fig. 2.1 *Diagram of a decapod crustacean* (From Hegner and Stiles, 1963).

The majority of the *Nephrops* shell waste obtained consisted only of the abdominal shell segments (Fig. 2.2). However, an occasional head (cephalothorax) with legs attached was also present in each batch of waste.

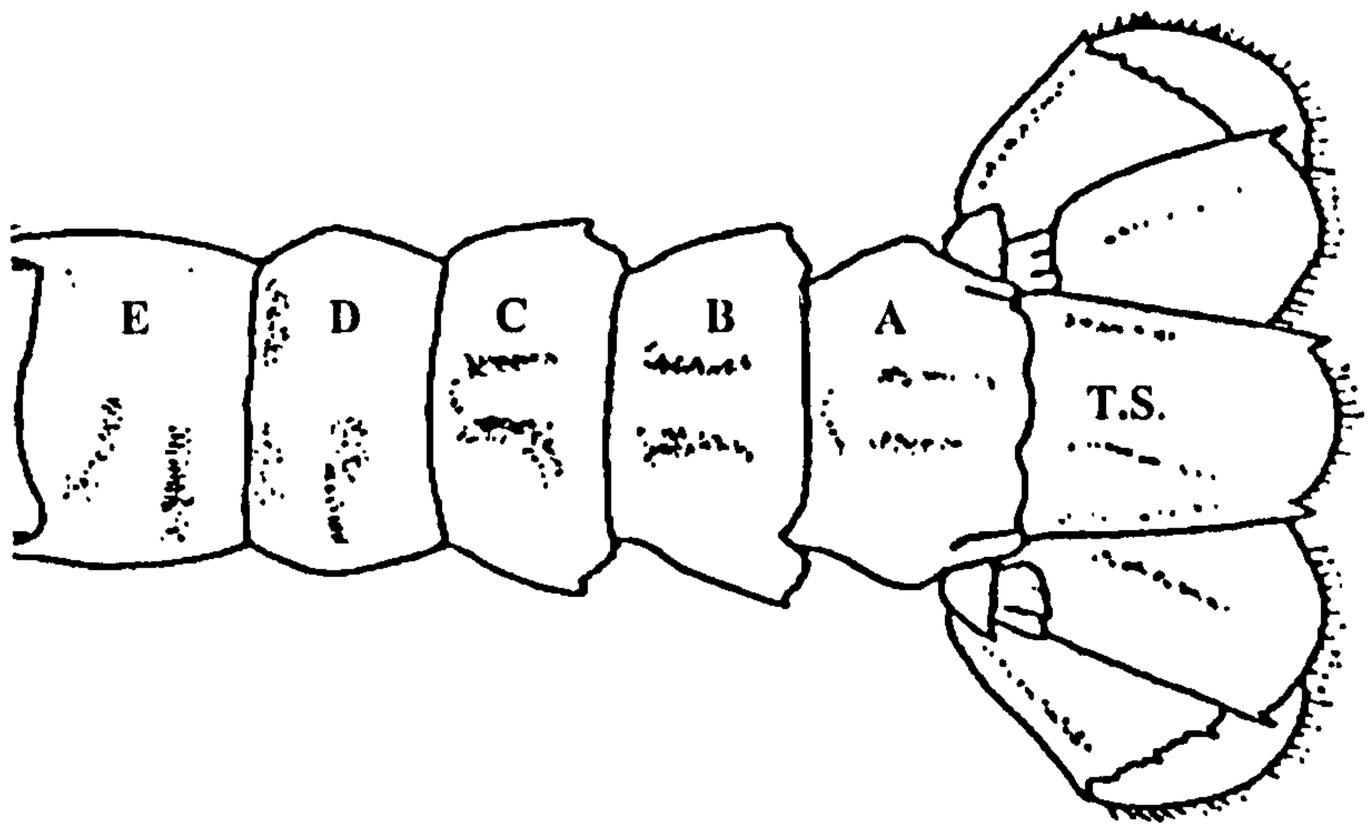


Fig. 2.2 *Diagram of the abdominal segments present in the shell waste.* T.S = tail segment, A - E = abdominal segments

The tail segment was made up of five separate pieces that could be spread out into a fan shape as seen in Fig. 2.2.

A clear membrane was visible between the segments. This membrane allowed movement at the joints when the shell was wet. Each abdominal segment consisted of a convex dorsal plate, the tergum, a ventral transverse bar, the sternum, and plates projecting down at the sides, the pleura (Fig. 2.3). The swimmerets were present attached to the abdominal segments.

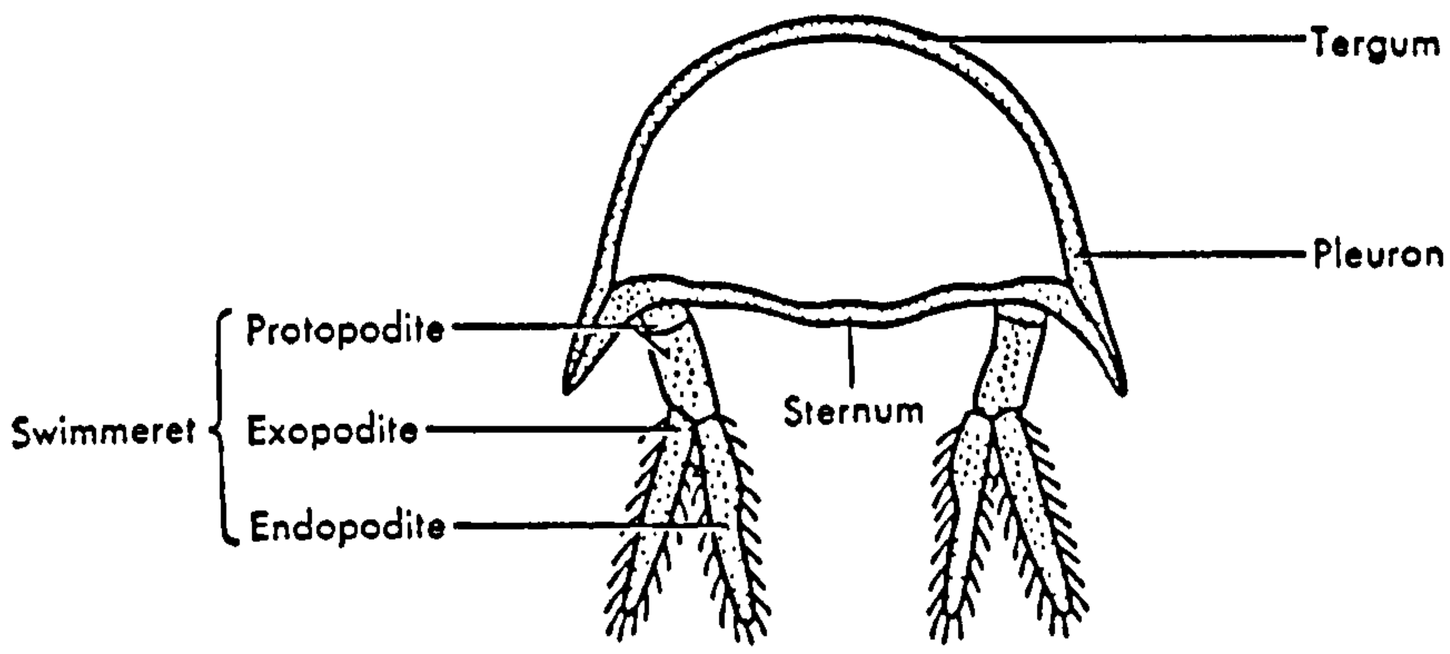


Fig. 2.3 *Diagram of a cross section of a typical abdominal segment* (From Hegner and Stiles, 1963).

2.3.2 Ultra-Structure of the Shell Waste.

Scanning electron microscopy along the epicuticle (outer surface) of the shell showed widespread variation in the ultrastructure of different areas of the abdominal shell segments. Feathery fibres were located at the end of the tail segments (Fig. 2.4). These were visible with the naked eye (Fig. 2.2).

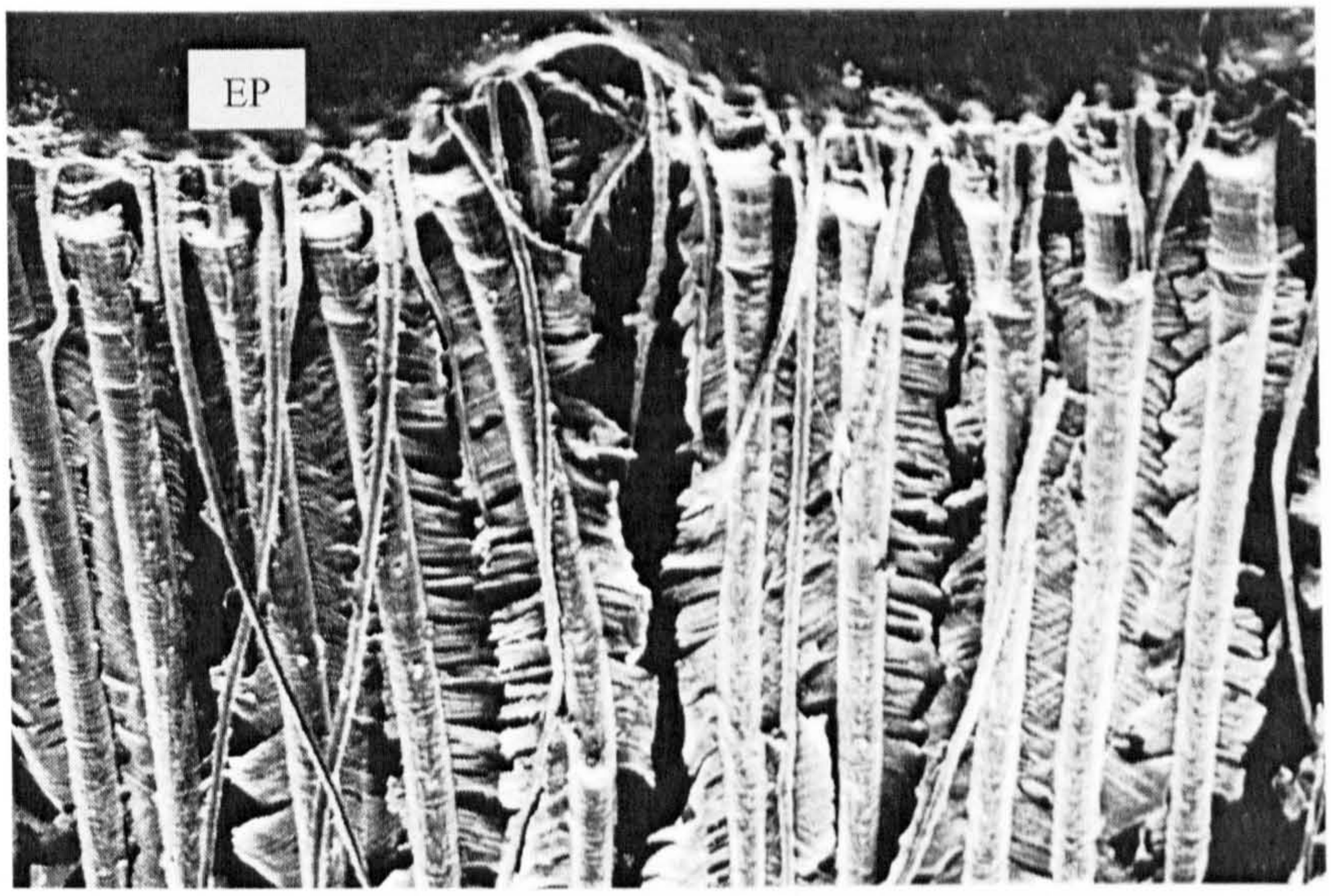


Fig. 2.4 *Scanning electron micrograph of fibres present at the end of the tail segment* (x90). EP = epicuticle

Moving along a tail segment in the direction of the cephalothorax the uppermost surface was smooth but penetrated by pores with protruding fibres (Fig. 2.5).

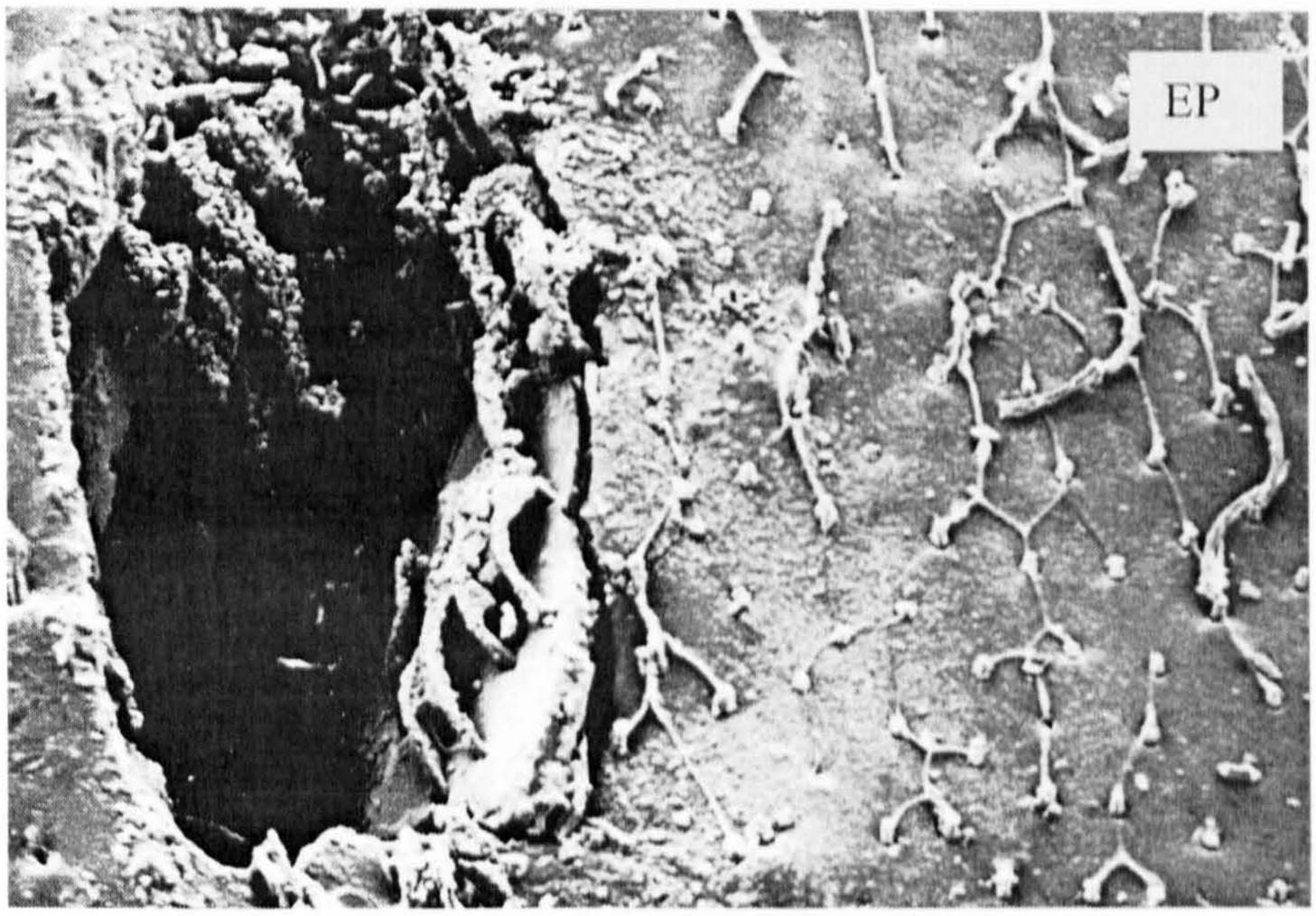


Fig. 2.5 *Scanning electron micrograph of the dorsal surface of the tail segment* (x90). EP = epicuticle

Some of these fibres are shown in higher magnification (Fig. 2.6).

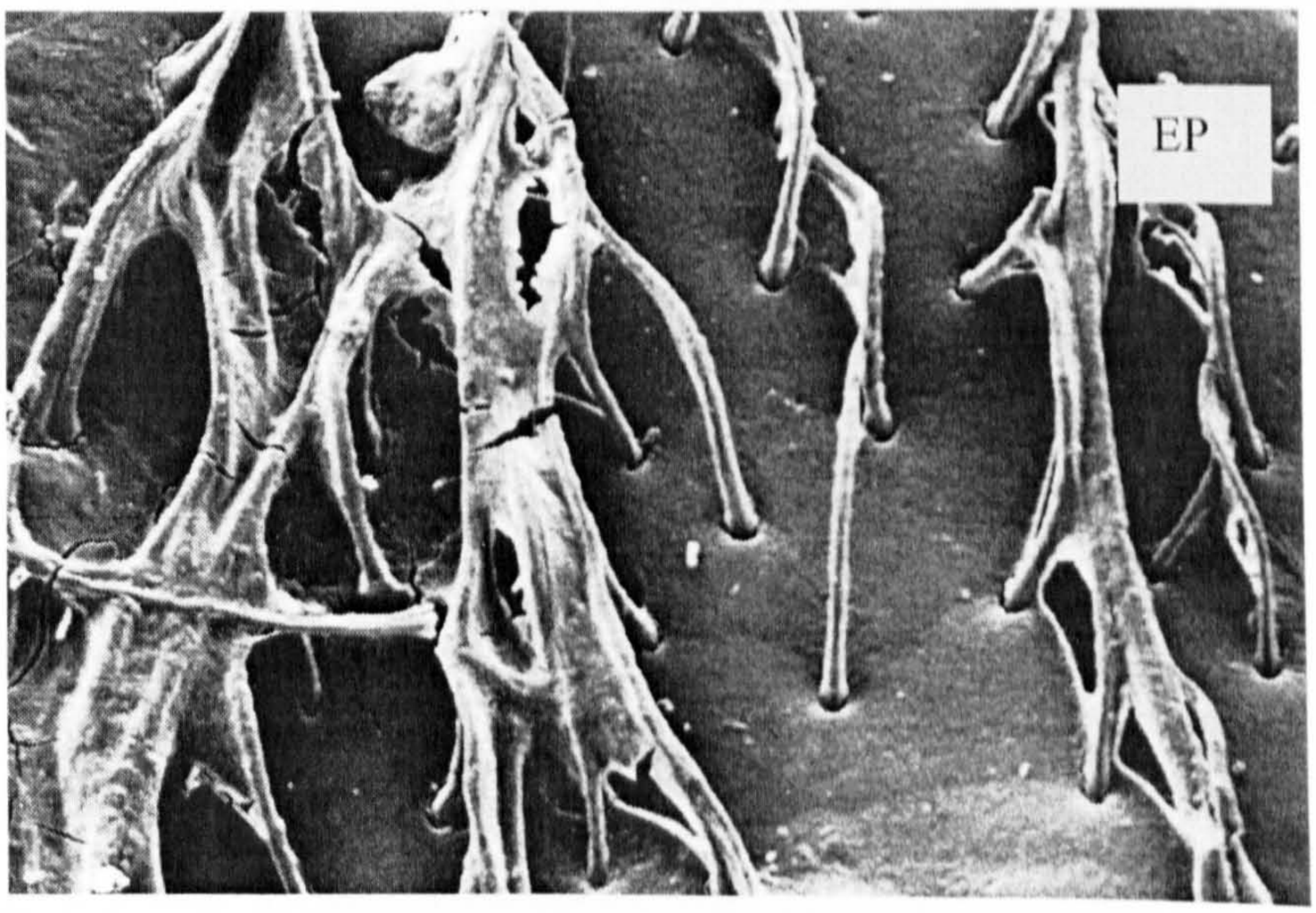


Fig. 2.6 *Scanning electron micrograph of the dorsal surface of the tail segment* (x180). EP = epicuticle

The area of attachment between segments possessed fibres running perpendicular to the segment (Fig. 2.7). These fibres fitted over the top of the next segment. At higher magnification they were seen to protrude from pores and some of the fibres appeared broken (Fig. 2.8).

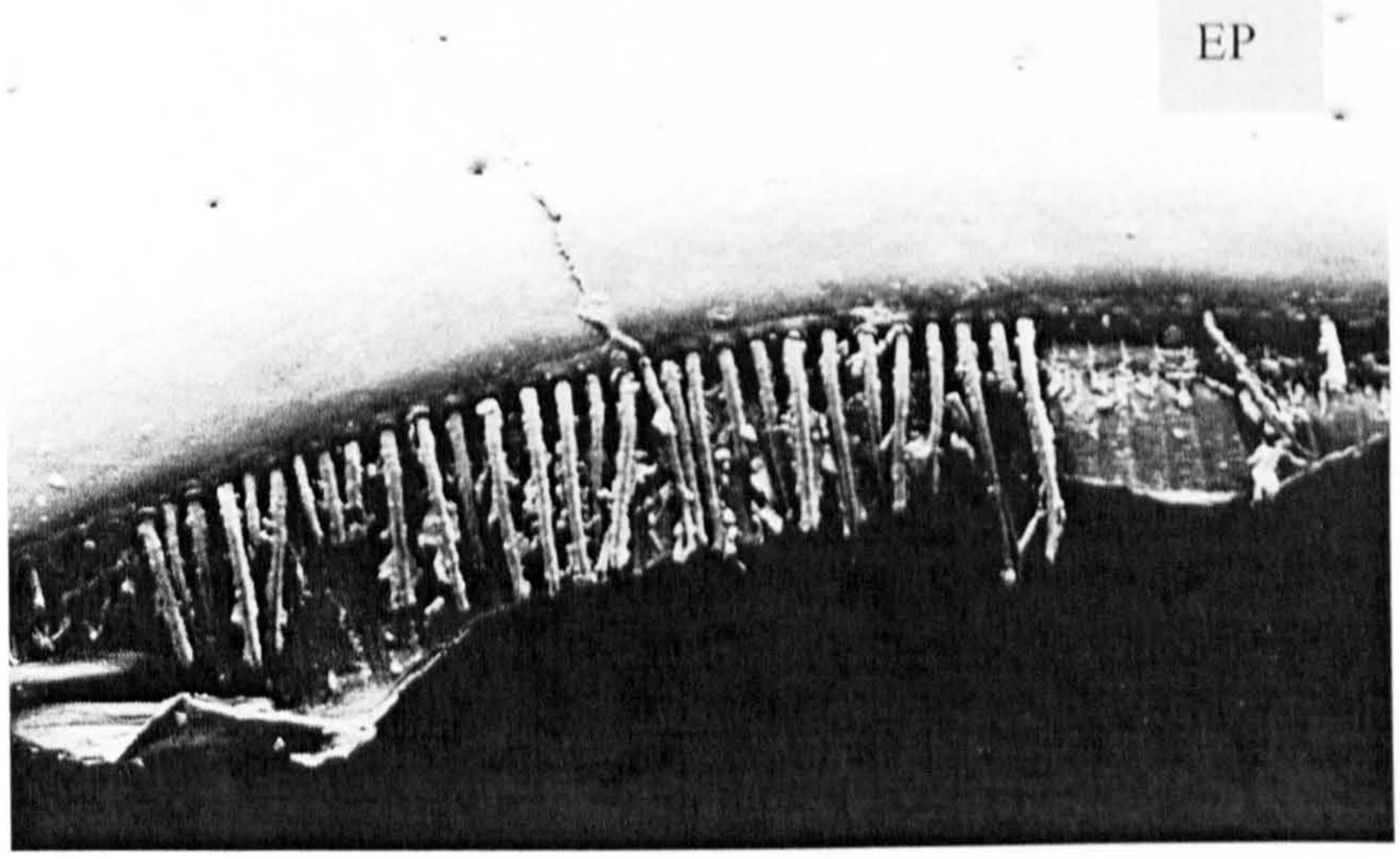


Fig. 2.7 *Scanning electron micrograph showing area of attachment between two segments* (x90). EP = epicuticle

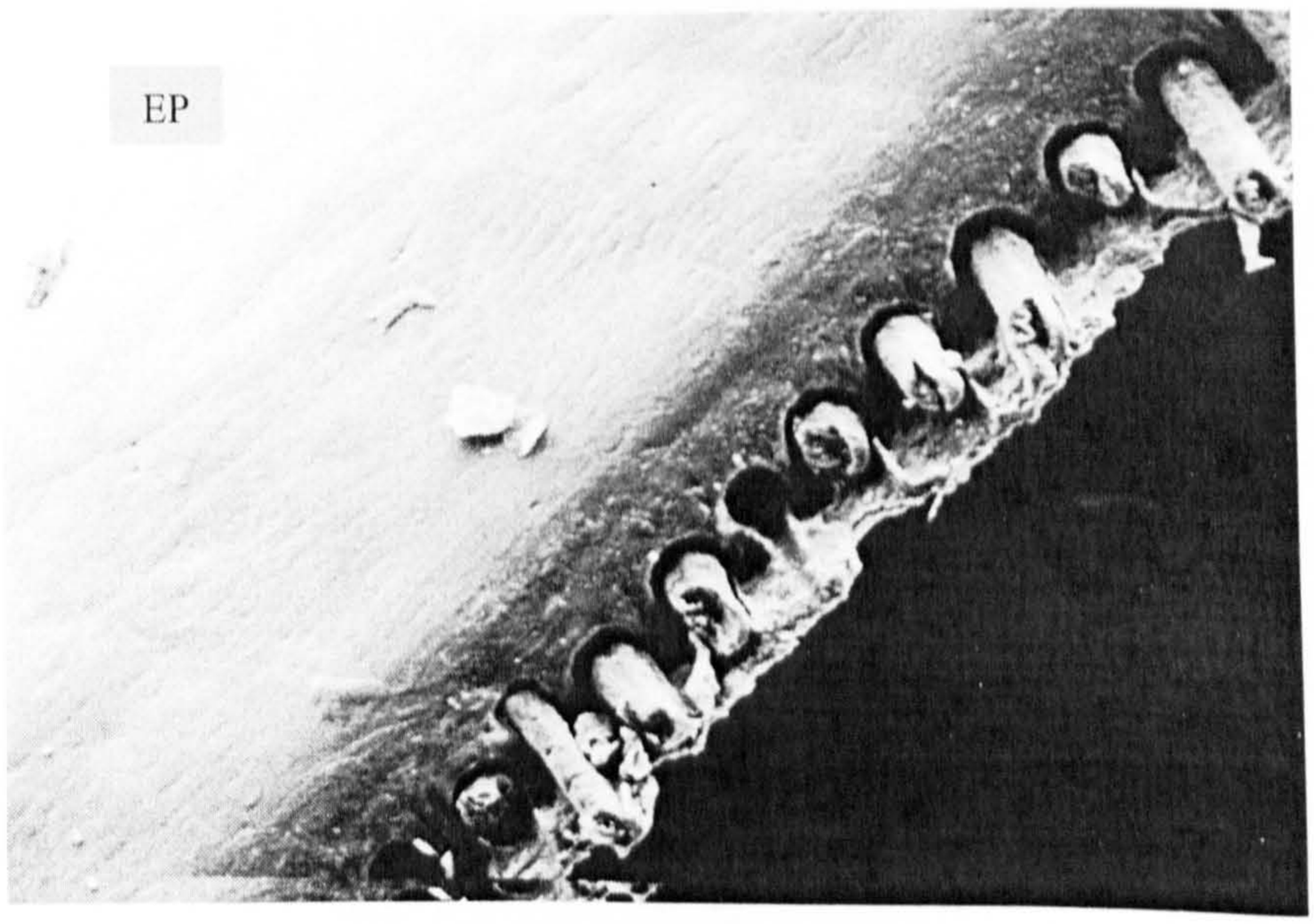


Fig. 2.8 *Scanning electron micrograph showing area of attachment between two segments* (x360). EP = epicuticle

The segment adjacent to the tail segments (segment A, Fig. 2.2) presented with fibres similar to those found on the tail segments (Figs. 2.9 and 2.10). These fibres stopped abruptly where segment A fitted underneath the next segment (segment B) (Fig. 2.9).

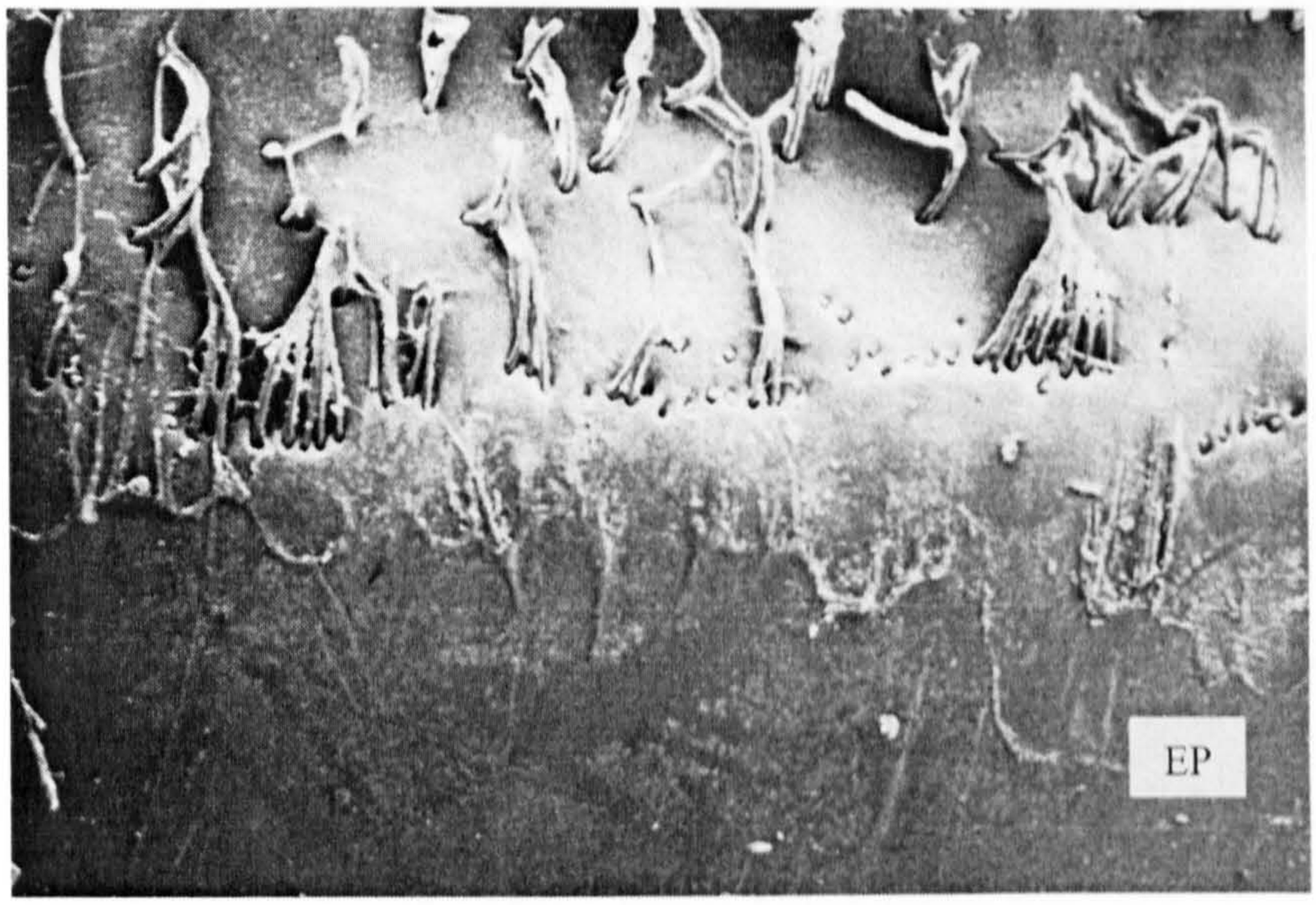


Fig. 2.9 *Scanning electron micrograph of the dorsal surface of segment A* (x90). EP = epicuticle

Fig. 2.10 *Scanning electron micrograph of the dorsal surface of segment A* (x720). EP = epicuticle

A separate region of segment A possessed no fibres. Instead it showed a smooth cuticular appearance (Fig. 2.11).

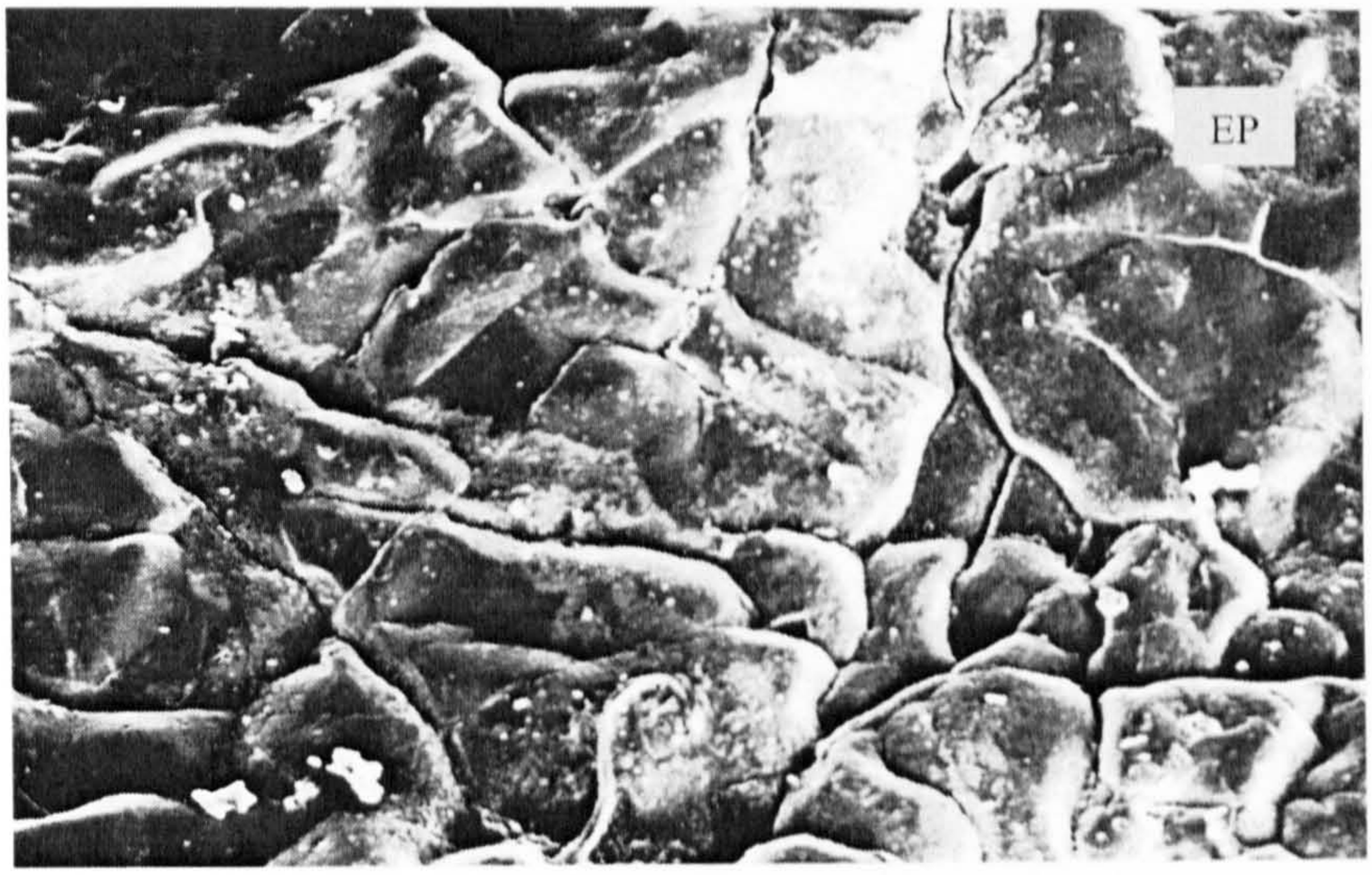


Fig. 2.11 *Scanning electron micrograph of the dorsal surface of segment A* (x180). EP = epicuticle

The other segments (segments B - E) displayed a different morphology from the tail segments and segment A. They possessed smooth areas interupted by pores (Fig. 2.12 and 2.13).

Fig. 2.12 *Scanning electron micrograph of the dorsal surface of segment B* (x180). EP = epicuticle

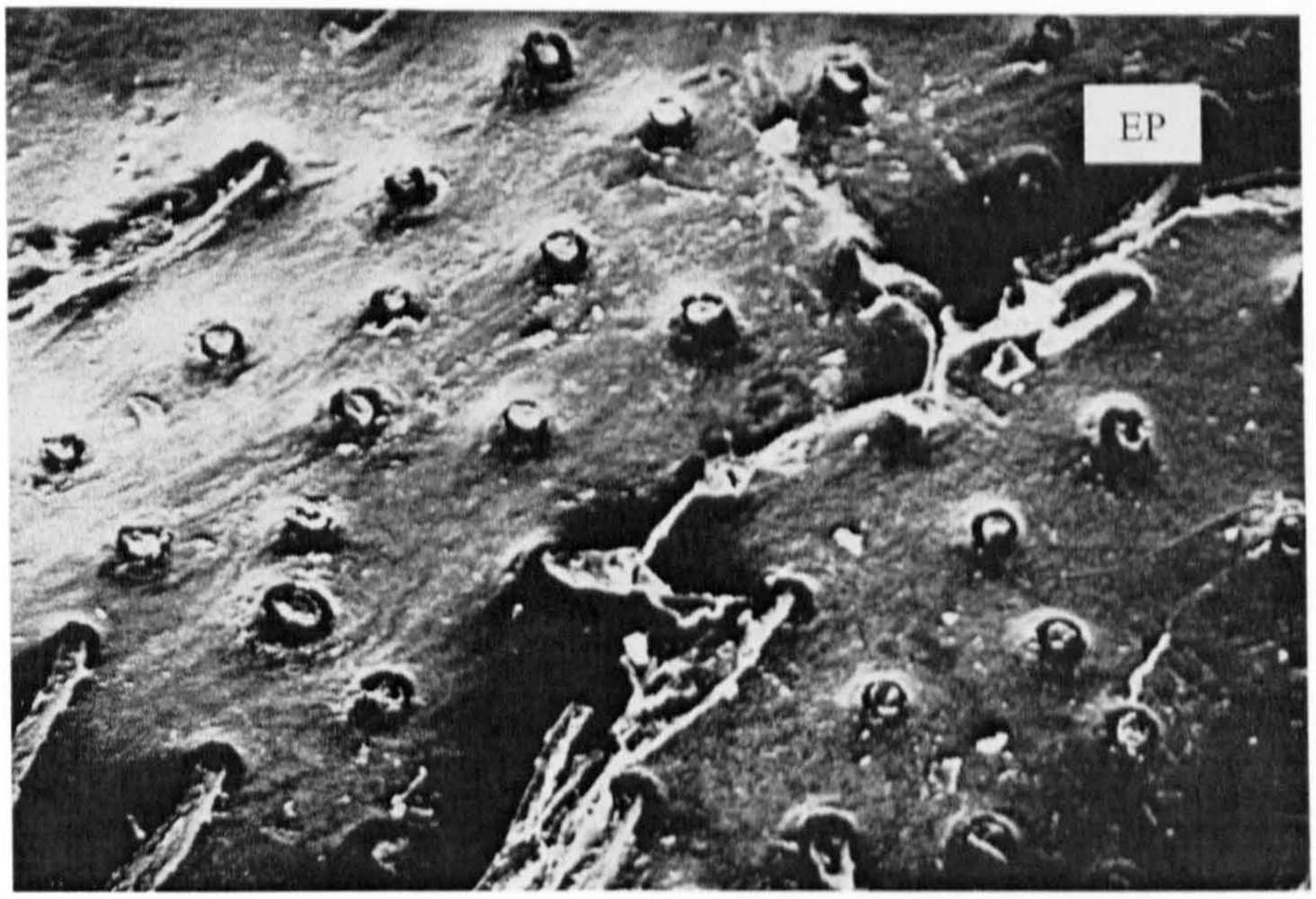


Fig. 2.13 *Scanning electron micrograph of the dorsal surface of segment B* (x360). EP = epicuticle

Protruding from the pores were spines (Figs. 2.12 and 2.13), large feathery fibres (Figs. 2.14 and 2.15) and hairy fibres (Figs. 2.16 and 2.17).

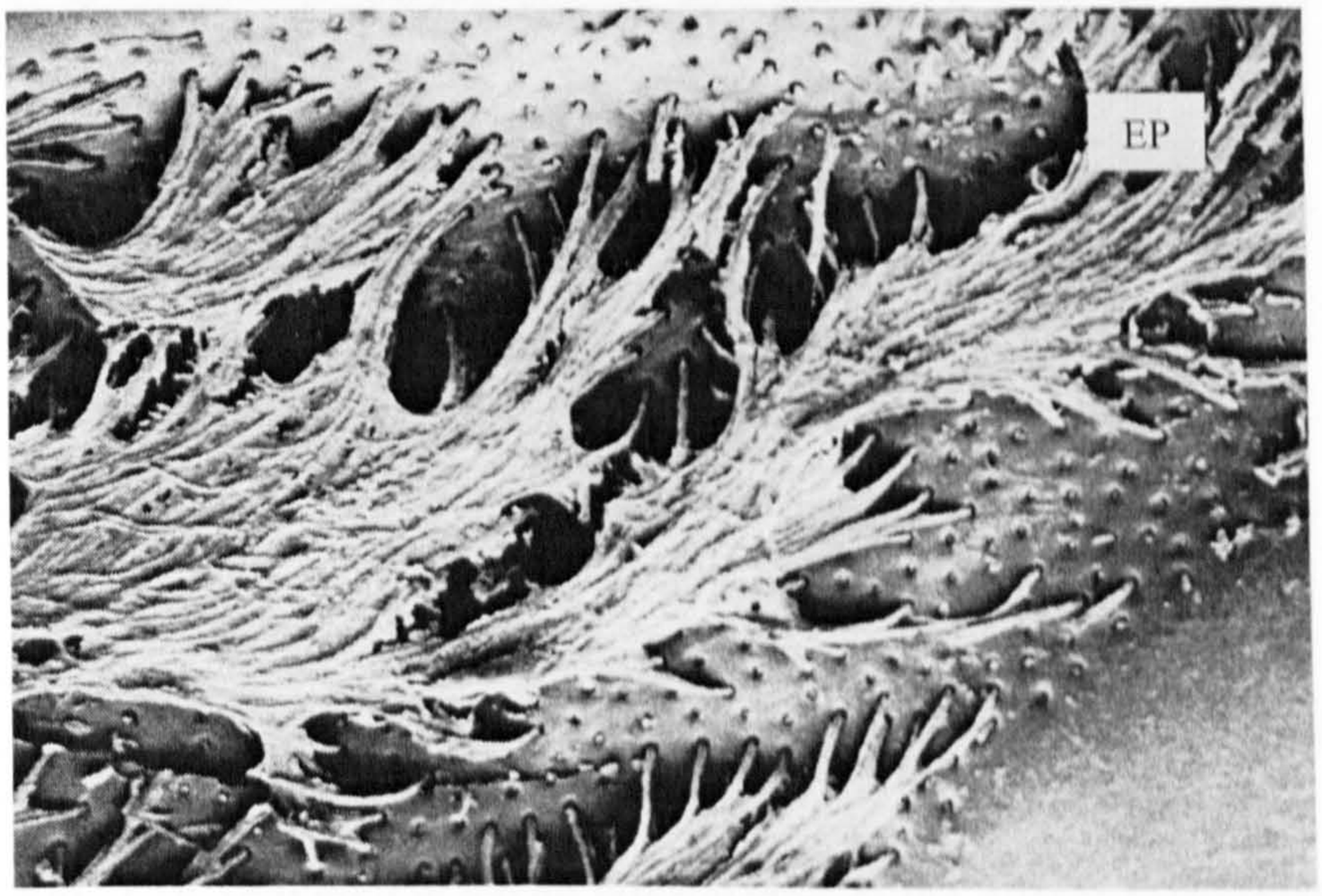


Fig. 2.14 *Scanning electron micrograph of the dorsal surface of segment B* (x90). EP = epicuticle

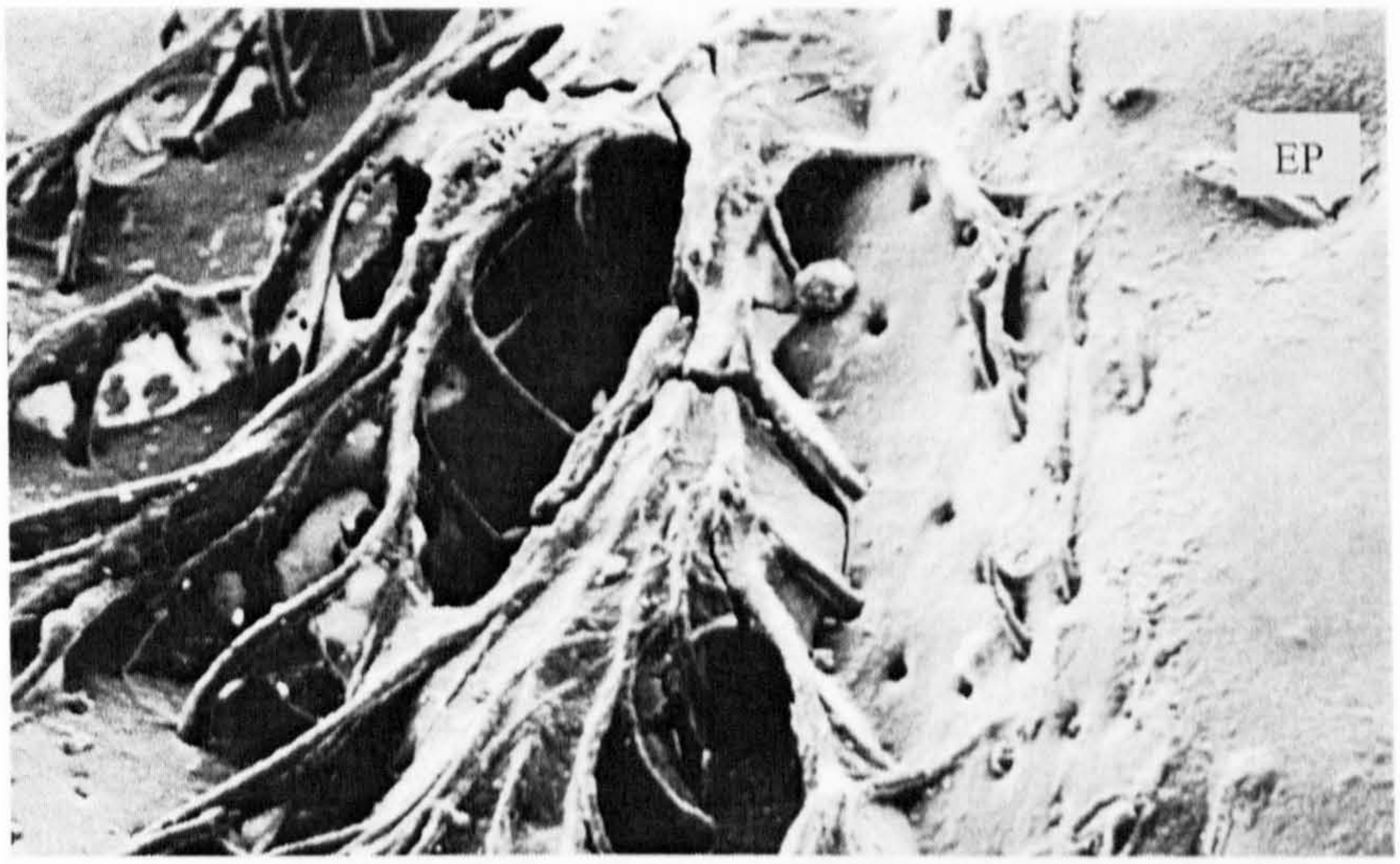


Fig. 2.15 *Scanning electron micrograph of the dorsal surface of segment B* (x180). EP = epicuticle

Fig. 2.16 *Scanning electron micrograph of the dorsal surface of segment B* (x360). EP = epicuticle

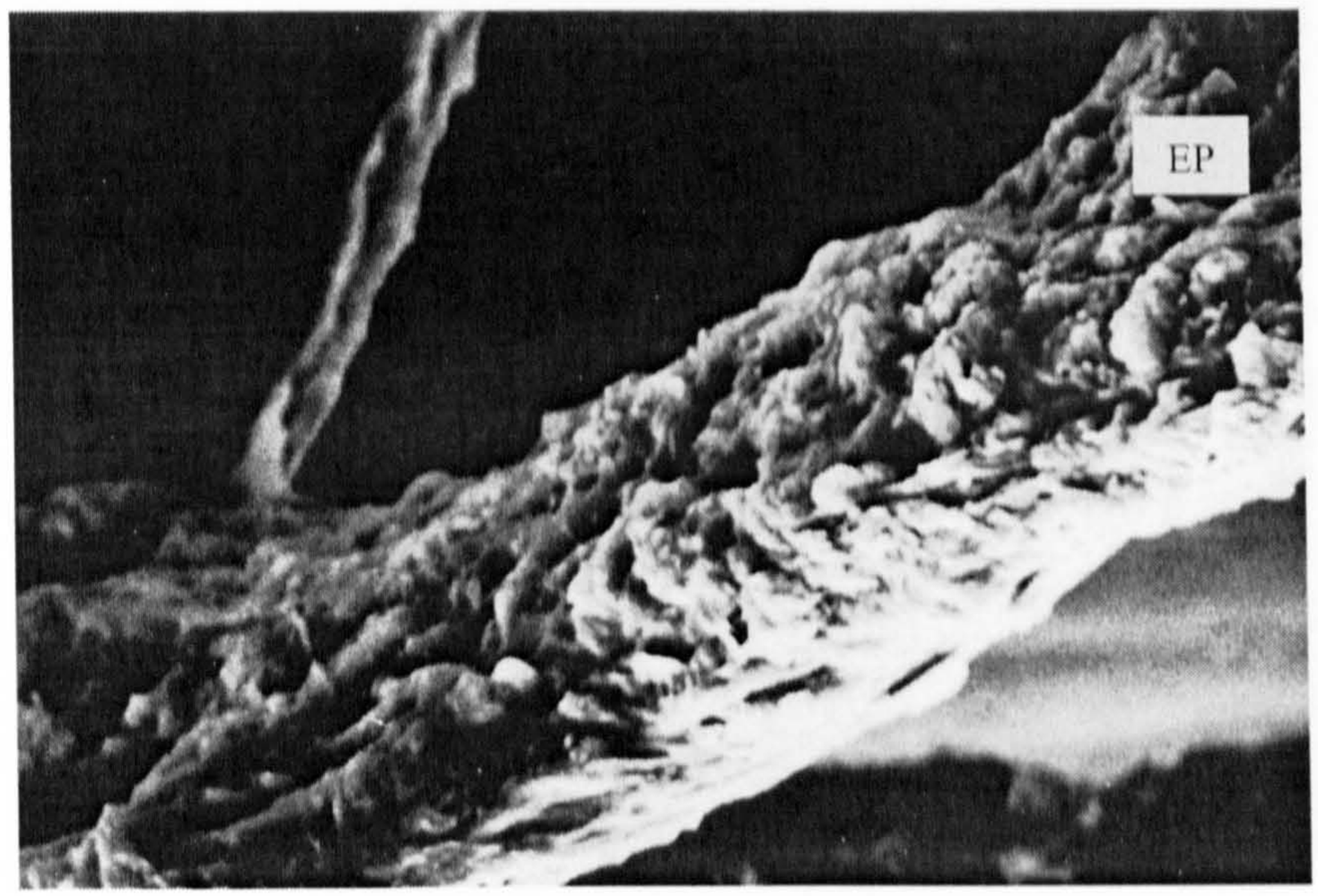


Fig. 2.17 *Scanning electron micrograph of the dorsal surface of segment B* (x2800). EP = epicuticle

In a separate region segments B – E were smooth but contained nodules (Figs. 2.18 and 2.19) and parallel fibres (Fig. 2.20).

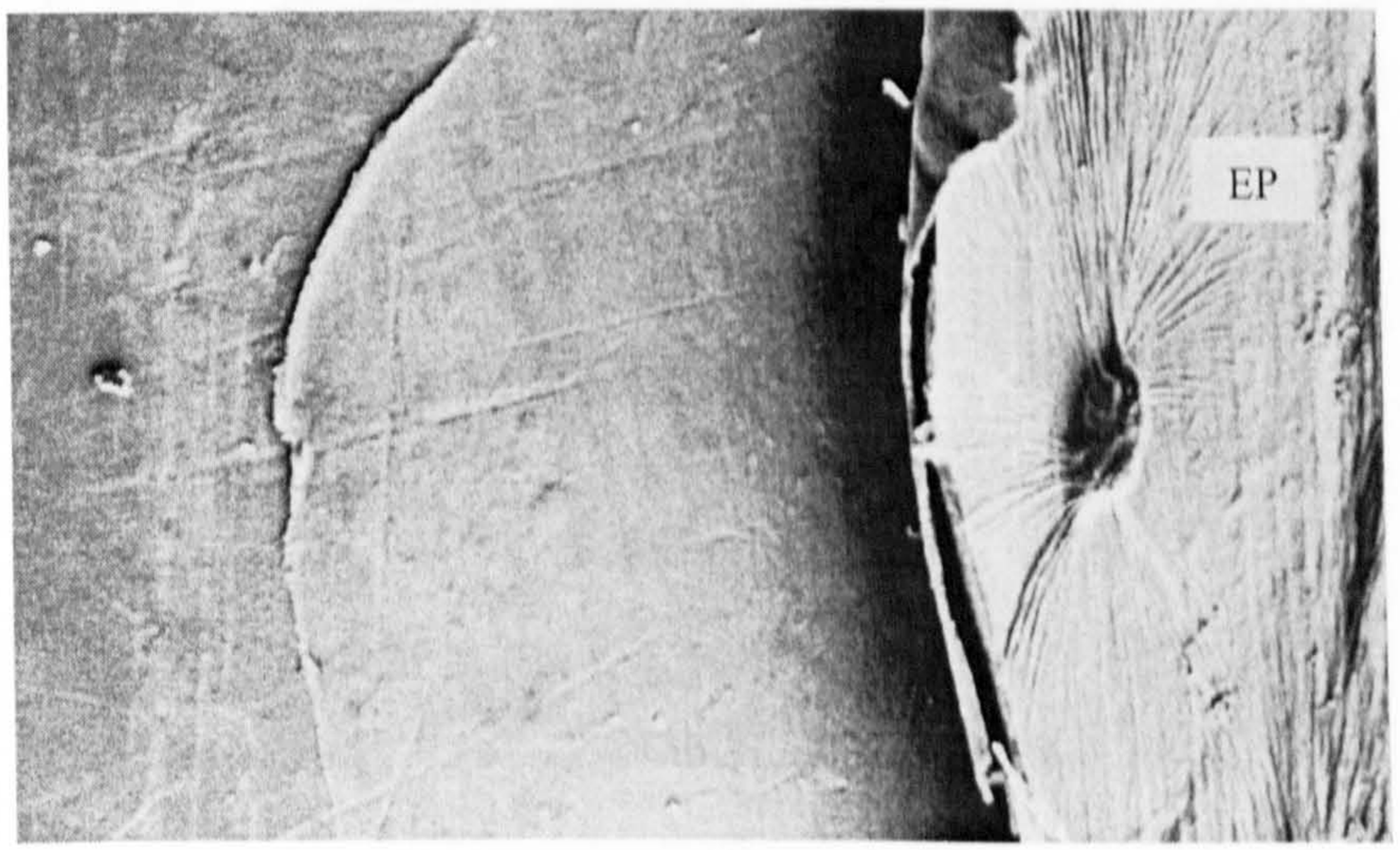


Fig. 2.18 *Scanning electron micrograph of the dorsal surface of segment B* (x180). EP = epicuticle

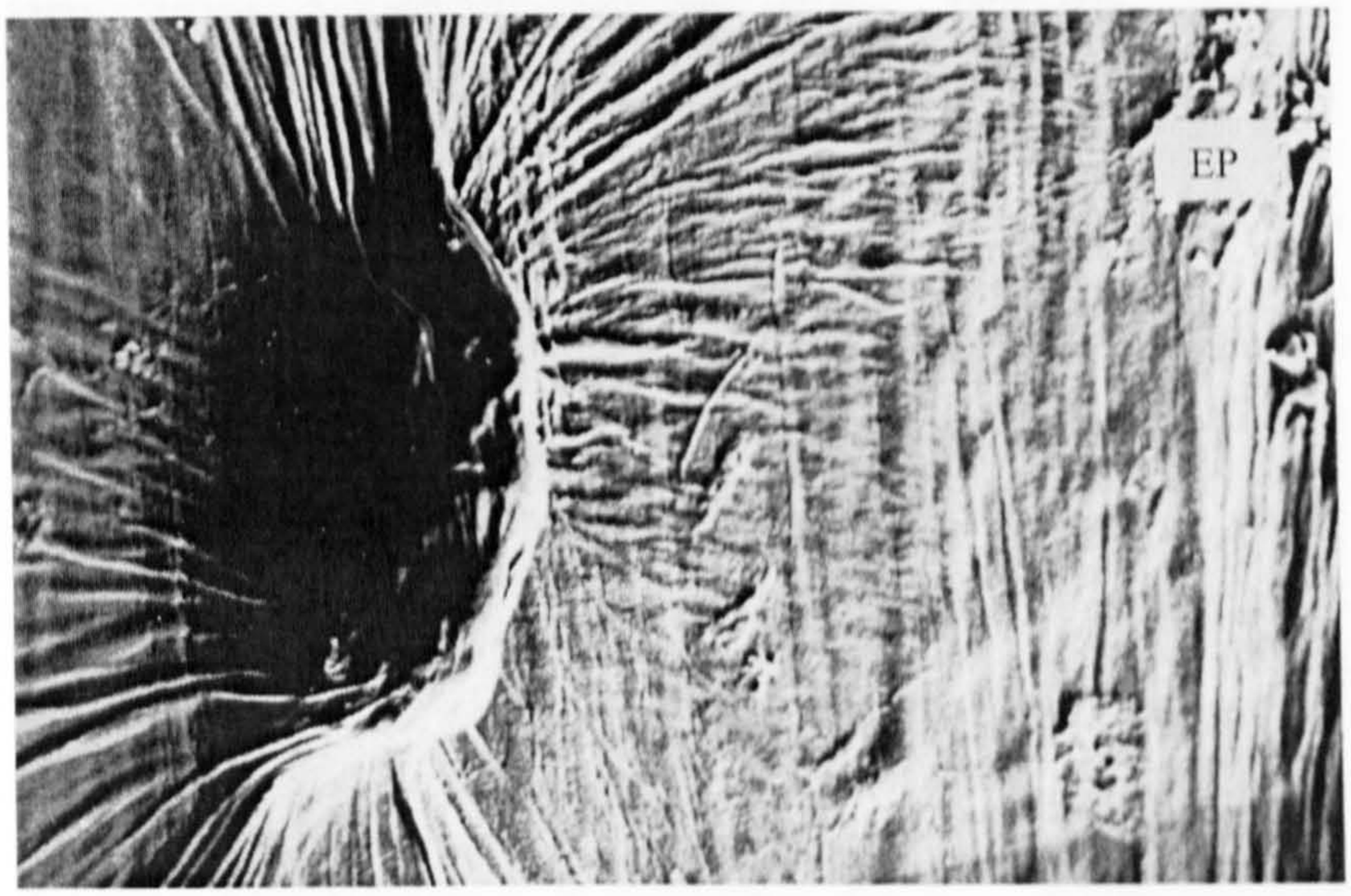


Fig. 2.19 *Scanning electron micrograph of the dorsal surface of segment B* (x720). EP = epicuticle

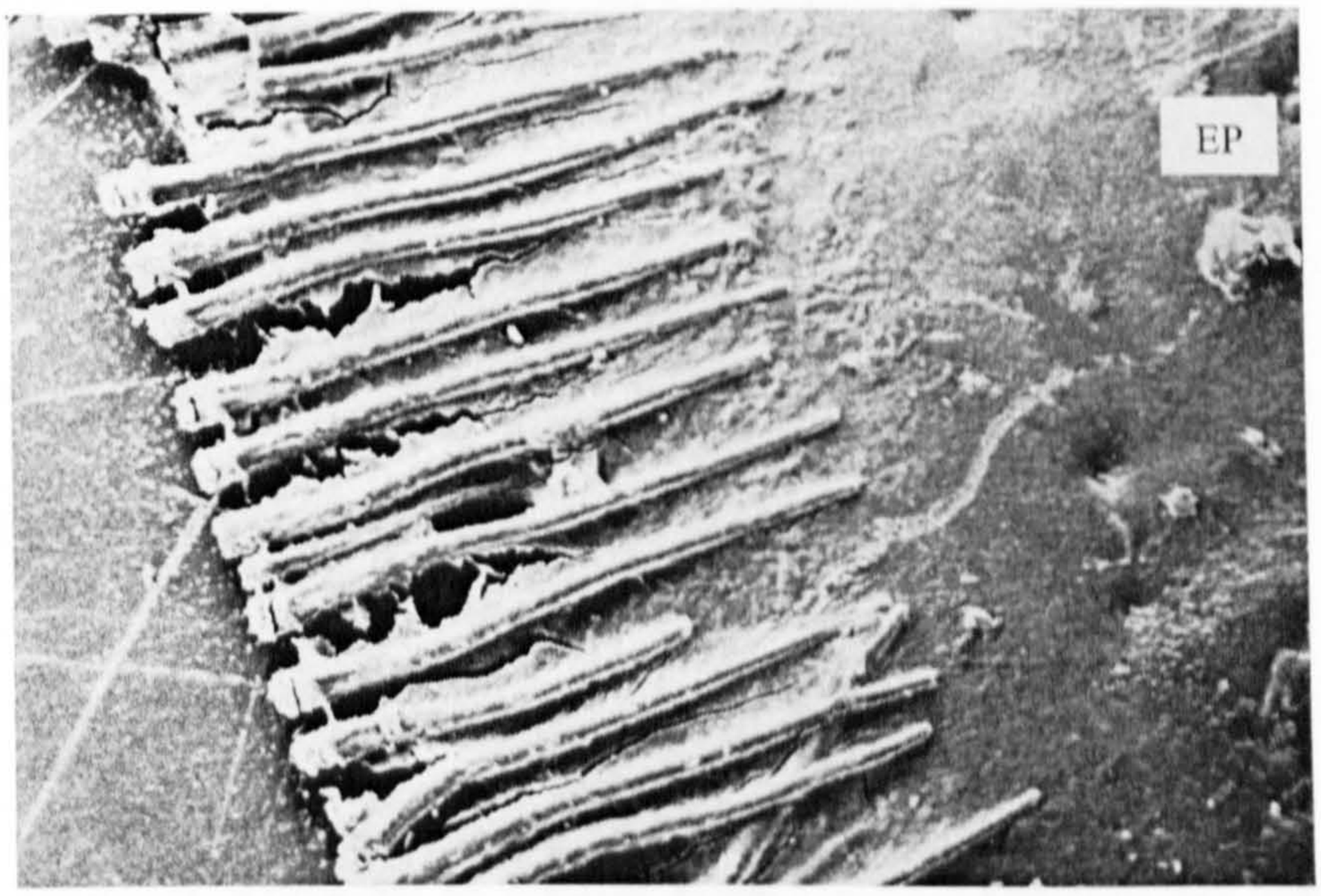


Fig. 2.20 *Scanning electron micrograph of the dorsal surface of segment B* (x180). EP = epicuticle

The various layers in the shell were visible in a broken region of the shell (Fig. 2.21).

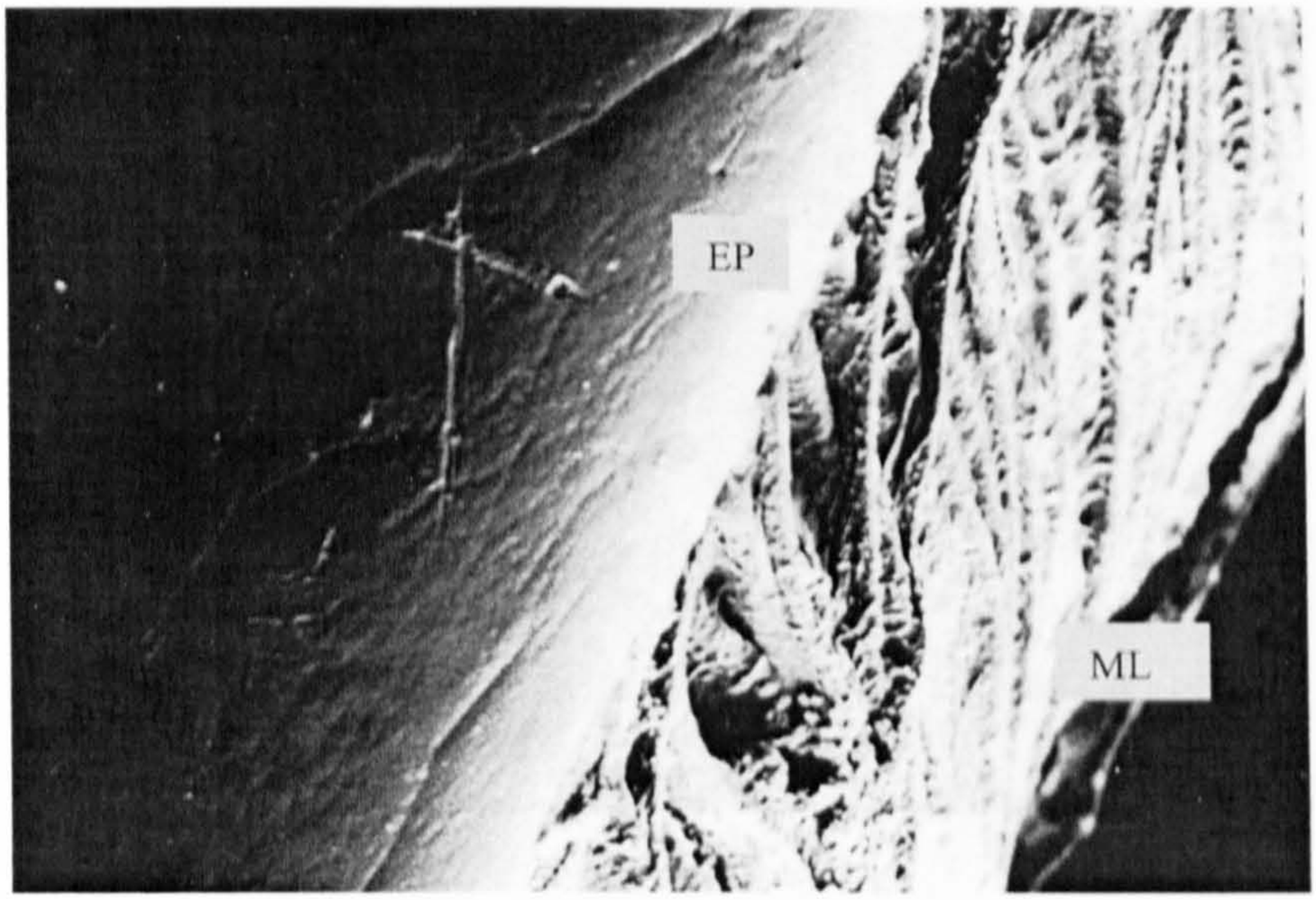


Fig. 2.21 *Scanning electron micrograph of a broken section of the shell* (x360). EP = epicuticle, ML = membranous layer

These separate layers were more apparent when the shell was studied in transverse section (Figs. 2.22 – 2.30). Figs. 2.22, 2.23 and 2.24 show the different layers of the shell (epicuticle, exocuticle, endocuticle and membranous layer).

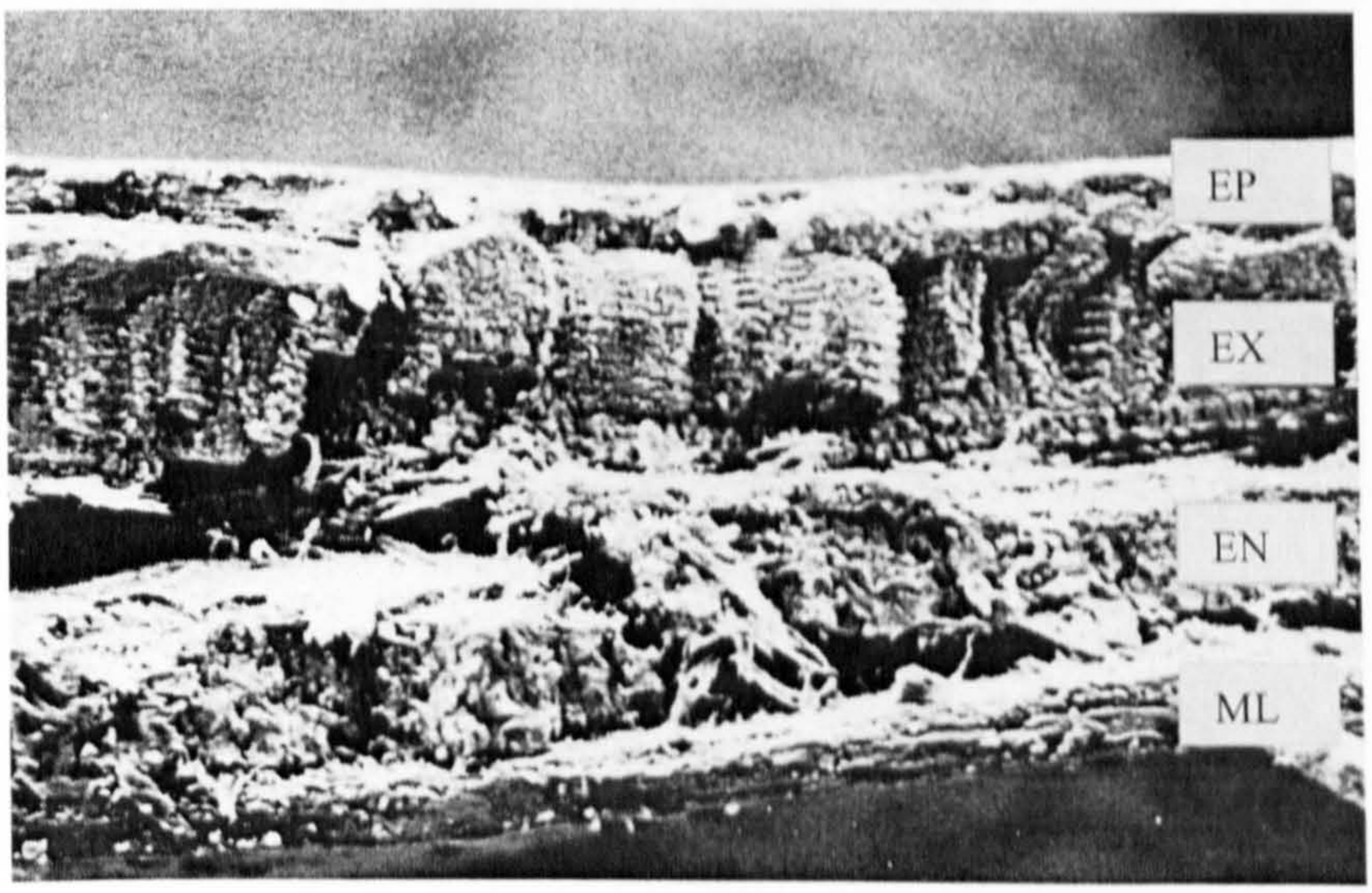


Fig. 2.22 *Scanning electron micrograph of a transverse section of the cuticle* (x360). EP = epicuticle, EX = exocuticle, EN = endocuticle, ML = membranous layer

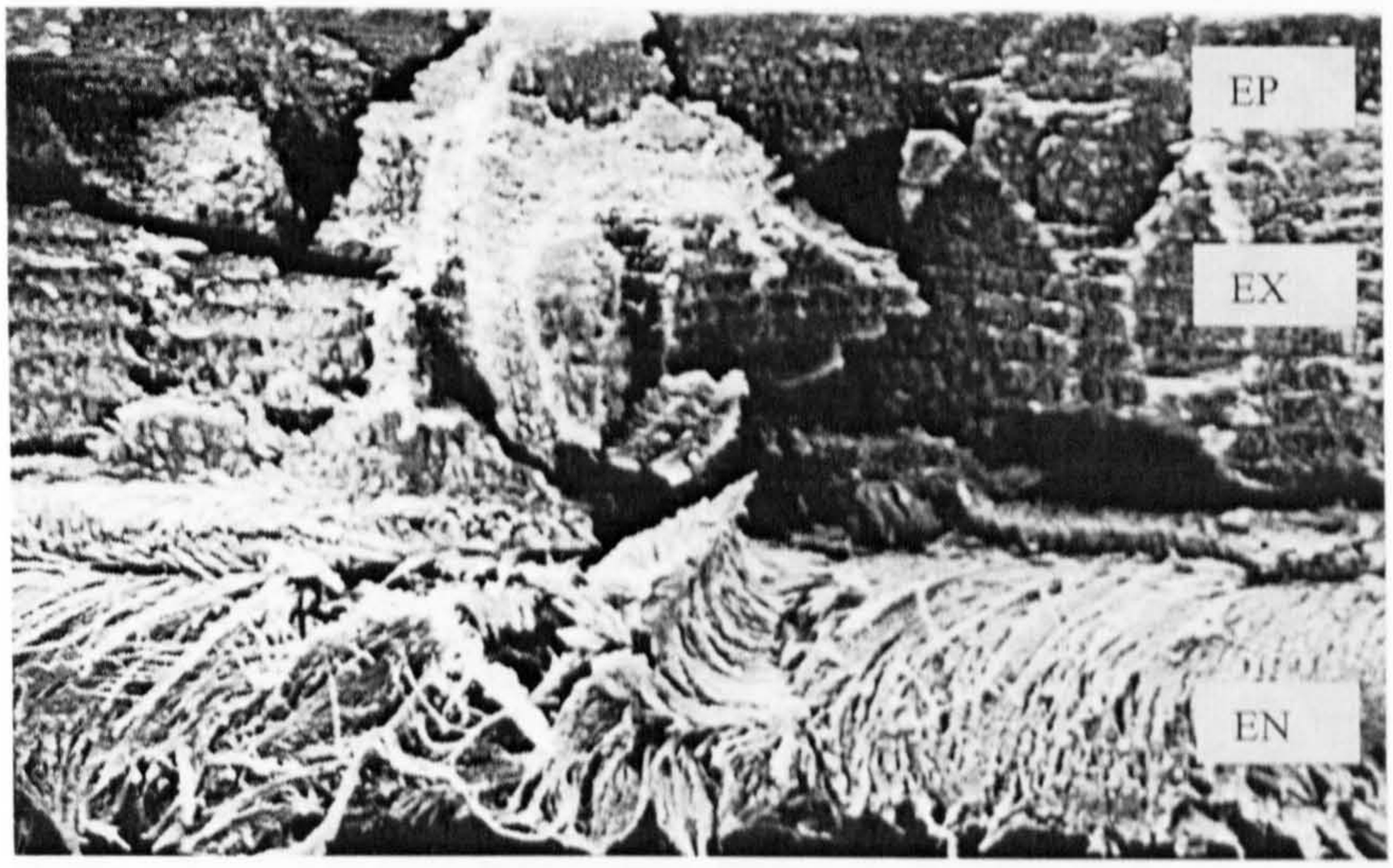


Fig. 2.23 *Scanning electron micrograph of a transverse section of the cuticle* (x720). EP = epicuticle, EX = exocuticle, EN = endocuticle

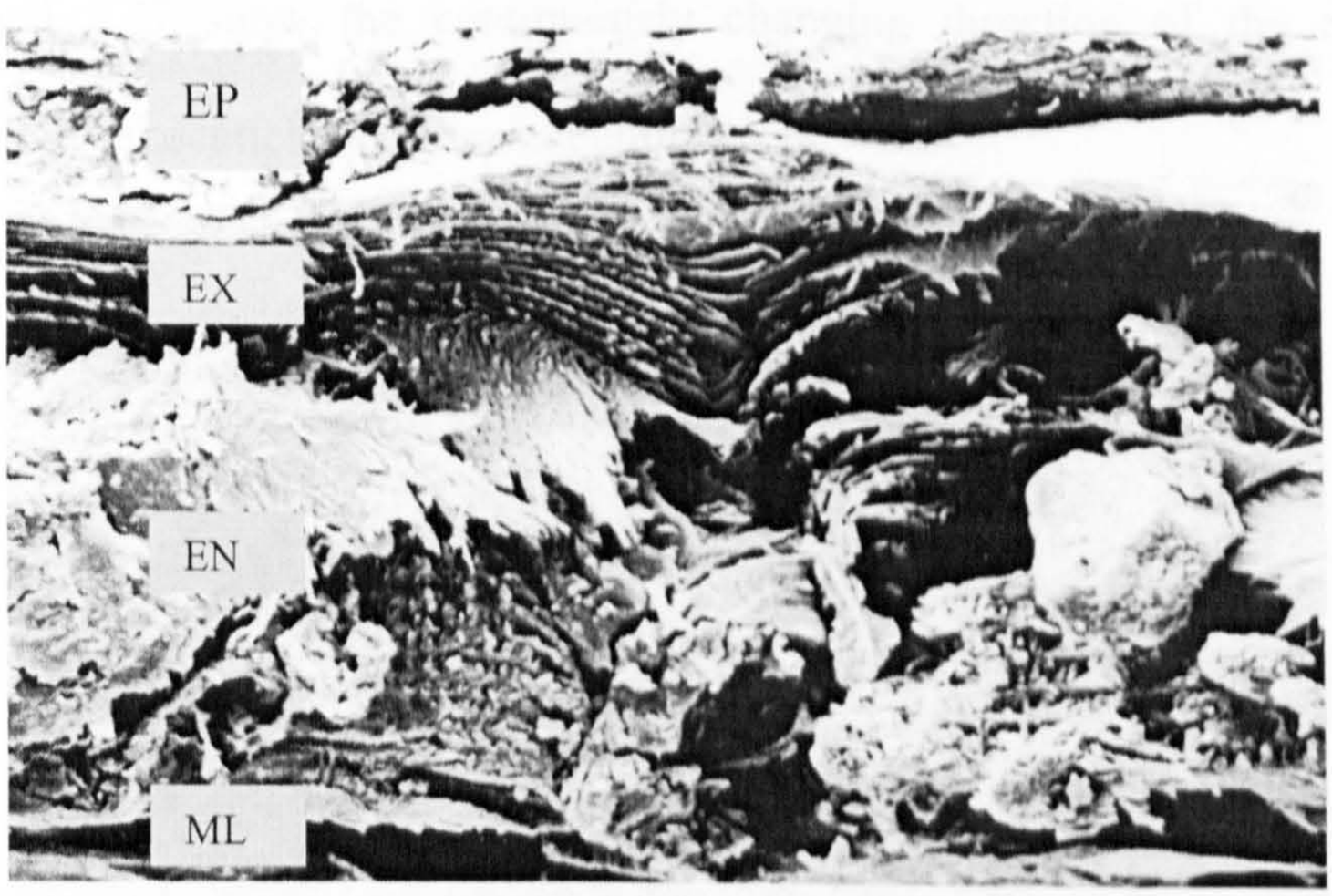


Fig. 2.24 *Scanning electron micrograph of a transverse section of the cuticle* (x720). EP = epicuticle, EX = exocuticle, EN = endocuticle, ML = membranous layer

The epi-, exo- and endocuticular layers as seen in Fig. 2.24 are shown at higher magnification in Figs. 2.25 – 2.27. Fig. 2.25 shows the outermost layer, the epicuticle.

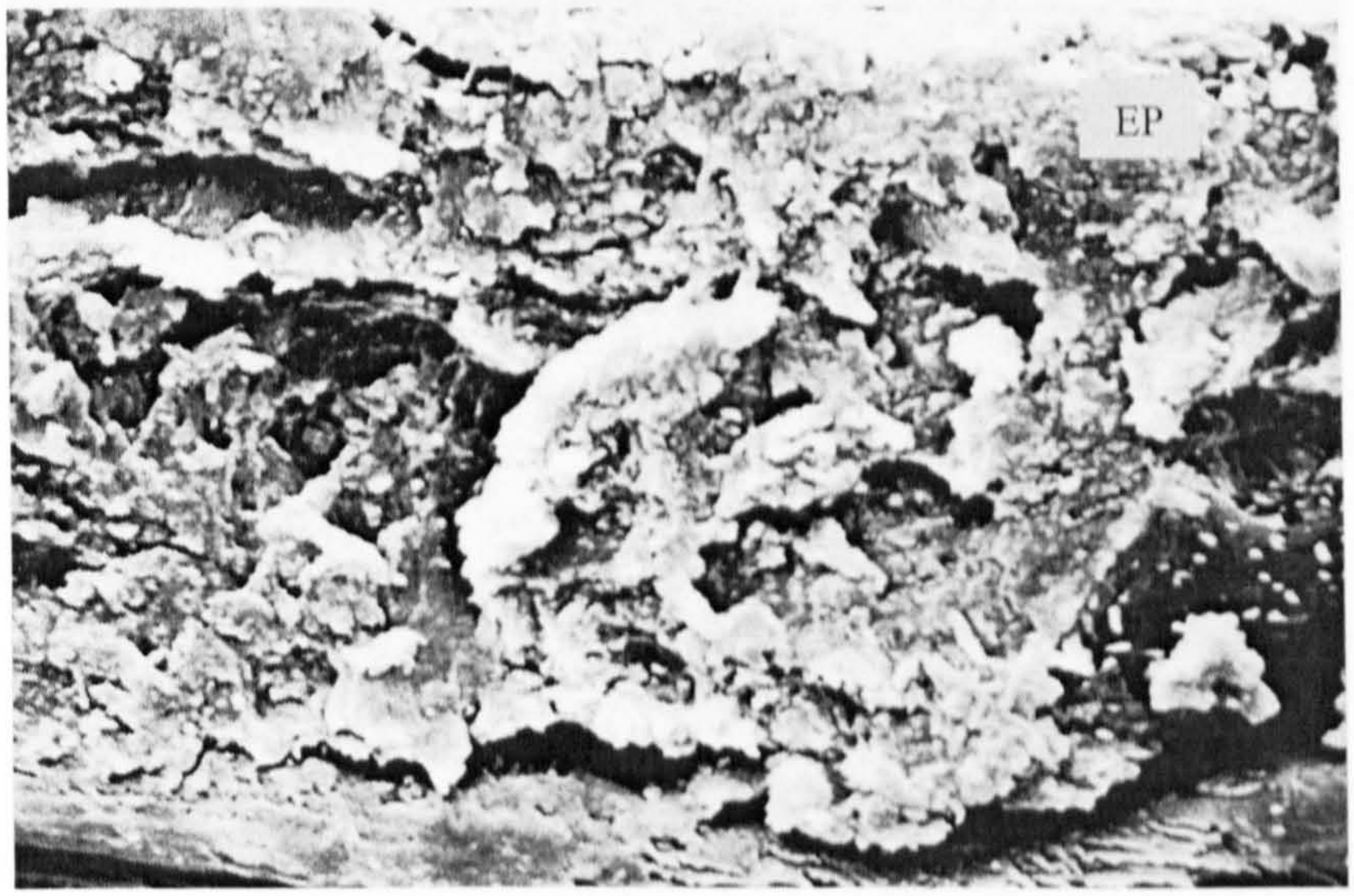


Fig. 2.25 *Scanning electron micrograph of a transverse section of the epicuticle* (x2800). EP = epicuticle

Figs. 2.26 and 2.27 show the continuously changing direction of the chitin-protein microfibrils in the exocuticle and the endocuticle.

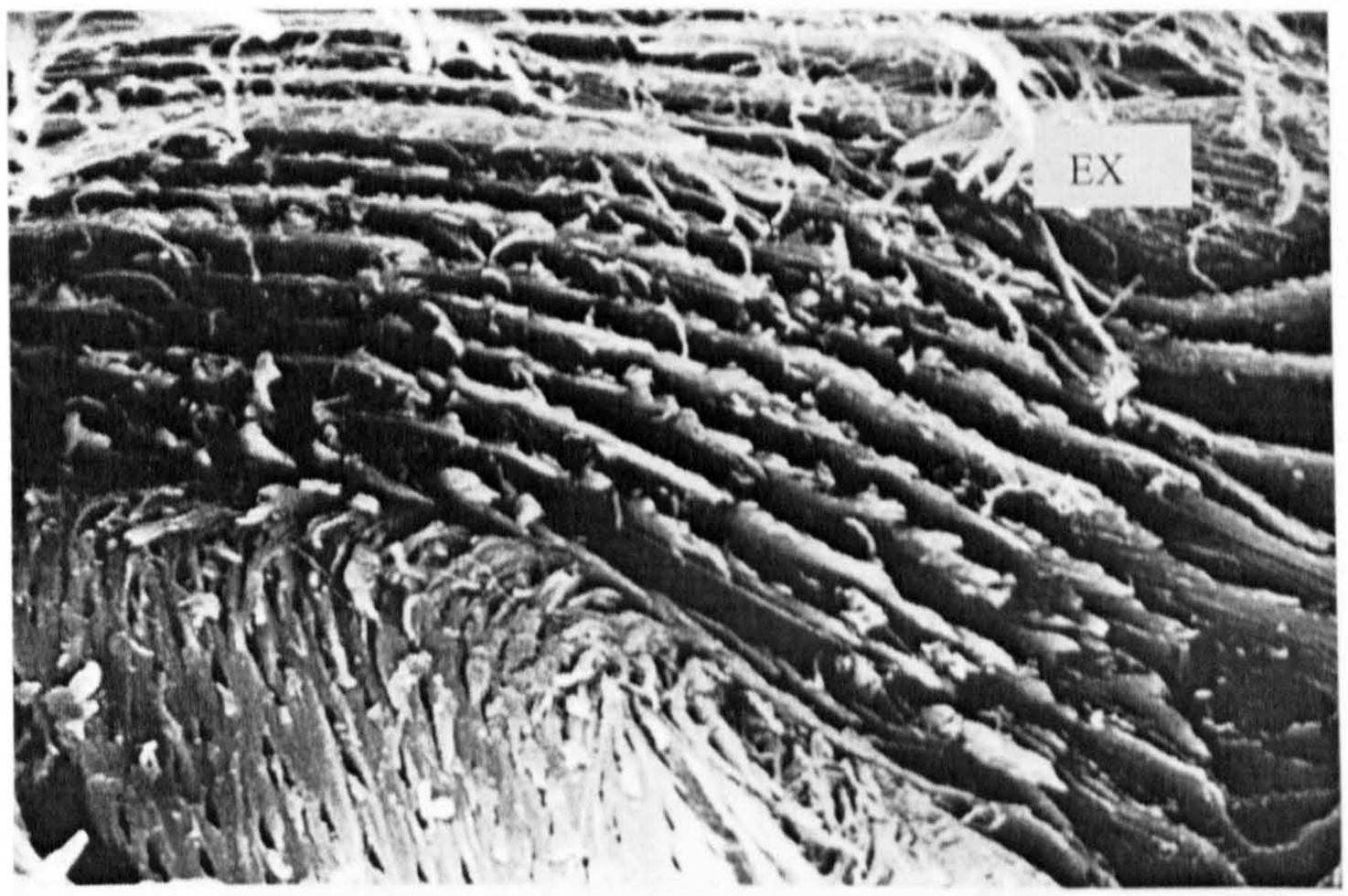


Fig. 2.26 *Scanning electron micrograph of a transverse section of the exocuticle* (x2800). EX = exocuticle

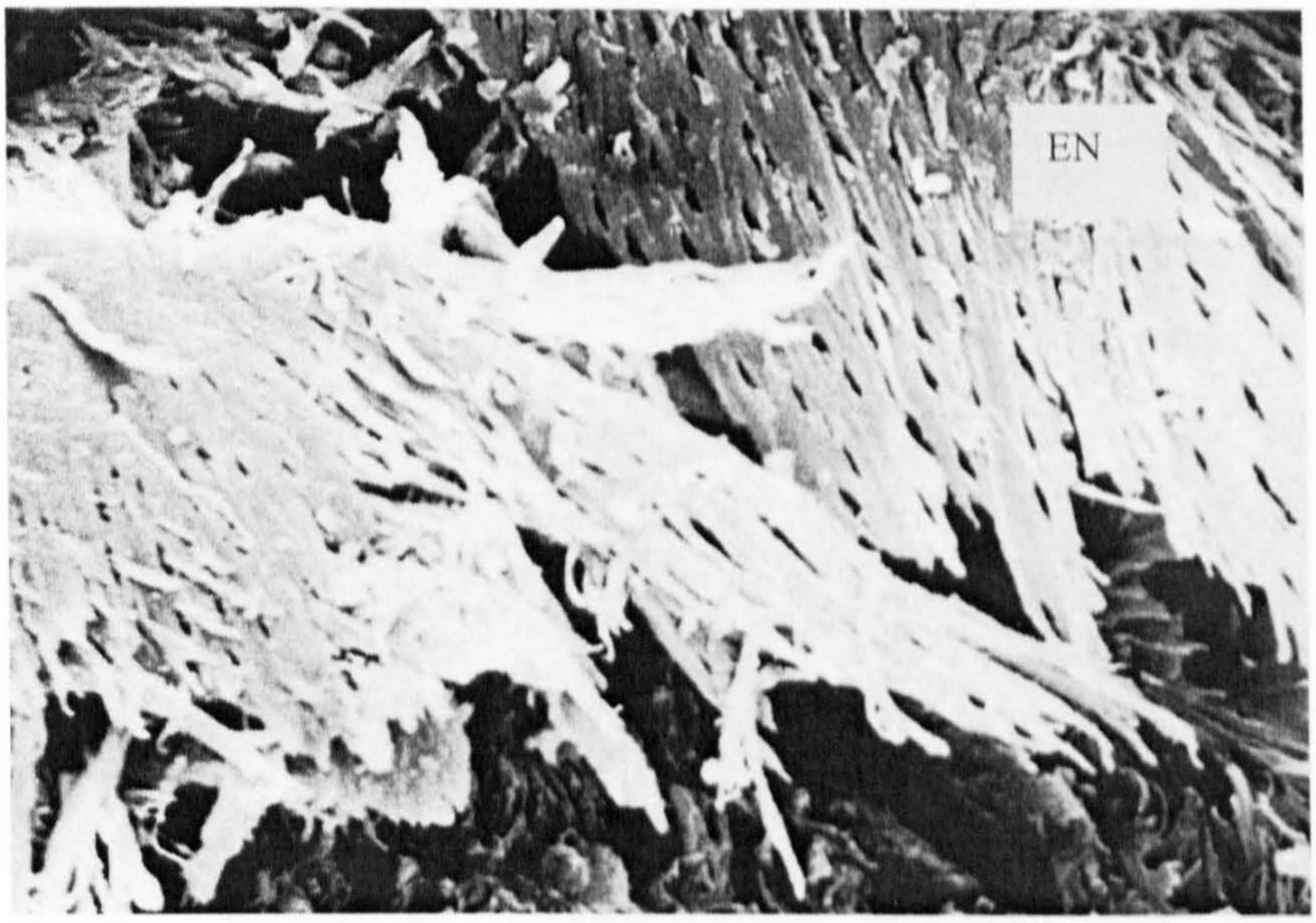


Fig. 2.27 *Scanning electron micrograph of a transverse section of the endocuticle* (x2800). EN = endocuticle

In Fig. 2.28 the layers are shown from a different perspective. Underneath the epicuticle the closely packed lamellae of the exocuticle appear. The exocuticle is also pictured in Fig. 2.29.

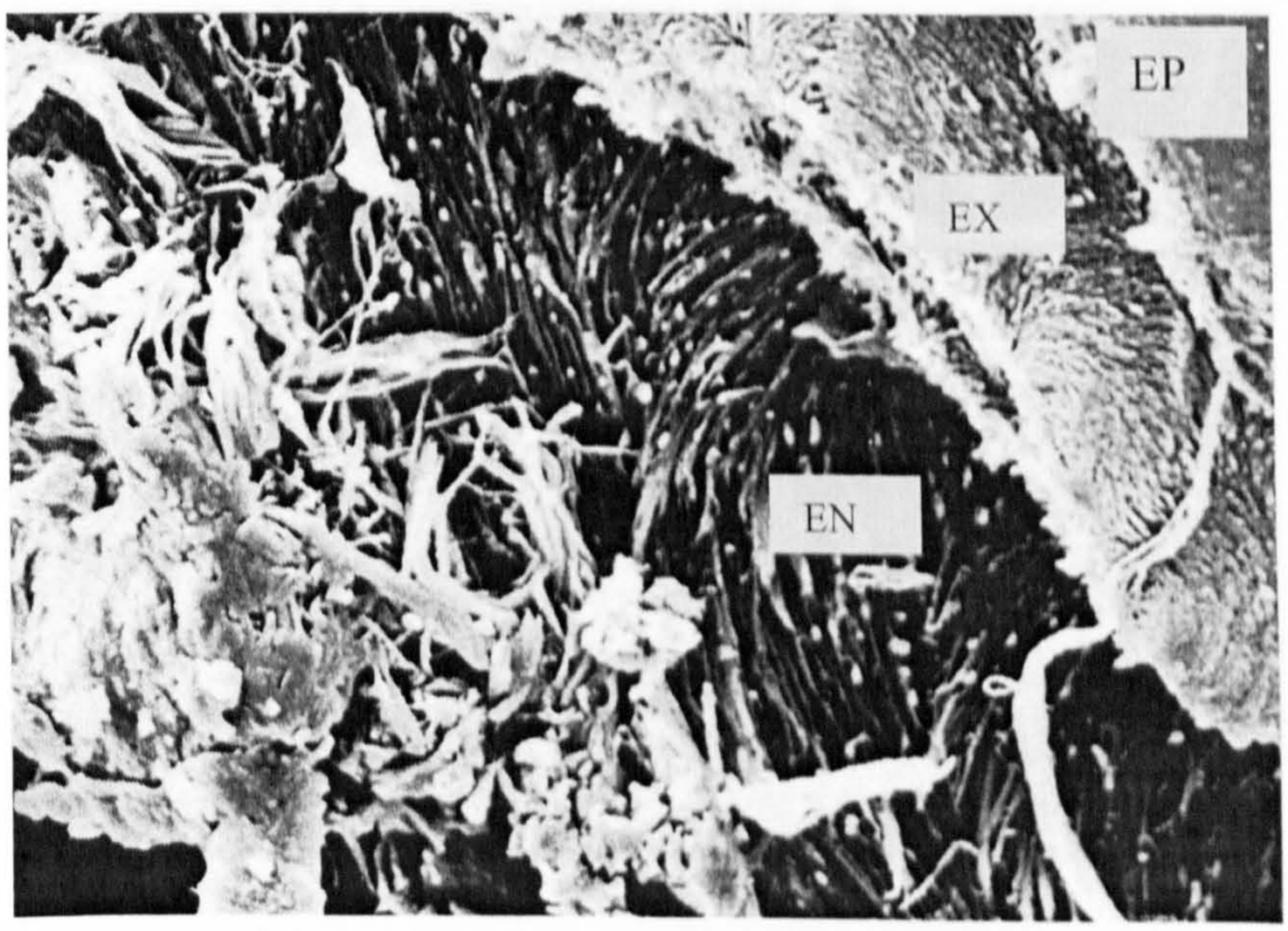


Fig. 2.28 *Scanning electron micrograph of a transverse section of the cuticle* (x1440). EP = epicuticle, EX = exocuticle, EN = endocuticle

Underneath the exocuticle in Fig. 2.28 the lamellae appear more widely spaced in the endocuticle. The endocuticle is shown at a higher magnification in Fig. 2.30.

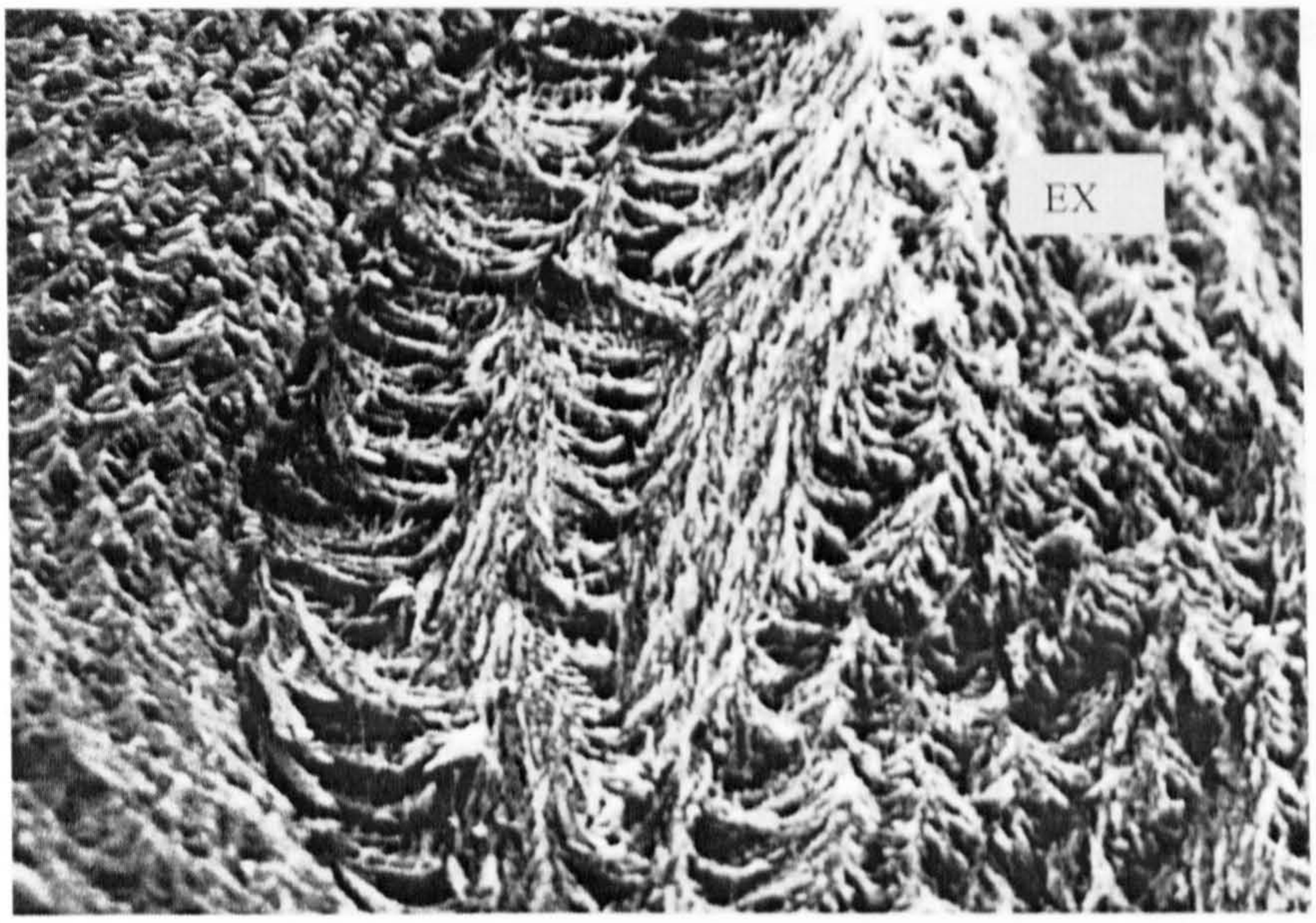


Fig. 2.29 *Scanning electron micrograph of a transverse section of the exocuticle* (x720). EX = exocuticle

Fig. 2.30 *Scanning electron micrograph of a transverse section of the endocuticle* (x5600). EN = endocuticle

2.4 DISCUSSION

The ultra-structure of prawn shell has not been widely studied. The scanning electron micrographs of the epicuticular surface and the lamellar regions within the shell provided an overall picture of the cuticle and the location of chitin within that cuticle. They also provided views with which demineralised and deproteinised shell could be compared.

Wide variability was present in the different areas of the epicuticle, with smooth and fibrous regions making up the full picture. Many of the fibres seen were possibly sensory in nature and the spines in Figs. 2.12 and 2.13 were of particular interest. These correlated with spines noted in a study of the common crab (Hegdahl *et al.*, 1977c). In the crab the epicuticular spines were shown to contain mineral and were probably extensions of the pore canals present in lower layers.

Scanning electron microscopy of the transverse sections of the shell expanded the initial work carried out by Bustos (1996) on *Nephrops* cuticle. The different layers in the cuticle were easily visible (Fig. 2.28). The lamellae were tightly packed in the exocuticle but became more loosely organised in the endocuticle. Similar results were seen in the shore crab and the European clawed lobster where the lamellae found in the exocuticle were more closely packed than those in the endocuticle (Mutvei, 1974).

Horizontal chitin-protein lamellae were visible in close association with vertical lamellae (Fig. 2.26). This too was similar to the scanning electron micrographs of *Cancer maenas* and *Homarus gammarus* (Mutvei, 1974). Pore canals pierced these lamellae, again as observed in other decapods.

Time was not available to study components of the shell waste other than the upper region of the abdominal segments such as the sternum, the swimmerets and the membrane between the segments. This brief examination has however served to highlight the position of chitin within the shell and to emphasise the diverse structure of the shell itself.

CHAPTER THREE

THE CHEMICAL COMPOSITION OF NEPHROPS SHELL WASTE

3.1 INTRODUCTION

The shells of decapod crustaceans contain ash, which consists mainly of calcium carbonate, chitin, protein, lipids and pigments. The concentration of these components varies depending on the species of crustacean in question. For example, shrimp (*Pandulas borealis*) contains 17.0% chitin, 41.9% protein and 29.2% ash (Synowiecki and Al-Khateeb, 2000) whereas Louisiana crawfish contains 23.5% chitin, 16.9% protein and 63.6% ash (No *et al.*, 1989).

The levels of shell components vary not only between species but also within the one species as the moulting stage changes. Crustaceans grow in size at moult as measured by change in carapace length (González-Gurriarán *et al.*, 1998). This growth must be facilitated by the synthesis of shell components in greater abundance at each ecdysis. Hornung and Stevenson (1971) used incorporation of ^{14}C labelled glucose to show that chitin synthesis occurred at all stages of the moult cycle but reached its peak post-moult in crayfish - a species of decapod crustacean very closely related to *Nephrops norvegicus*.

During pre-ecdysis the old cuticle is degraded. Some of the components are resorbed whilst others are lost. Nicol *et al.*, (1992) have shown variations in the levels of chemical components of the Antarctic krill (*Euphasia superba*) at different stages of the moult cycle. For example, the levels of ash, lipid, chitin and calcium are at their lowest immediately after ecdysis. The crustacean shell is therefore not a static commodity but rather changes continuously so the chemical composition will vary throughout the stages of the moult cycle.

No definite moult season has been observed for *Nephrops norvegicus*. Two major moulting periods were noted for Norway lobsters from Northwest Spain: the first in autumn-winter and the second in spring-summer (González-Gurriarán *et al.*, 1998). In the Irish Sea, sexually mature females moult between May and August, just after the eggs hatch (Farmer, 1974). This occurs after an egg incubation of approximately 8 - 9 months.

In other parts of the world egg incubation occurs for different periods of time. The length of time of incubation may be dependent on ambient sea temperature. In Iceland, the incubation period is 12 - 13 months, whilst further south in Portugal, where the sea is warmer, the incubation period is 6 -7 months (Eiríksson, 1993). As a result, the moult, which occurs immediately after hatching, will take place in different months of the year depending on location. Sexually mature males moult twice a year until they are 6 - 7 years old. Older males moult once a year, during winter. Juveniles, on the other hand, may undergo up to 3 moults each year (Sardà, 1995).

These differences in moult period depending on age, sex and location, suggest that little difference in shell composition should be noted throughout the year since a random batch of shell will contain males, females and juveniles all at different stages of moult. Bustos (1996) stated that only small variations between the compositions of shells collected at different months of the year were observed.

The chemical composition of shell waste obtained at different times of the year, for use in this project, was analysed to determine if batch-to-batch variation should be taken into account in subsequent experiments. The results obtained were compared with shell of different particle sizes, shell from different sources and shell that had begun to perish before drying.

3.2 MATERIALS AND METHODS

3.2.1 Initial Treatment of Shell

The shell waste was dried at 40°C, then ground to particles approximately 0.5 - 1mm^2 in size, using an Apex grinder Type 314. The particles were sieved, using Laboratory Test Sieves with apertures 1.00mm and 500μm (BS410/1986), and separated according to size (<0.5mm, 0.5 - 1.0mm and >1.0mm). A dust mask was worn during grinding of shell and handling of shell particles. Particles in the size range 0.5 - 1.0mm were used for analysis unless otherwise stated.

3.2.2 Chemical Analyses of Solid Samples

Solid samples were analysed singly for each component determined. For ash, calcium, phosphorus, chitin and moisture analyses samples of known value were run periodically and one sample in thirty was run in duplicate to check the validity of the results obtained. For C, H, N analysis reference standards were run between every ten samples.

All results reported were obtained from analyses carried out on samples previously oven dried at 40°C. Statistical analyses (section 3.2.3) were carried out on these results. Moisture content was determined for the same samples (section 3.2.2.8) and the results recorded. To verify that the variation in moisture content did not cause reported statistical significance all results were re-calculated on a dry matter basis and the statistical analyses repeated. Any significant effects calculated to be due to the moisture content alone have been stated in the results section.

3.2.2.1 Ash Determination

Samples (0.5 - 2g) were placed in pre-weighed porcelain crucibles. The crucibles were incubated in a muffle furnace at 550°C for 4 - 6 hours until a grey/white ash remained. The

crucibles were allowed to cool in a desiccator. The crucibles were re-weighed and the mass of residual material determined.

3.2.2.2 Dissolution of Ash Samples for Calcium and Phosphorus Determination

Ten ml of hydrochloric acid (50% v/v) was added to each crucible taking care that losses due to effervescence did not occur and the crucibles baked at 105°C to dehydrate the silica. The residue was moistened with concentrated HCl (1ml) and distilled H_2O (3ml) and boiled for 2 minutes. Approximately 5ml distilled H_2O was then added and the samples boiled again. Samples were quantitatively transferred to a 100ml volumetric flask by filtration through a 9cm Whatman No. 50 paper. Filtrate and washings were made up to 100ml with distilled H_2O.

3.2.2.3 Calcium Determination

Calcium standards were prepared from stock calcium carbonate (dried) (500mg/l) in the range 50 - 300mg/litre. Standards were diluted 1/10 in lanthanum chloride. Samples were diluted 1/100 in lanthanum chloride. Lanthanum chloride was used to correct for any interference due to the presence of phosphate. Standards and samples were read at an absorbance of 422.7nm on an atomic absorption spectrophotometer (Perkin Elmer 2380) using an air-acetylene flame.

3.2.2.4 Phosphorus Determination

Sample solutions (10ml) (prepared as in section 3.2.2.2) were pipetted into 50ml volumetric flasks. Ten ml of 5M hydrochloric acid was added and the total volume made up to 50ml with deionised H_2O to give a final concentration of 1M HCl. Phosphorus standard solutions were prepared, in the concentration range 0 - 0.03mg/ml, from stock $KH_2PO_{4\ (aq)}$ (0.25mg/ml) as follows. Stock solution (1, 2, 3, 4, 5 and 6ml) was placed in

50ml volumetric flasks. Ten ml of 5M HCl was added to each flask and the volume made up to 50ml with deionised H_2O.

Standards and samples (5ml) were pipetted into test-tubes. Mixed reagent (5ml) containing 10mg/ml ammonium molybdate and 0.5mg/ml ammonium vanadate was added to each tube. The tubes were mixed by vortexing and left at 20°C for 15 minutes. Absorption was measured at 400nm on a visible spectrophotometer (Pye/Unicam SP8-500).

3.2.2.5 Chitin Determination

Chitin content was determined using an adaptation of the method of Black and Schwartz (1950). The sample (1 or 2g) was mixed with 1M HCl (40ml) on a magnetic stirrer at room temperature for 2h. The sample was filtered under vacuum through a porous sintered glass disc of porosity 1 and washed to neutrality with tap water. The sample was then washed off the sintered glass disc, into a beaker, with 5% (w/v) NaOH (40ml) and stirred at 100°C for 2 hours. The resultant chitin was filtered through ashless filter paper (Whatman No. 41) in a porcelain funnel and washed to neutrality with tap water. The funnel and filter paper had been weighed prior to filtering. Chitin, funnel and filter paper were dried at 105°C overnight, allowed to cool in a desiccator and re-weighed to determine percentage chitin.

3.2.2.6 C, H, N Analysis

The carbon, hydrogen and nitrogen content were determined using a Perkin-Elmer 2400 CHN Elemental Analyser. Accurately weighed samples (usually 2mg to 4 significant figures) were sealed in tin capsules and the capsules inserted into the analyser. In the analyser the samples were combusted completely in the presence of excess oxygen and combustion reagents to form carbon dioxide, water and nitrogen. This gas mixture was

carried in a flow of helium through a reducing agent. After separation into the three component gases the level of each was determined using a thermal conductivity detection system. The percentage weight for each element was calculated automatically by a microprocessor contained in the analyser. Baseline values, determined by running empty capsules, and calibration factors, determined by analysing materials of known C, H, N composition, were used in the calculations.

3.2.2.7 Calculation of Residual Protein

Residual protein was quantified by calculation. The theoretical percentage nitrogen in chitin is 6.9%. The chitin nitrogen (i.e. 6.9% of the percentage chitin determined as in section 3.2.2.5) was subtracted from the total nitrogen obtained by C, H, N analysis. The corrected nitrogen value was multiplied by a factor of 6.25 (the Kjeldahl conversion factor for meat protein assuming that protein has 16% nitrogen) to give the protein concentration in the solid residue.

3.2.2.8 Moisture Analysis

Samples (1-3g) were dried at 105°C for 18 hours. Samples were weighed before and after drying. After drying the samples were cooled in a desiccator before weighing. Moisture (g/kg) was determined by weight difference.

3.2.3 Statistical Analysis

All statistical tests were carried out using GraphPad Prism, version 3.0 (GraphPad Software, Inc.). Unpaired t-tests were used to compare two sets of data. Where three or more groups were compared one-way analysis of variance was followed by Tukey's post test.

3.3 RESULTS

3.3.1 Shell Composition

The shell waste was collected at four different times in one year (January, May, September and November). The results of the chemical composition of each batch are shown in Figs. 3.1 and 3.2.

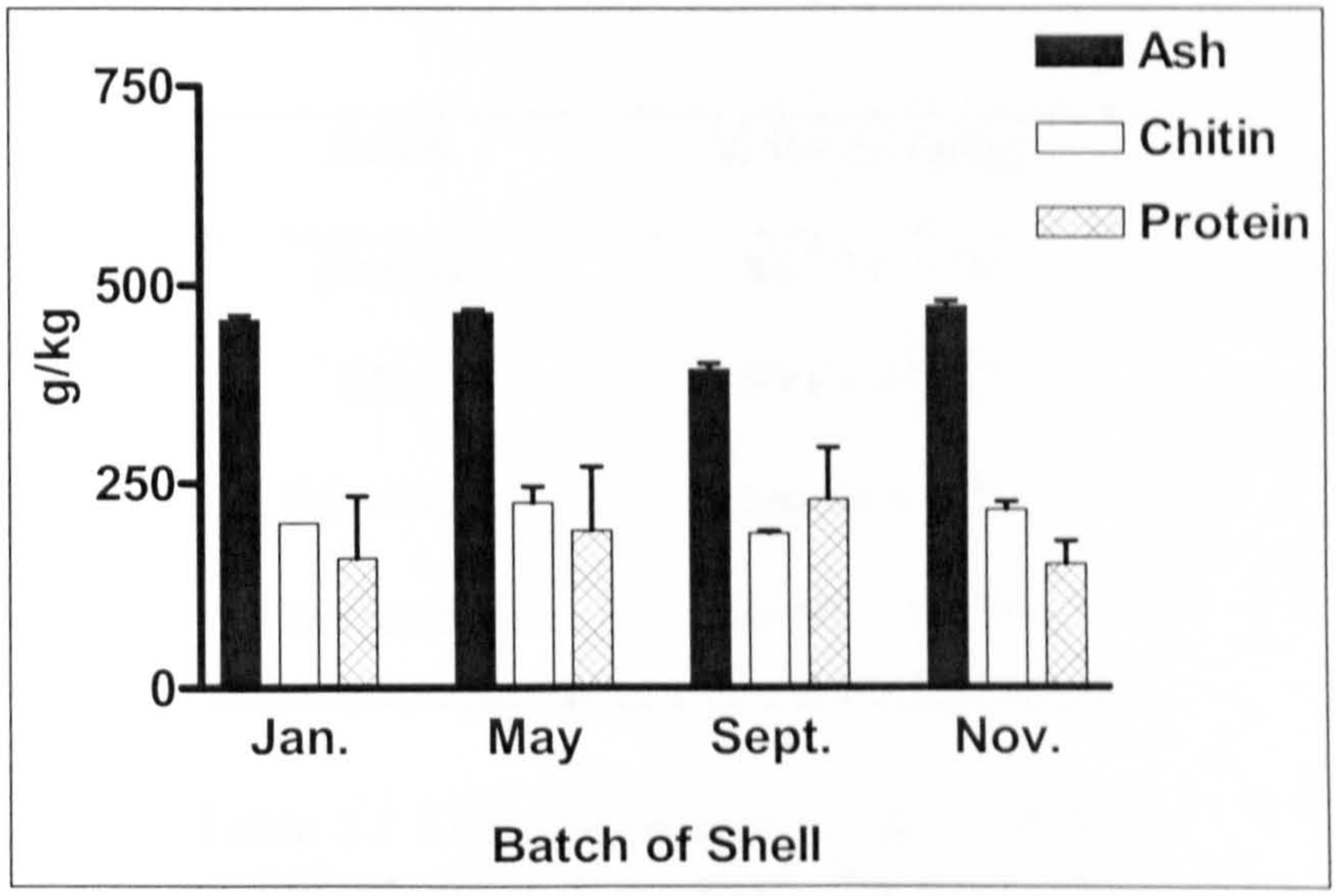


Fig. 3.1 *Ash, chitin and protein content of shell obtained at different times of the year.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

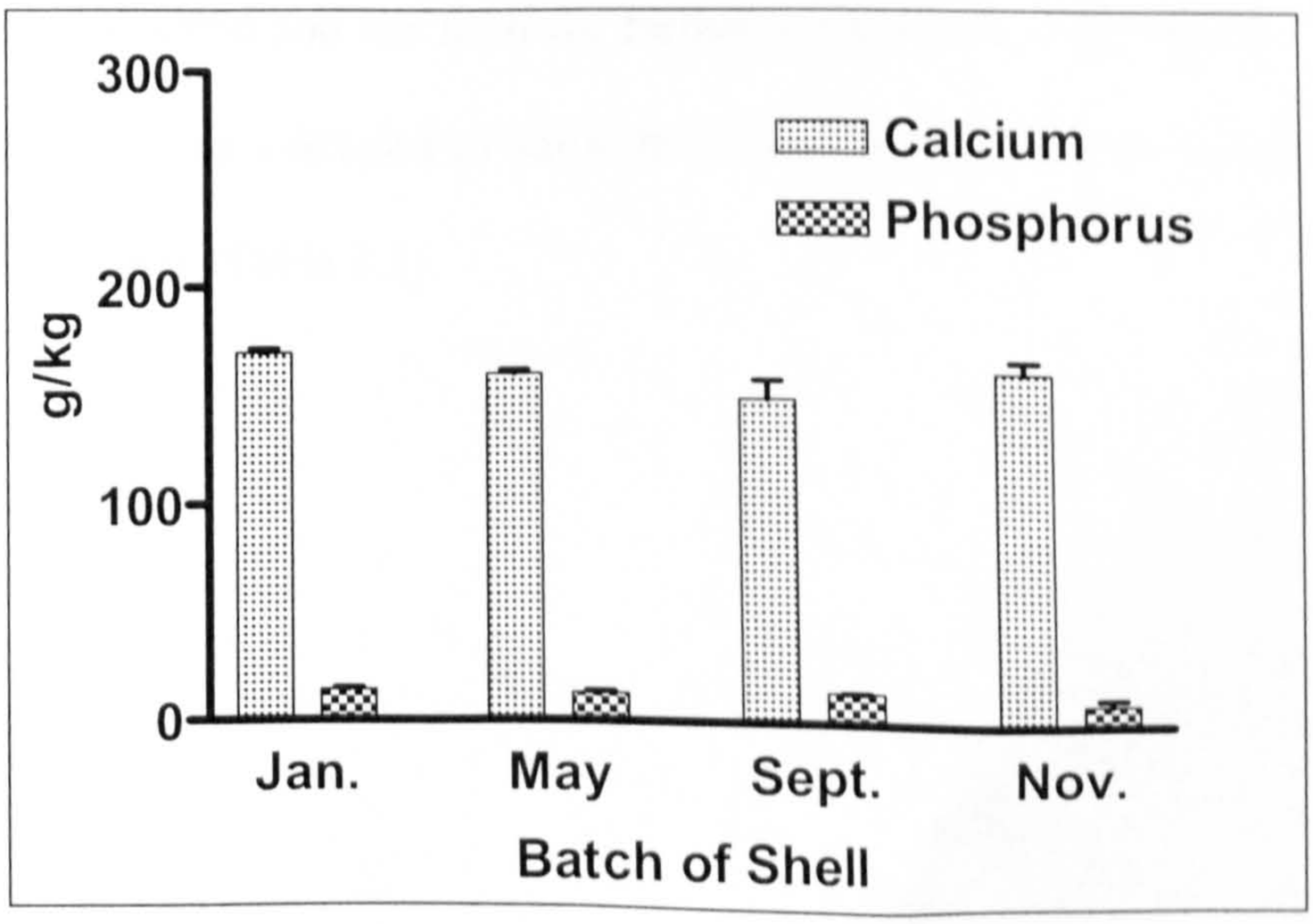


Fig. 3.2 *Calcium and phosphorus content of shell obtained at different times of the year.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

The only significant difference observed was a lower percentage ash found in the September batch ($p<0.01$ when compared with batches obtained in January, May and November).

The moisture content of each batch is shown in Table 3.1. Moisture contents of shell obtained at different times of the year did not differ significantly.

Batch	Moisture (g/kg)
January	88.20 ± 9.90
May	89.60 ± 8.77
September	106.30 ± 9.33
November	89.05 ± 10.39

Table 3.1 *Moisture content of shell obtained at different times of the year.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

In an attempt to obtain a homogeneous starting material the batch of shells obtained in September was discarded and not used for further experiments. The figures for the three remaining batches were averaged to show the chemical composition of fresh *Nephrops norvegicus* shell waste (Table 3.2).

Component	g/kg shell waste[a]
Ash	465.58 ± 8.88
Chitin	214.17 ± 15.63
Protein	165.66 ± 22.27
Moisture	88.95 ± 7.55
Others[b] (fats, pigments, etc.)	59.65 ± 27.0
Minerals:	
Calcium	163.21 ± 5.45
Phosphorus	14.14 ± 2.29

(a) Average of three different batches ± standard deviation
(b) Determined by difference

Table 3.2 *The chemical composition of Nephrops norvegicus shell waste.*

Phosphorus analysis was discontinued at this stage due to the low levels contained in the waste (1.4% w/w).

3.3.2 Comparison of Shell from Different Sources

The chemical composition of shell waste, obtained in the springtime, from the Irish Sea was compared with shell waste obtained from the North Sea at the same time of year (Figs. 3.3 and 3.4). The North Sea shells were larger than those from the Irish Sea but the chemical composition of the waste from the two sources was similar.

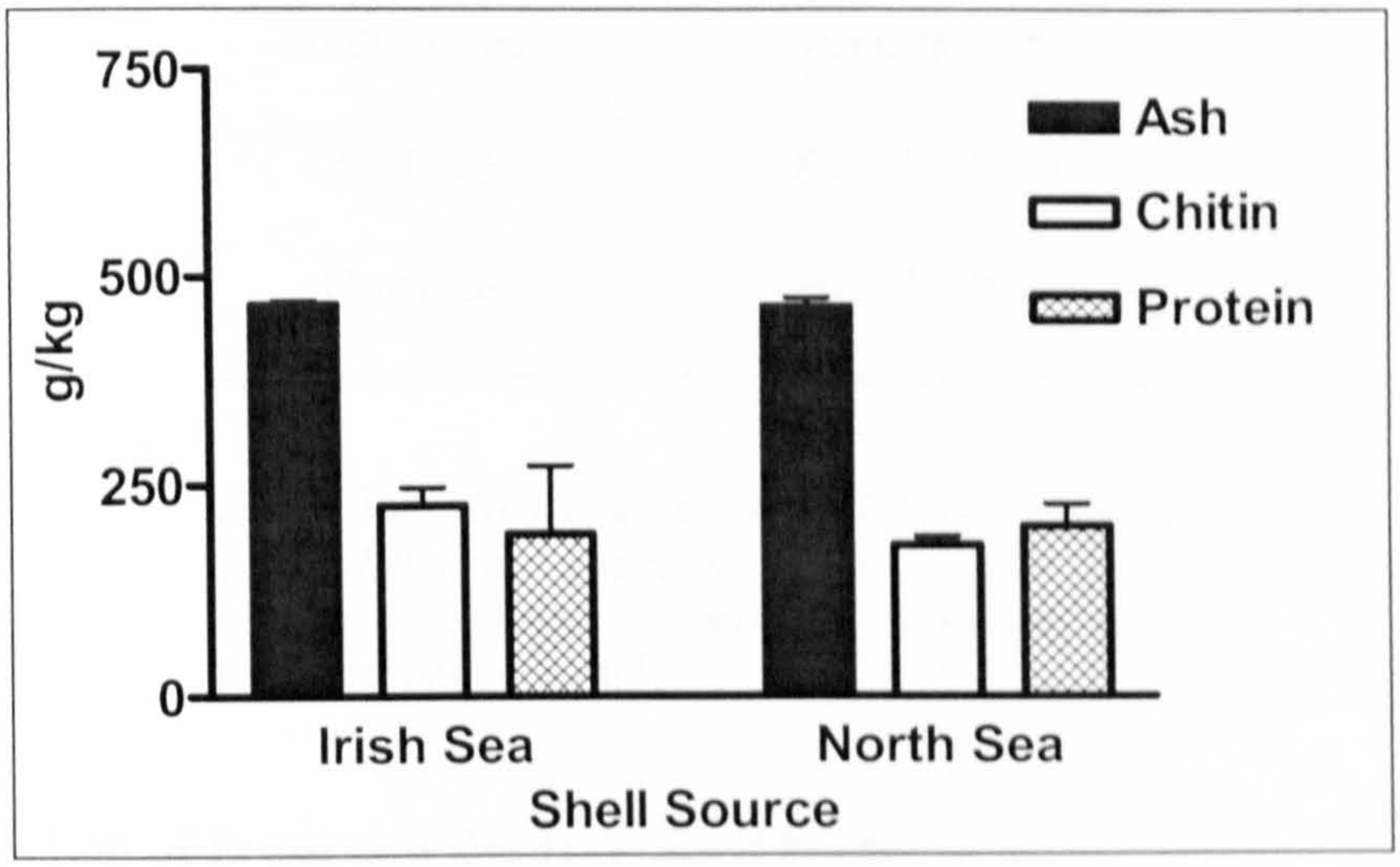


Fig. 3.3 *Ash, chitin and protein content of shell obtained from different sources.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

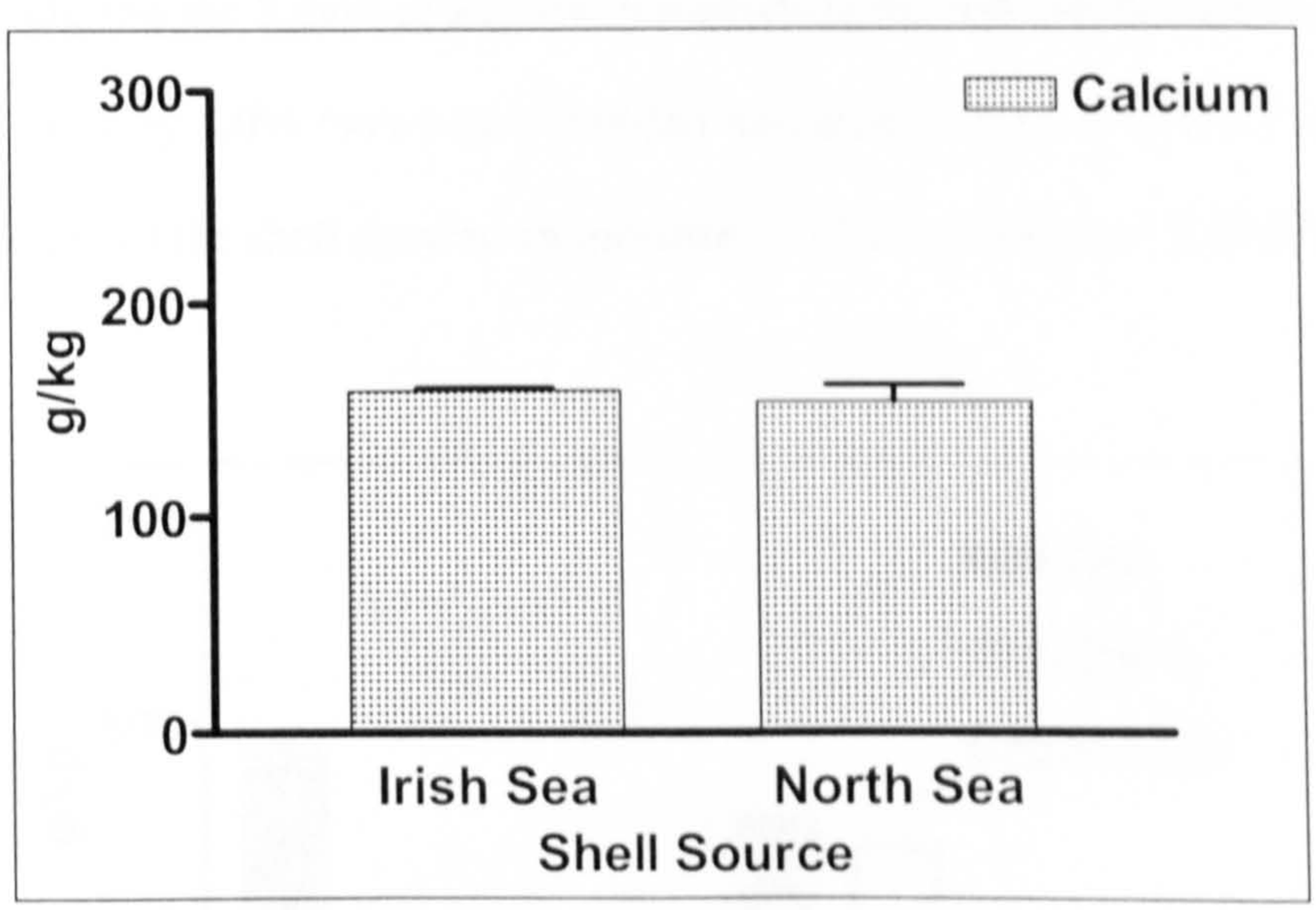


Fig. 3.4 *Calcium content of shell obtained from different sources.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

The moisture content of the shells from the two different sources was also similar (Table 3.3).

Shell Source	Moisture (g/kg)
Irish Sea	89.60 ± 8.77
North Sea	95.99 ± 6.21

Table 3.3 *Moisture content of shell obtained from different sources.* The results shown represent the mean ± standard deviation of 2 determinations per sample

3.3.3 Comparison of Fresh and Decayed Shell Waste

The composition of shell waste washed approximately one hour after collection and dried immediately at 40°C for 5 days (fresh waste) was compared with shell waste kept at ambient temperature for 5 days, before washing and drying at 40°C (decayed waste) (Figs. 3.5 and 3.6). During the 5 days at ambient temperature the ash and protein levels dropped considerably - ash by 8.0% (w/w) ($p = 0.0086$) and protein by 9.4% (w/w) ($p = 0.0343$). The chitin content of the shell showed an increase of 17.0% (w/w) ($p = 0.0022$).

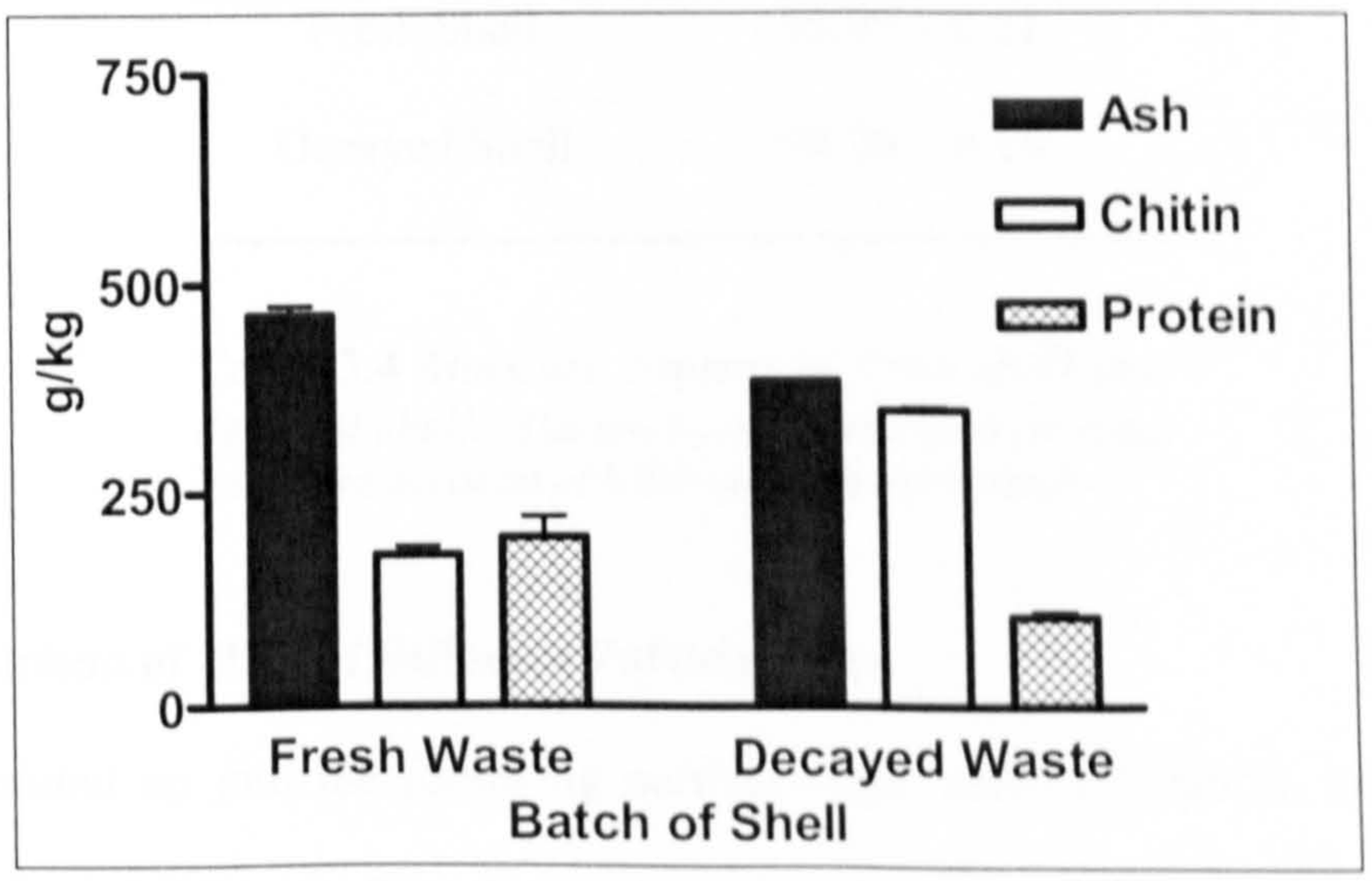


Fig. 3.5 *Ash, chitin and protein content of fresh shell and decayed shell.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

Levels of calcium and moisture did not vary significantly between fresh and decayed shell waste ($p = 0.2317$) (Fig. 3.6 and Table 3.4).

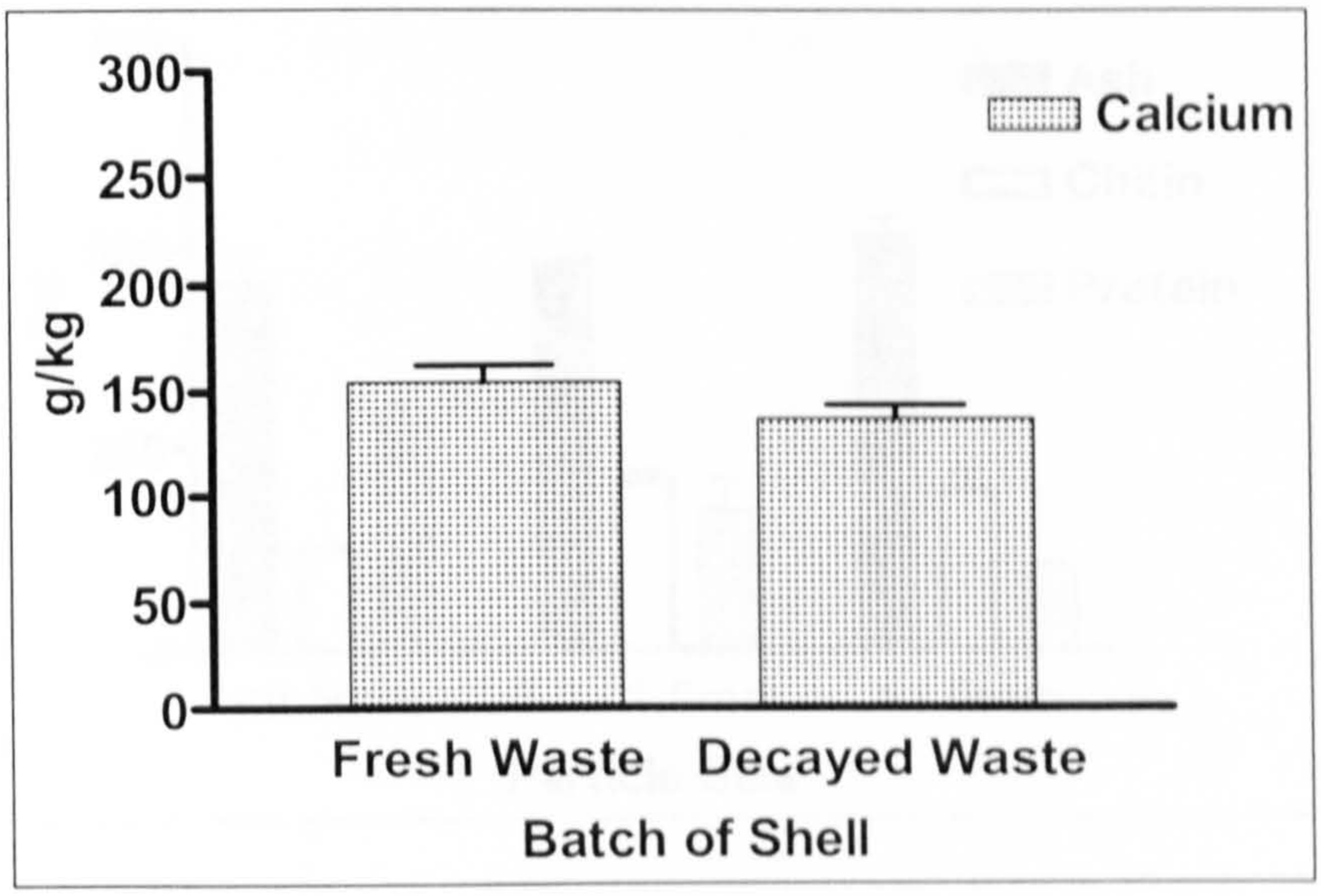


Fig 3.6 *Calcium content of fresh shell and decayed shell.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

Batch	Moisture (g/kg)
Fresh Shell	95.99 ± 6.21
Decayed Shell	94.20 ± 6.36

Table 3.4 *Moisture content of fresh shell and decayed shell.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

3.3.4 Comparison of Shell of Different Particle Sizes.

Shell was ground up into the following particle sizes: small (<0.5mm), medium (0.5 - 1.0mm) and large (>1.0mm). The chemical composition of each particle size was analysed (Figs. 3.7 and 3.8). Ash levels did not vary between the different batches. However, large particles (>1.0mm) and medium particles (0.5 - 1.0mm) contained significantly more chitin

($p < 0.05$) than small particles (<0.5mm). Large particles contained less protein than small particles ($p<0.05$).

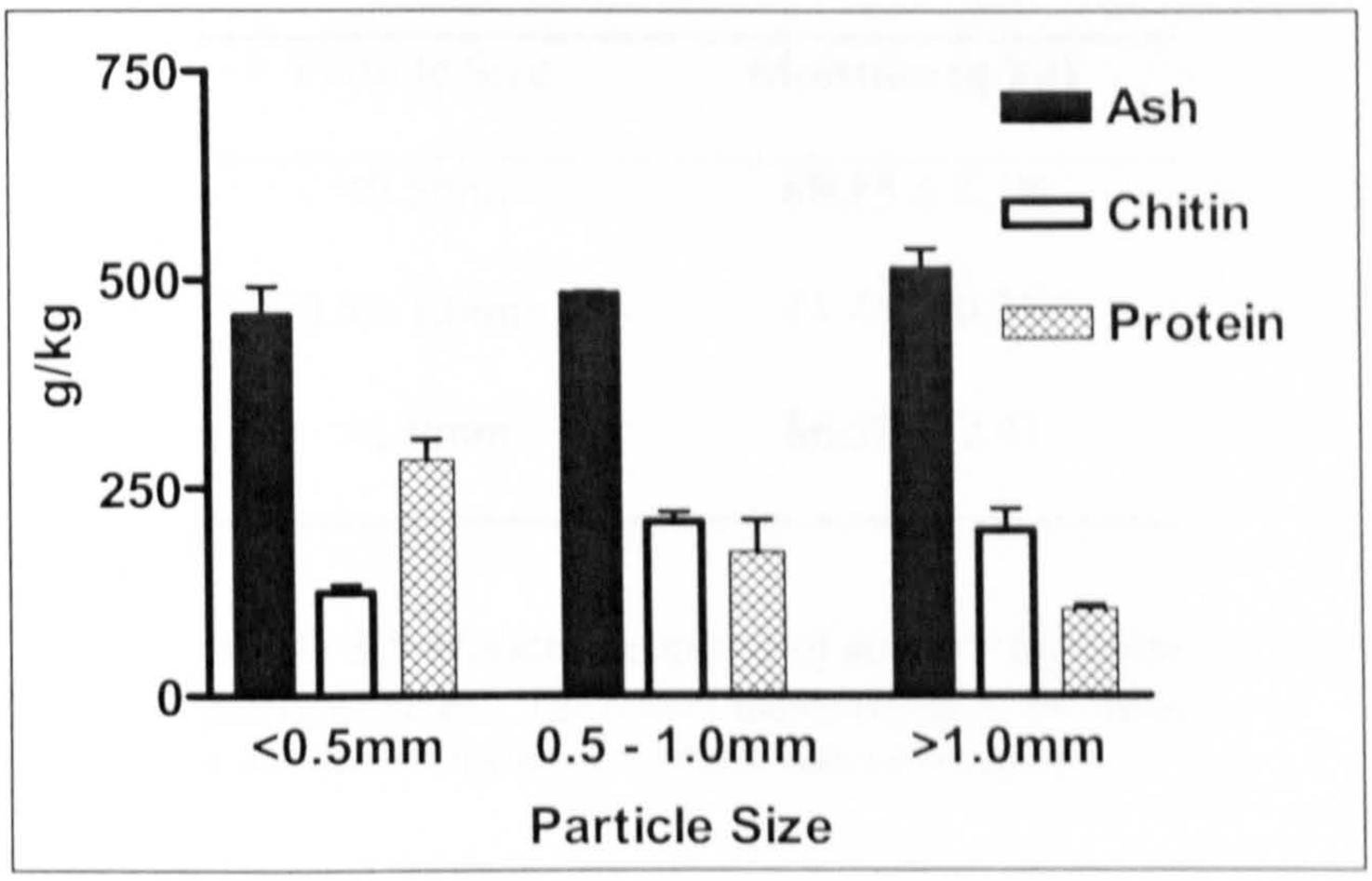


Fig. 3.7 *Ash, chitin and protein content of shell of different particle sizes.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

Calcium levels did not differ significantly between the different sizes but this may have been due to the wide variation seen at the smallest particle size ($p>0.05$) (Fig 3.8).

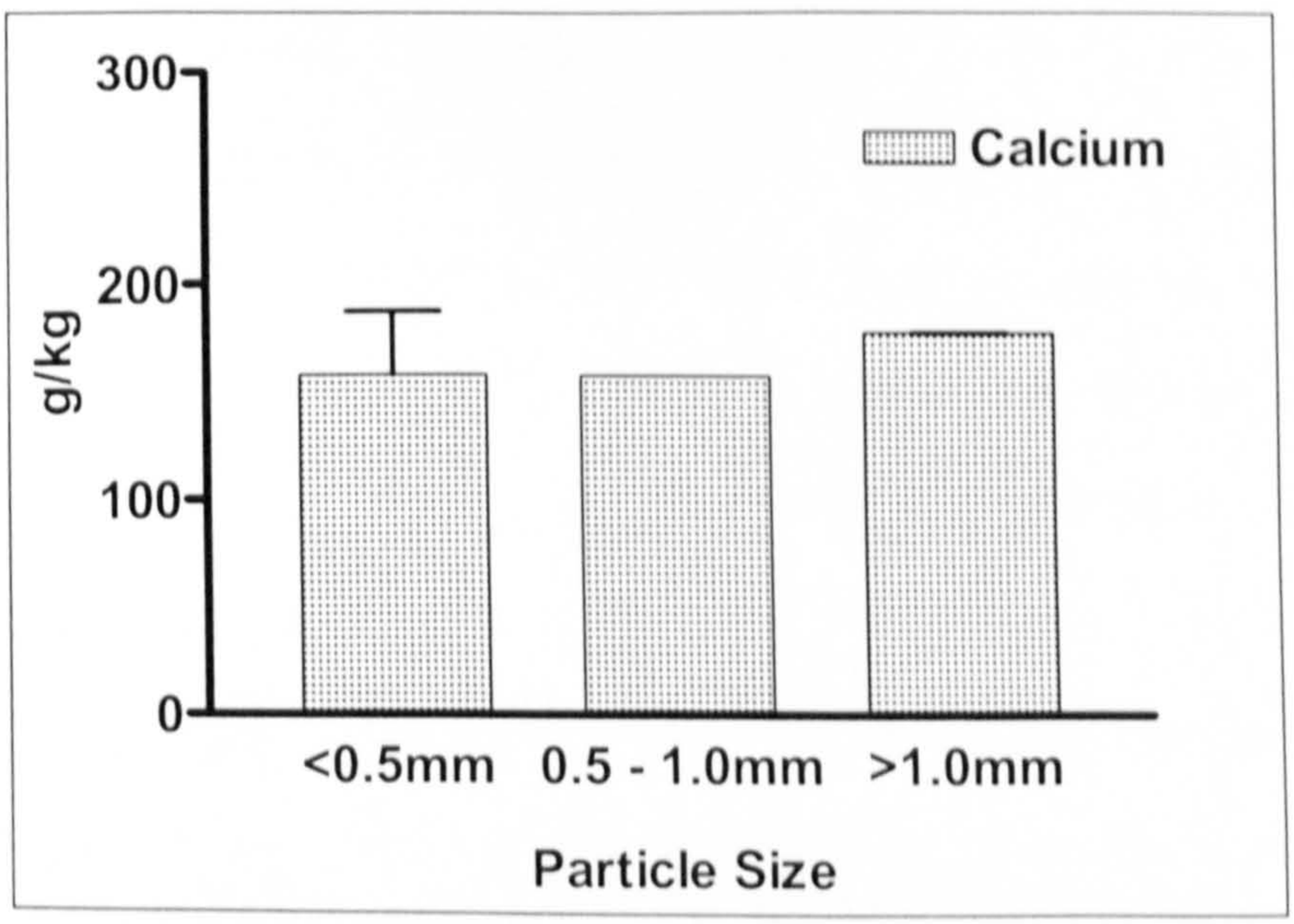


Fig. 3.8 *Calcium content of shell of different particle sizes.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

Moisture contents did not vary significantly between the three different particle sizes analysed ($p>0.05$) (Table 3.5).

Particle Size	Moisture (g/kg)
<0.5mm	88.15 ± 3.18
0.5 - 1.0mm	81.45 ± 0.35
>1.0mm	86.50 ± 1.41

Table 3.5 *Moisture content of shell of different particle sizes.* The results shown represent the mean ± standard deviation of 2 determinations per sample.

3.4 DISCUSSION

The storage and drying facilities available meant that one large batch of shell waste could not be obtained, washed and dried before the shells started to perish, nor could a sufficient quantity of waste be stored frozen. Therefore, the shells were obtained at four different time points throughout the year. The shells obtained for this project were a random mixture, from prawns harvested and stored frozen at the fish factory during the six months prior to shell waste collection. Due to the random nature of each collection, batch-to-batch variation was not expected to be large. Nevertheless, each batch was analysed to ensure that any variation between batches was not going to affect subsequent experiments.

The lower percentage ash noted in the September batch may have been a result of the moulting pattern of the prawns. Throughout the year catches contain a larger proportion of male *Nephrops* than females but in July and August the ratio of females has been seen to increase to approximately 50% (Farmer, 1974). Older females in the Irish Sea moult from May-August, between egg-hatching and egg-laying (Farmer, 1974). Therefore, the shells in the September batch were probably harvested during this moult season. Prior to moult, pre-ecdysial moulting fluid is secreted by cuticle-secreting epidermal cells into the ecdysial space between the cuticle and the epidermis and hydrogen ions are secreted into the moulting fluid. This causes the breakdown of $CaCO_3$ to Ca^{2+} and CO_3^{2-} or $H^+CO_3^{2-}$. Resorption of calcium and bicarbonate ions occurs by transport through the cuticle and hypodermis (Roer and Dillaman, 1993). Not all the ions will be resorbed however and some will be lost to the environment. This dissolution of calcium carbonate could lead to a lower percentage ash being detected in the shells of female *Nephrops*.

As the batch-to-batch variation was not significant for waste obtained in January, May and November the waste obtained in September was discarded and further experiments carried out using the remaining batches. For each component the mean for the three batches was used as an initial standard to which subsequent results could be

compared. However, there is no reason why shell obtained at any time of year could not be used for chitin isolation.

The variation in size observed in shells from different sources can be attributed to population biology and growth rate of the *Nephrops* in a particular area (Briggs, 1997). However, the similarity in chemical composition between shells from two different sources suggests that the size and/or origin of the shell will not affect degradation. On the basis of this preliminary study it appears that the degradative methods applied will not have to be modified when shell from different regions is used.

Slightly decayed shell contained 17% (w/w) more chitin than fresh shell. This increase in chitin was due to a decrease in ash and protein. Approximately 9% (w/w) of the protein had been broken down in the five days prior to drying. If the shell protein is to be isolated at the same time as chitin any delay in preserving the shell will result in a lower protein yield. This means that the transportation of the waste for long distances for processing will not be possible, as is the case for crab processing waste (Abazinge *et al.*, 1994), or indeed any fish/shellfish processing waste, unless the waste can be transported in the frozen state. On the other hand, if chitin is the only component required from the shell, it may be beneficial to allow the waste to decay slightly before use. However, it should be noted that the results provided herein do not show if the chitin had been degraded to any extent. Stankiewicz *et al.*, (1998) studied the biodegradation of a stomatopod crustacean under anoxic conditions and showed that after 8 weeks of decay some chitin still remained but structural degradation had occurred. No structural degradation was noted in the first two weeks so it is unlikely that the chitin in the prawn shell will have been degraded in the five days prior to drying. All further studies in this project were carried out using shell that had been dried immediately after collection from the fish factory. This minimised degradation of any of the shell components and so provided a similar starting material for all experiments.

The variation in composition of shell waste with different particle sizes was not unexpected. No *et al.*, (1989) compared crawfish meals with different particle sizes. Similar results ensued. Particles smaller than 0.5mm contained more protein, less calcium and less fibre than larger particles. For all further experimentation on the shell waste, a substrate particle size range of 0.5 - 1.0mm was used. This was the size range determined by Bustos (1996) to be the optimum for the microbial demineralisation of prawn shell waste.

CHAPTER FOUR

CHEMICAL EXTRACTION OF CHITIN FROM NEPHROPS SHELL WASTE

4.1 INTRODUCTION

Traditionally, chitin has been isolated from crustacean shell using chemicals to remove the other components of the shell (ash, protein, lipids and pigments); of these the two main components to be removed are the ash and the protein. A range of chemicals has been employed successfully. For demineralisation, acids including hydrochloric acid (HCl) (Hackman, 1954; No *et al.*, 1989), ethylene-diamine-tetra-acetic acid (EDTA) (Foster and Hackman, 1957; Brine and Austin, 1981a and b), and formic acid (Horowitz *et al.*, 1957) have been used. For deproteinisation sodium hydroxide (Horowitz *et al.*, 1957) and sodium carbonate (Giles *et al.*, 1958) have been applied. In general, hydrochloric acid and sodium hydroxide are the most widely used. Fig. 4.1 shows a simplified flow diagram for the isolation of chitin from crustacean shell.

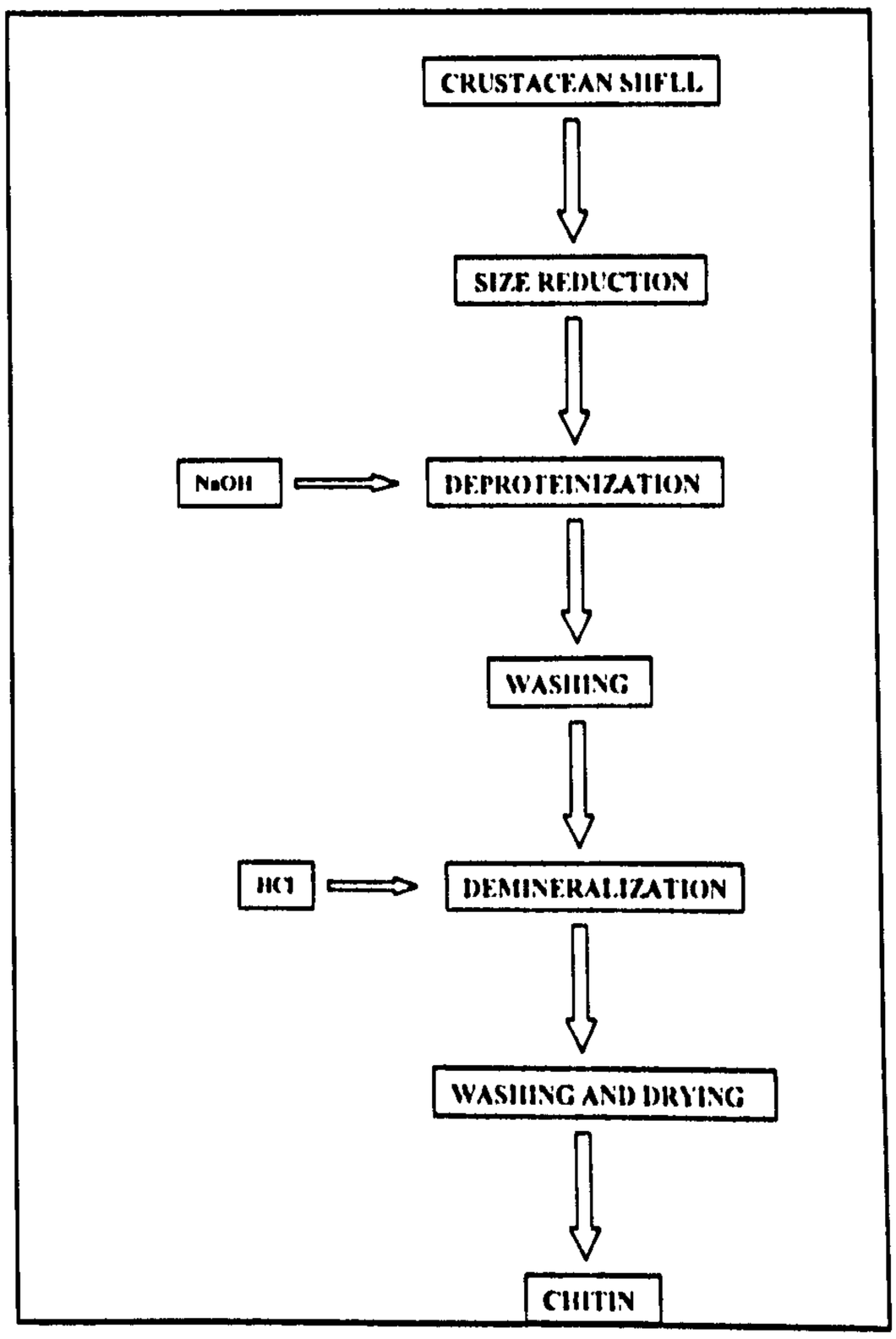


Fig. 4.1 *The isolation of chitin from crustacean shell* (No and Meyers, 1995).

The intermediate washing steps involved in chitin extraction, using HCl and NaOH, lead to the production of large volumes of aqueous acidic and alkaline waste. The difficulty in disposing of this waste has necessitated the search for biotechnological methods to isolate chitin. Another drawback of this process is that the harsh chemicals employed degrade potentially valuable shell components, such as the protein and the pigment astaxanthin. Therefore, abolishing the need for chemicals in the process would allow products other than chitin to be isolated from the shell and at the same time decrease expenditure on waste disposal and reduce environmental pollution. The chemical method was used in this project as a means of providing traditionally prepared chitin for comparison with chitin isolated by a bioprocessing technique.

Ideal conditions have not been optimised for the chemical isolation of chitin from *Nephrops norvegicus* shell waste. Kamasastri and Prabhu (1961) prepared chitin from prawn shell waste by refluxing in acetone for 45 minutes to remove pigments and lipids; HCl (5%) at ambient temperature for 2 hours was used for demineralisation and NaOH (5% w/v) under reflux for 2 hours caused deproteinisation. Madhavan and Nair (1974) also isolated chitin from prawn waste (*Metapenaeus dobsoni*). Protein was removed by boiling the waste with 3% (w/v) sodium hydroxide solution. For demineralisation, the deproteinised residue was then immersed in 1.25M hydrochloric acid at room temperature for 1 hour. Pigments were bleached using cold hypochlorite solution containing 0.3 to 0.5% available chlorine for 30 minutes. The same methods were utilised with prawn waste from other species such as *Penaeus indicus* and *Penaeus monodon*.

Bustos (1996) carried out a preliminary demineralisation study on *Nephrops norvegicus* shell. The effects of concentration of HCl and the solid to liquid ratio (SLR) were considered. The ash content obtained was less than 5% of the final product. The parameters stipulated in the methods of Bustos (1996) have been employed in this project. These methods used 1M HCl and a 10:1 solid to liquid ratio at room temperature for 6

hours for demineralisation and 4% (w/v) NaOH at 90°C for 4 hours for deproteinisation. These conditions provided a standard chitin with which future bioprocessed shell could be compared.

4.2 MATERIALS AND METHODS

4.2.1 Demineralisation

Ground shell waste (200g), of particle size 0.5 - 1.0mm, was mixed with 1M HCl (2L), in a 5 litre reactor vessel (Fig. 4.2), for 6 hours at room temperature. The lid for the vessel, which was held in place with a stainless steel clip, possessed a number of open ports. The mixture was stirred constantly during this period with a motorised stirrer (RW 20 IKA®-Labortechnik) set at 200rpm. At least one neck of the adapter lid was left open to allow escape of carbon dioxide gas.

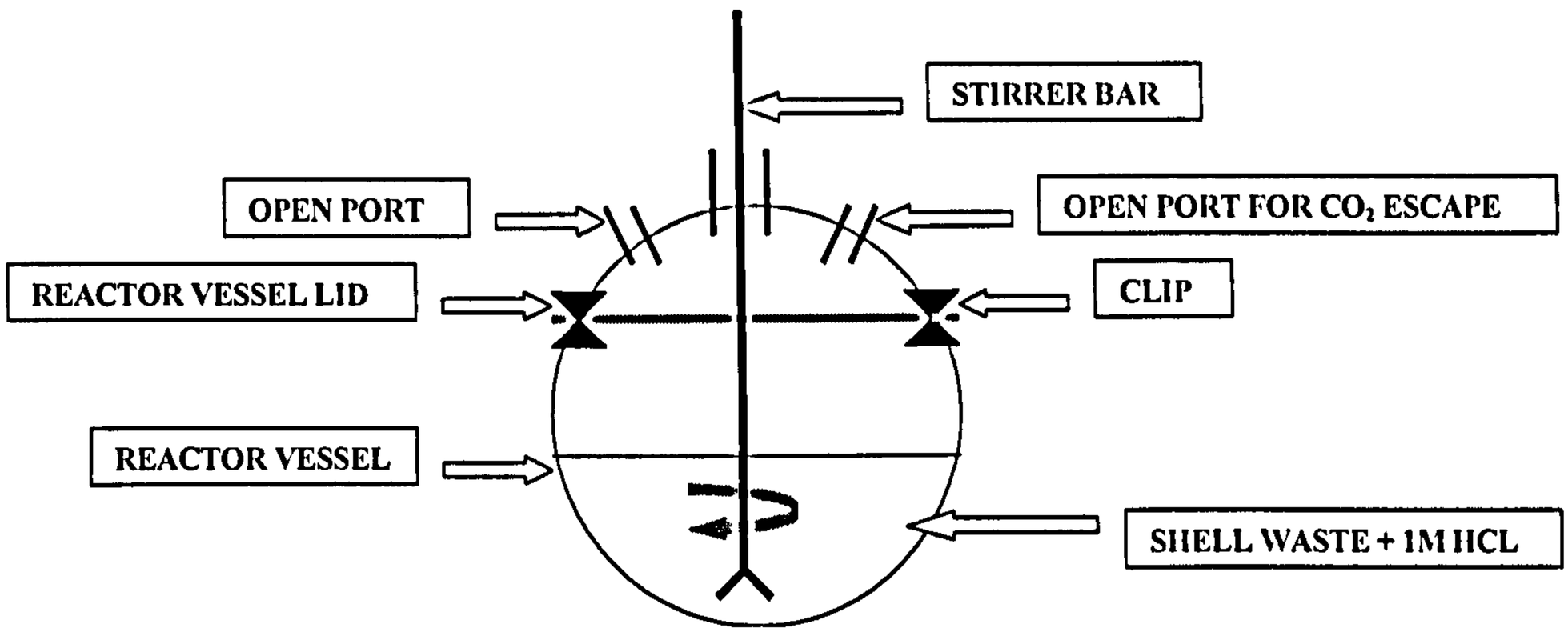


Fig. 4.2 *Schematic diagram showing apparatus for shell waste demineralisation.*

The treated material was filtered through two layers of pre-weighed muslin. The material was washed with tap water until neutral pH had been attained. Residual water was squeezed out of the muslin and sample by hand. Muslin and sample were dried at 40°C until constant weight. Percentage weight loss was then determined. The residue was brushed off the muslin and stored in a foil bag until use. A sample (approximately 5 - 10g) was kept for further compositional analysis.

4.2.2 Deproteinisation

The demineralised shell was deproteinised with 4% (w/v) NaOH, in a 1:20 (w/v) solid to liquid ratio, at 90°C and 200rpm for 4 hours. The reaction was carried out in a 2 litre reactor vessel. The vessel was heated with an electric mantle (Electromantle EM1000/CE) and agitated with a motorised stirrer (RW 20 IKA®- Labortechnik). The lid for the vessel was fitted with a condenser. A thermocouple attached to a digital thermometer (Digitron) was inserted through the condenser for temperature measurement. All unused necks of the adapter remained closed. The reaction was carried out in a fume hood. After 4 hours the temperature was allowed to drop to 60°C before the condenser was turned off in order to prevent evaporation.

The residue was filtered, washed, dried and stored in an identical manner to the demineralised product. A sample (5 - 10g) was kept for analysis. At this point the solid could be termed chitin.

4.2.3 Analyses

The chemical composition of each batch of demineralised and deproteinised shell waste was analysed using the methods described in section 3.2.2.

4.2.4 Scanning Electron Microscopy

Residual material, post demineralisation and deproteinisation of shell waste, was investigated using scanning electron microscopy. The method was as described in section 2.2.2.2 except that the particles viewed were <1.0mm in size.

4.3 RESULTS

4.3.1 Demineralisation

Ash (99.3% w/w) was removed from the *Nephrops* waste by the chemical demineralisation treatment (Fig. 4.3). After demineralisation chitin accounted for 52.9% (w/w) of the shell. The remainder of the product could be attributed mainly to protein (36.4% w/w). The shell retained its brown colour so it is unlikely that pigments were removed by this treatment.

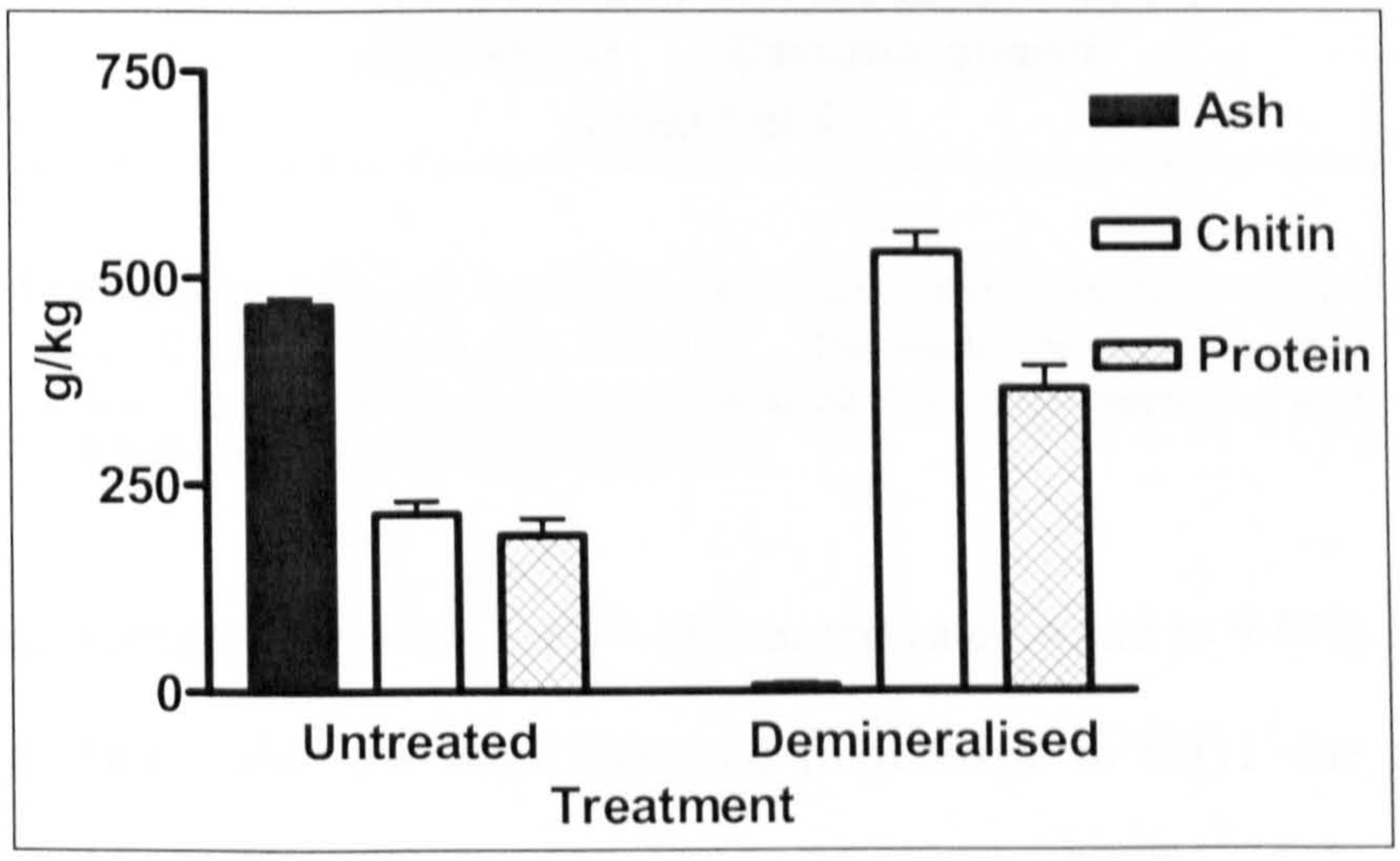


Fig. 4.3 *The chemical composition of untreated shell waste and shell waste demineralised with 1M HCl.* The results shown represent the mean ± standard deviation of values obtained from three batches of waste. Each batch was demineralised in duplicate.

Calcium was reduced to less than 1% (w/w) (Fig. 4.4).

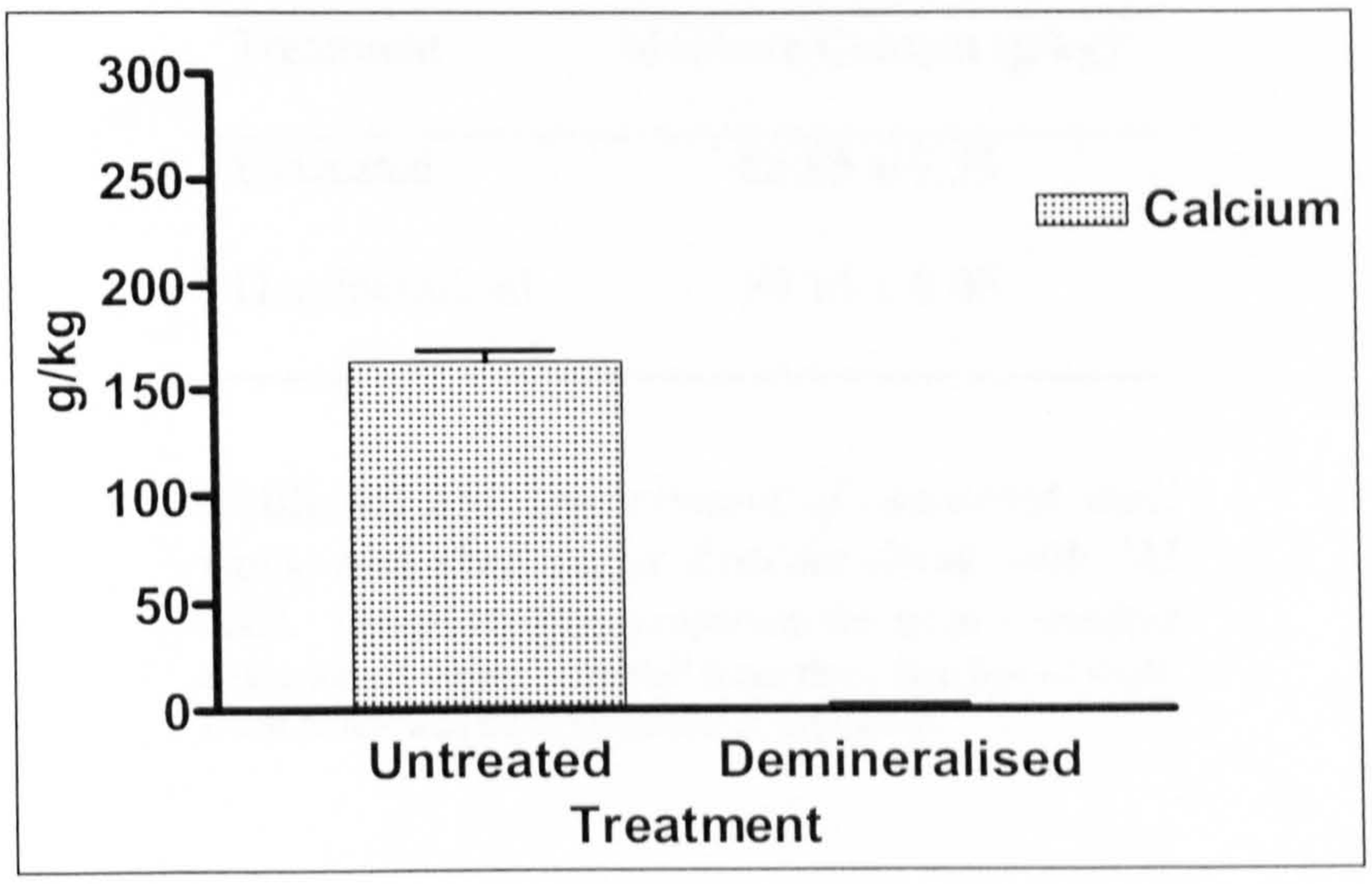


Fig. 4.4 *The calcium content of untreated shell waste and shell waste demineralised with 1M HCl.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was demineralised in duplicate.

The percentage nitrogen increased from 4.13% in untreated waste to 9.47% in chemically demineralised shell. The N:C ratio increased from 0.167 to 0.211 due to the raised nitrogen level (Table 4.1).

Treatment	%C (w/w)	%H (w/w)	%N (w/w)	N:C ratio
Untreated	24.49 ± 2.81	3.73 ± 0.36	4.13 ± 0.94	0.167 ± 0.021
Demineralised	44.82 ± 1.93	6.83 ± 0.17	9.47 ± 0.45	0.211 ± 0.005

Table 4.1 *Elemental composition and N:C ratio of untreated shell waste and shell waste demineralised with 1M HCl.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was demineralised in duplicate.

The moisture content of the untreated shell waste and deproteinised shell waste are shown in Table 4.2.

Treatment	Moisture Content (g/kg)
Untreated	88.95 ± 7.55
Demineralised	69.14 ± 8.05

Table 4.2 *Moisture content of untreated shell waste and shell waste demineralised with 1M HCl.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was demineralised in duplicate.

4.3.2 Deproteinisation

After deproteinisation the resultant residue was white, indicating pigment removal. The ash was reduced to 0.2% (w/w) and protein to 0.64% (w/w). The remaining residue was almost exclusively chitin (Fig. 4.5).

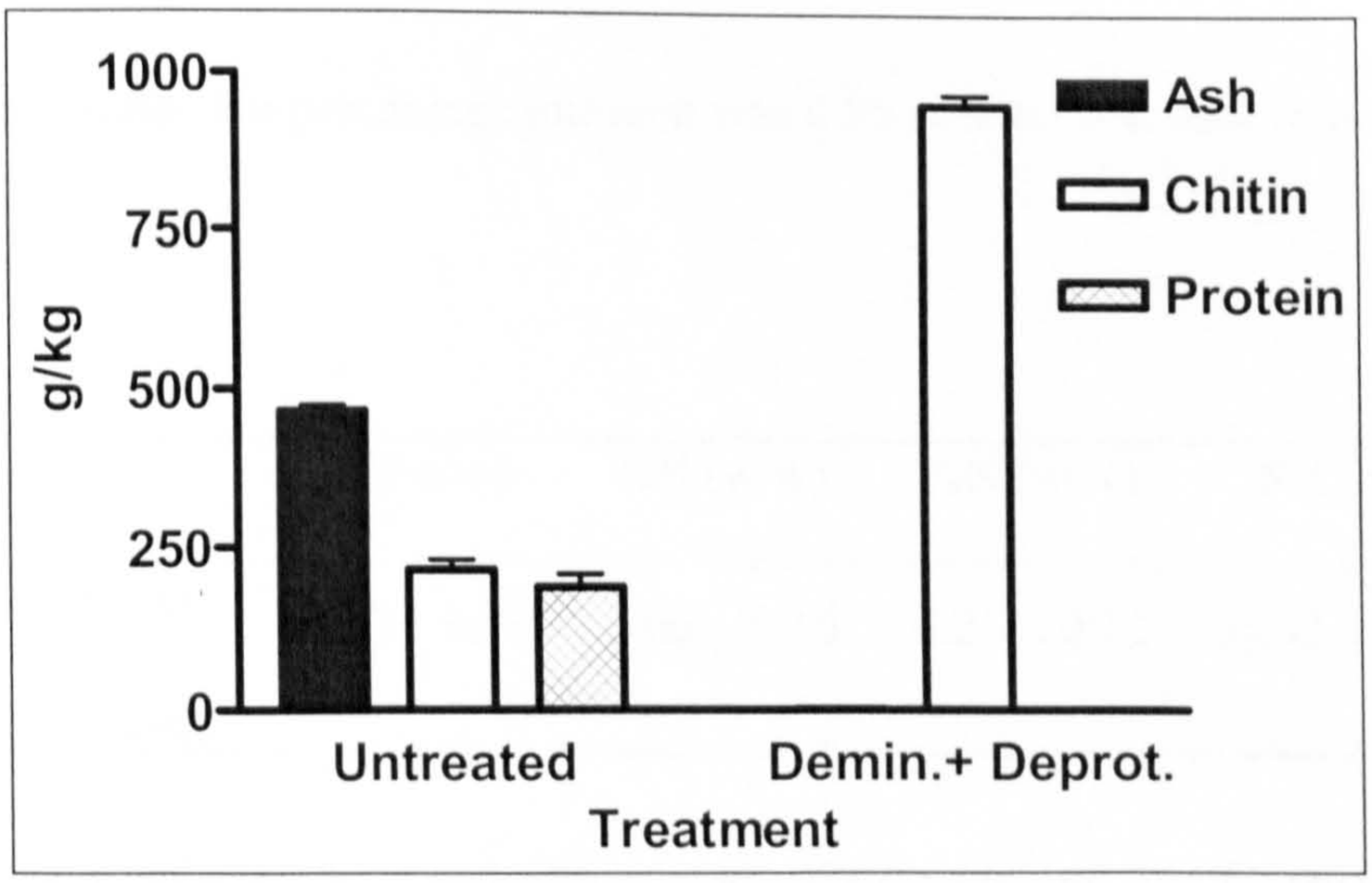


Fig. 4.5 *The chemical composition of untreated shell waste and shell waste after demineralisation with 1M HCl (demin.) and deproteinisation with 4% (w/v) NaOH (deprot.).* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was deproteinised in duplicate.

Calcium content was very low, 0.26% (w/w)(Fig. 4.6).

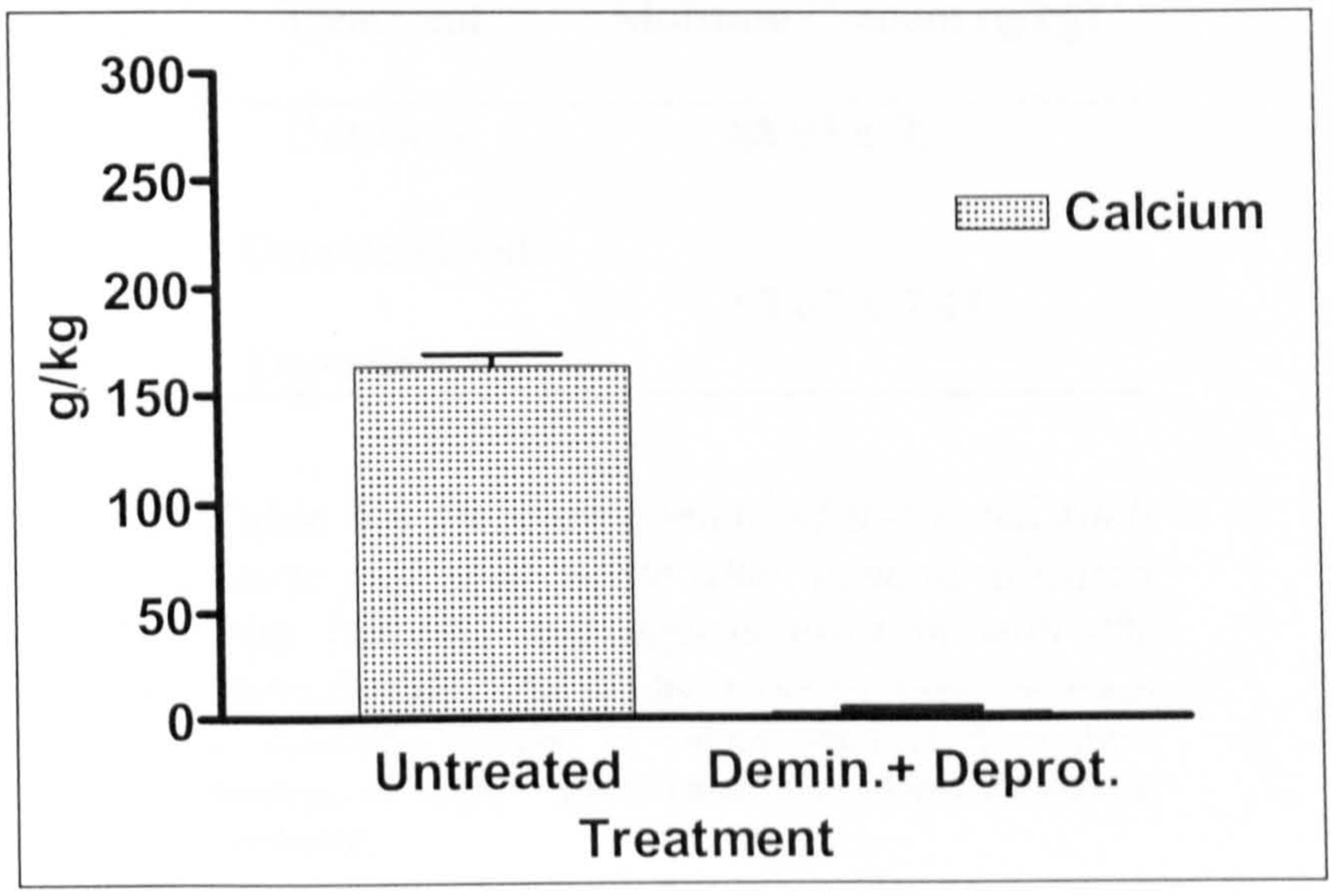


Fig. 4.6 *The calcium content of untreated shell waste and shell waste after demineralisation with 1M HCl (demin.) and deproteinisation with 4% (w/v) NaOH (deprot).* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was deproteinised in duplicate.

After deproteinisation the percentage nitrogen was 6.25 and the N:C ratio was 0.142 (Table 4.3).

Treatment	%C (w/w)	%H (w/w)	%N (w/w)	N:C ratio
Demineralised + Deproteinised	44.00 ± 0.31	6.66 ± 0.16	6.25 ± 0.12	0.142 ± 0.003

Table 4.3 *Elemental composition and N:C ratio of shell waste after demineralisation with 1M HCl and deproteinisation with 4% (w/v) NaOH.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was deproteinised in duplicate.

The moisture content of the untreated shell waste and the deproteinised shell waste are shown in Table 4.4.

Treatment	Moisture Content (g/kg)
Untreated	88.95 ± 7.55
Demineralised + Deproteinised	63.02 ± 7.41

Table 4.4 *Moisture content of untreated shell waste and shell waste after demineralisation with 1M HCl and deproteinisation with 4% (w/v) NaOH.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was deproteinised in duplicate.

Scanning electron micrographs of the deproteinised shell are shown in Figs. 4.7 - 4.8. Microfibrils of chitin are visible.

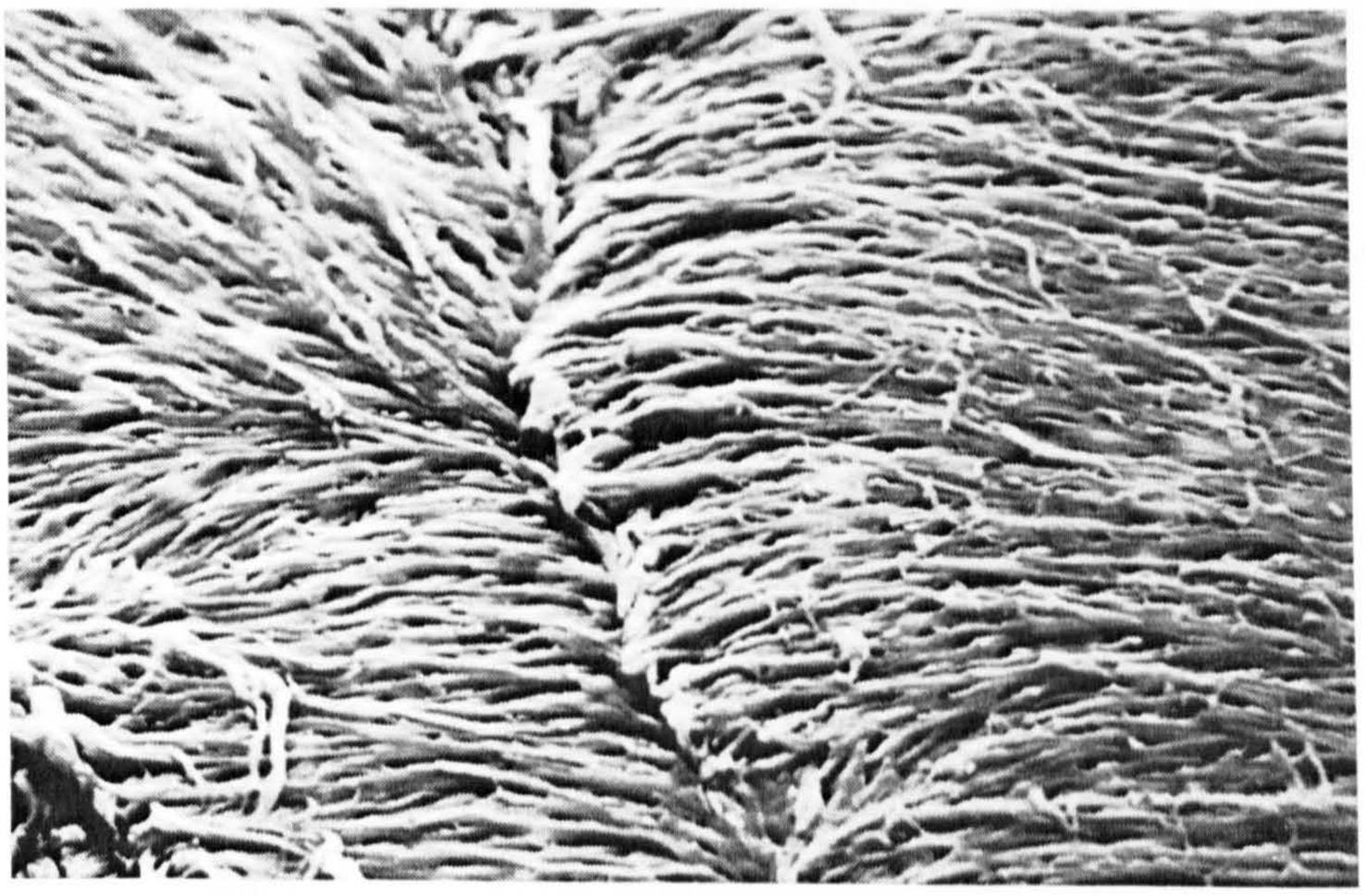


Fig. 4.7 *Scanning electron micrograph of chemically isolated chitin* (x2800).

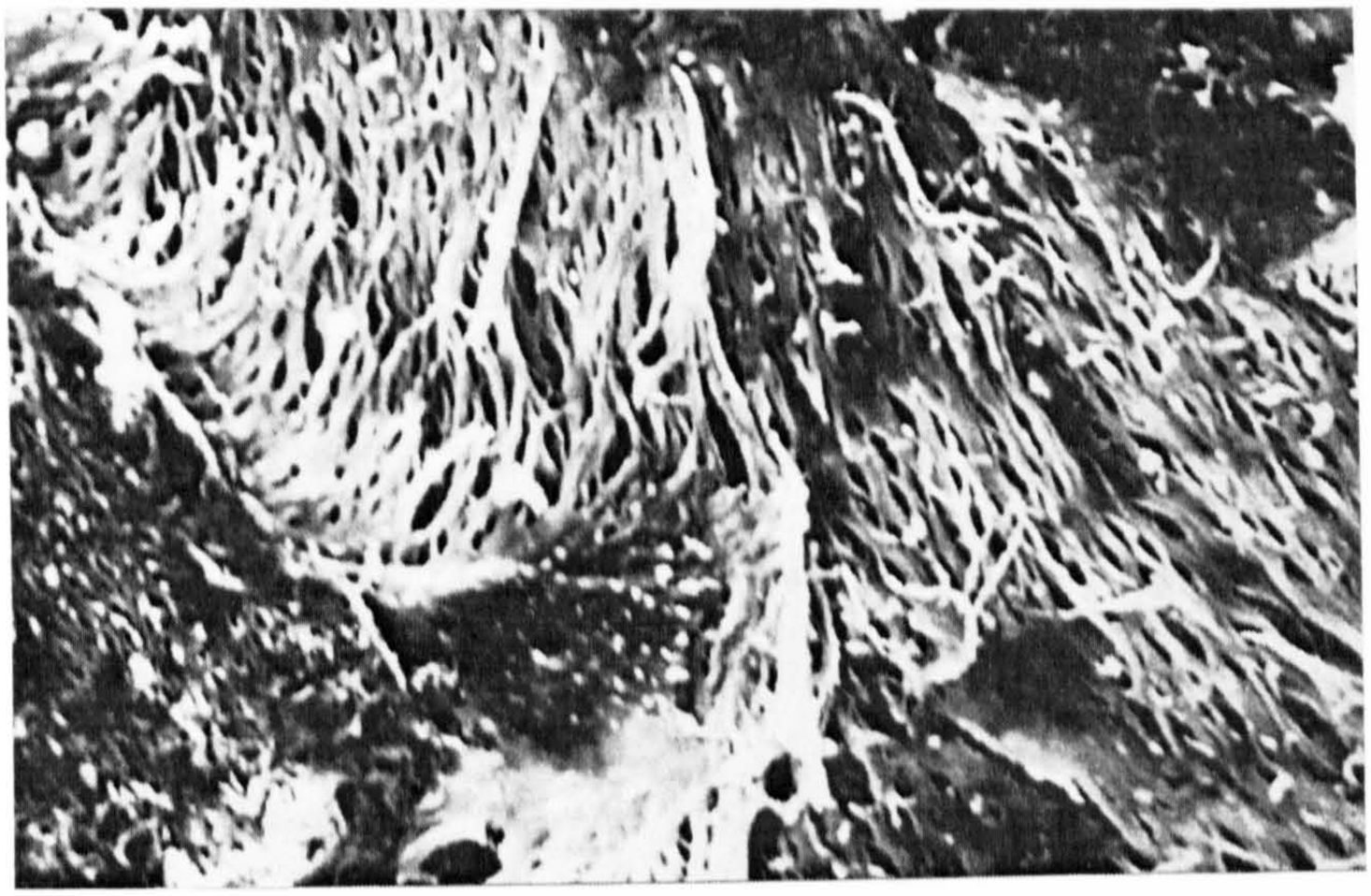


Fig. 4.8 *Scanning electron micrograph of chemically isolated chitin* (x2800).

4.3.3 Summary of Chemical Demineralisation and Deproteinisation Results.

Fig. 4.9 and Table 4.5 show the chemical composition of the shell waste after both the demineralisation and the deproteinisation procedures. It can be clearly seen that the demineralisation procedure was effective in removing ash and the deproteinisation procedure effective in removing protein.

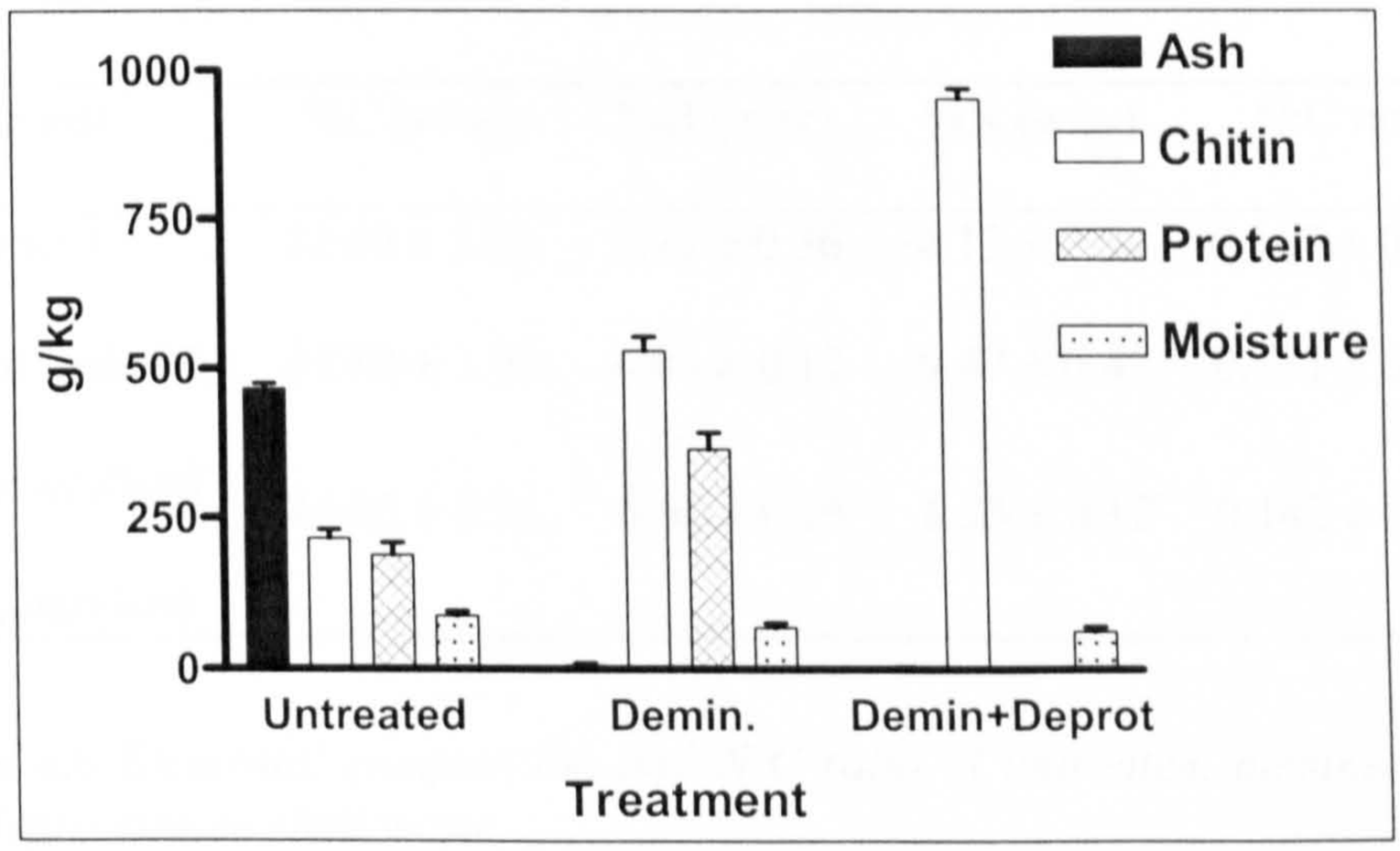


Fig. 4.9 *The chemical composition of untreated shell waste, and shell waste after chemical demineralisation (demin.) and after chemical demineralisation plus chemical deproteinisation (demin. + deprot.).*

The final product consisted mainly of chitin and moisture.

Component	Demineralised (g/kg)	Demineralised + Deproteinised (g/kg)
Chitin	529.40 ± 24.68	951.6 ± 16.89
Protein	363.564 ± 27.49	0.64 ± 1.43
Ash	6.66 ± 3.10	1.66 ± 2.49
Moisture	69.94 ± 6.72	63.21 ± 6.30
Calcium	1.58 ± 1.02	2.56 ± 1.96

Table 4.5 *The chemical composition of chemically demineralised and chemically deproteinised shell waste.*

The elemental compositions of untreated, demineralised and deproteinised shell waste are compared in Table 4.6. The deproteinised waste had an elemental composition typical of chitin.

Treatment	%C (w/w)	%H (w/w)	%N (w/w)	N:C ratio
Untreated	24.49 ± 2.81	3.73 ± 0.36	4.13 ± 0.94	0.167 ± 0.021
Demineralised	44.82 ± 1.93	6.83 ± 0.17	9.47 ± 0.45	0.211 ± 0.005
Demineralised + Deproteinised	44.00 ± 0.31	6.66 ± 0.16	6.25 ± 0.12	0.142 ± 0.003

Table 4.6 *Elemental composition and N:C ratio of untreated, demineralised and deproteinised shell waste.*

4.4 DISCUSSION

Chitin isolation by chemical treatment resulted in a white, flaky product. This residue was characterised by analysis of its overall chemical composition and by C, H, N determination. Contamination by ash and protein was very low, 0.2% (w/w) and less than 0.01% (w/w) respectively. Ash content of isolated chitin is usually less than 1% (w/w) (reviewed by No and Meyers, 1995). The variations in ash levels seen are normally due to differences in demineralisation methods used - differences in the acid type and concentration employed, the temperature and the time involved will all lead to chitin isolates with different degrees of contamination/degradation. Residual protein is another normal contaminant of chemically isolated chitin, due to the difficulty in removing all of the covalently linked amino acids (Brine and Austin, 1981b). Prolonged alkaline hydrolysis (1M NaOH for 48 h) has been shown to result in a chitin with amino acid residues still attached (Brine and Austin, 1981b).

After the demineralisation treatment the percentage nitrogen had increased from 4.13% in untreated shell to 9.47% in chemically demineralised shell. This increase was due to the higher proportions of protein and chitin present in the degraded shell (Fig. 4.3) since both chitin and protein contain nitrogen. After deproteinisation, the percentage nitrogen was 6.25%. The theoretical percentage nitrogen in chitin is 6.9%, but some figures quoted are lower probably due to the presence of moisture in the sample, as is the case here. The N:C ratio at this point was 0.142. This is similar to the theoretical N:C value of 0.146. When no residual protein remains on the sample prior to elemental analysis, the N:C ratio can be used to give an indication of the degree of N-acetylation of the chitin obtained (Roberts, 1992). However, since small levels of residual protein were still present this method was not utilised here and the degree of N-acetylation was not determined. Neither was any attempt made to determine the molecular weight of the product due to the difficulty of dissolving chitin with residual contaminants.

The average moisture content of the final product was 6.3% (w/w) as the samples were dried at a relatively low temperature of 40°C. This temperature was chosen for drying the initial shell waste and for drying the intermediate and final products in an attempt to reduce the degradation of the protein component of the shell. Any protein present will have been hydrolysed by the chemicals used for the extraction of chitin but the products were still dried at 40°C so that they could be compared with future bioprocessed samples.

The scanning electron micrographs show layers of chitin fibres remaining after chemical degradation of the shell (Figs. 4.7 and 4.8). The fibres in Fig. 4.7 resemble the vertical fibres seen in the exocuticle of untreated shell in the lower part of Fig. 2.26. In Fig. 4.8 some of the bundles have started to unravel and show similarities to the endocuticular fibres depicted in Fig. 2.30.

CHAPTER FIVE

BIOPROCESSING OF NEPHROPS SHELL WASTE BY LACTIC ACID FERMENTATION

5.1 INTRODUCTION

In recent times, micro-organisms have been utilized instead of chemicals, to isolate chitin from shell-fish waste (Hall and De Silva, 1992; Hall *et al.*, 1994; Healy *et al.*, 1994; Bustos and Healy, 1994; Hall and Reid, 1995; Guerrero-Legarreta *et al.*, 1996; Shirai *et al.*, 1997; Rao and Stevens, 1997; Zakaria *et al.*, 1998; Rao *et al.*, 1998; Shirai *et al.*, 1998 and 2001). In these studies lactic acid, produced by lactic acid bacteria, dissolved the calcium carbonate in the shell, thereby demineralising it. Deproteinisation took place in the same system under the action of proteolytic enzymes and microorganisms indigenous to the waste.

Lactic acid bacteria belong to the genera *Streptococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*. All members of these genera produce lactic acid from hexoses and all are described as facultative anaerobes, i.e. they grow in the presence or absence of oxygen. However, differences between the genera are also apparent. Homofermentative members produce lactic acid as the major or sole product of glucose fermentation. Heterofermentative members produce equal molar amounts of lactate, CO_2 and ethanol from hexoses. Differences in cell metabolism lead to the production of D(-) lactic acid, L(+) lactic acid or DL-lactic acid (a mixture of both D- and L- lactic acid). They are also morphologically different. Some are rods and others are coccoid in shape. Table 5.1 lists some lactic acid bacteria with their characteristic features.

Genus	Glucose fermentation	Morphology	Lactate	Species
Lactobacillus	Homofermentative	Rod	DL	*L. acidophilus*
			L(+)	*L. casei*
			D(L)*	*L. coryniformis*
			DL	*L. curvatus*
			DL	*L. plantarum*
			L(+)	*L. salivarius*
	Heterofermentative	Rod	DL	*L. brevis*
			DL	*L. buchneri*
			DL	*L. fermentum*
			DL	*L. viridescens*
Pediococcus	Homofermentative	Coccus	DL	*P. acidilactici*
			DL	*P. damnosus (cerevisiae)*
			DL	*P. pentosaceus*
Enterococcus	Homofermentative	Coccus	L(+)	*E. faecalis*
			L(+)	*E. faecium*
Lactococcus	Homofermentative	Coccus	L(+)	*L. lactis*
Streptococcus	Homofermentative	Coccus	L(+)	*S. bovis*
Leuconostoc	Heterofermentative	Coccus	D(-)	*L. mesenteroides*

*About 15 to 20% of the total lactic acid is the L(+) isomer.

Table 5.1 *Some lactic acid bacteria of importance during ensiling* (McDonald *et al.*, 1991).

Hall and De Silva (1992) used lactic acid fermentation to isolate chitin from tropical prawn waste, *Penaeus monodon*. A starter culture of Stabisil powder, a commercial bacterial aid used in the ensilation of fish offal, was added to minced shrimp waste. This waste contained shrimp heads and shrimp tail shell. The active organisms in the inoculant were *Lactobacillus plantarum, Pediococcus acidilactici* and *Enterococcus faecium* (Strain M74). The bacteria were provided with lactose as a source of carbohydrate. Combinations of more than 2% (w/w) Stabisil and more than 10% (w/w) lactose, mixed with the waste, caused a drop in pH from an initial 7.3 pH units to around 4.3-4.5 pH units in 5 days. After this time, most of the chitin from the waste was found in the silage sediment whereas autolysed protein gave rise to a protein-rich liquor suggesting that the chitin and protein could be easily separated.

Various adaptations of this study have since been employed. Hall *et al.*, (1994) isolated lactic acid bacteria from shrimp waste. Their action on shell waste was compared with various forage and meat commercial starters (Guerrero-Legarreta *et al.*, 1996). All inoculants tried were successful in reducing the pH to values inhibitory to spoilage organisms except the forage starters Biomax and VegeStart. The fermentation of tropical prawn waste from *Penaeus monodon* has been compared with cold-water prawn waste from *Nephrops norvegicus* (Hall *et al.*, 1994). The fermentation of the cold-water waste required greater levels of glucose than the tropical prawn waste to keep the pH low. This was attributed to the larger proportion of calcium carbonate in the *Nephrops norvegicus* waste compared to the *Penaeus monodon* waste that caused a greater buffering capacity as the shell dissolved. Zakaria *et al.*, (1998) scaled up the lactic acid fermentation of scampi waste in a rotating horizontal bioreactor. After 5 days fermentation at 30°C, 77.5% (w/w) of the protein and 61.0% (w/w) of the calcium present in the waste were solubilised. Shirai *et al.*, (2001) examined the effect of the initial glucose concentration and the inoculation level of lactic acid bacteria in the ensilation of shrimp waste using a response surface methodology (RSM) to help describe correlations between the different variables. Bautista *et al.*, (2001) produced 'low cost' lactic acid from whey and used it to demineralise crayfish shell waste.

The common denominator in the above studies is that <u>whole</u> shell waste was utilised, i.e. waste containing tail shell plus head. The system was therefore rich in proteolytic enzymes from head tissue allowing for additional solubilisation of shell protein. Healy *et al.*, (1994) employed lactic acid fermentation, in a solid-state fermentation system, with tail shell alone. This system lacked proteolytic enzymes from prawn heads. Ground shell was mixed with 15% lactose and 2 - 4% Stabisil at 25°C for 7 days. However, the lack of proteolytic enzymes in the system negated the solubilisation of enough protein - approximately 40% of the initial protein was removed. Various proteolytic microorganisms

were subsequently selected and evaluated on their ability to deproteinise demineralised prawn shell (Bustos and Healy, 1994; Bustos, 1996). BAFP 202, a grass silage inoculant containing *Bacillus subtilis*, *Pediococcus pentosaseus* and *Enterococcus faecium,* consistently gave the most deproteinisation. However, the product obtained still contained a higher level of nitrogen (>8% (w/w) nitrogen) than theoretical chitin (6.9% (w/w) nitrogen) showing that there was still residual protein. Bustos found that the residual amino acid content was greater than 10% (w/w) compared with 2% (w/w) for chemically treated shell and 0.8% (w/w) for a commercially available crab shell chitin (Sigma, Poole, Dorset).

In the present project the lactic acid fermentation of prawn tail shell was investigated further in an attempt to produce a 'cleaner' chitin. The inoculant Stabisil was no longer obtainable. However, a similar inoculant - H/M Inoculant (Lactosil®) (Nutrimix, U.K.) was used. Lactosil® was sold as 'a bacterial aid to the fermentation of forage intended for silage' and contained the same active ingredients as Stabisil - *Lactobacillus plantarum*, *Enterococcus faecium* Strain M74 and *Pediococcus acidilactici*. As chitin has a very similar structure to cellulose (Figs. 1.4 and 1.5) it was anticipated that the use of a grass silage inoculant would prove beneficial to the isolation of chitin.

The bacteria in Lactosil are important in the ensilation of grass. All show homofermentative metabolism and ferment glucose and fructose by way of the metabolic pathway shown in Fig. 5.1.

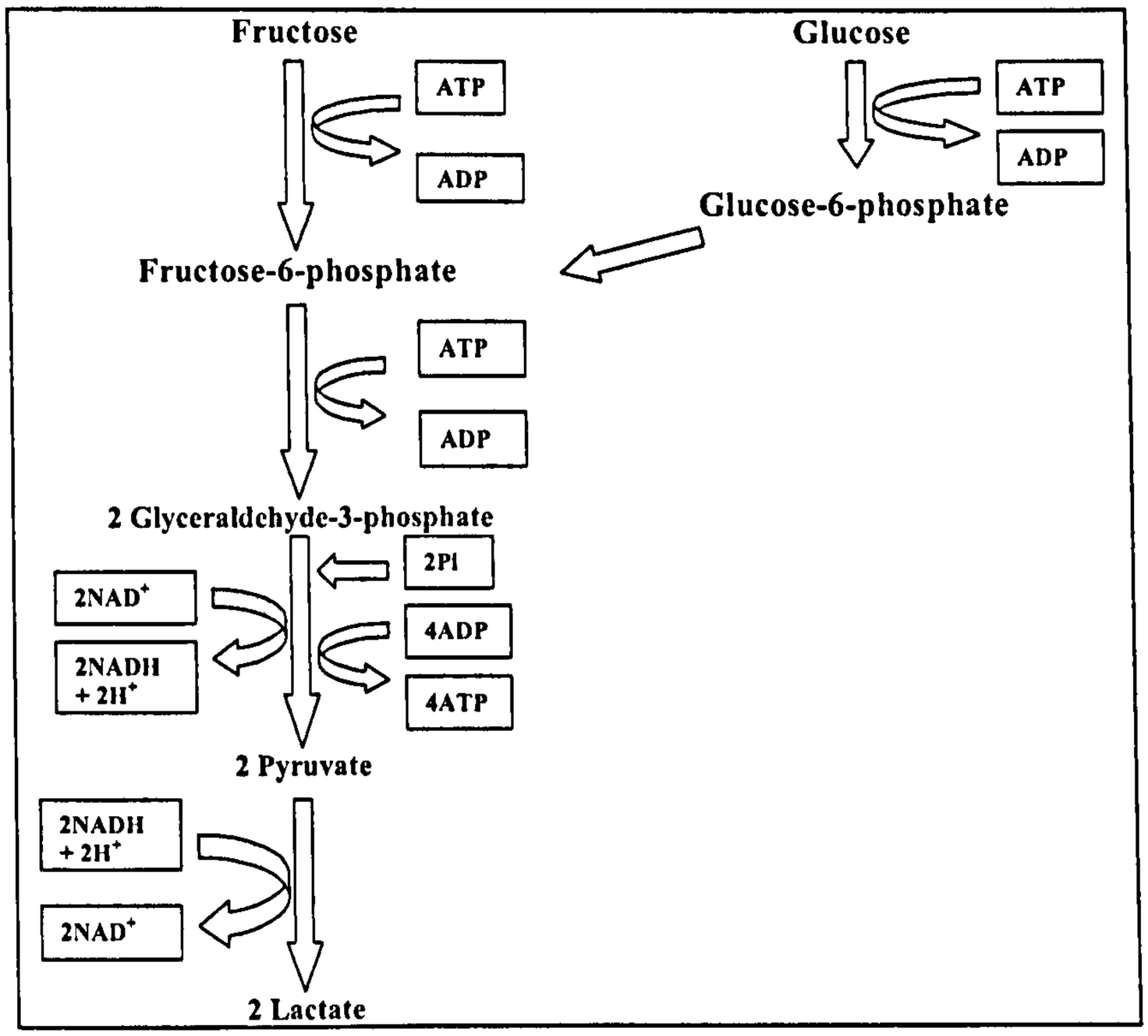


Fig. 5.1 *Fermentation of glucose and fructose by homofermentative lactic acid bacteria* (McDonald *et al.*, 1991).

Lactobacilli grow best in slightly acidic media with an initial pH of 6.4 to 4.5 and can decrease the pH as low as 3.5. In contrast *Enterococcus faecium* is able to initiate growth at pH 9.6 and can reduce the pH to a value of 4.0. *Pediococcus acidilactici* has a pH optimum of 6.0 to 6.5 and can reduce the pH to below 4.0 (Sneath *et al.*, 1986). A combination of these bacteria, when provided with a suitable source of carbohydrate, should therefore be effective at the start of fermentation when the pH is relatively high and at the end when a lower pH has been achieved - regardless of the nature of the substance being preserved.

5.2 MATERIALS AND METHODS

5.2.1 Characterisation of Inoculant

5.2.1.1 Bacterial Growth Curve

de Man, Rogosa, Sharpe (MRS) broth was prepared by dissolving 52.2g MRS granules in 1 litre distilled water. MRS broth/1.5% (w/v) agar was prepared by dissolving MRS granules (52.2g) and purified agar (15g) in 1 litre distilled H_2O. MRS broth and MRS/agar solutions were autoclaved at 121°C for 15 minutes, in 100 or 200ml aliquots and stored at 4°C.

Sterile MRS broth (100ml or 200ml) in 100ml or 200ml wide-mouthed autoclavable bottles was inoculated with H/M Inoculant (Lactosil®) (Nutrimix Ltd., U.K.) (0.1 or 0.2g respectively). The dry Lactosil silage inoculant was stored in a sealed polythene bag at room temperature. The inoculated MRS broth was incubated (LTE incubator) statically at 30°C for 72 hours. At 0, 8, 24, 48 and 72 hours this inoculant was diluted serially (10^{-1} to 10^{-10}) with autoclaved maximum recovery diluent (1% (w/v) Peptone and 8.5% (w/v) NaCl in distilled water). Each dilution (100µl) was pipetted aseptically onto plastic, sterile, labelled petri dishes (Sterilin). Hand hot MRS agar was poured onto the dishes to cover the base. The plate was mixed gently to disperse the bacteria. Once the plate had set it was over-laid with more MRS agar. When the second layer of agar had set, the plates were incubated at 30°C. After 2 days, visible colonies on each plate were counted. The count was multiplied by 10 and by the appropriate dilution factor to give the number of colony forming units per ml (cfu/ml).

5.2.1.2 Bacterial Growth on a Range of Media

Sterile MRS broth was inoculated with Lactosil silage inoculant (0.1g/100ml) and incubated at 37°C for 24 hours. The culture broth was inoculated onto plates containing Nutrient agar, MRS agar, MacConkey agar and Sheep blood agar (blood agar plus 7%

defibrinated sheep blood, E&O Labs.). All plates were incubated at 37°C under aerobic conditions, anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) and microaerophilic conditions (5% H_2, 5% O_2 and 85% N_2) for 48 hours.

5.2.1.3 Gram Staining

Smears of bacteria from plates grown under the aforementioned conditions were fixed to microscope slides. Crystal violet solution was applied to each smear for one minute. After washing with running tap water, Gram's Iodine was applied for 1 minute. The slide was again washed in running tap water then decolourised by treating with acetone for approximately 5 seconds until the decolouriser flowed colourless. The slide was washed gently with running tap water then counterstained with 0.1% (w/v) carbol fuchsin for 1 minute. After washing with tap water the slide was blotted dry. Once dry, the smear was viewed under the microscope.

5.2.1.4 Bacterial Preparation for the Microbial Treatment of Shell

MRS broth (100ml or 200ml) was inoculated with Lactosil silage inoculant (0.1g or 0.2g respectively). The inoculated broth was incubated for 48 hours at 30°C. At this stage the inoculant contained 10^9 - 10^{10}cfu/ml. Aliquots of inoculated broth were used to inoculate mixtures of glucose and *Nephrops* shell waste. The inoculant was used at a 15% (v/v) ratio except where indicated otherwise. Hereafter the term Lactosil is used to refer to Lactosil silage inoculant cultured as above in MRS broth.

5.2.2 Microbial Treatment of Shell

5.2.2.1 Microbial Treatment in 100ml Bottles

Shell waste (6.67g), of particle size 0.5 - 1.0mm, was placed in a 100ml screw capped bottle (Duran). Glucose solution (15% (w/v) in deionised H_2O) which had been filter

sterilised through a reusable bottle top filter (Nalgene) fitted with a 0.45μm cellulose nitrate filter (Nalgene), was aseptically poured on top of the shell. Inoculant, 0.1% (w/v) Lactosil in MRS broth, 48 hour culture, was aseptically added to the flask (1% (v/v)). The bottle was incubated in an orbital shaker (New Brunswick G-25) at 30°C and 150rpm for 7 days. The experiment was carried out in duplicate. A control containing shell and glucose but no Lactosil was also set up. Samples of culture broth (10ml) were removed daily and analysed for pH and total titratable acid (TTA) according to the methods described in sections 5.2.4.1, 5.2.4.2 and 5.2.4.3. Analysis of TTA was carried out in duplicate.

At the end of the fermentation period the culture broth was filtered off through muslin and kept for analysis. The remaining residue was washed on the muslin with tap water, dried and stored in an identical manner to the chemically demineralised and chemically deproteinised products (sections 4.2.1 and 4.2.2). The solid residue was analysed for ash, calcium, carbon, hydrogen and nitrogen using the methods in section 3.2.2. Analysis of solid product was carried out once for each component. The culture broth was analysed, in duplicate, for total titratable acid according to the methods described in sections 5.2.4.1 and 5.2.4.3.

5.2.2.2 Optimisation of the Parameters for Lactic Acid Fermentation

The following parameters were optimised for lactic acid fermentation of prawn shell waste in 1 litre conical flasks: effect of inoculant concentration, effect of agitation speed, effect of temperature, effect of glucose concentration, effect of length of time of fermentation and effect of number of fermentation treatments. A comparison between the effects due to Lactosil and the effects due to bacteria indigenous to the shell was also carried out.

Shell waste (0.5 - 1.0mm particle size) was placed in 1 litre conical flasks (58g shell). Each flask was fitted with an autoclavable silicone stopper. The stopper possessed a small hole bored through the centre. The hole was blocked with non-adsorbent cotton

wool. The vessel and shell were autoclaved for 15 minutes at 121°C for all experiments except where the effects of the bacteria indigenous to the shell were studied. Glucose solution (0 - 15% (w/v) in deionised H_2O), which had been filter sterilised through a reusable bottle top filter (Nalgene) fitted with a 0.45μm cellulose nitrate filter (Nalgene), was aseptically poured on top of the shell. Inoculant, 0.1% (w/v) Lactosil in MRS broth, 48-hour culture, was aseptically added to the relevant flasks (0 - 15% (v/v)). The culture contained approximately 10^9 - 10^{10}cfu/ml. The final solid:liquid ratio was 1:15. A plastic bubble fermentation lock was sterilised by soaking in sodium metabisulphite solution (10g/litre distilled H_2O) overnight. It was then partially filled with sterile water and inserted through the hole in the stopper after removal of the cotton wool plug. The flasks/bottles were incubated in an orbital shaker (New Brunswick G-25) at 30°C or 40°C and 100, 150 or 200rpm for 0 - 7 days, as each experiment dictated.

At the end of the fermentation period the culture broth was filtered off through muslin and kept for analysis. The remaining residue was washed on the muslin with tap water, dried and stored in an identical manner to the chemically demineralised and chemically deproteinised products (sections 4.2.1 and 4.2.2).

The actual parameters adhered to in each individual experiment are shown in Tables 5.2 - 5.6. Parameters which varied in each experiment are shown in italics in the tables. The final parameters adhered to for lactic acid fermentation are shown in Table 5.7. Each experiment was carried out in triplicate unless indicated otherwise in the results section. Analysis of solid product was carried out once for each component, according to the methods in section 3.2.2. Analysis of total titratable acid in the culture broth was carried out in duplicate, according to the methods in sections 5.2.4.1 and 5.2.4.3. The results reported represent the mean ± standard deviation of the 3 results for each solid component and the mean ± standard deviation of the 6 results for the total titratable acidity, unless indicated otherwise.

5.2.2.3 Experimental Parameters

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	*0 - 15% (v/v)*
Agitation Speed	200rpm
Temperature	30°C
Glucose Concentration	15% (w/v)
Time	7 days

Table 5.2 *Parameters used to study the effect of inoculant concentration.*

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	15% (v/v)
Agitation Speed	200rpm
Temperature	*30°C and 40°C*
Glucose Concentration	15% (w/v)
Time	7 days

Table 5.3 *Parameters used to study the effect of temperature.*

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	15% (v/v)
Agitation Speed	*100, 150, 200 rpm*
Temperature	30°C
Glucose Concentration	15% (w/v)
Time	7 days

Table 5.4 *Parameters used to study the effect of agitation speed.*

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	15% (v/v)
Agitation Speed	200rpm
Temperature	30°C
Glucose Concentration	*0 - 20% (w/v)*
Time	7 days

Table 5.5 *Parameters used to study the effect of glucose concentration.*

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	15% (v/v)
Agitation Speed	200rpm
Temperature	30°C
Glucose Concentration	15% (w/v)
Time	*0, 3, 4, 5, 7 days*

Table 5.6 *Parameters used to study the effect of time.*

Experimental Conditions	
Particle Size of Shell	0.5 - 1.0mm
Shell pre-treatment	Autoclaving
Solid:Liquid Ratio	1:15
Inoculant Concentration (48 hour inoculant)	15% (v/v)
Agitation Speed	200rpm
Temperature	30°C
Glucose Concentration	15% (w/v)
Time	7 days

Table 5.7 *Parameters adopted for lactic acid fermentation*

5.2.3 Chemical Analyses of Solid Samples

Ash, calcium, chitin, protein, C, H, N and moisture levels of solid residues were determined using the methods for the determination of chemical composition (section 3.2.2).

5.2.4 Chemical Analyses of Culture Broth

Total titratable acid was analysed in all broth samples. Selected broth samples were also analysed for pH, lactic acid and glucose.

5.2.4.1 Sample Preparation

Culture broth (50ml), or 10ml for experiments in 100ml volumes, was centrifuged at 2333 x g for 10 minutes (MSE Mistral 1000). Culture supernatant was poured off and the pellet discarded.

5.2.4.2 pH Determination

pH in broth supernatant was determined using a Gelplas electrode.

5.2.4.3 Total Titratable Acid Determination

A known volume of the culture supernatant (usually 10ml) was diluted to 100ml using deionised H_2O. Aliquots (25ml) of the diluted supernatant were titrated against NaOH (0.02M) using phenolphthalein as indicator until the equivalence point was reached. Results were expressed as mg lactic acid per g dry shell.

5.2.4.4 Lactic Acid Determination

Lactic acid in the culture broth supernatant was measured using Sigma's Diagnostic Lactate reagents (Procedure No. 826-UV). A calibration curve was prepared by diluting

the lactate standard provided with deionised H_2O, using the dilutions indicated in the protocol. The supernatant sample was diluted approximately 2,000 fold using deionised water. Working reagent was prepared and used as described in the protocol. Working reagent was added to the diluted samples and standards in 4ml cuvettes. The cuvettes were covered with parafilm, mixed by inversion and incubated for 15 minutes in a water bath at 37°C. Absorbance values were read at 340nm (Perkin Elmer Spectrophotometer) as described in the protocol. Reagents were prepared and used in a fume cupboard where possible.

5.2.4.5 Glucose Determination

Glucose levels in the culture broth supernatant were determined using a Boehringer Mannheim D-Glucose assay kit, Cat. No. 716 251. Culture supernatant was placed in a water-bath at 80°C for 15 minutes to stop enzymatic reactions. After adjusting the pH to pH 8.0 with NaOH (4% w/v) the supernatant was diluted 100 fold with deionised water. Reagents were made up and the reaction carried out following the kit procedure sheet. Sample volumes were varied where necessary and the volume of deionised water adjusted accordingly.

5.2.5 Isolation of Solid Material from Refrigerated Culture Broth

Culture broth was refrigerated, at 4°C, for 72 hours. During refrigeration some of the liquid solidified. The remaining liquid was filtered off and the solid dried at 60°C until a constant weight was obtained. The dry solid was subjected to Fourier Transform Infrared Spectroscopy (FTIR) according to the method in section 5.2.5.1.

5.2.5.1 Fourier Transform Infrared Spectroscopy (FTIR)

Each sample to be investigated (approximately 2mg) was ground with potassium bromide (100mg, spectroscopic grade, BDH) and compressed (10000kg) to a semi-transparent disc (thickness 0.5 - 1mm). FTIR spectra were recorded, in the wavenumber range 4000 - 500 cm^{-1}, using a Perkin-Elmer Spectrum RXI FTIR spectrophotometer.

5.3 RESULTS

5.3.1 Characterisation of Inoculant

5.3.1.1 Bacterial Growth on a Range of Media

Lactosil silage inoculant was grown in four different types of agar (Nutrient agar, MacConkey agar, MRS agar and Sheep Blood agar) under aerobic, microaerophilic and anaerobic conditions. Only one type of bacteria was observed under the conditions used. This bacterium was Gram positive, coccoid in shape and appeared as grey or white colonies.

5.3.1.2 Bacterial Growth in MRS Broth

The growth of the silage inoculant, Lactosil, in MRS broth, was measured using the plate count method. The bacteria showed exponential growth between 8 and 48 hours (Fig. 5.2). At 48 hours, the culture achieved a growth optimum of approximately 10^{10} colony forming units/ml (cfu/ml). Forty-eight hour cultures were subsequently used in experiments for the investigation of *Nephrops* shell degradation.

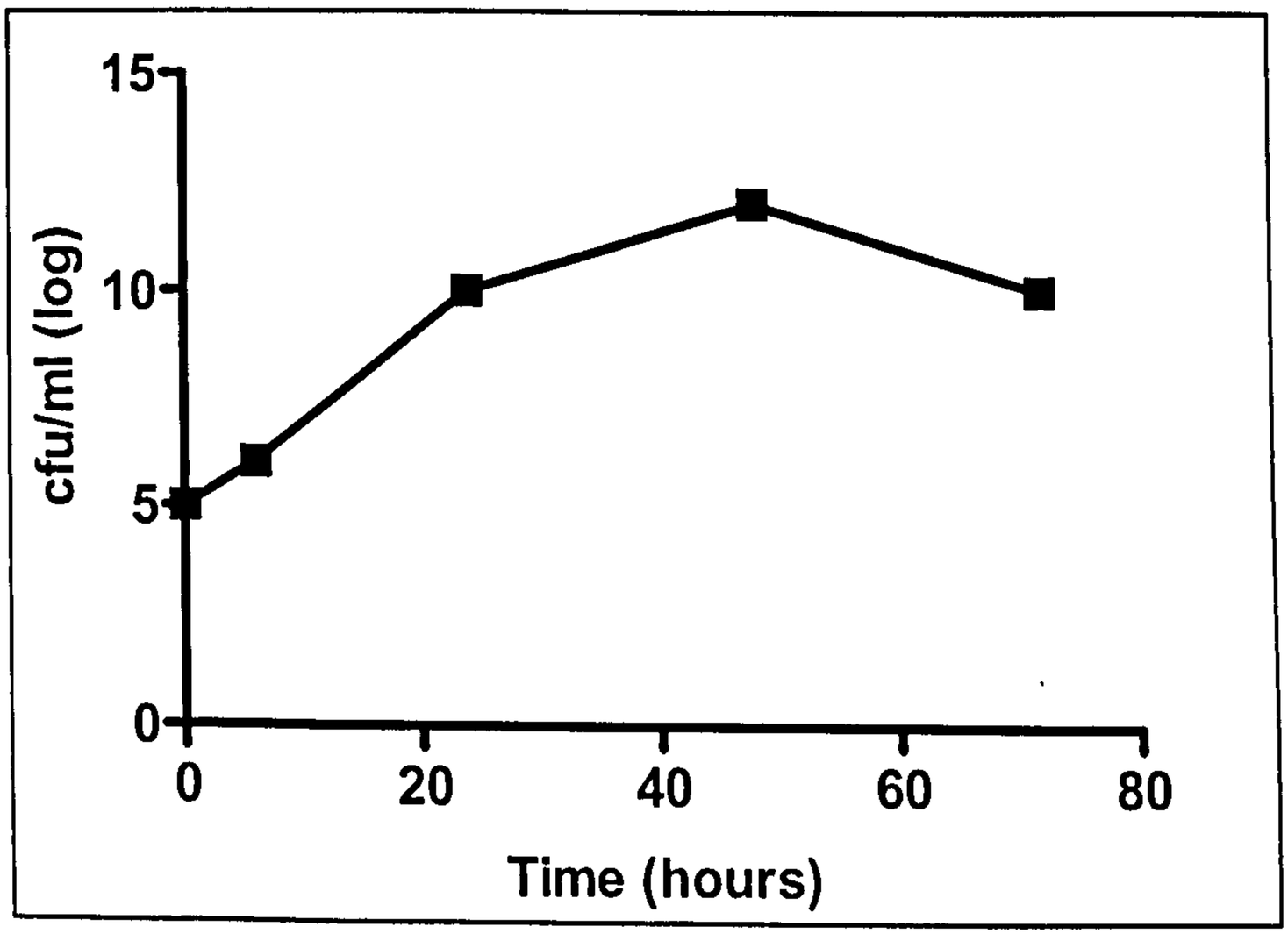


Fig. 5.2 *Growth curve from 0 - 72 hours for Lactosil in MRS broth.*

5.3.2 Microbial Treatment of Shell

5.3.2.1 Microbial Treatment in 100ml Bottles

Similar pH values were reached in samples with Lactosil (pH 3.69) and without Lactosil (pH 3.68) (Fig. 5.3) due to similar amounts of lactic acid being produced (Fig. 5.4).

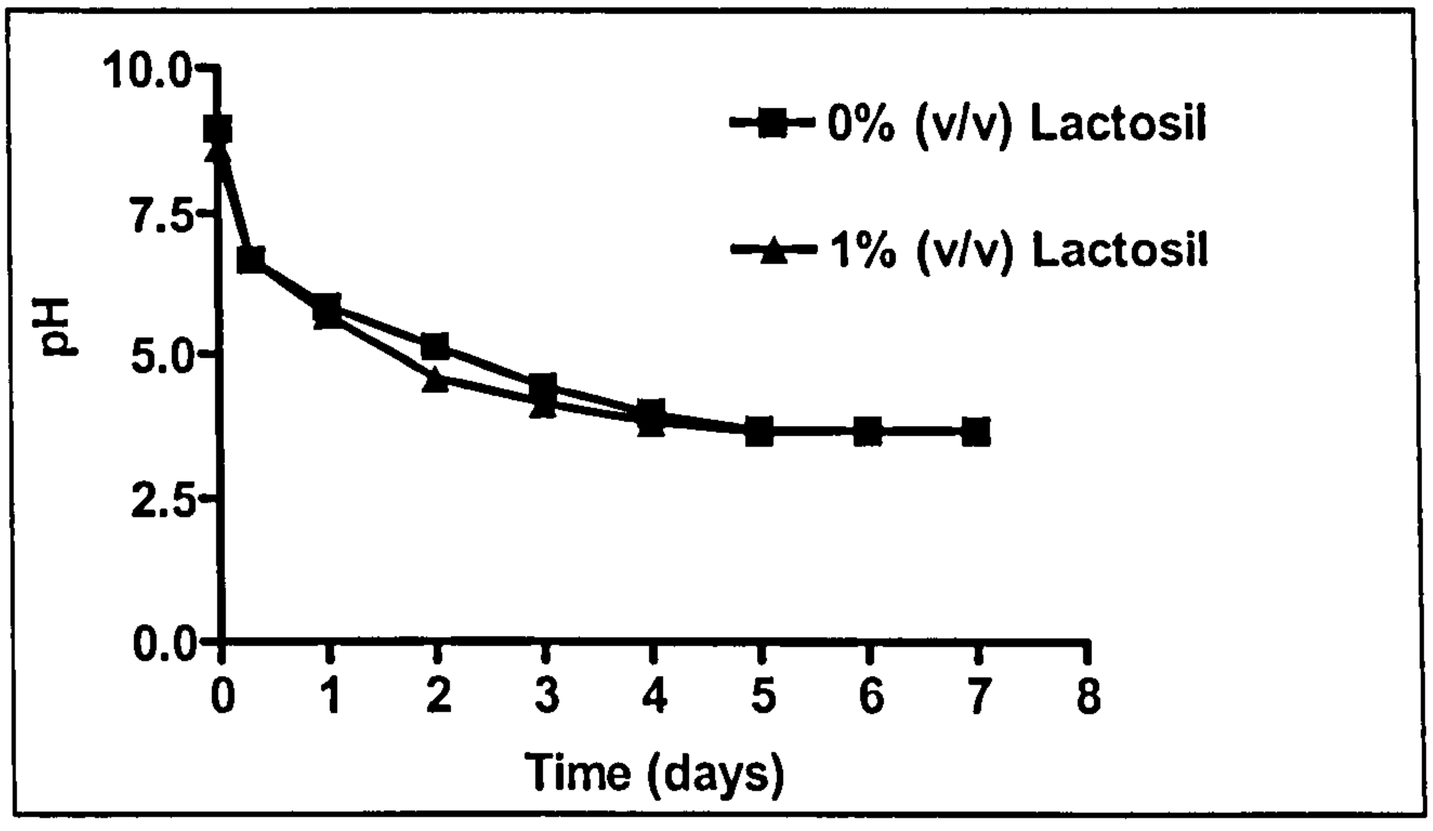


Fig. 5.3 *pH changes during lactic acid fermentation with and without added inoculant.* The results plotted represent the mean ± standard deviation of values obtained from duplicate assays.

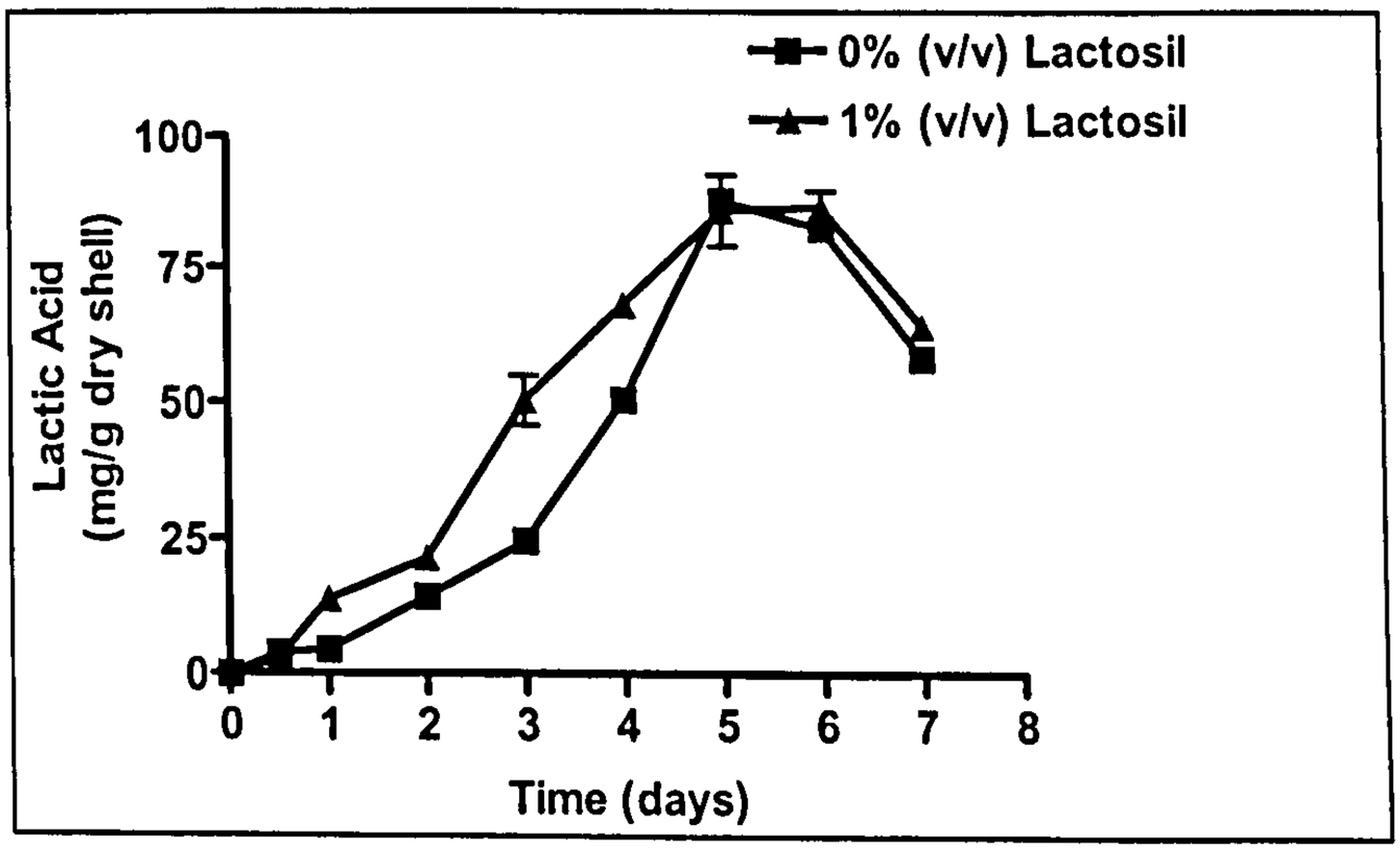


Fig. 5.4 *Lactic acid production during lactic acid fermentation with and without added inoculant.* The results plotted represent the mean ± standard deviation of values obtained from duplicate assays.

The lactic acid produced reduced the ash content of the untreated shell from 465.58 ± 8.88g/kg shell to 152.56 ± 31.81g/kg shell when Lactosil was present. In the control

sample (0% Lactosil) the ash was reduced from 465.58 ± 8.88g/kg shell to 219.09 ± 41.87g/kg shell (Fig. 5.5).

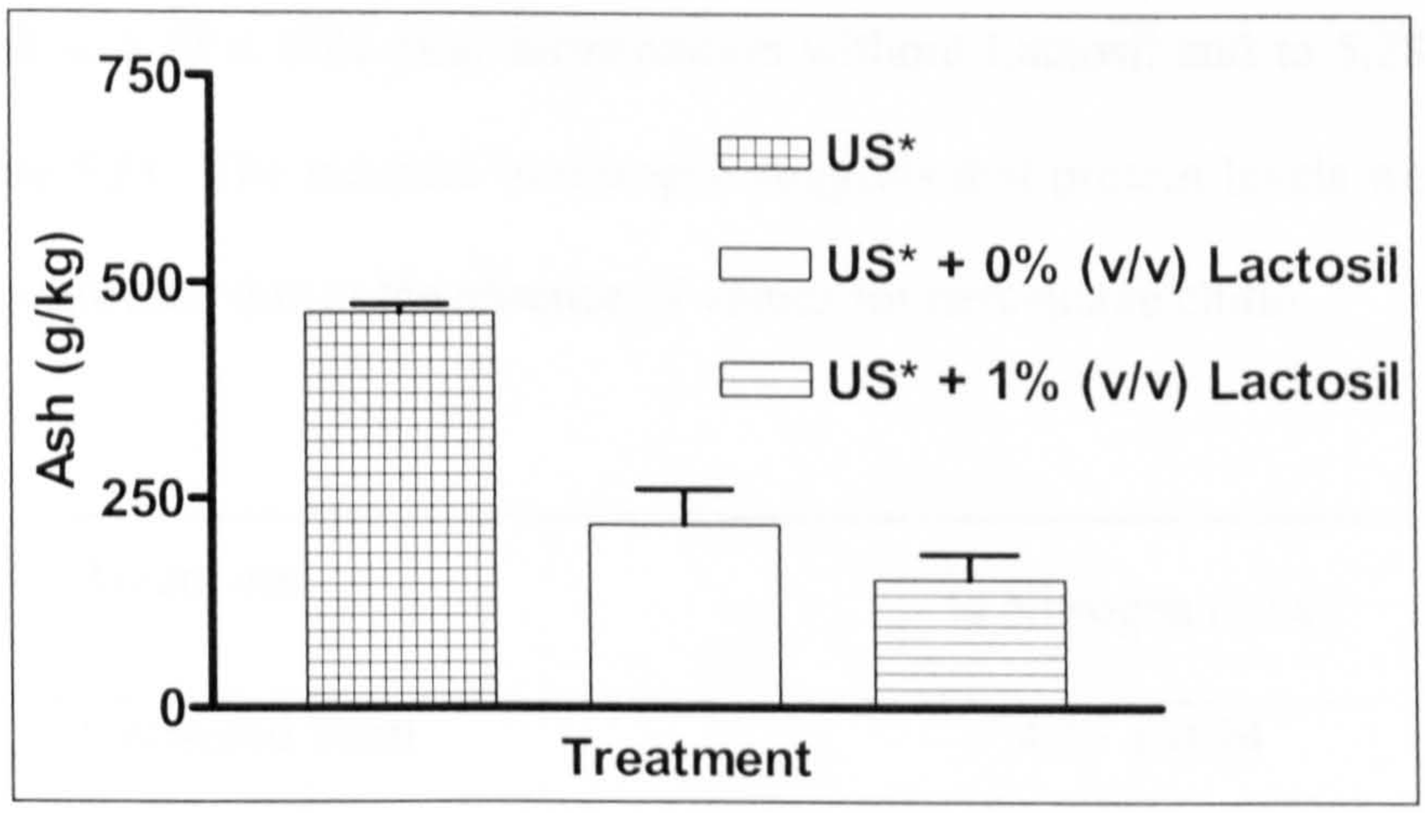


Fig. 5.5 *Comparison between levels of ash in untreated shell and shell microbially treated with Lactosil (1% v/v) and without Lactosil.* The results shown represent the mean ± standard deviation of values obtained from duplicate samples. *US = untreated shell

Similarly, the level of calcium in the shell was reduced after treatment by lactic acid fermentation with and without added inoculant (Fig. 5.6).

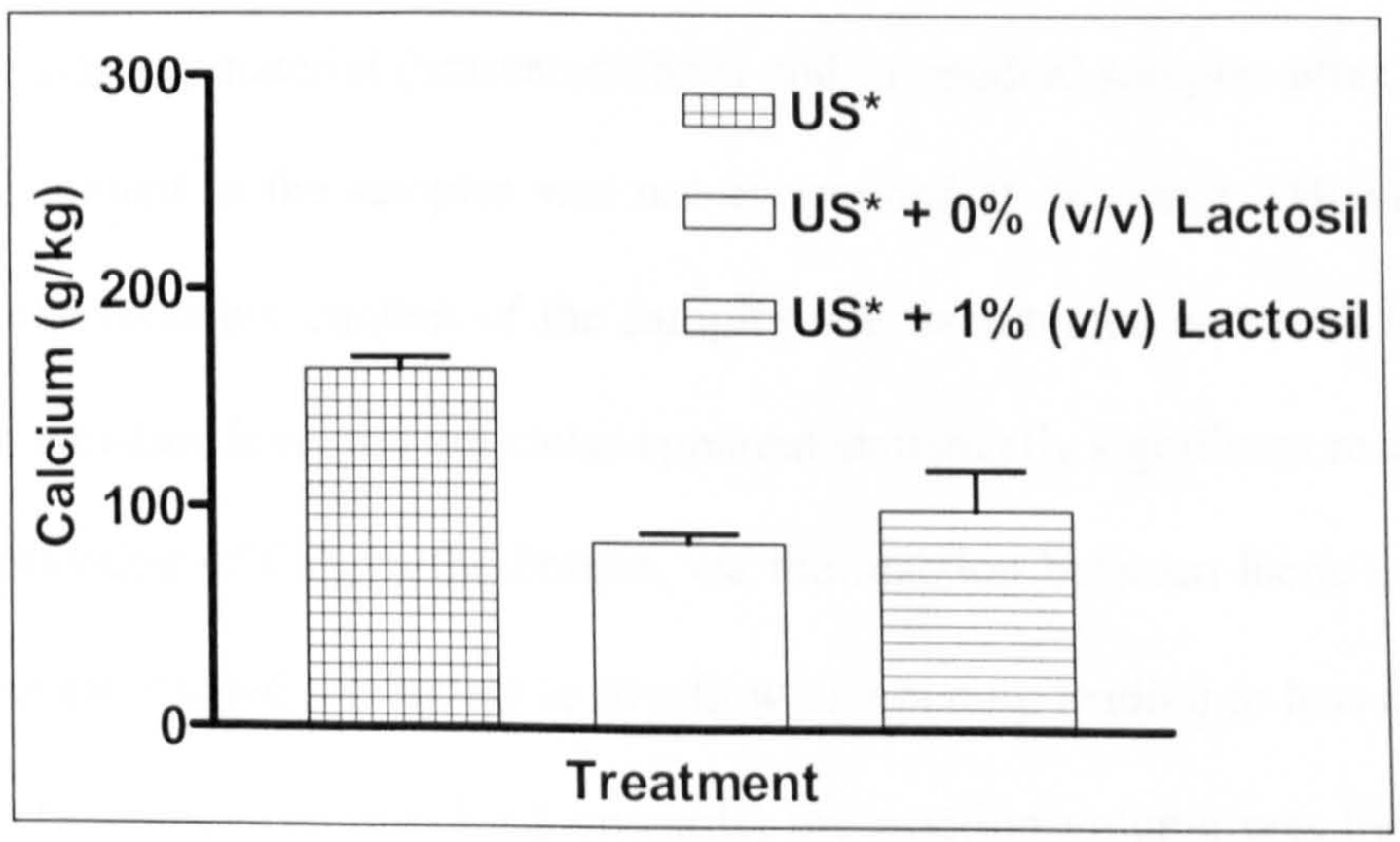


Fig. 5.6 *Comparison between levels of calcium in untreated shell and shell microbially treated with Lactosil (1% v/v) and without Lactosil.* The results shown represent the mean ± standard deviation of values obtained from duplicate samples. *US = untreated shell

Sufficient residual material, post microbial treatment, was not produced to allow for the determination of chitin content as well as ash and nitrogen levels. Therefore no protein results could be calculated. The percentage nitrogen (w/w) increased from 4.13 ± 0.94 in untreated shell to 5.45 ± 0.21 after fermentation without Lactosil and to 5.28 ± 0.04 with Lactosil (Table 5.8). The increase in nitrogen suggests that protein levels were raised but this was not confirmed due to the absence of values for percentage chitin.

Treatment	% Nitrogen (w/w)
Untreated Shell	4.13 ± 0.94
Untreated Shell + 0% (v/v) Lactosil	5.45 ± 0.21
Untreated Shell + 1% (v/v) Lactosil	5.28 ± 0.04

Table 5.8 *Nitrogen content of untreated shell and shell microbially treated with and without Lactosil.* The results shown represent the mean ± standard deviation of values obtained from duplicate samples.

Due to all material being dried at a relatively low temperature of 40°C, moisture was present in the starting material (untreated shell) and in residual samples after degradation. The moisture content of the samples was not determined at this stage. However, in later experiments, the moisture content of the samples was ascertained and used to verify that differences in moisture level did not cause apparent statistically significant results.

The production of CO_2 in the bottles, via the reaction between lactic acid and shell calcium carbonate, caused the bottles to overflow on opening leading to loss of sample. In subsequent experiments (section 5.3.3 onwards) the reaction volume was increased and a vent used to inhibit build up of CO_2 (see method section 5.2.2.2).

5.3.2.2 The Chemical Composition of Autoclaved Shell

It was not possible to distinguish the effects due to Lactosil from those caused by bacteria indigenous to the shell. Therefore, the shell was autoclaved to eliminate the native bacteria. The chemical composition of autoclaved shell was determined and compared with untreated shell (Figs. 5.7 and 5.8).

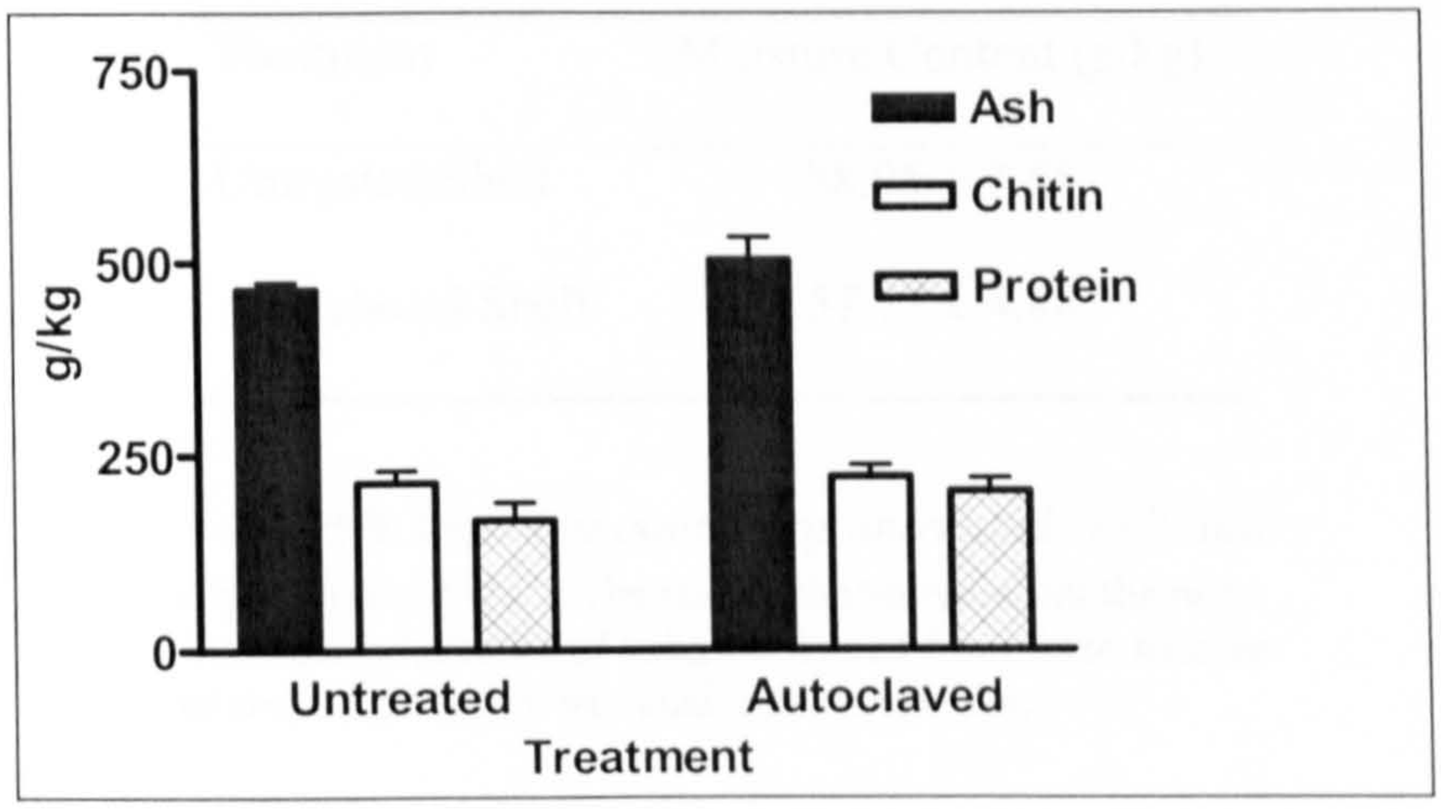


Fig. 5.7 *The chemical composition of untreated shell and autoclaved shell.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was analysed in duplicate.

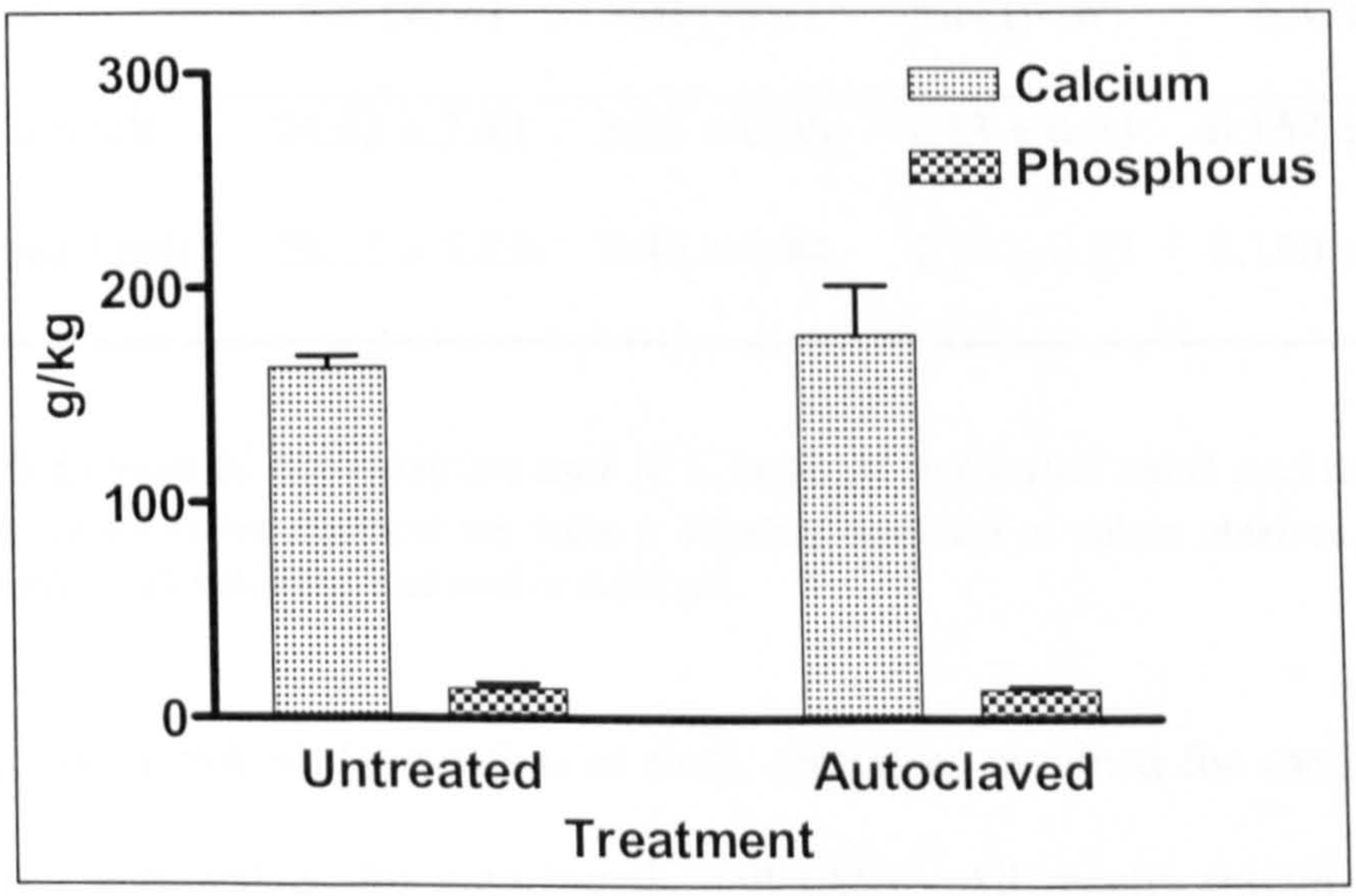


Fig. 5.8 *The calcium and phosphorus levels in untreated shell and autoclaved shell.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was analysed in duplicate.

Autoclaving appeared to cause an increase in ash of 3.6% (w/w). However, after autoclaving moisture levels measured in the samples had dropped by 3.1% (w/w) (Table 5.9). When the results were corrected for moisture content no significant differences in ash, calcium, chitin or protein content were noted between untreated and autoclaved shell waste.

Treatment	Moisture Content (g/kg)
Untreated Shell	88.95 ± 7.55
Autoclaved Shell	57.75 ± 4.88

Table 5.9 *Moisture content of untreated shell and autoclaved shell.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was analysed in duplicate.

Similarly, levels of carbon, hydrogen, nitrogen and N:C ratio were not affected by autoclaving (Table 5.10).

Treatment	%C (w/w)	%H (w/w)	%N (w/w)	N:C ratio
Untreated Shell	24.62 ± 2.83	3.73 ± 0.36	4.13 ± 0.94	0.167 ± 0.008
Autoclaved Shell	28.25 ± 5.25	3.46 ± 0.44	4.78 ± 0.83	0.180 ± 0.011

Table 5.10 *Elemental composition and N:C ratio of untreated shell and autoclaved shell.* The results shown represent the mean ± standard deviation of values obtained from three batches of shell. Each batch was analysed in duplicate.

After subsequent microbial degradation of shell, all values obtained for shell components were compared with values for autoclaved shell (AS). All results shown hereafter for autoclaved shell are mean results of the three batches of shell obtained at different times of the year.

5.3.2.3 Comparison between Lactic Acid Results obtained by Titration (a) and by Lactate Assay Kit (b)

After seven days of fermentation, culture broth from Lactosil treated samples was analysed for total titratable acidity (TTA) by titration with sodium hydroxide. The same broth samples were also analysed for lactic acid using Sigma's lactate kit. The results from the titration and the commercial kit were compared (Table 5.11). The two different methods gave similar results so it was assumed that TTA was due to lactic acid alone. All further results for lactic acid were therefore determined by titration.

Result from Titration TTA (g/litre)	Result from Lactate Kit Lactic acid (g/litre)
10.86	13.00
14.87	14.50

Table 5.11 *Comparison of results obtained by titration and by a commercial lactate assay kit.* Analysis was carried out on two different samples.

5.3.3 Optimisation of Parameters for Lactic Acid Fermentation of *Nephrops* Shell Waste

5.3.3.1 Effect of Inoculant Concentration

Lactosil, at a concentration of 8% (v/v), lead to the production of lactic acid in the culture broth at levels greater than 200mg/g dry shell (Fig. 5.9).

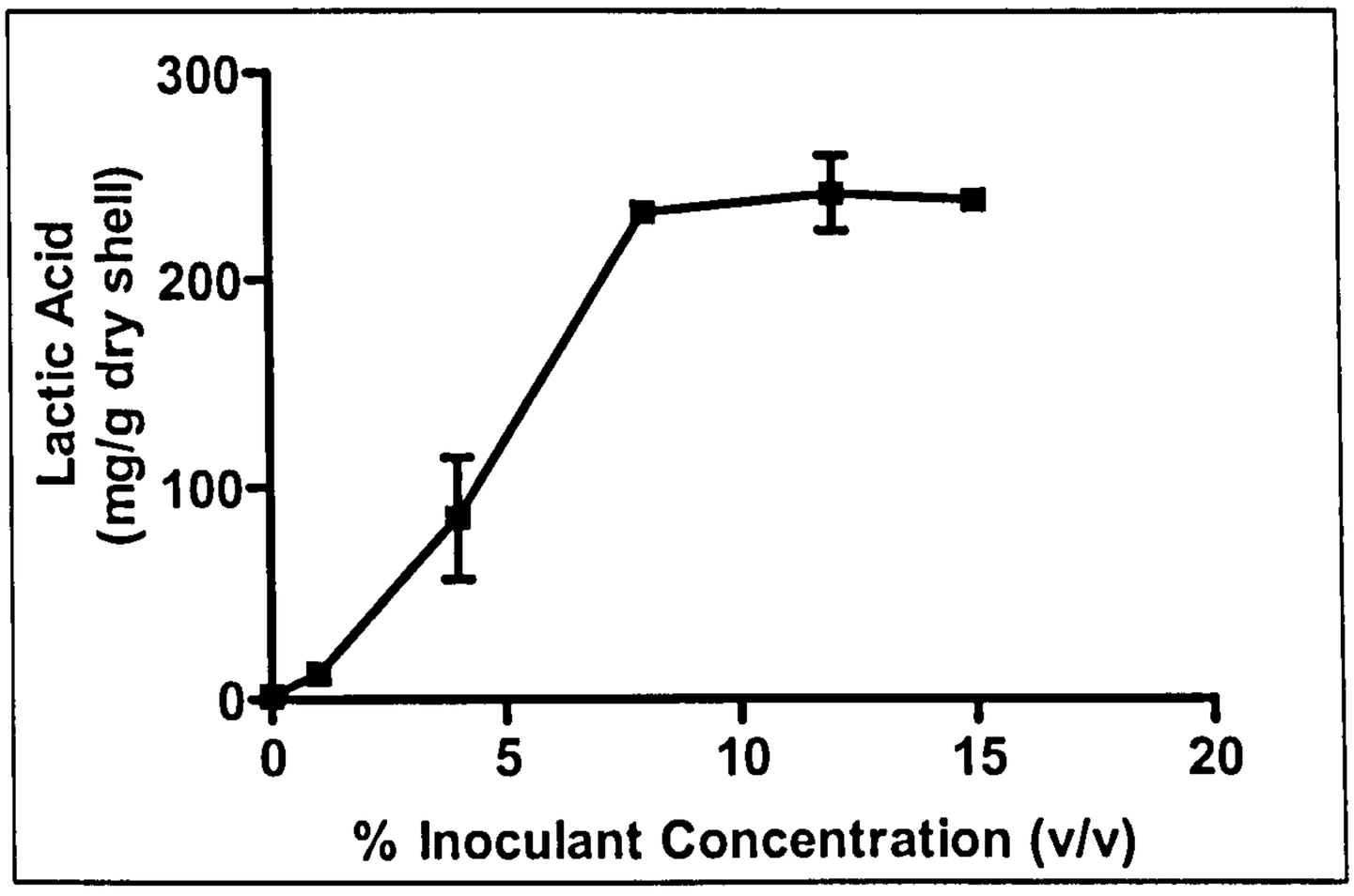


Fig. 5.9 *Effect of inoculant concentration on the production of lactic acid.* The results plotted represent the mean ± standard deviation of values obtained from three separate experiments.

Increasing the inoculant concentration to 12% (v/v) and 15% (v/v) failed to cause any further increase in lactic acid production. As the lactic acid production increased, the levels of ash in the starting material (autoclaved shell) dropped from 501.90 ± 31.27g/kg to 115.99 ± 2.31g/kg shell at an 8% (v/v) concentration of Lactosil (Fig. 5.10). Further increases in inoculant concentration to 12% (v/v) and 15% (v/v) led to no further reduction in ash content.

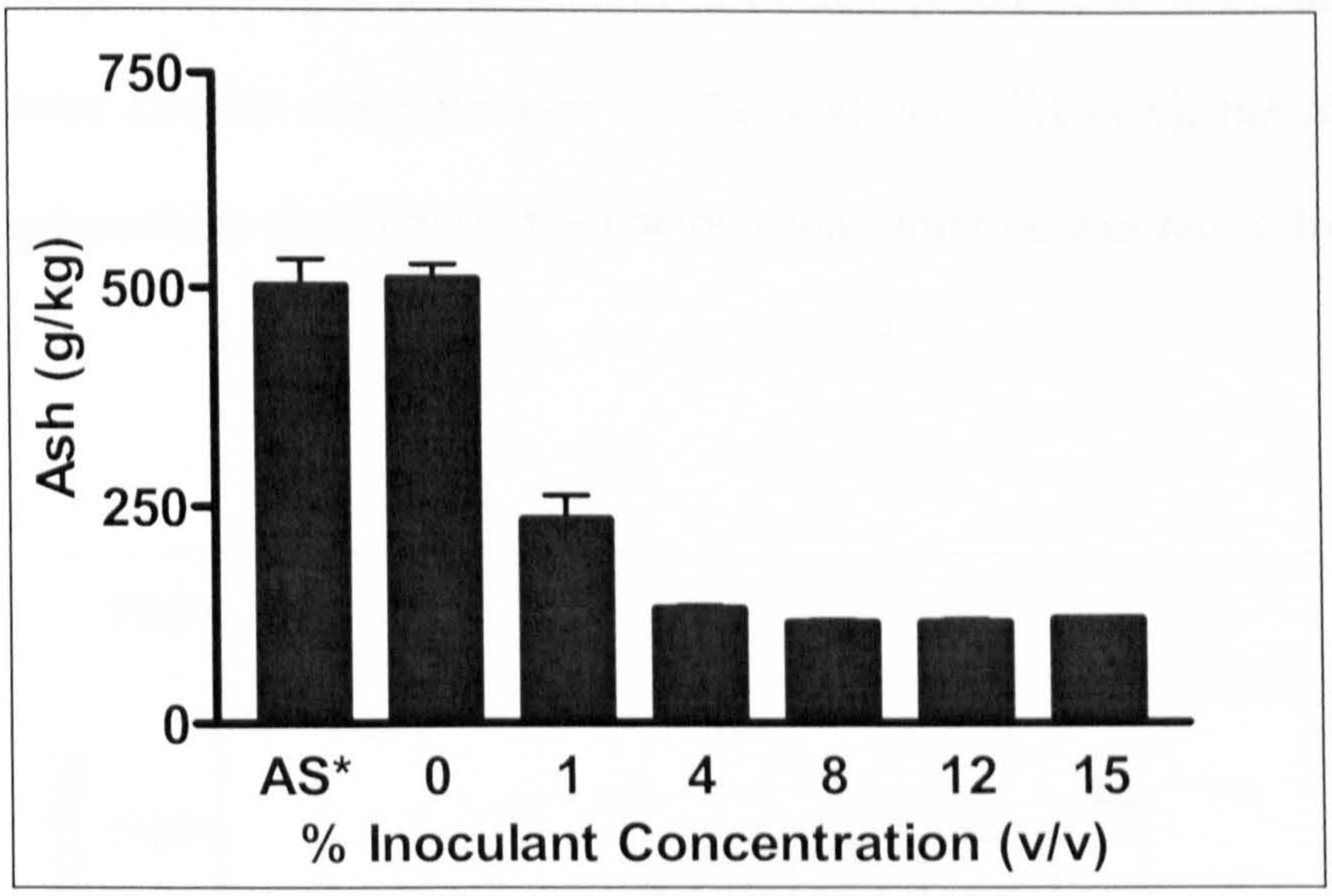


Fig. 5.10 *Effect of inoculant concentration on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Calcium levels followed a similar pattern to ash. They fell from 178.64 ± 21.63g/kg in autoclaved shell to 33.65 ± 0.91g/kg at an 8% (v/v) concentration of Lactosil (Fig. 5.11).

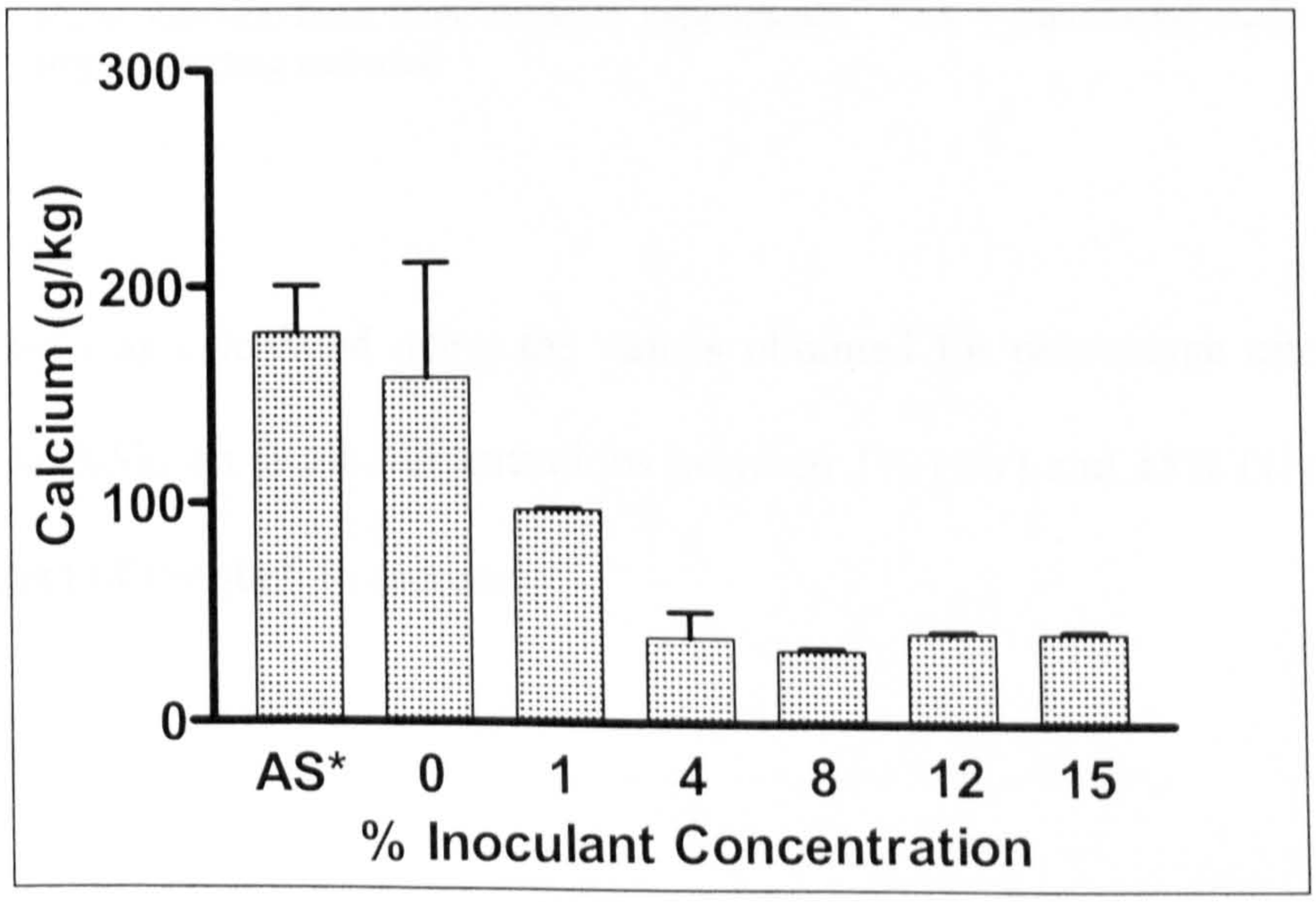


Fig. 5.11 *Effect of inoculant concentration on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Chitin levels increased steadily as the concentration of the inoculant was raised (Fig. 5.12). Despite no change occurring in the amount of lactic acid produced or in the ash content of the shell between Lactosil concentrations of 8% (v/v) and 15% (v/v), the chitin content increased significantly ($p<0.05$) when the Lactosil concentration was raised from 8% (v/v) to 15% (v/v).

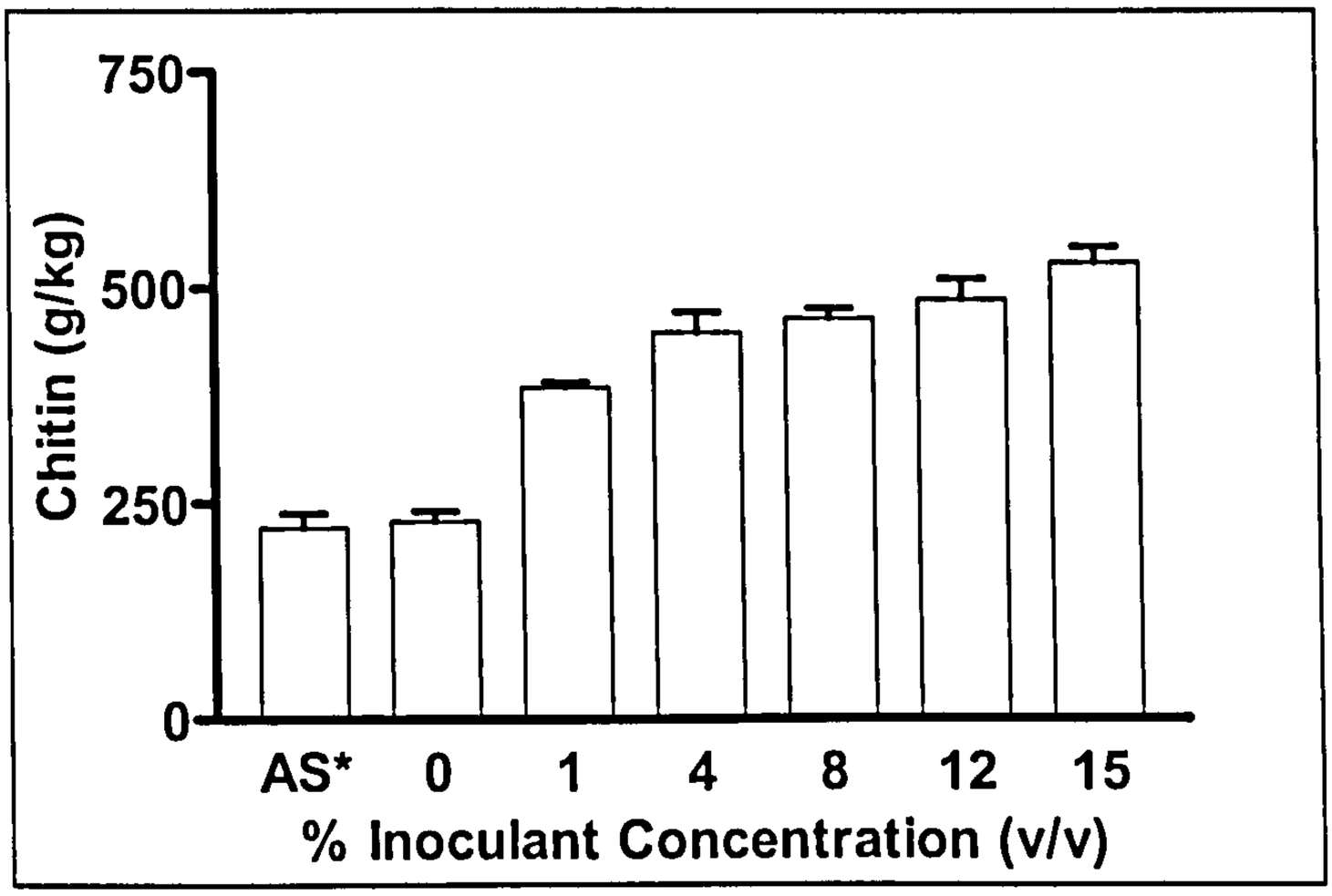


Fig. 5.12 *Effect of inoculant concentration on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Protein content was calculated using the values obtained for percentage nitrogen (Table 5.12). Lactosil addition at all concentrations between 1% (v/v) and 15% (v/v) caused the nitrogen content of the shell to increase.

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Lactic Acid Fermentation with 0% (v/v) Lactosil	3.60 ± 0.56
Lactic Acid Fermentation with 1% (v/v) Lactosil	7.70 ± 1.54
Lactic Acid Fermentation with 4% (v/v) Lactosil	9.50 ± 0.56
Lactic Acid Fermentation with 8% (v/v) Lactosil	7.56 ± 0.50
Lactic Acid Fermentation with 12% (v/v) Lactosil	8.11 ± 0.80
Lactic Acid Fermentation with 15% (v/v) Lactosil	7.68 ± 0.85

Table 5.12 *Effect of inoculant concentration on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments

Protein levels in the shell were significantly different at Lactosil concentrations of 1% (v/v) ($p<0.05$) and 4% (v/v) ($p<0.01$) compared with 0% (v/v) Lactosil (Fig. 5.13). At 4% (v/v) Lactosil the protein concentration doubled.

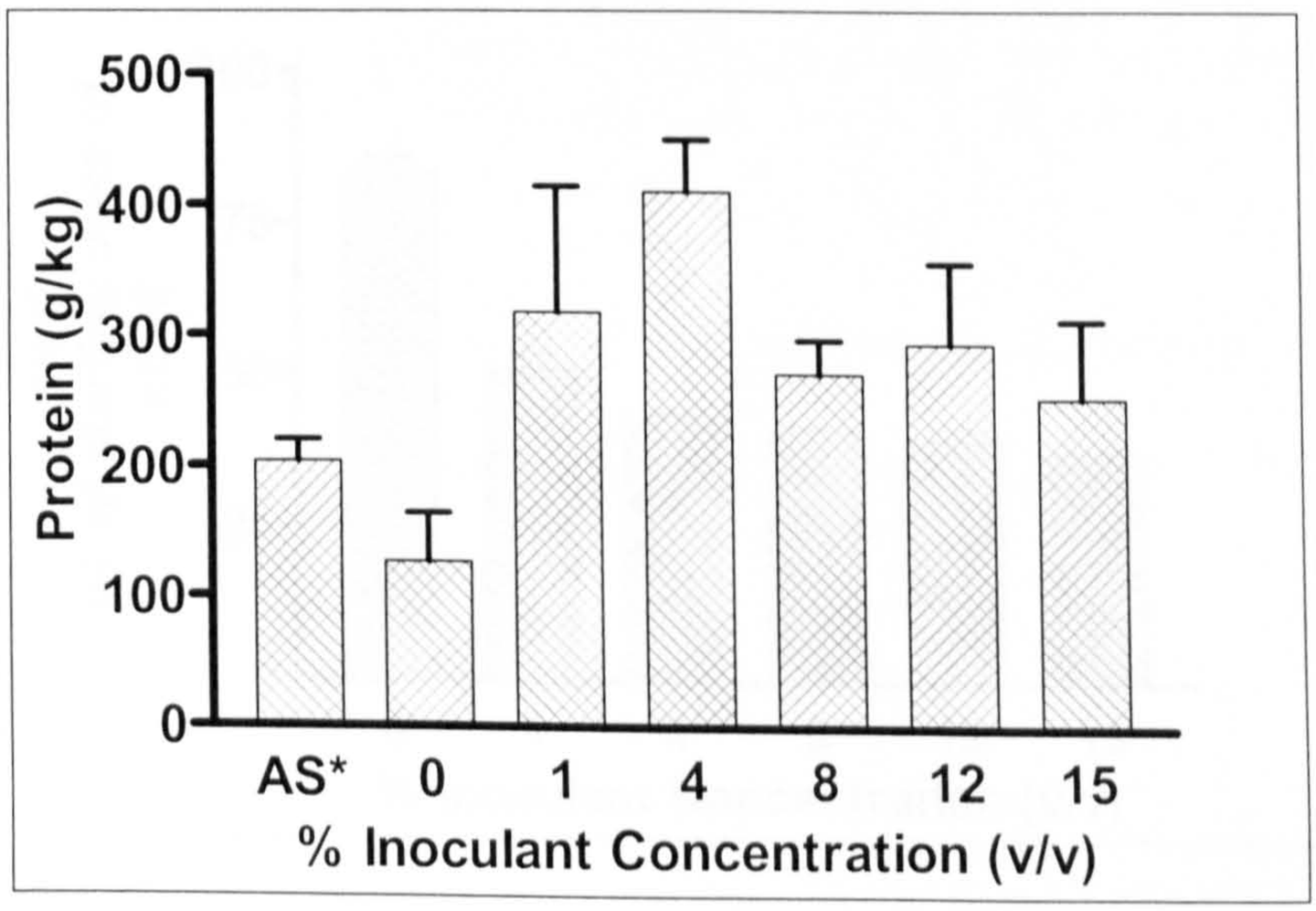


Fig. 5.13 *Effect of inoculant concentration on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

At Lactosil concentrations of 8%, 12% and 15% (v/v) the protein concentration had started to decrease and was no longer significantly different from protein levels at 0% (v/v) Lactosil (Fig. 5.13). This loss in protein explains the continued increase in chitin levels between 8 and 15% (v/v) Lactosil (Fig. 5.12). Increasing the inoculant concentration beyond 15% (v/v), in an attempt to reduce protein levels, was not feasible since no further ash reduction would have been achieved in this manner.

In Fig. 5.13 it can be seen that when the concentration of inoculant was 0% (v/v) protein levels were lower than in the starting material (AS*). No lactic acid was produced in the samples when Lactosil was 0% (v/v) (Fig. 5.9), indicating the absence of lactic acid bacteria. This loss in protein was therefore unlikely to be caused by microbial degradation.

Neither the ash content, the calcium content nor the chitin content differed significantly from the levels of ash, calcium and chitin in the original starting material at a 0% (v/v) inoculant level (Figs. 5.10, 5.11 and 5.12).

Fig. 5.14 shows the overall yield as a percentage of the starting material when different concentrations of Lactosil were used to degrade the shell.

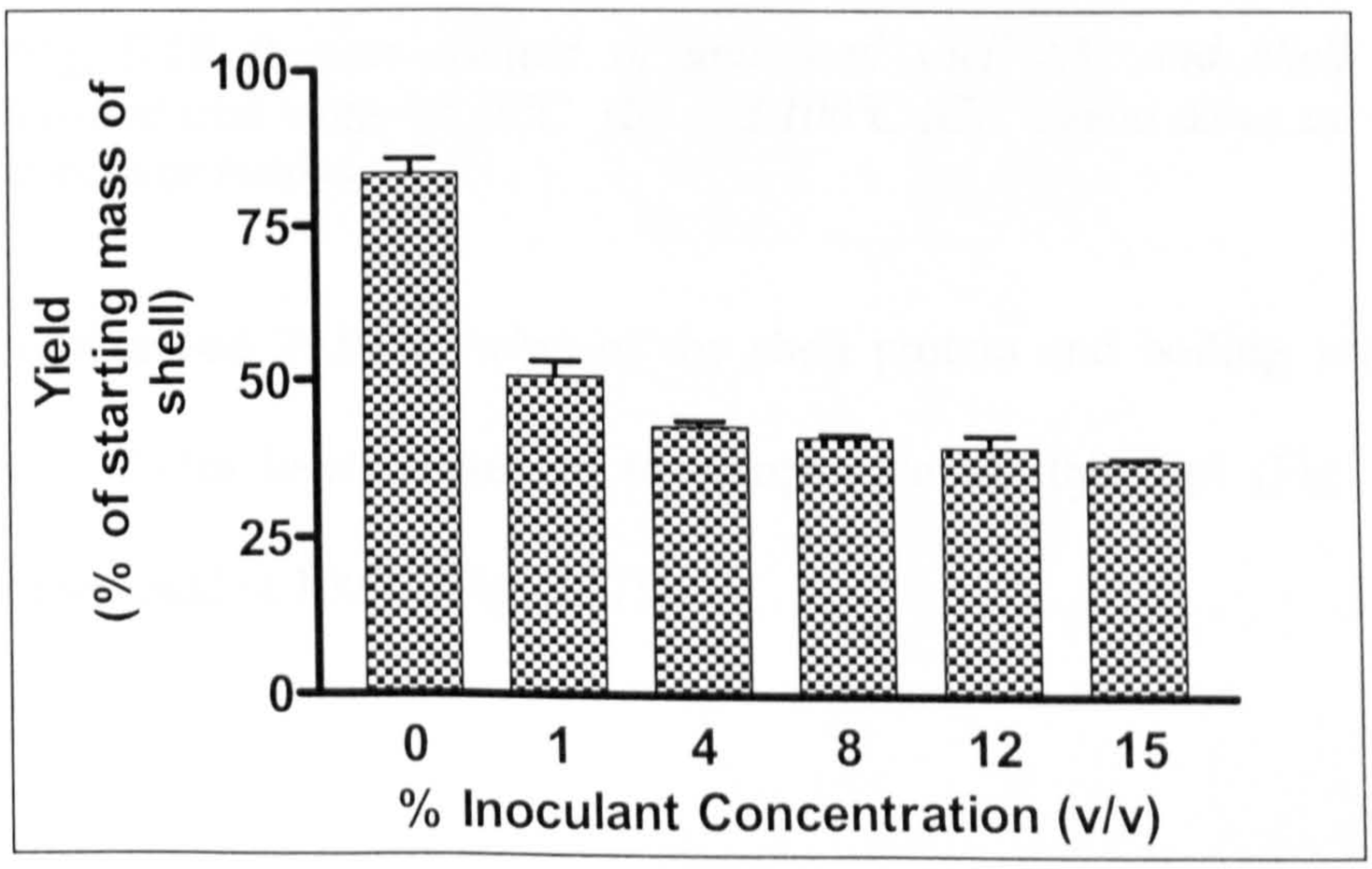


Fig. 5.14 *Effect of inoculant concentration on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

When the shell was fermented with 15% (v/v) Lactosil, 37.37 ± 0.41% of the shell mass remained. Therefore, 62.63% of the shell mass was lost. However, 16.51% of the shell mass was lost when the shell was just mixed with glucose, i.e 0% (v/v) inoculant. Therefore, only 46.12% (62.63% - 16.51%) of the 62.63% loss can be attributed to microbial action.

Untreated shell (A) was washed in warm water (B) and in boiling water (C) and the protein component of the shell quantified before and after washing (Fig. 5.15).

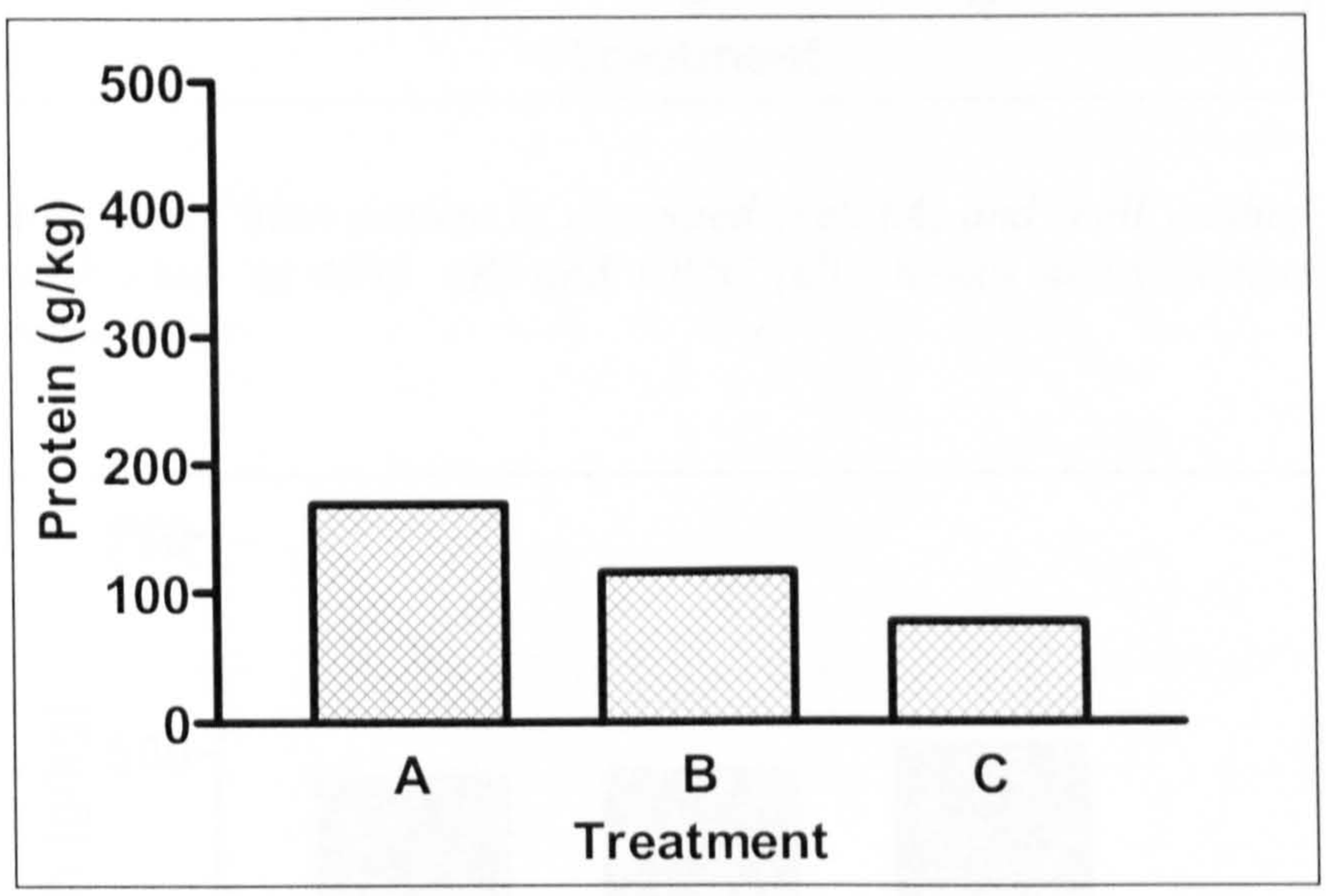


Fig. 5.15 *Protein content of untreated shell (**A**) and shell washed with water at 40°C (**B**) and 100°C (**C**).* Results shown are from single samples.

Water at 40°C removed 31.93% (w/w) of the shell protein and boiling water removed 54.72% (w/w). Chitin levels increased to compensate for this loss (Fig. 5.16). Ash concentration increased at 100°C (Fig. 5.17).

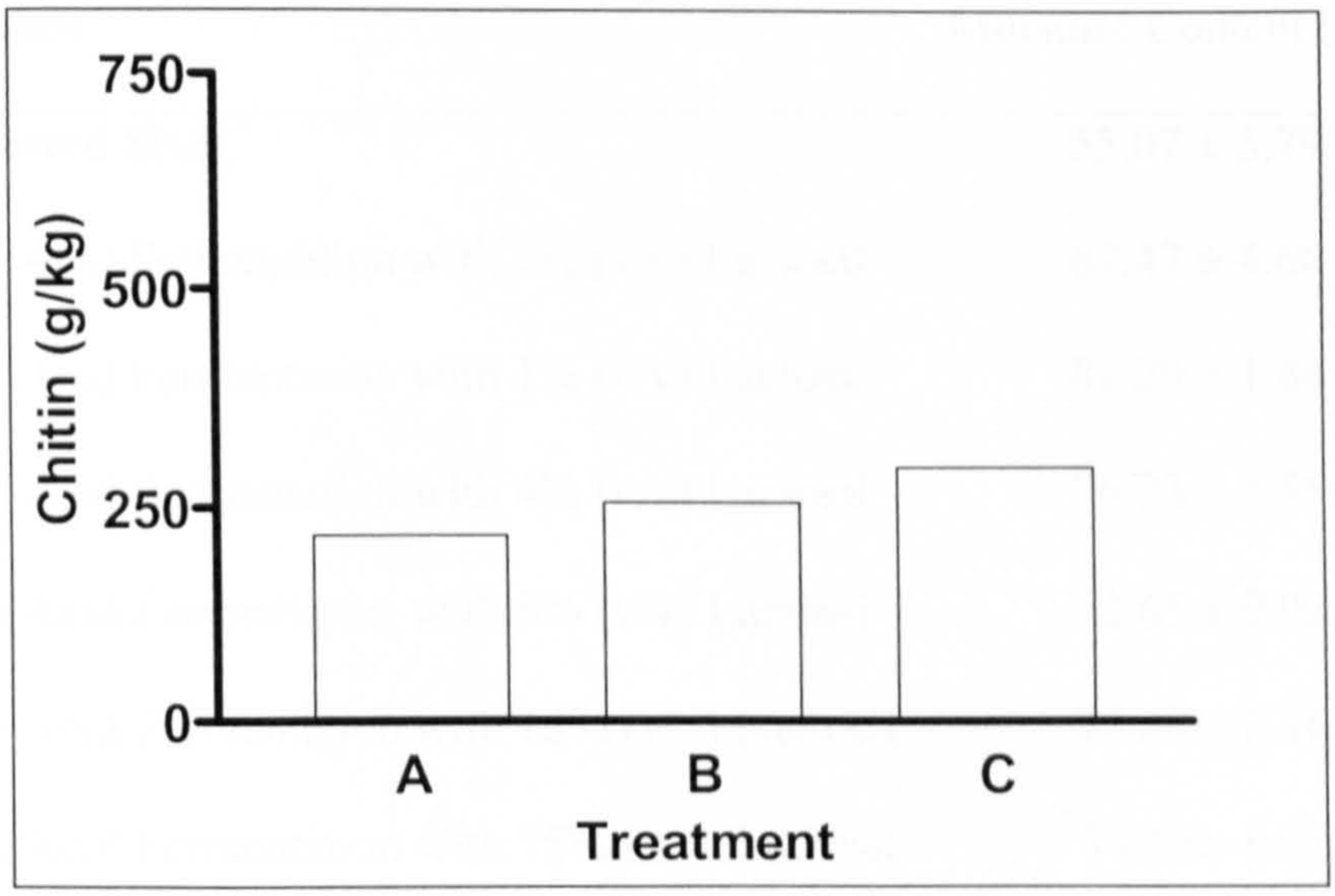


Fig. 5.16 *Chitin content of untreated shell (**A**) and shell washed with water at 40°C (**B**) and 100°C (**C**).* Results shown are from single samples.

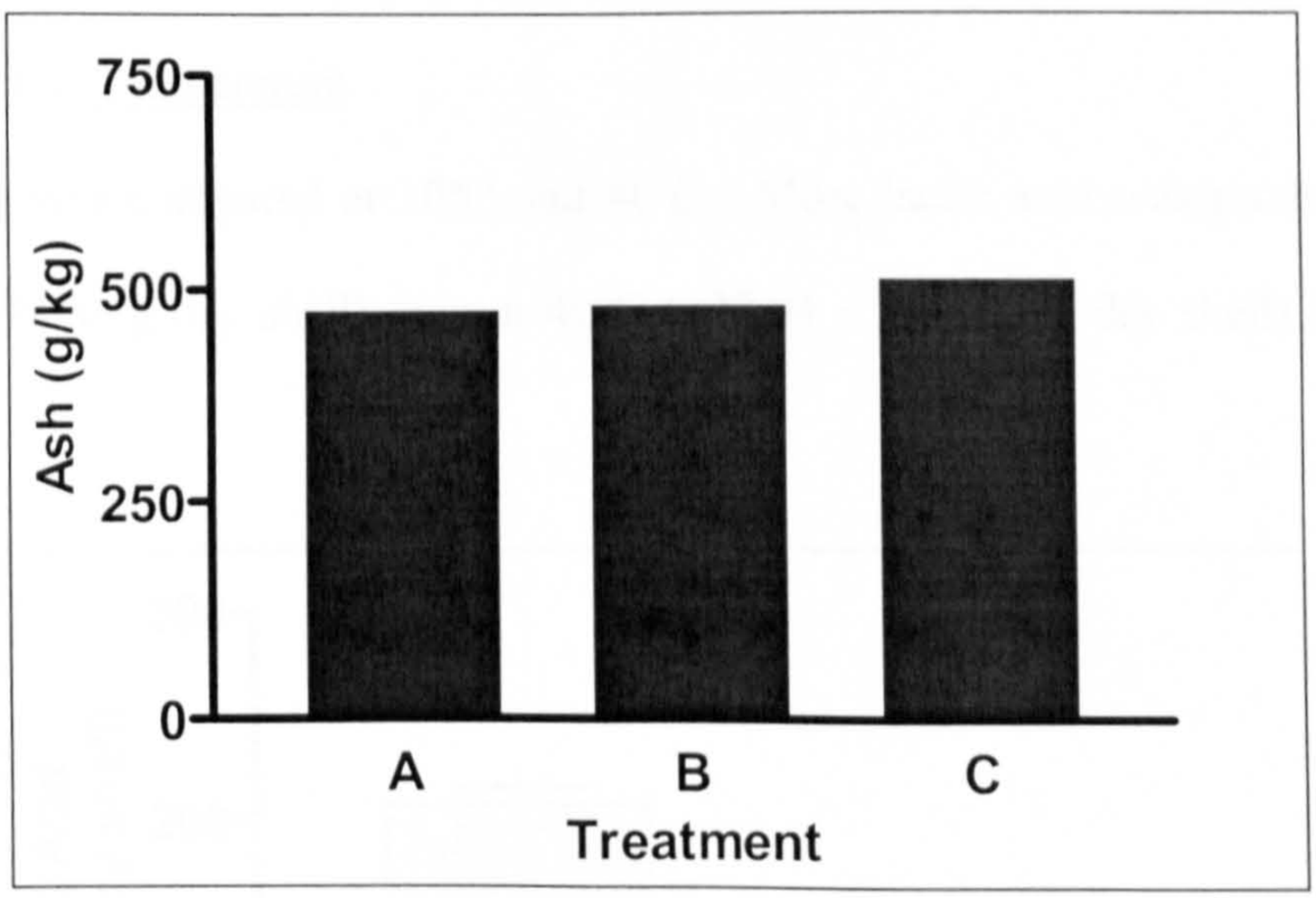


Fig. 5.17 *Ash content of untreated shell (**A**) and shell washed with water at 40°C (**B**) and 100°C (**C**).* Results shown are from single samples.

It therefore appears that protein solubilisation may have accounted for the loss in protein that occurred when no inoculant was included in the system. However, the expected compensating increase in ash or chitin was not seen. Variation in moisture content did not account for the drop in protein at 0% Lactosil (Table 5.13).

Treatment	Moisture Content (g/kg)
Autoclaved Shell	55.07 ± 5.79
Lactic Acid Fermentation with 0% (v/v) Lactosil	67.47 ± 4.60
Lactic Acid Fermentation with 1% (v/v) Lactosil	81.20 ± 1.84
Lactic Acid Fermentation with 4% (v/v) Lactosil	76.23 ± 1.55
Lactic Acid Fermentation with 8% (v/v) Lactosil	72.65 ± 0.92
Lactic Acid Fermentation with 12% (v/v) Lactosil	75.33 ± 2.16
Lactic Acid Fermentation with 15% (v/v) Lactosil	74.53± 1.27

Table 5.13 *Moisture content of shell after lactic acid fermentation with different concentrations of Lactosil.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

5.3.3.2 Effect of Temperature

Fermentation was compared at 30°C and 40°C. More lactic acid was produced at 30°C (207.42 ± 8.94mg/g dry shell) than at 40°C (137.04 ± 6.34mg/g dry shell) ($p = 0.0004$) (Fig. 5.18).

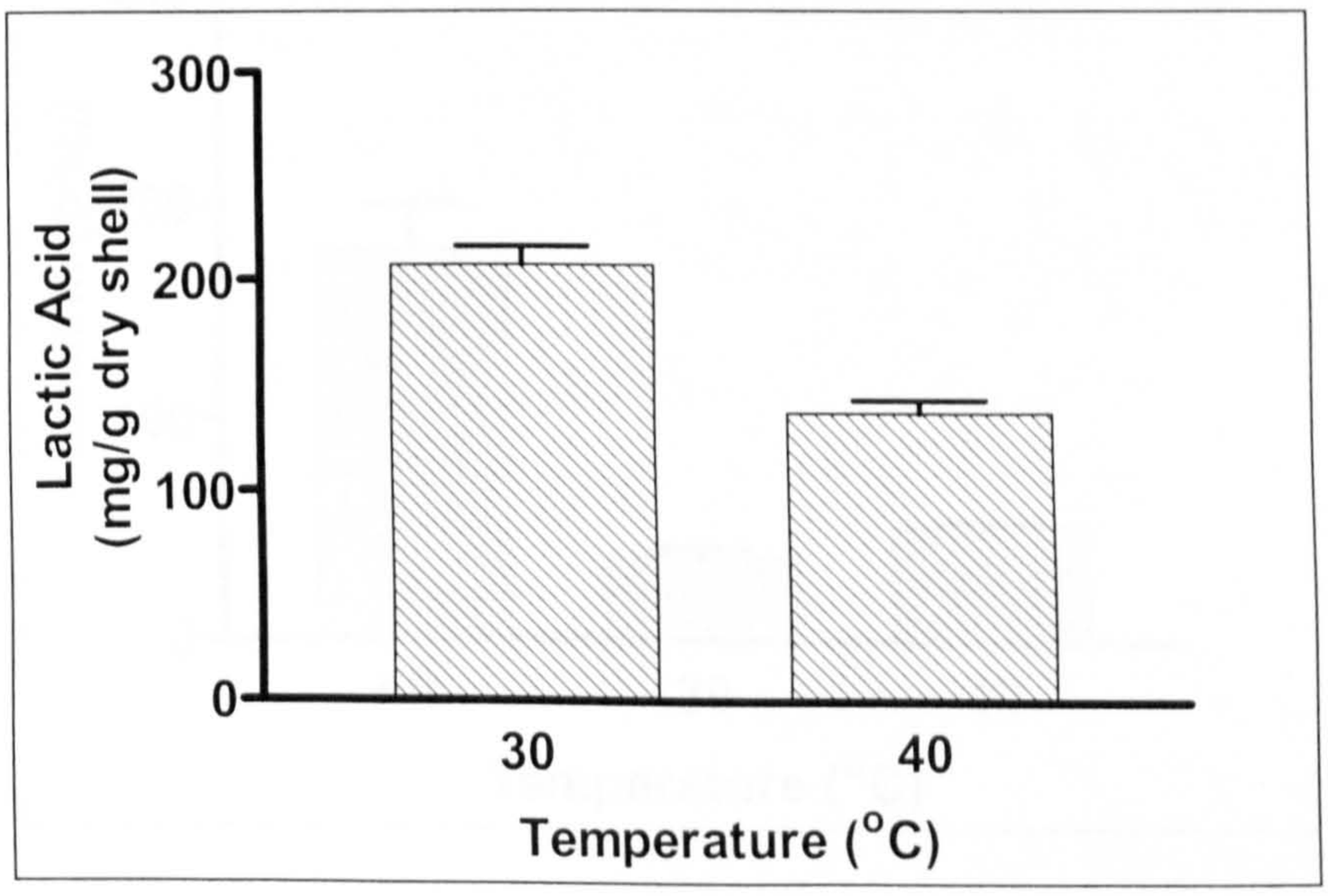


Fig. 5.18 *Effect of temperature on the production of lactic acid.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

A significantly lower ash content was therefore seen in samples treated at 30°C than in samples treated at 40°C ($p = 0.0022$) (Fig. 5.19).

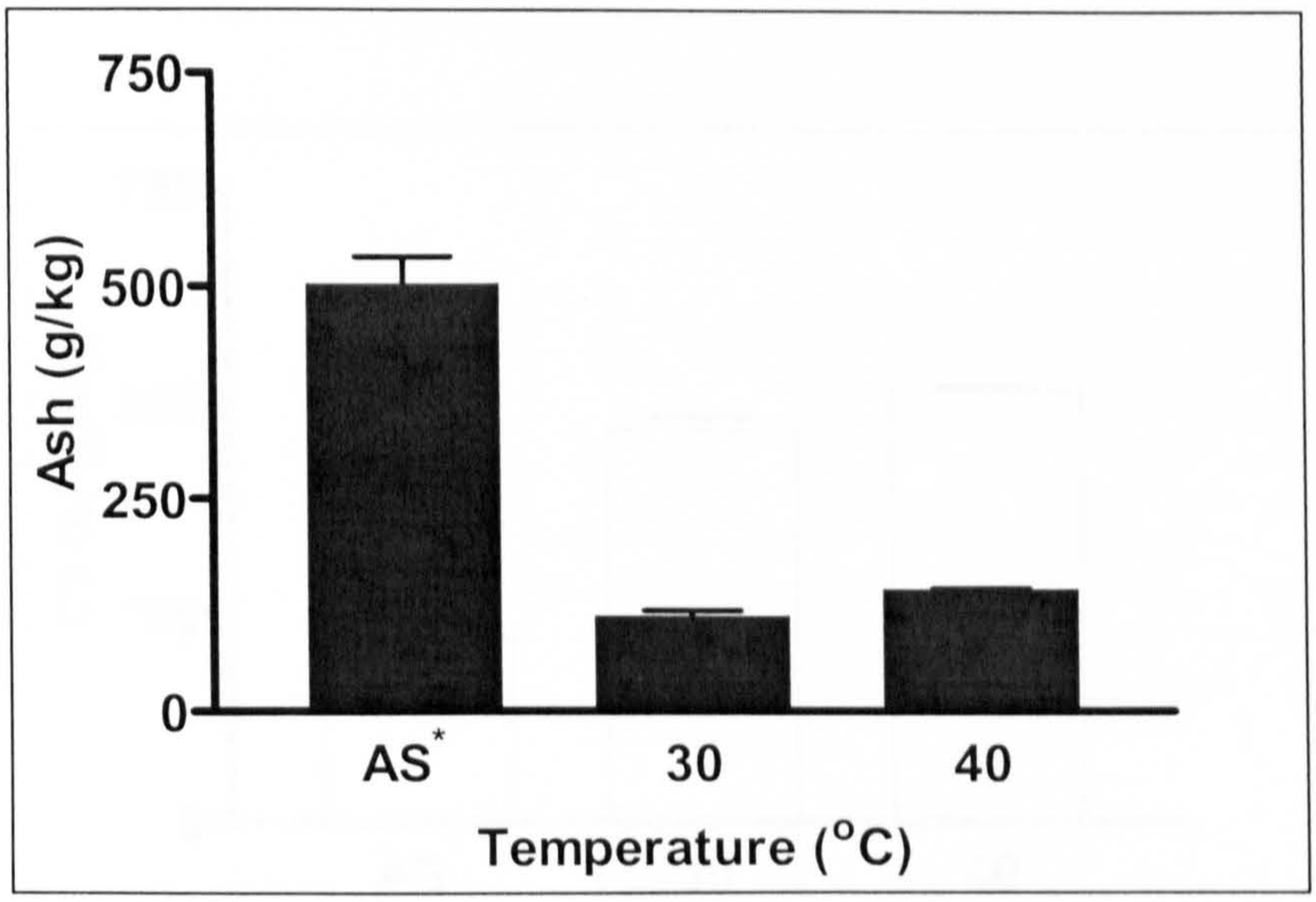


Fig. 5.19 *Effect of temperature on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Calcium content followed a similar pattern to ash levels. A lower calcium content was present at 30°C than at 40°C ($p = 0.0039$) (Fig. 5.20).

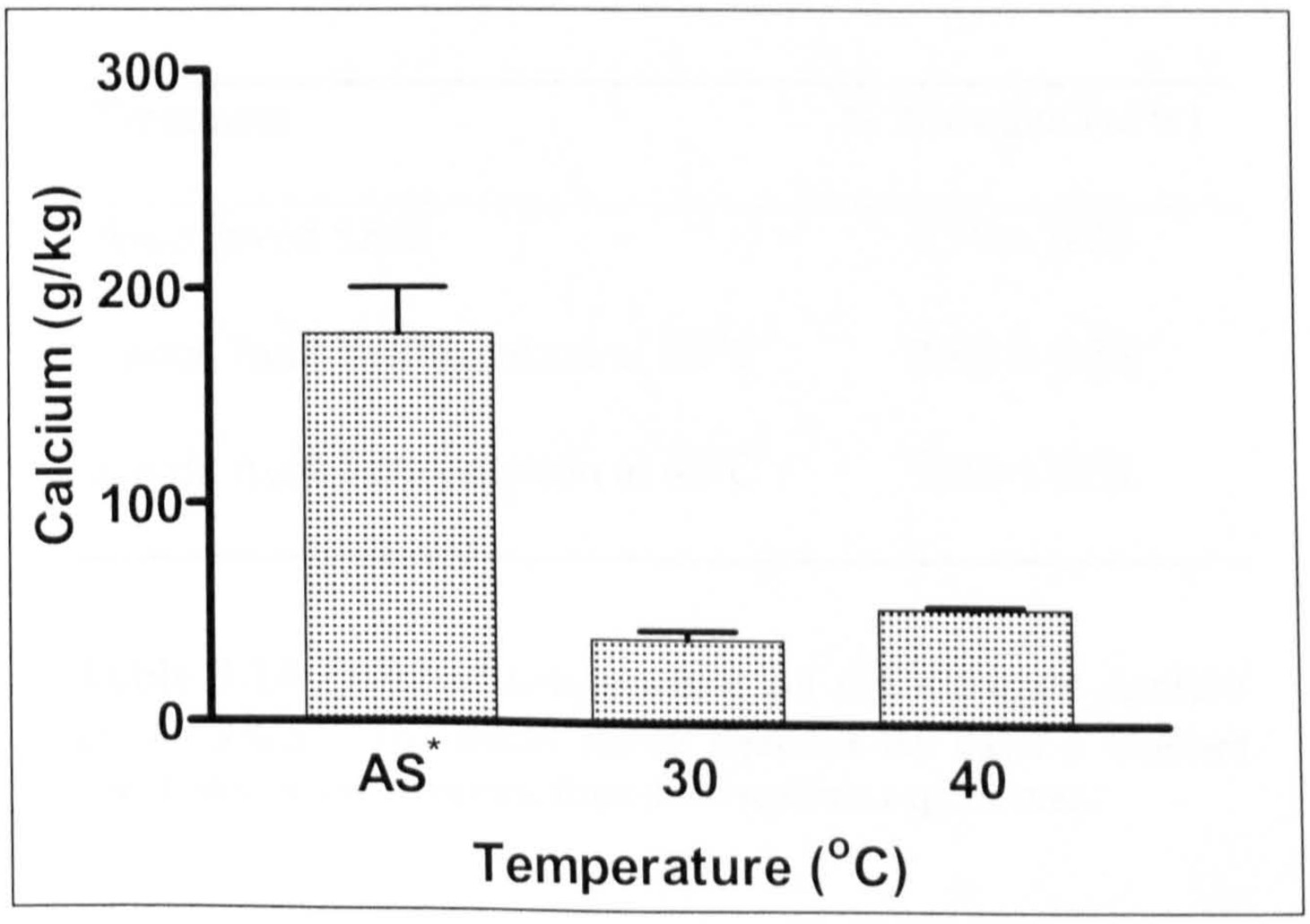


Fig. 5.20 *Effect of temperature on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

The chitin component was also significantly higher at 40°C (515.00 ± 5.00g/kg) than at 30°C (470.00 ± 13.23g/kg) ($p = 0.0053$) (Fig. 5.21).

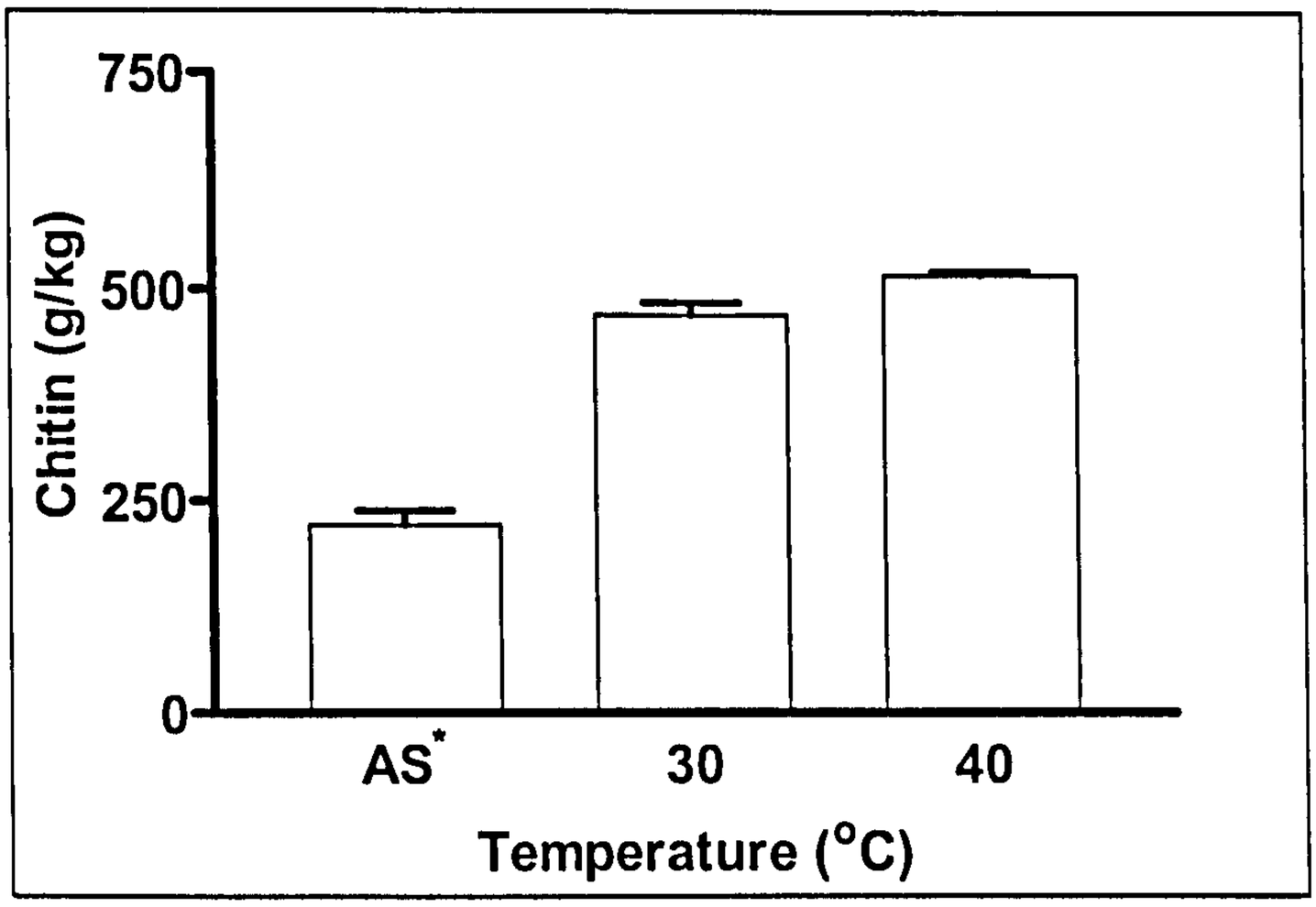


Fig. 5.21 *Effect of temperature on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Percentage nitrogen was lower at 40°C than at 30°C (Table 5.14).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Lactic Acid Fermentation at 30°C	8.66 ± 0.58
Lactic Acid Fermentation at 40°C	7.05 ± 0.51

Table 5.14 *Effect of temperature on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Protein content reflected the results for percentage nitrogen. Protein levels were significantly lower at 40°C than at 30°C ($p = 0.117$) (Fig. 5.22). However, despite the reduced protein seen after lactic acid fermentation at 40°C (Fig. 5.22) shell washed at 40°C (Fig. 5.15) showed lower protein levels.

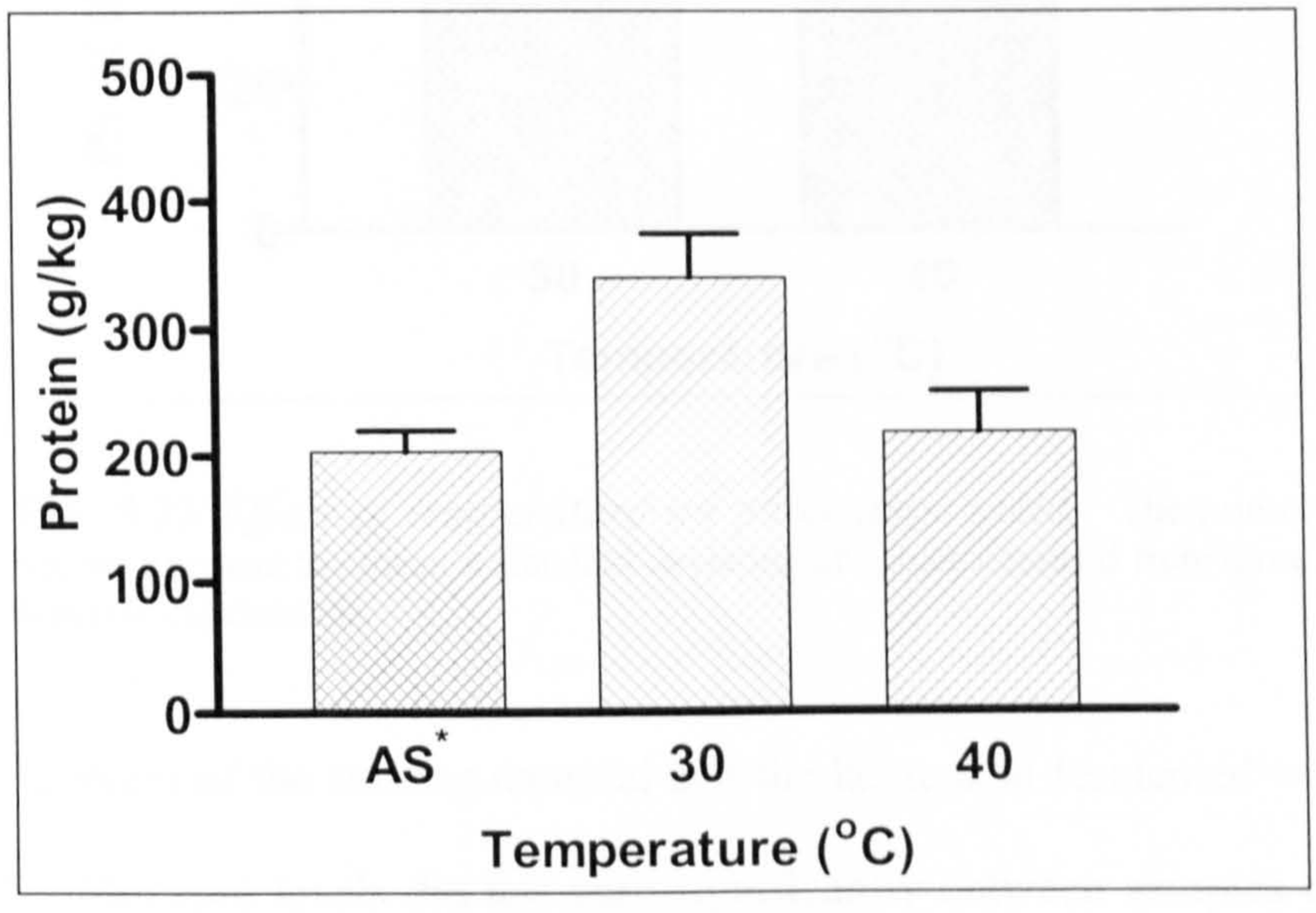


Fig. 5.22 *Effect of temperature on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

The overall percentage yield was 42.04 ± 1.36 at 30°C and 40.89 ± 0.03 at 40°C (Fig. 5.23). Therefore, approximately 60% of the total shell mass was lost at fermentation regardless of whether the temperature used was 30°C or 40°C.

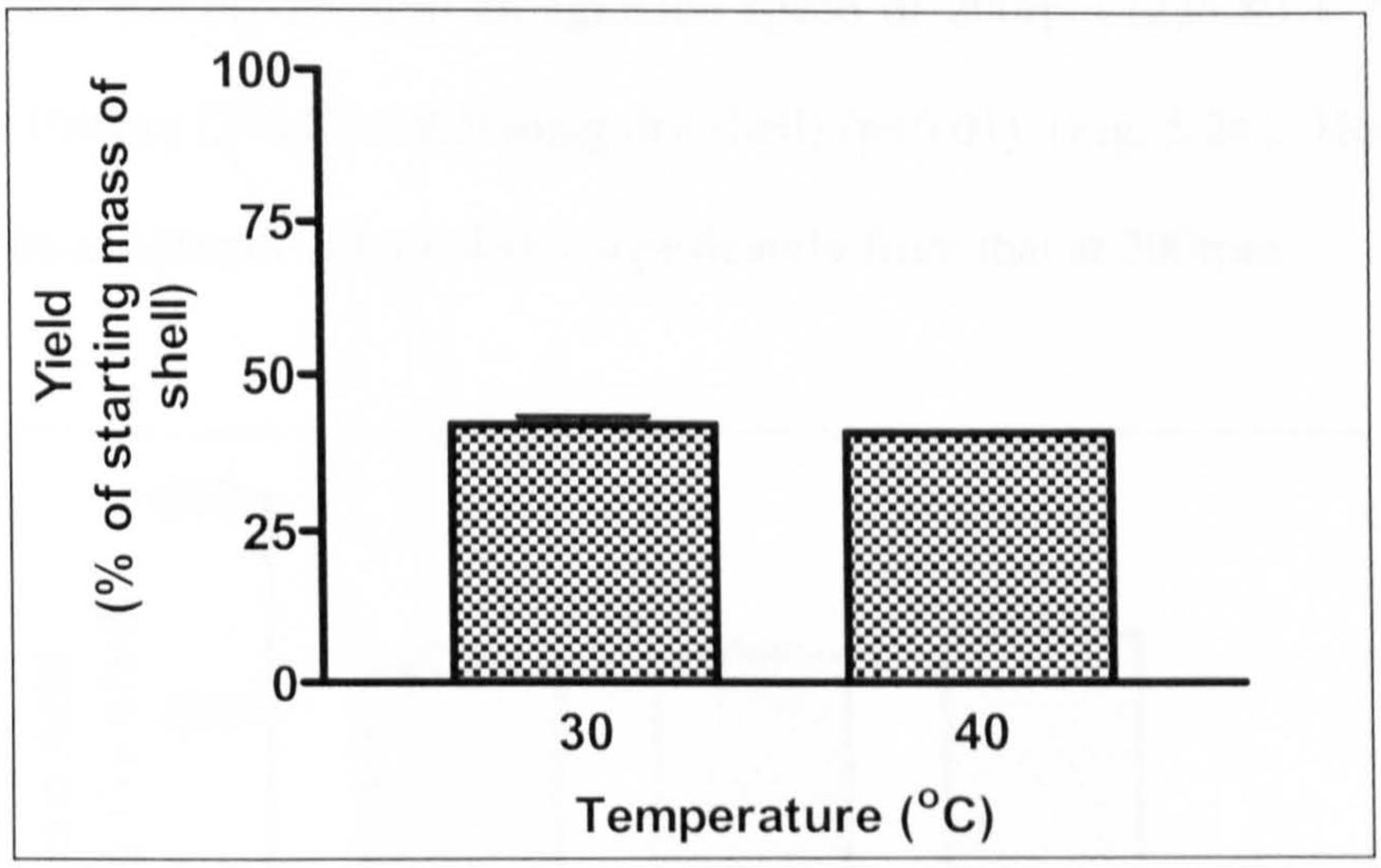


Fig. 5.23 *Effect of temperature on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The moisture content of the starting material and the lactic acid fermented waste is shown in Table 5.15. Moisture levels did not vary significantly between samples of autoclaved shell and autoclaved shell subjected to lactic acid fermentation at 30°C and 40°C.

Treatment	Moisture Content (g/kg)
Autoclaved Shell	55.07 ± 5.79
Lactic Acid Fermentation at 30°C	62.97 ± 2.06
Lactic Acid Fermentation at 40°C	60.70 ± 0.71

Table 5.15 *Moisture content of shell after lactic acid fermentation at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

5.3.3.3 Effect of Agitation Speed (rpm)

More lactic acid was produced at an agitation speed of 200rpm (235.80 ± 7.99mg/g dry shell) than at 100rpm (218.70 ± 9.56mg/g dry shell) ($p<0.01$) (Fig. 5.24). However, lactic acid production at 150rpm did not differ significantly from that at 200rpm.

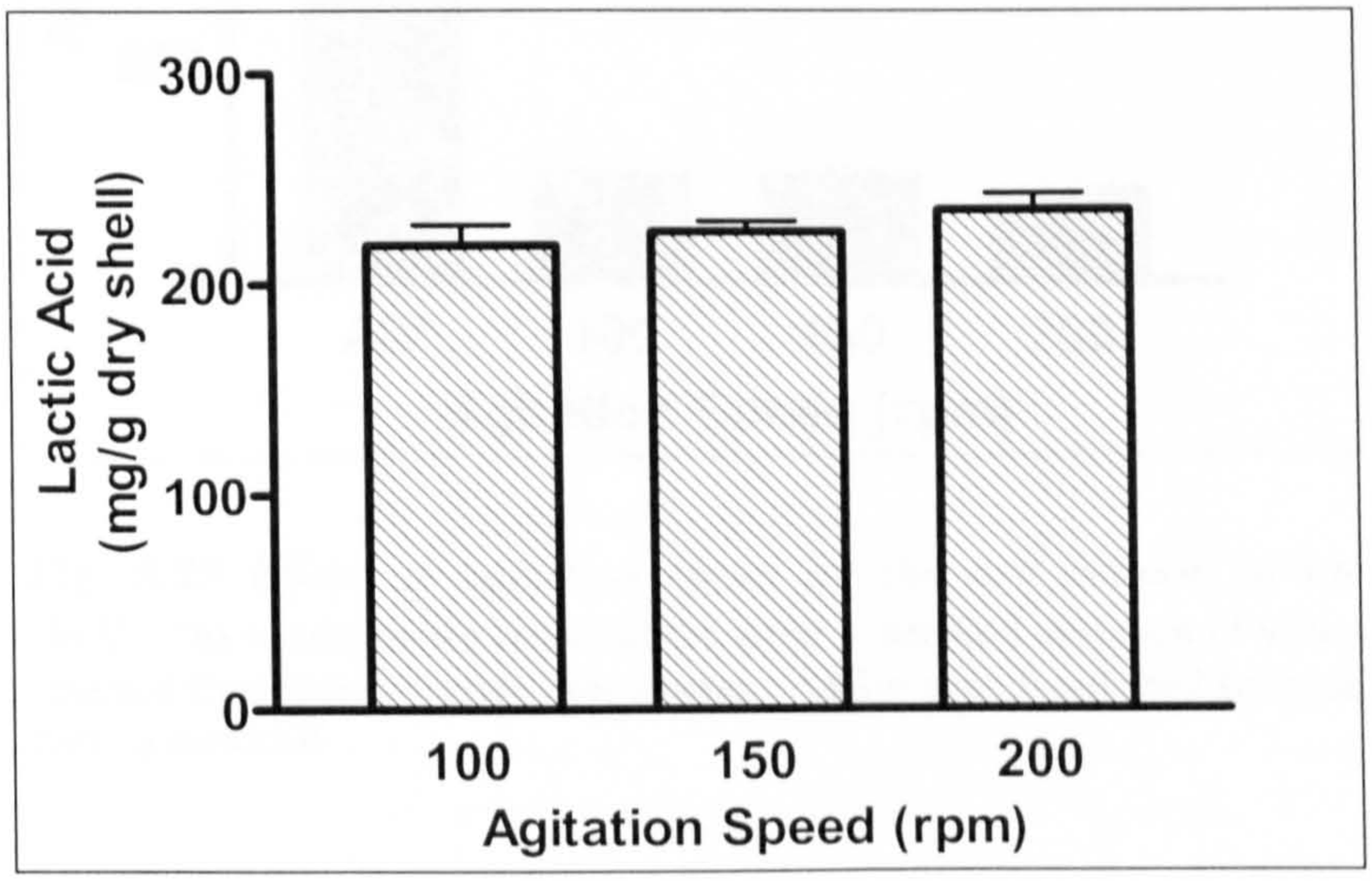


Fig. 5.24 *Effect of agitation speed on lactic acid production.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Ash levels in samples at 100rpm and 150rpm were significantly higher than those at 200rpm ($p<0.01$) (Fig. 5.25). This corresponded to the increase in lactic acid noted at 200rpm.

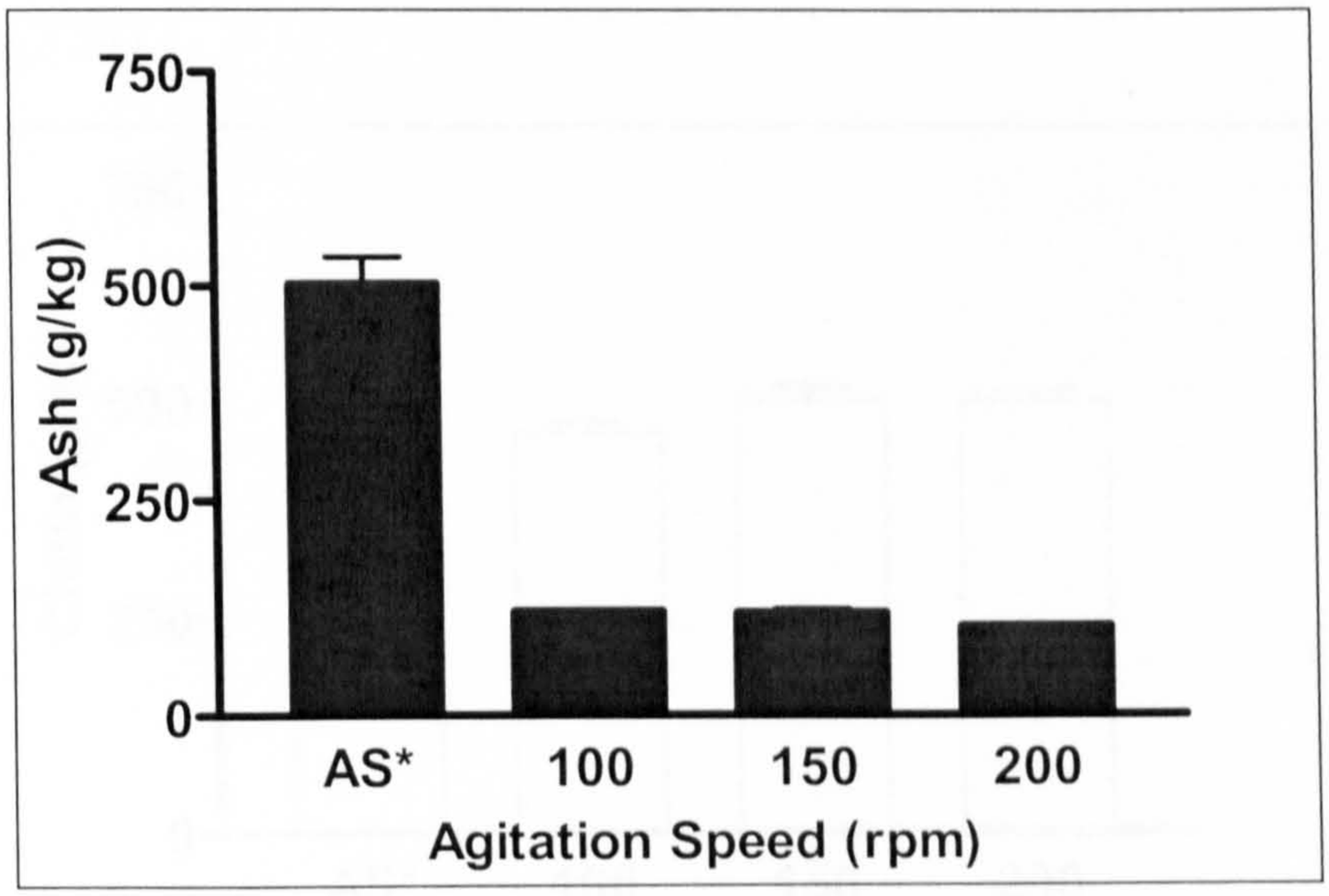


Fig. 5.25 *Effect of agitation speed on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

No variation was found in calcium content at different agitation speeds (Fig. 5.26).

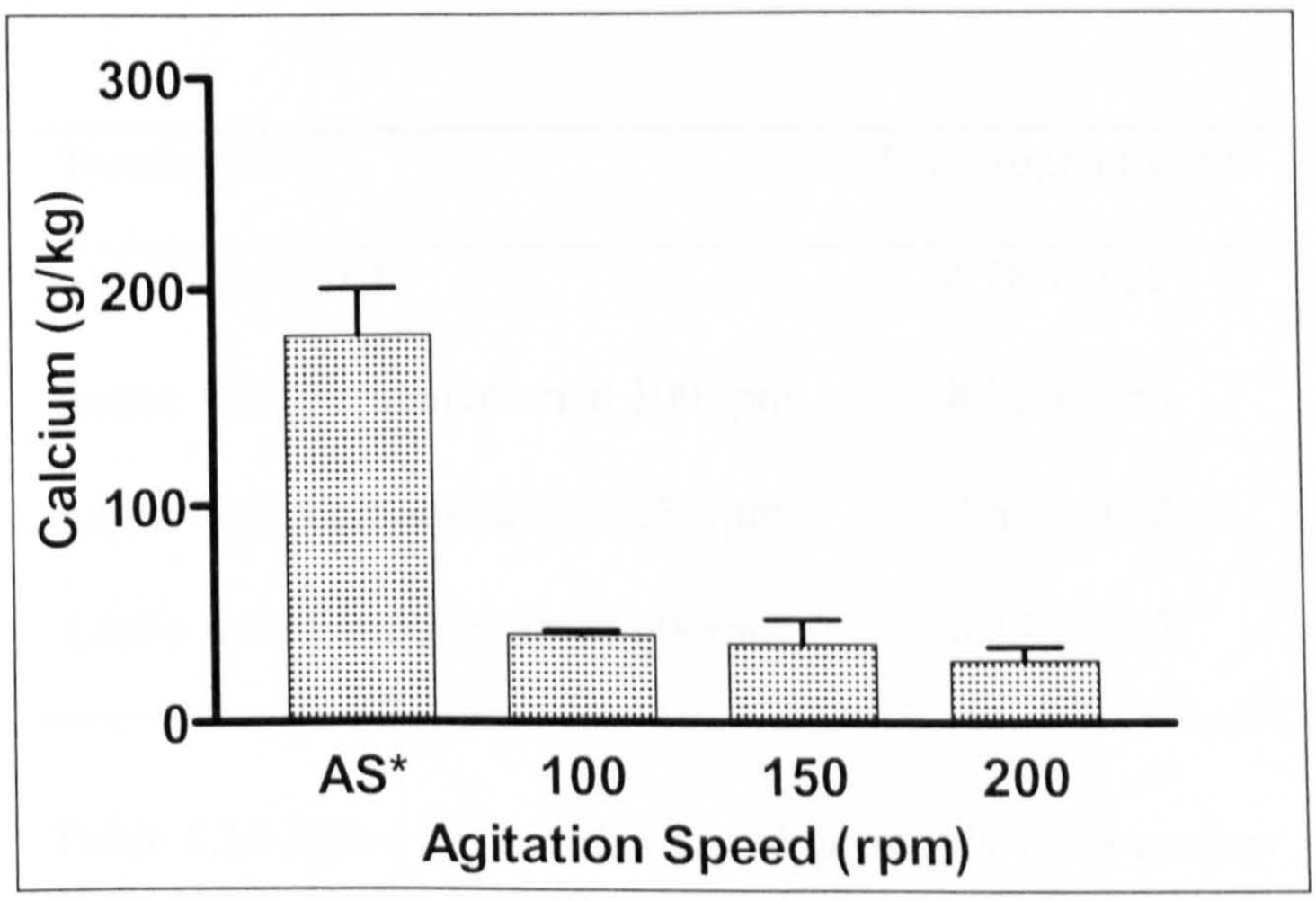


Fig. 5.26 *Effect of agitation speed on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Chitin content at 100rpm was significantly lower than chitin content at 150rpm or 200rpm ($p<0.05$) (Fig. 5.27).

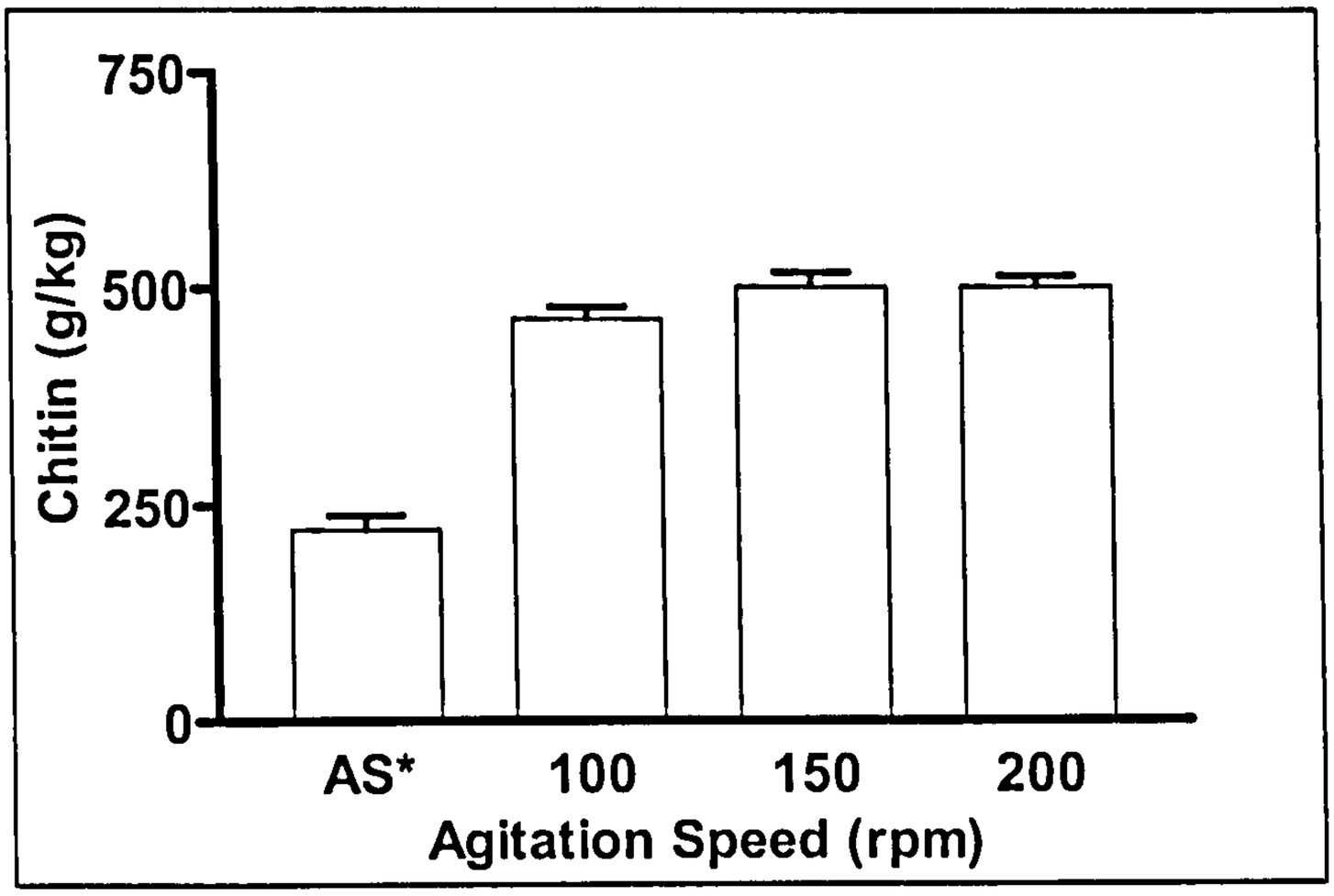


Fig. 5.27 *Effect of agitation speed on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Nitrogen levels were not affected by the change in agitation speed (Table 5.16).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Lactic Acid Fermentation at 100 rpm	8.26 ± 0.53
Lactic Acid Fermentation at 150rpm	7.67 ± 0.82
Lactic Acid Fermentation at 200rpm	8.13 ± 0.27

Table 5.16 *Effect of agitation speed on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Protein levels reflected the results for nitrogen content. No change in protein concentration occurred when agitation speed was varied from 100 to 150 and 200rpm (Fig. 5.28).

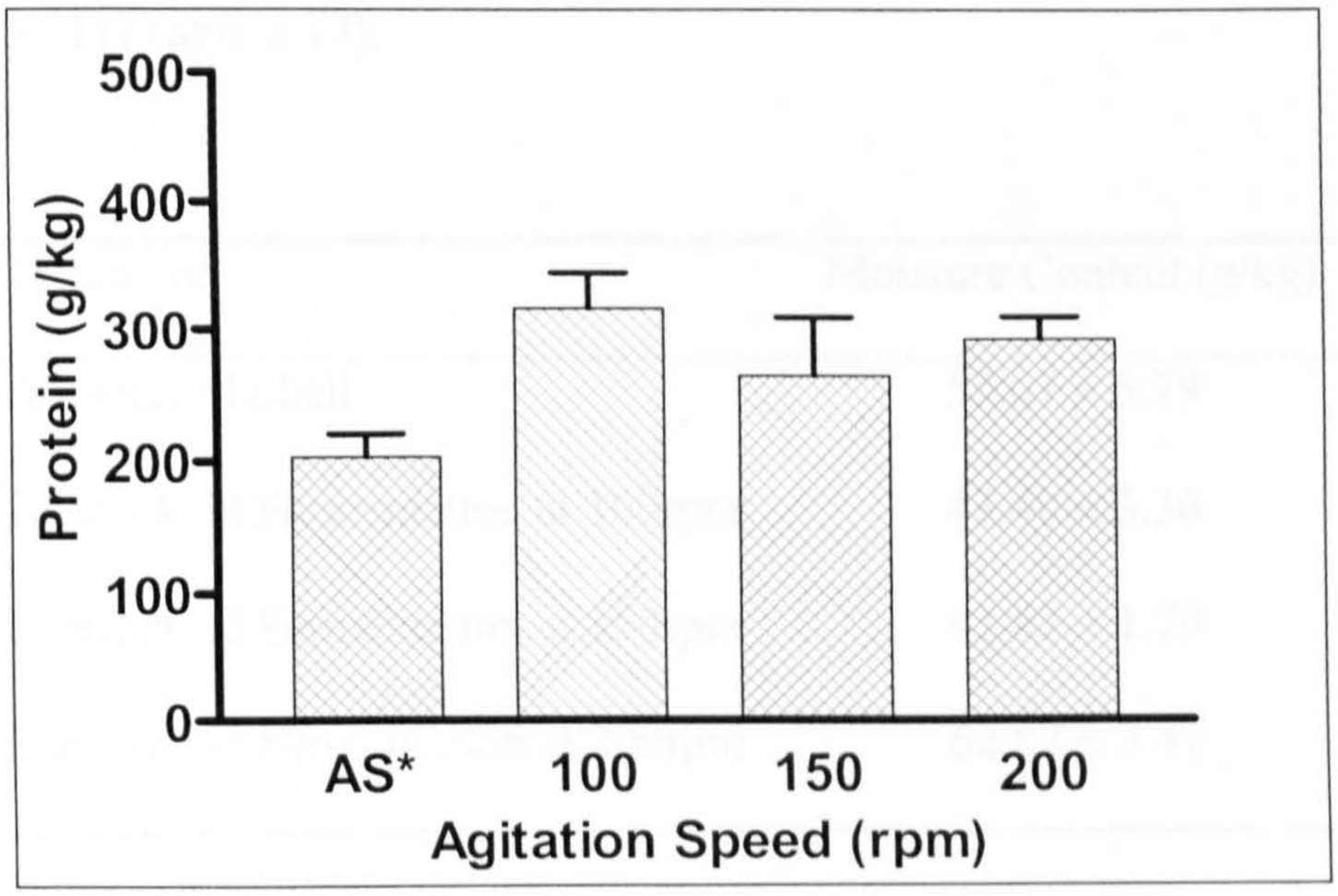


Fig. 5.28 *Effect of agitation speed on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

The overall yield obtained was between 40% (w/w) and 45% (w/w) at all agitation speeds studied (Fig. 5.29). Therefore approximately 55 - 60% (w/w) of the total shell mass was lost due to fermentation, irrespective of the agitation speed adopted.

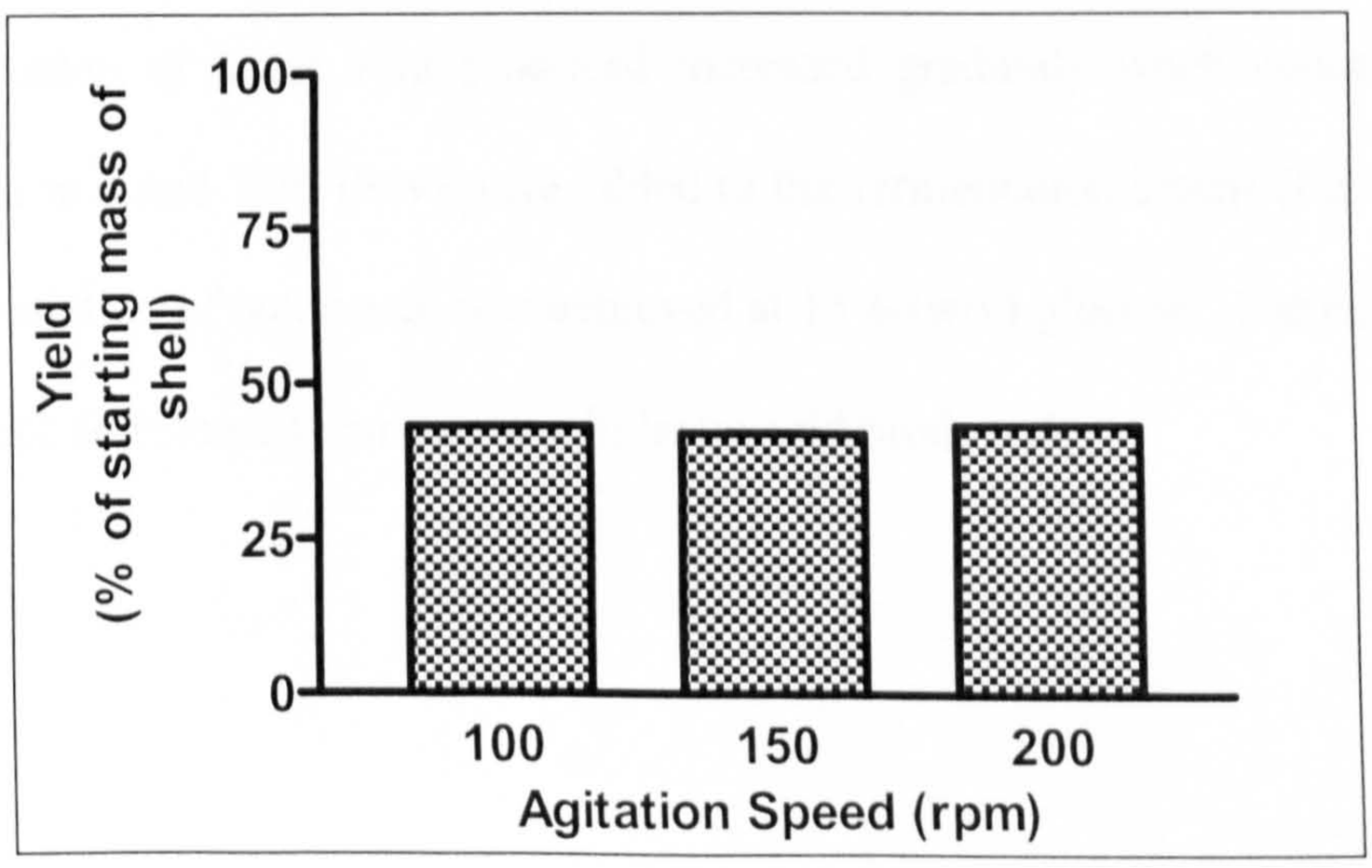


Fig. 5.29 *Effect of agitation speed on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Moisture content at 200rpm differed significantly from moisture content at 100rpm and 150rpm ($p<0.001$) (Table 5.17).

Treatment	Moisture Content (g/kg)
Autoclaved Shell	55.07 ± 5.79
Lactic Acid Fermentation at 100rpm	43.40 ± 3.36
Lactic Acid Fermentation at 150rpm	41.87 ± 1.70
Lactic Acid Fermentation at 200rpm	62.87 ± 3.47

Table 5.17 *Moisture content of shell after lactic acid fermentation at different agitation speeds.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Correction of all results for moisture levels indicated that only the ash results were affected by the moisture content. The level of statistical difference between ash at 150rpm and at 200rpm dropped from $p<0.01$ to $p<0.05$.

5.3.3.4 Effect of Glucose Concentration

The concentration of lactic acid produced increased gradually when concentrations of glucose between 0 and 15% (w/v) were added to the fermentation broths (Fig. 5.30). The optimum production of lactic acid was achieved at 15% (w/v) glucose. Further addition of glucose did not contribute to an increase in lactic acid produced.

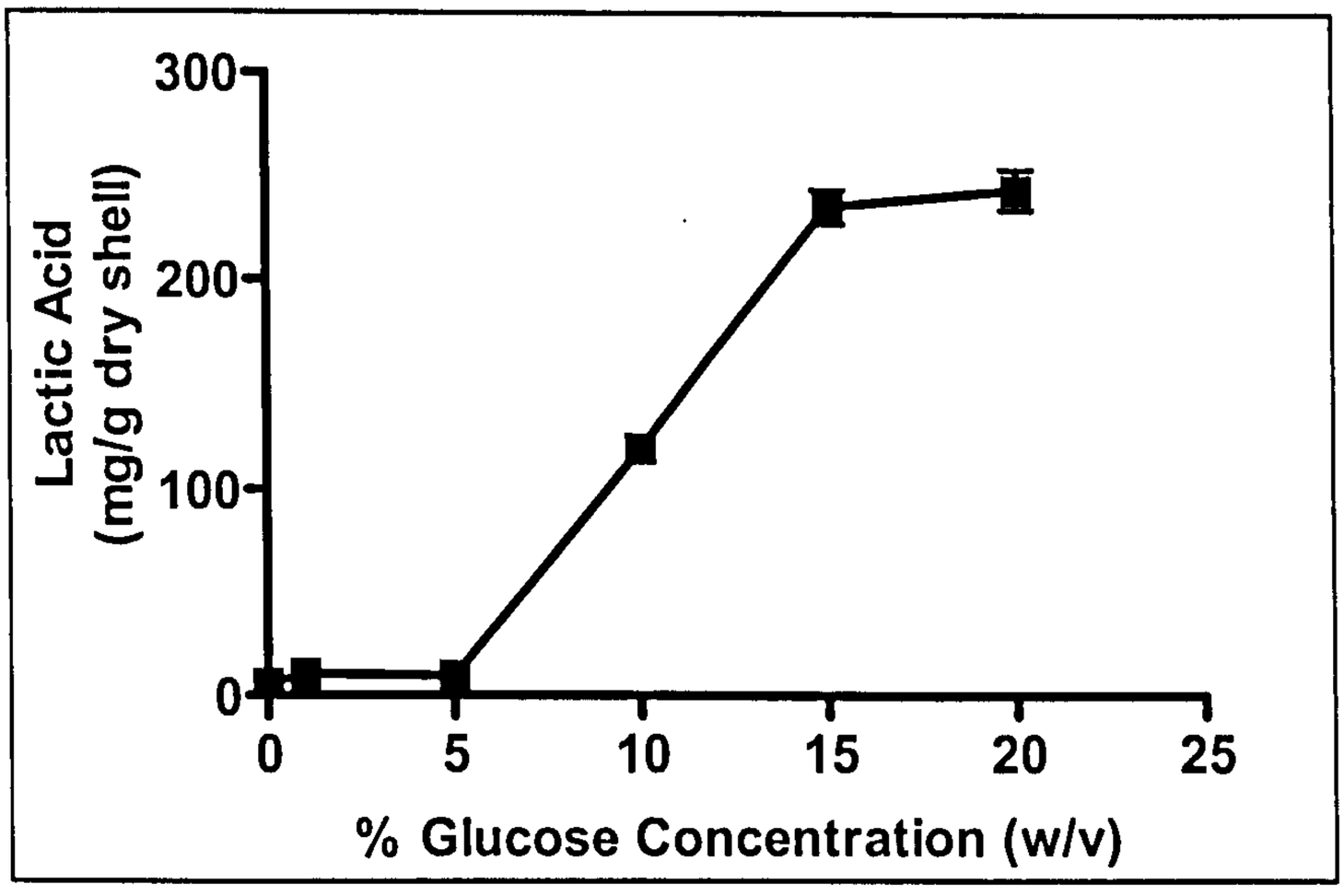


Fig. 5.30 *Effect of glucose concentration on lactic acid production.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Ash content fell when glucose concentration was increased from 1% to 5% and 10% (w/v) ($p<0.001$) (Fig. 5.31). Beyond 10% (w/v) glucose, no further significant decrease in ash was noted.

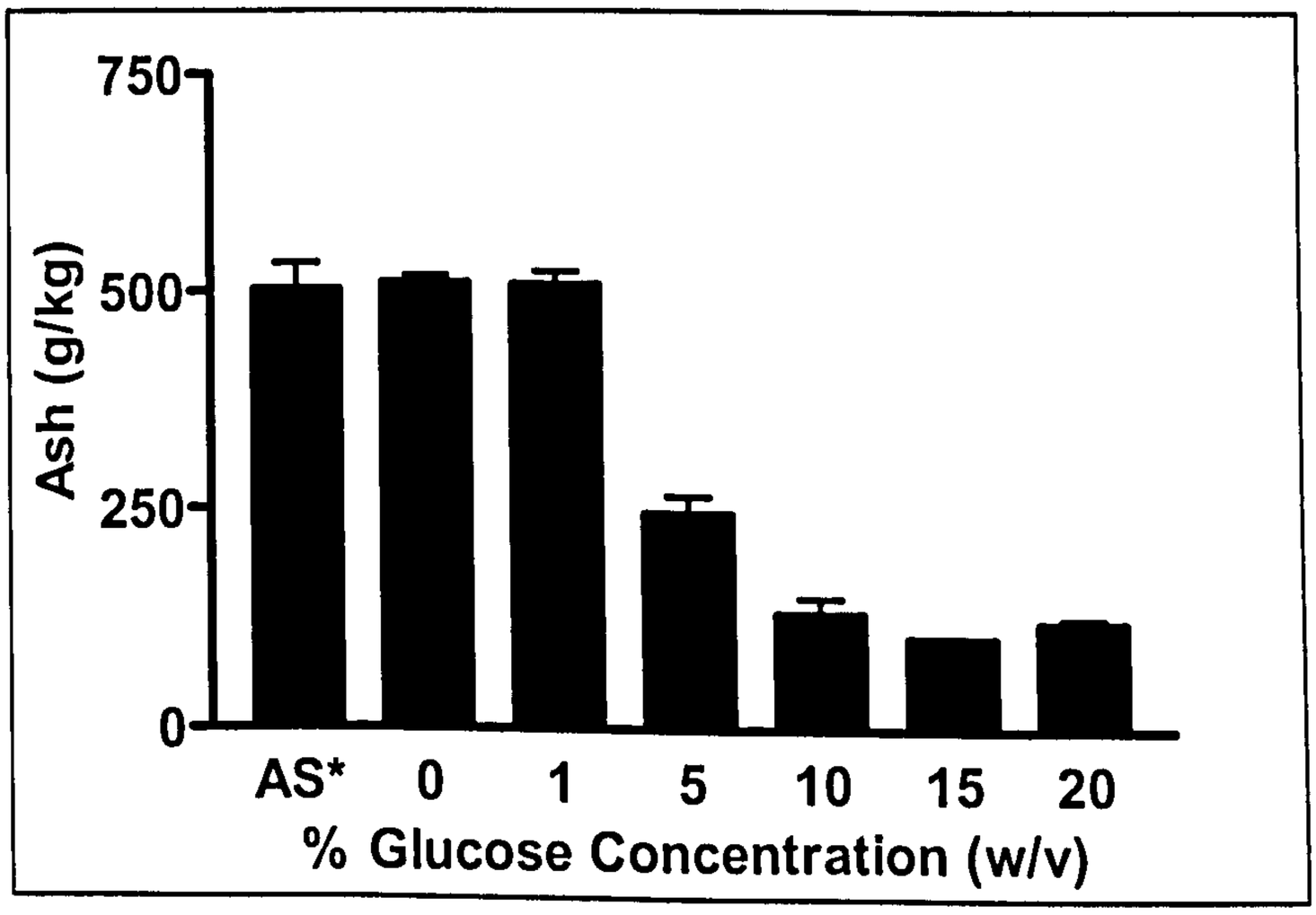


Fig. 5.31 *Effect of glucose concentration on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

At a concentration of 5% (w/v) glucose, ash content was significantly reduced ($p<0.001$) (Fig. 5.31) but lactic acid content had not yet started to rise (Fig. 5.30). pH levels however had started to fall (Fig. 5.32).

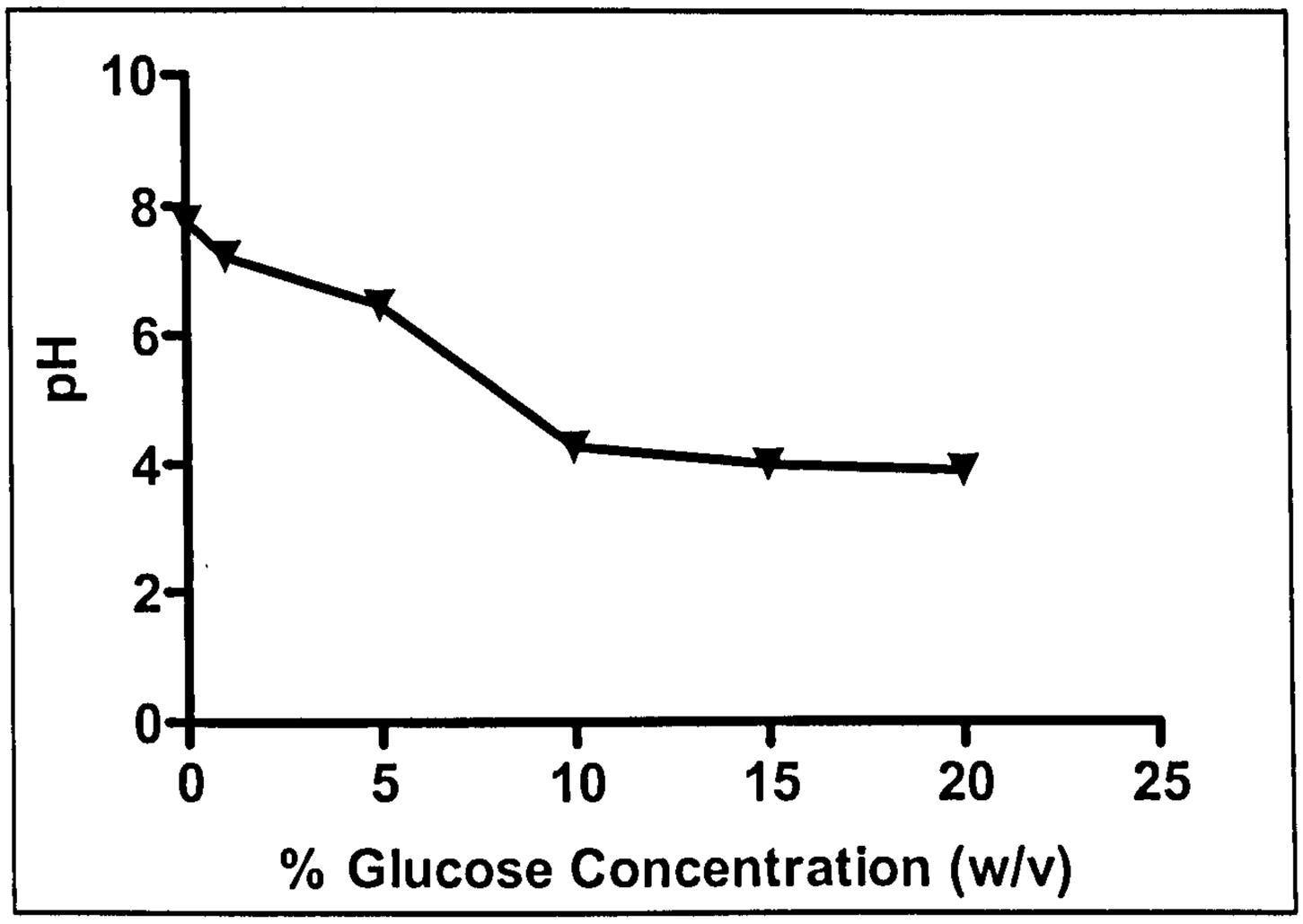


Fig. 5.32 *Effect of glucose concentration on the pH of the lactic acid fermentation culture broth.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Calcium content mirrored the ash content of the degraded shell. Calcium levels dropped significantly when glucose levels were raised from 1% to 5% and 10% (w/v) ($p< 0.001$) (Fig. 5.33). Glucose levels greater than 10% (w/v) caused no further significant decrease in calcium content.

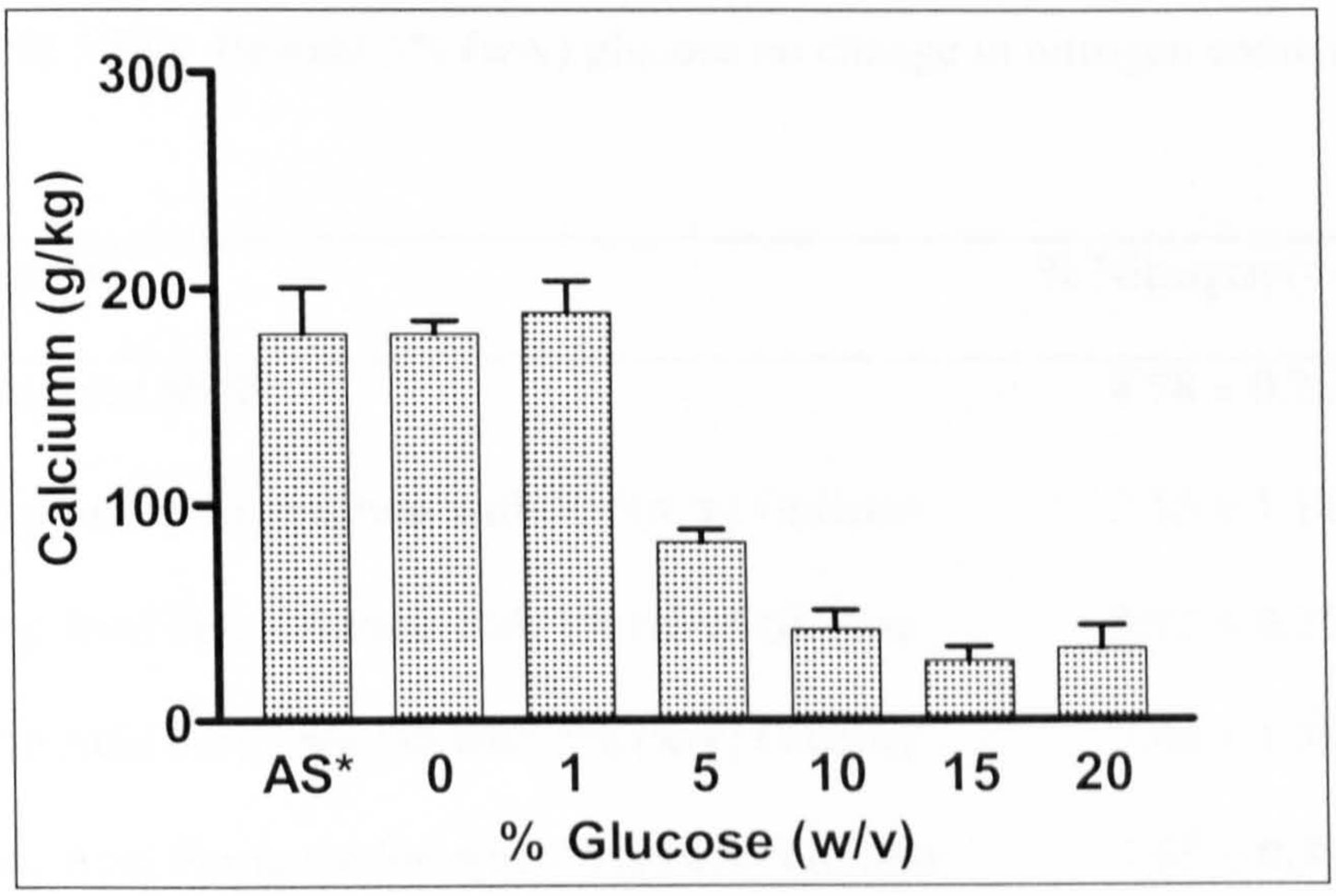


Fig. 5.33 *Effect of glucose concentration on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Chitin levels increased significantly as the glucose concentration increased from 0 to 5% (w/v) ($p<0.001$) (Fig. 5.34). Beyond 5% (w/v) glucose no further significant increase in chitin occurred.

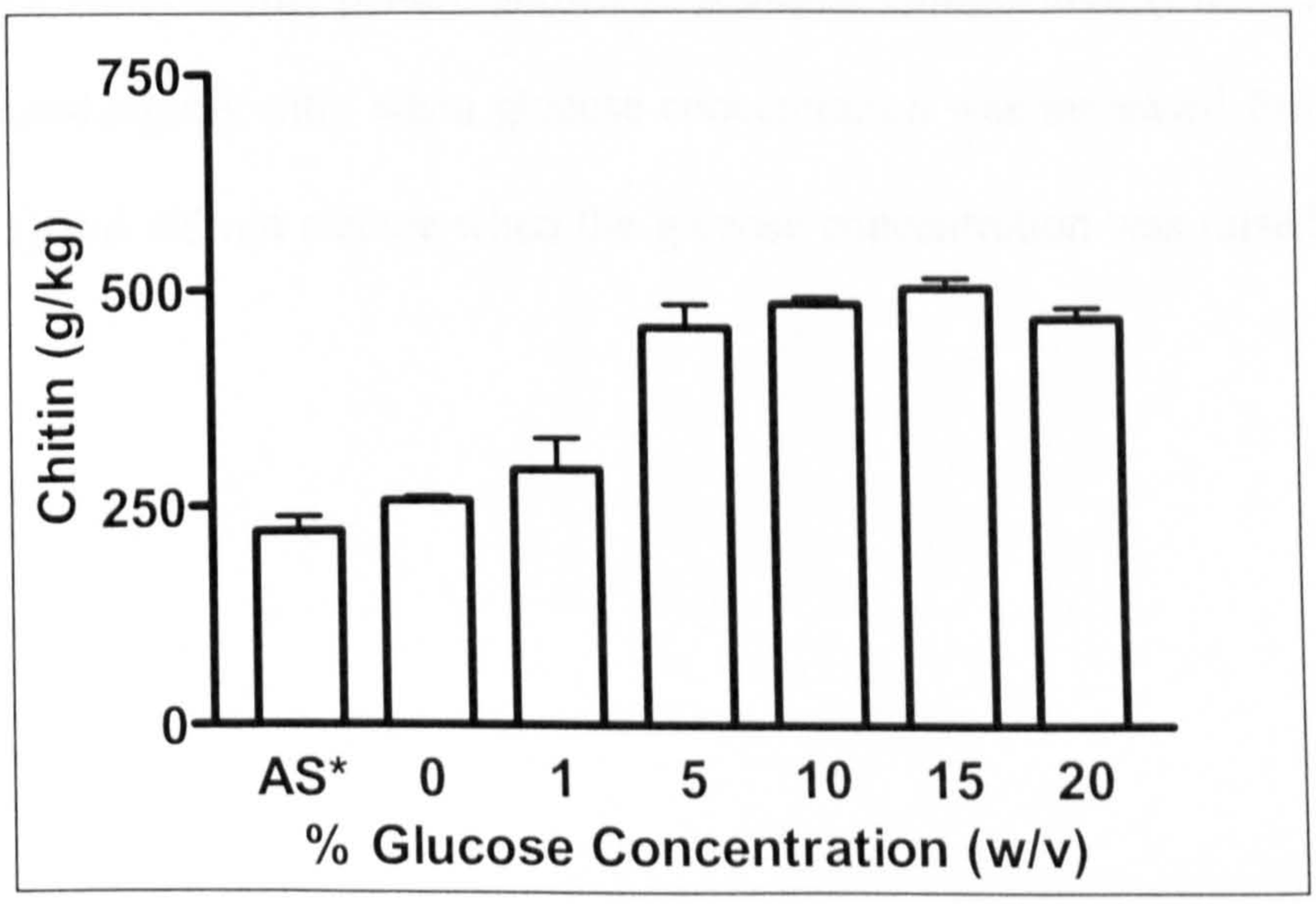


Fig. 5.34 *Effect of glucose concentration on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

The nitrogen content of the shell increased sharply between 1% and 5% glucose (w/v) ($p<0.01$) (Table 5.18). Beyond 5% (w/v) glucose no change in nitrogen content occurred.

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Lactic Acid Fermentation with 0% (w/v) Glucose	3.15 ± 1.14
Lactic Acid Fermentation with 1% (w/v) Glucose	3.12 ± 0.25
Lactic Acid Fermentation with 5% (w/v) Glucose	7.48 ± 1.30
Lactic Acid Fermentation with 10% (w/v) Glucose	7.65 ± 0.30
Lactic Acid Fermentation with 15% (w/v) Glucose	8.13 ± 0.27
Lactic Acid Fermentation with 20% (w/v) Glucose	7.59 ± 0.81

Table 5.18 *Effect of glucose concentration on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Protein content reflected the pattern shown in nitrogen composition (Fig. 5.35). Protein content increased significantly when glucose concentration was increased from 1% to 5% (w/v) ($p<0.01$) and did not change when the glucose concentration was raised beyond 5% (w/v).

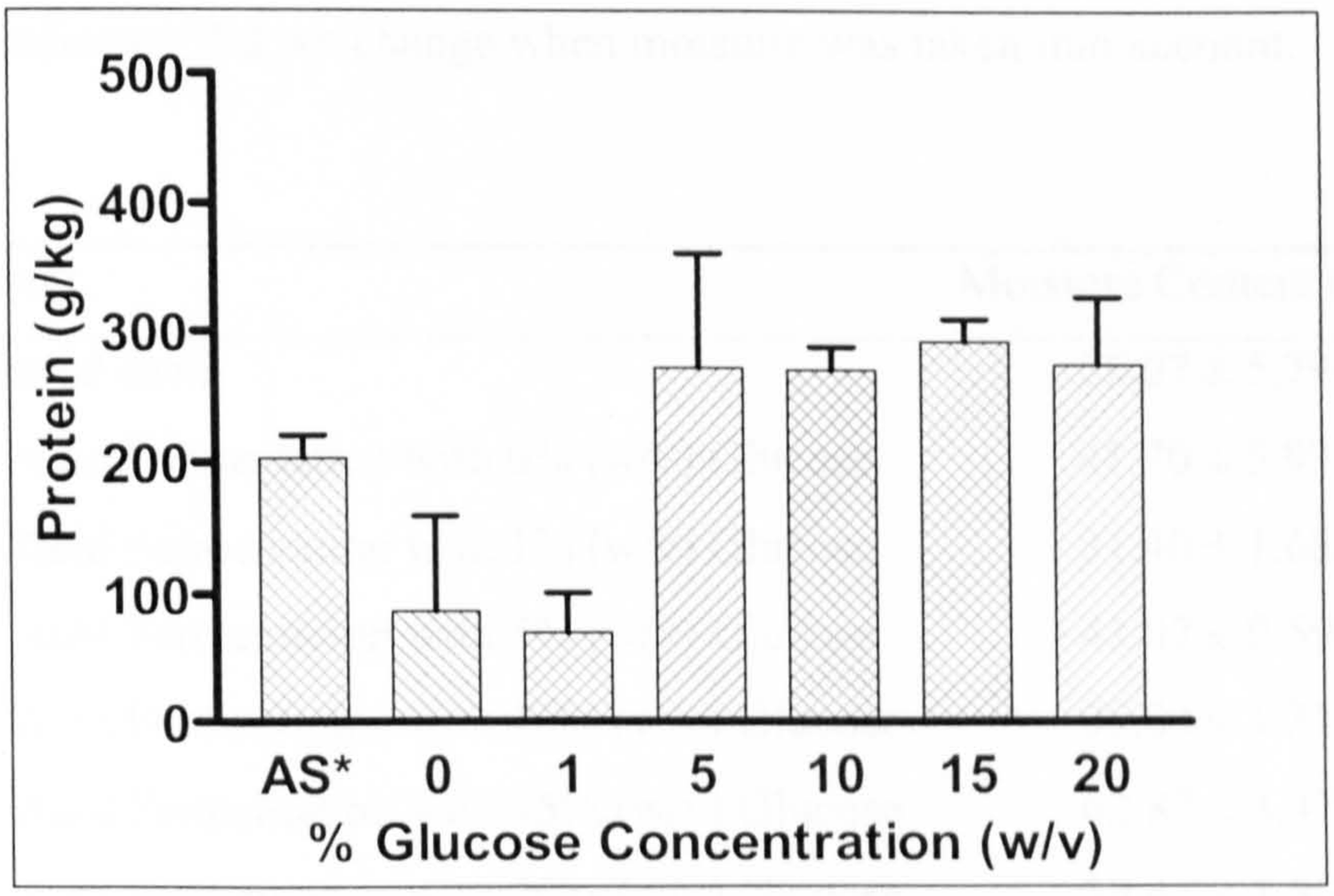


Fig. 5.35 *Effect of glucose concentration on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Concentrations of 5 - 20% (w/v) glucose caused an overall shell mass decrease of between 50 and 60% (w/w) (Fig. 5.36).

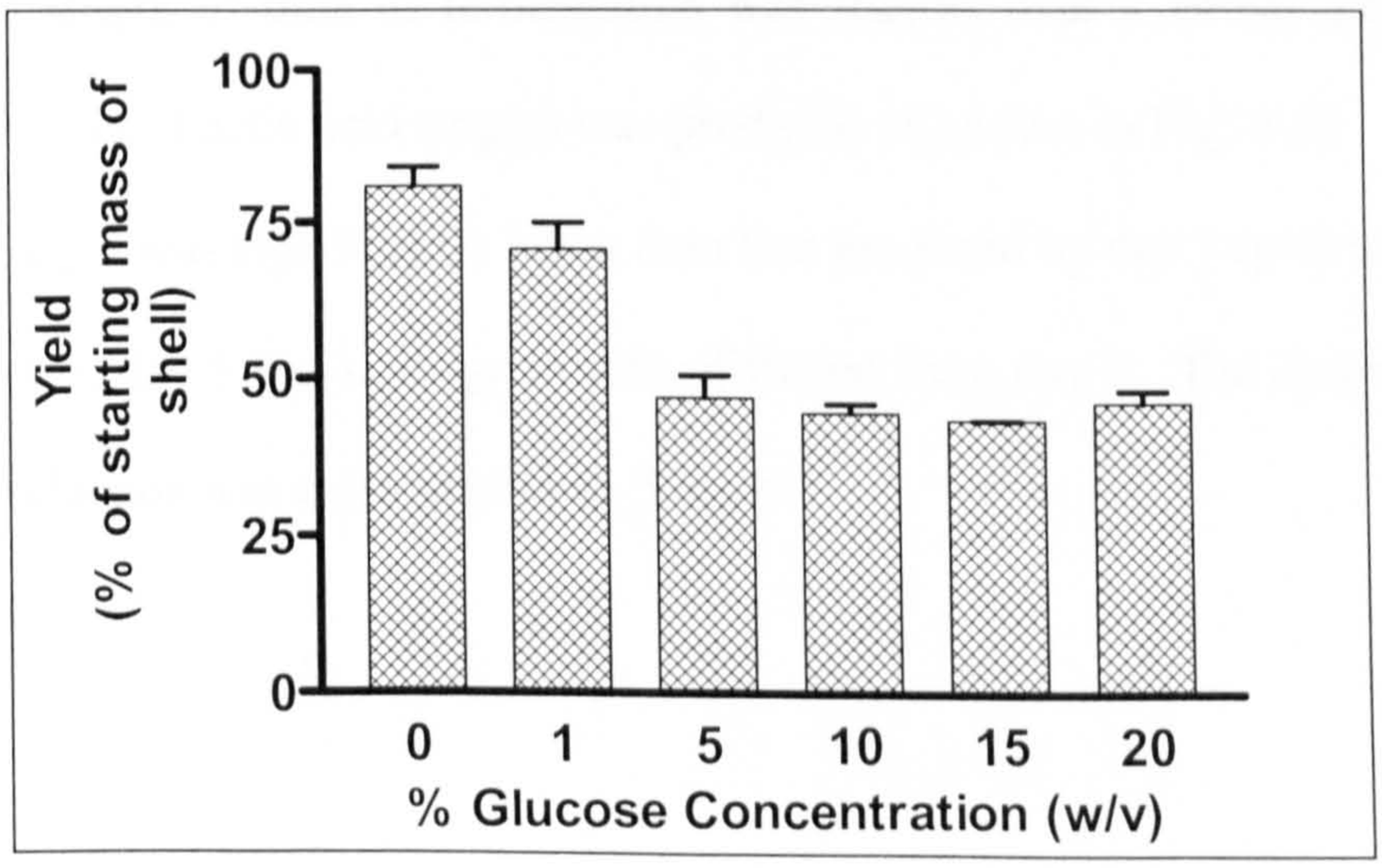


Fig. 5.36 *Effect of glucose concentration on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Moisture levels varied from 3.1% to 6.3% (w/v) (Table. 5.19) but the reported levels of statistical significance did not change when moisture was taken into account.

Treatment	Moisture Content (g/kg)
Autoclaved shell	55.07 ± 5.79
Lactic Acid Fermentation with 0% (w/v) Glucose	41.70 ± 5.99
Lactic Acid Fermentation with 1% (w/v) Glucose	31.40 ± 1.68
Lactic Acid Fermentation with 5% (w/v) Glucose	43.07 ± 0.59
Lactic Acid Fermentation with 10% (w/v) Glucose	39.83 ± 1.31
Lactic Acid Fermentation with 15% (w/v) Glucose	62.87 ± 3.47
Lactic Acid Fermentation with 20% (w/v) Glucose	39.43 ± 1.80

Table 5.19 *Moisture content of shell after lactic acid fermentation with different concentrations of glucose.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

5.3.3.5 Effect of Length of Time of Fermentation

The effect of length of time of fermentation was studied over a 14-day period on two separate occasions. Lactic acid (mg/g) was produced as shown in Fig. 5.37. The amount produced by day 4 was significantly lower than that produced by day 7 ($p<0.01$) but not by day 5. However, day 5 was not significantly different from day 7. The optimum time for lactic acid production was calculated to be 5 days.

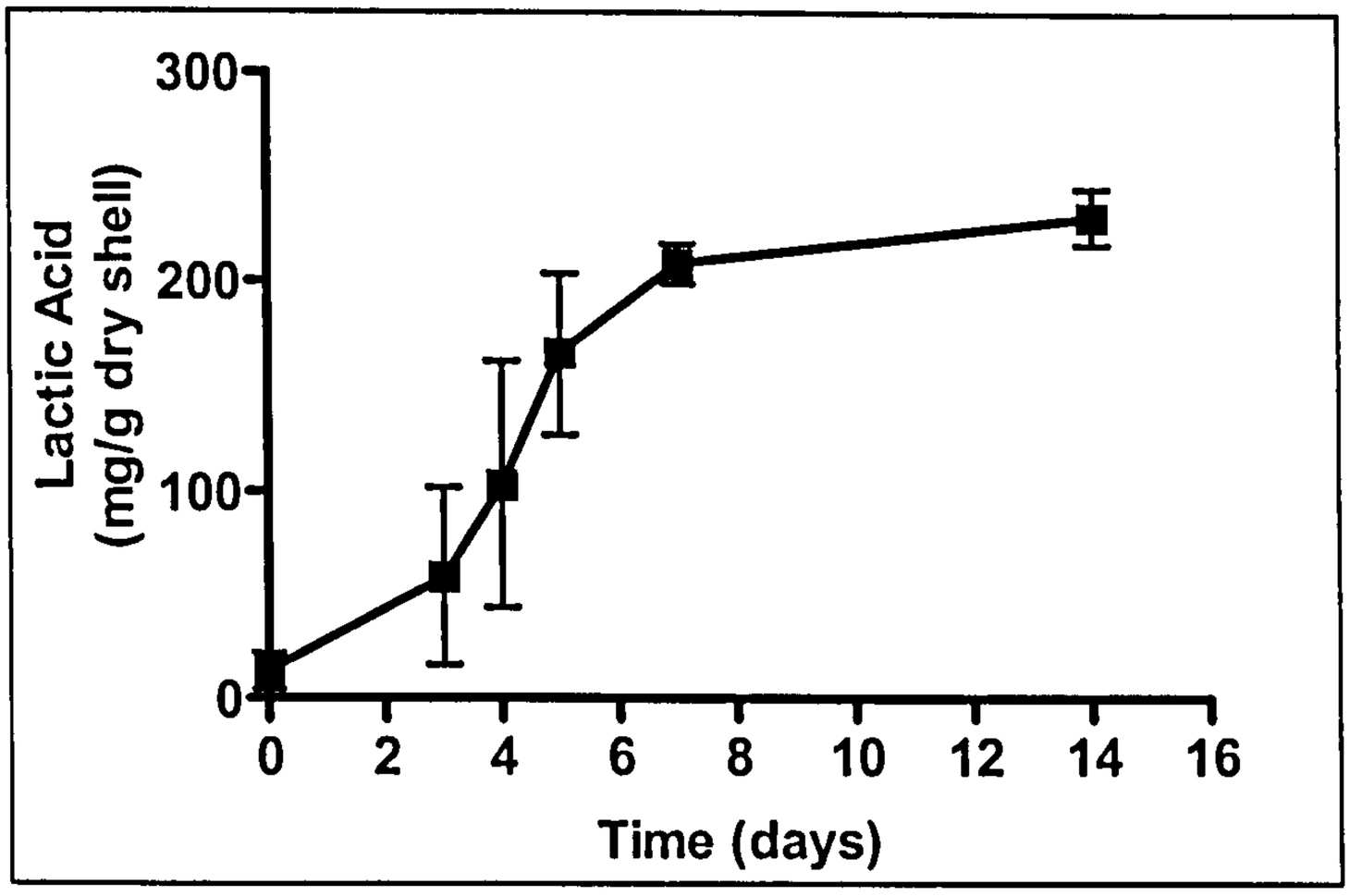


Fig. 5.37 *Effect of time on the production of lactic acid.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

The majority of the ash was solubilised by day 3 (Fig. 5.38). Ash measurements for day 3 were significantly different from day 0 ($p < 0.01$) but not from days 4, 5, 7 and 14.

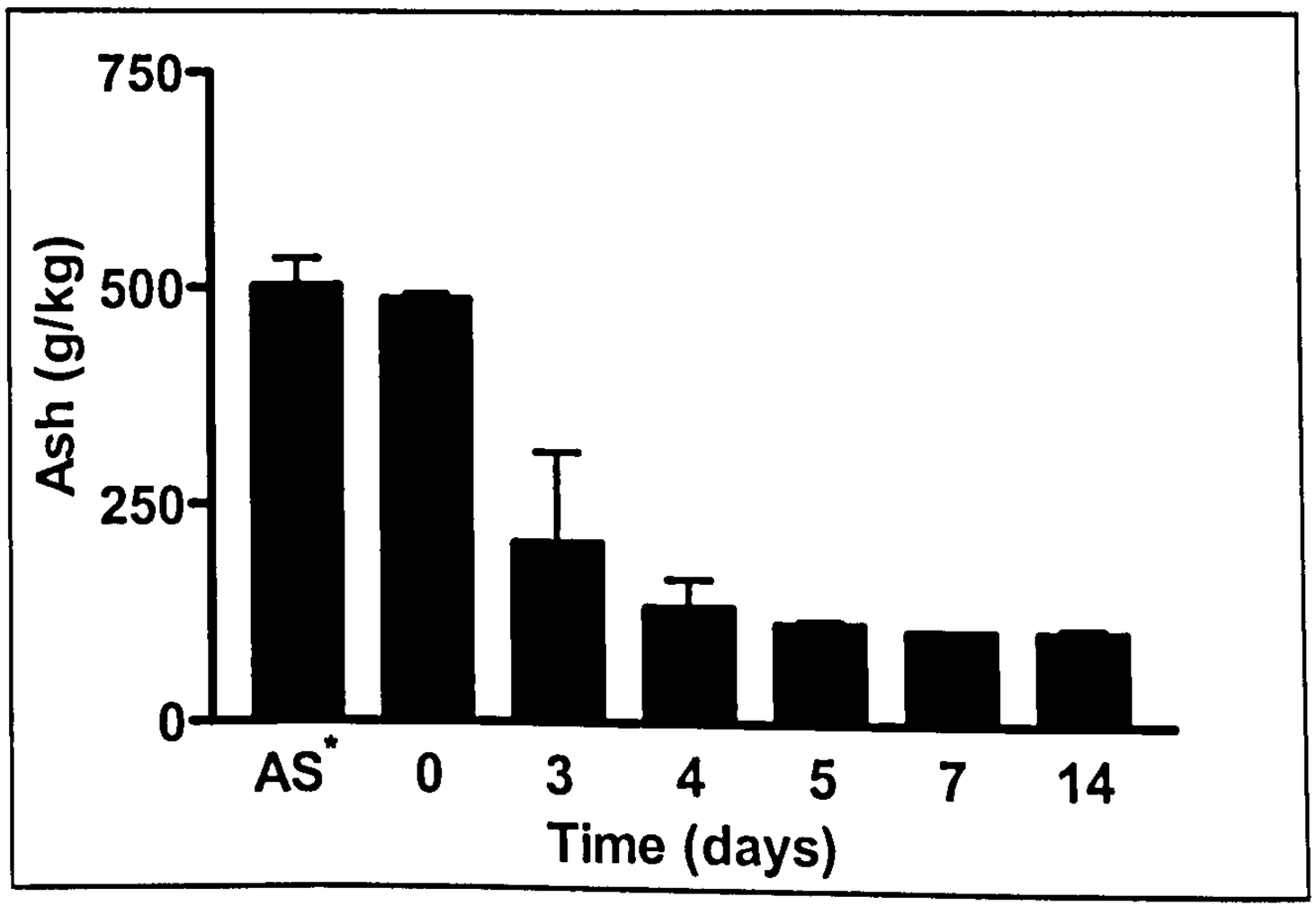


Fig. 5.38 *Effect of time on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

The calcium levels mirrored the ash results (Fig. 5.39). Calcium content dropped between days 0 and 3 ($p<0.01$). Beyond day 3 no further decrease in calcium content was noted.

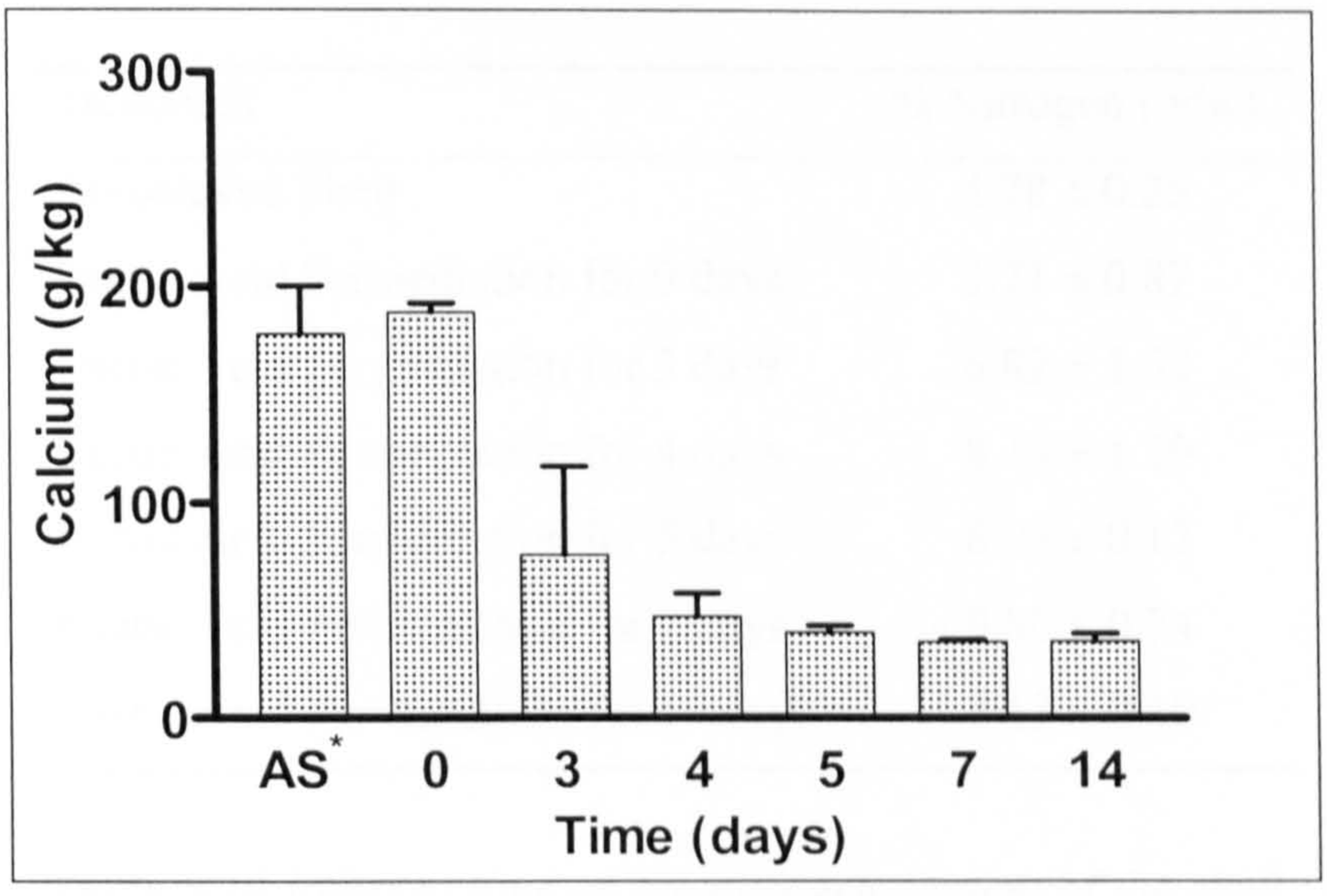


Fig. 5.39 *Effect of time on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Chitin levels increased between day 0 and day 3 ($p<0.01$) (Fig. 5.40). A 14 day fermentation period was required to increase the chitin concentration to levels significantly higher than day 3.

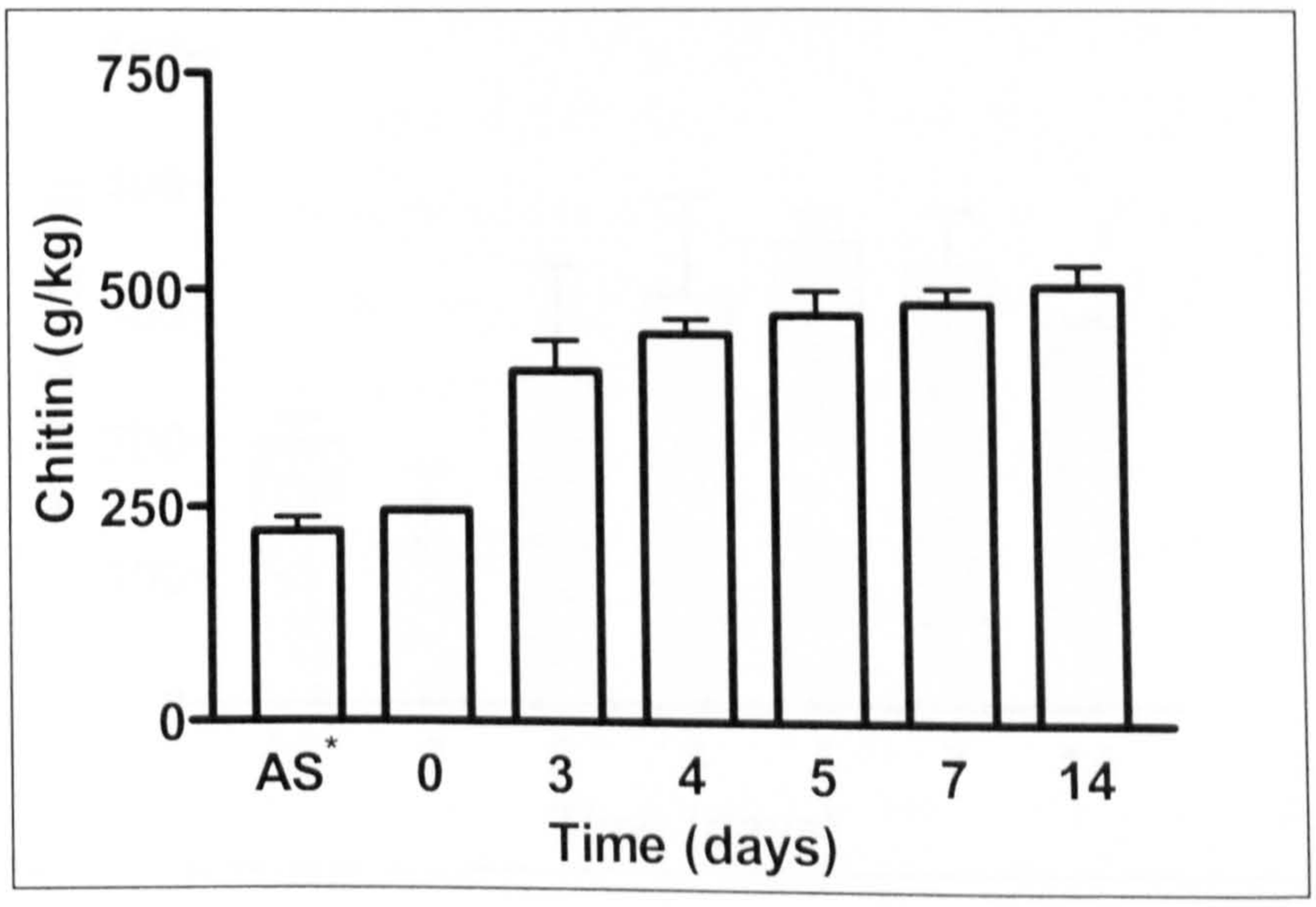


Fig. 5.40 *Effect of time on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Nitrogen content also increased between days 0 and 3 (Table 5.20). Beyond day 3 no further significant increase in nitrogen content was seen.

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Lactic Acid Fermentation for 0 days	3.71 ± 0.87
Lactic Acid Fermentation for 3 days	6.82 ± 1.62
Lactic Acid Fermentation for 4 days	8.13 ± 1.10
Lactic Acid Fermentation for 5 days	8.93 ± 0.17
Lactic Acid Fermentation for 7 days	9.80 ± 0.74
Lactic Acid Fermentation for 14 days	8.13 ± 0.10

Table 5.20 *Effect of time on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Protein levels increased between days 0 and 5 ($p<0.05$). No significant difference in protein content was noted between days 3, 4, 5, 7 and 14. This may have been due to the wide variation obtained in the data (Fig. 5.41).

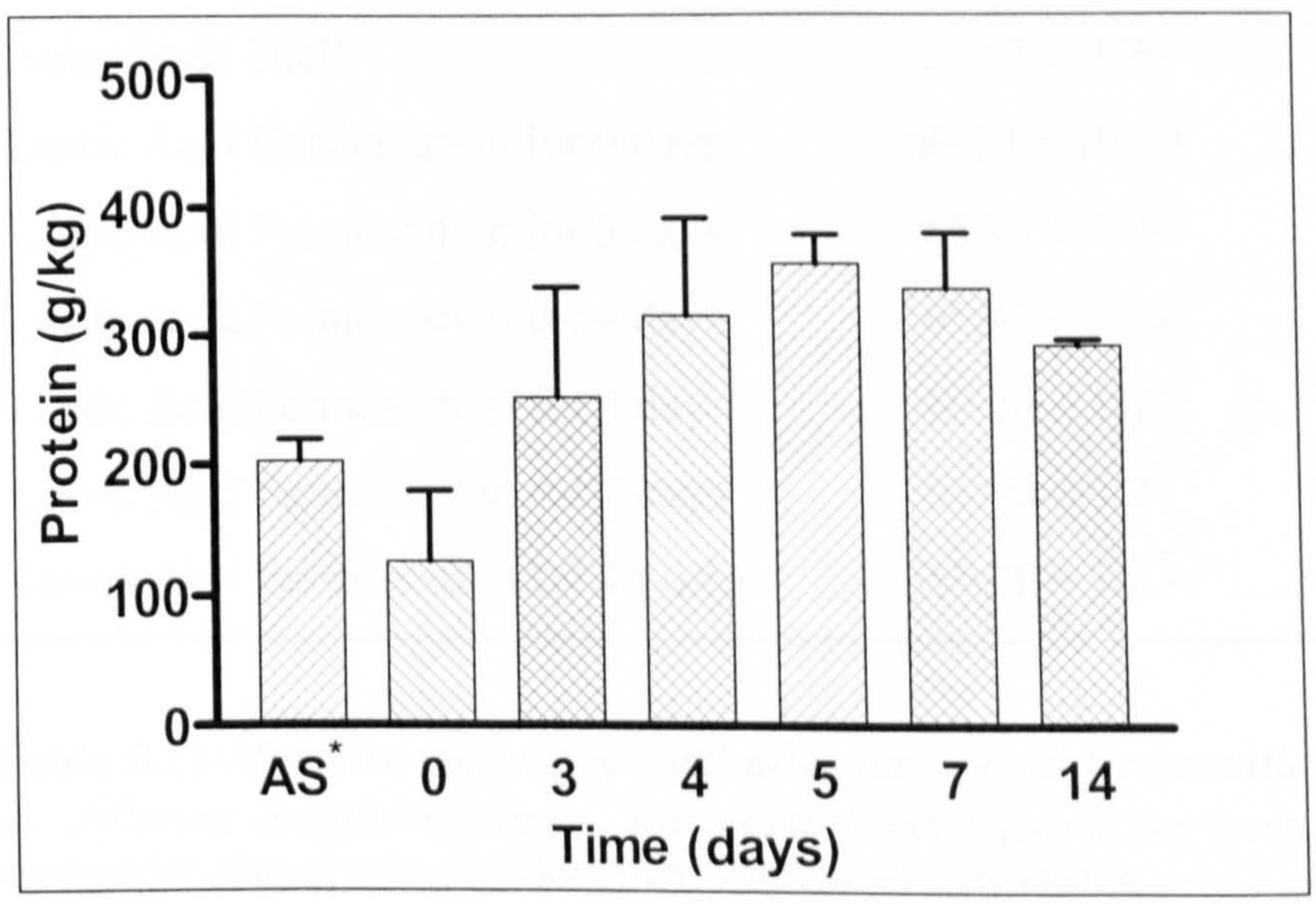


Fig. 5.41 *Effect of time on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Fig. 5.42 shows the change in percentage yield with time. After 7 days 41.76 ± 1.80% (w/w) of the shell mass remained.

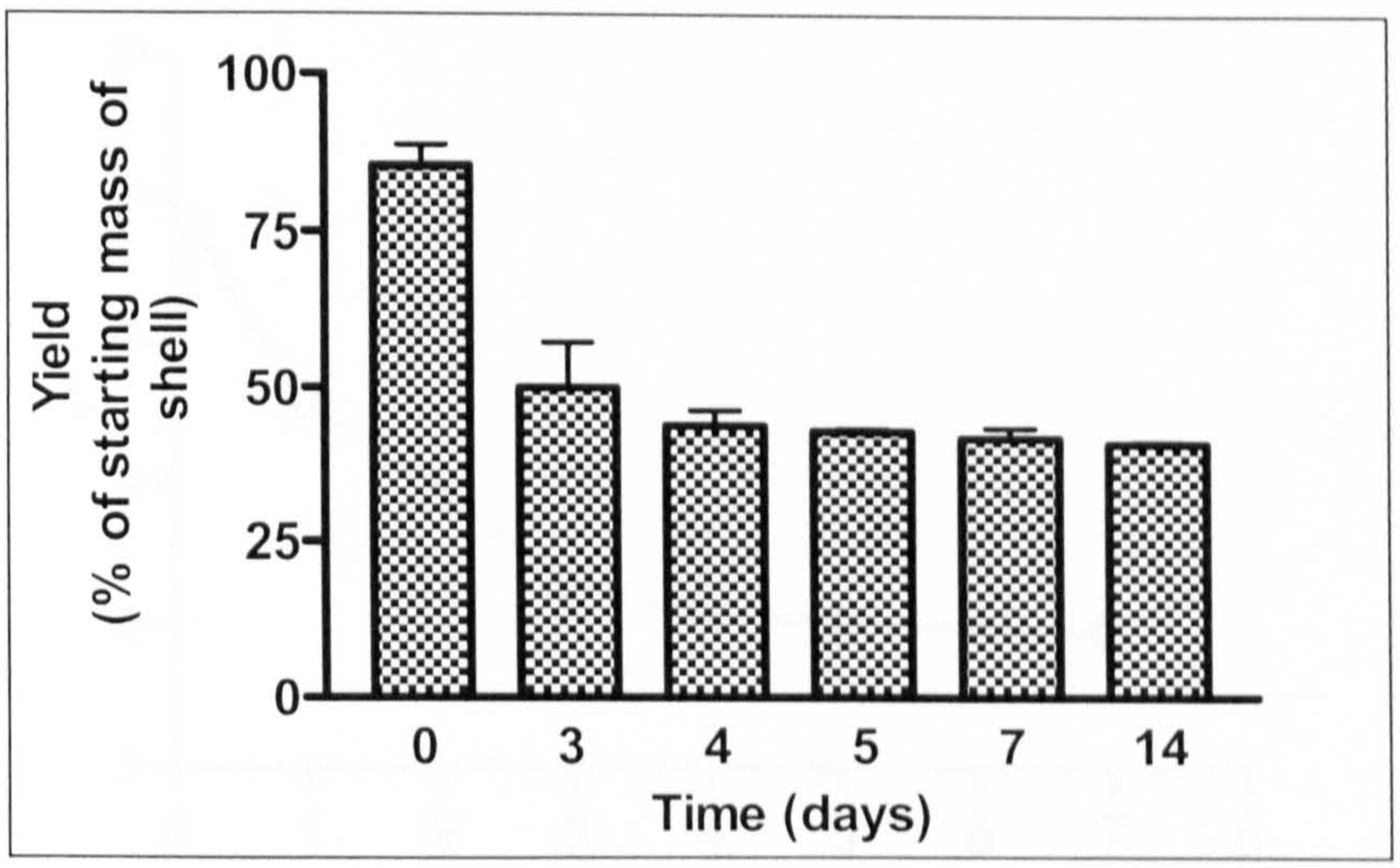


Fig. 5.42 *Effect of time on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Moisture content varied little between the samples (Table 5.21).

Treatment	Moisture Content (g/kg)
Autoclaved Shell	55.07 ± 5.79
Lactic Acid Fermentation for 0 days	60.50 ± 10.89
Lactic Acid Fermentation for 3 days	65.35 ± 7.99
Lactic Acid Fermentation for 4 days	65.85 ± 3.32
Lactic Acid Fermentation for 5 days	60.20 ± 1.13
Lactic Acid Fermentation for 7 days	61.90 ± 1.27
Lactic Acid Fermentation for 14 days	59.20 ± 2.83

Table 5.21 *Moisture content of shell after lactic acid fermentation for different lengths of time.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

A typical pH curve for the lactic acid fermentation is shown in Fig. 5.43. pH levels had dropped to their lowest value by day 4.

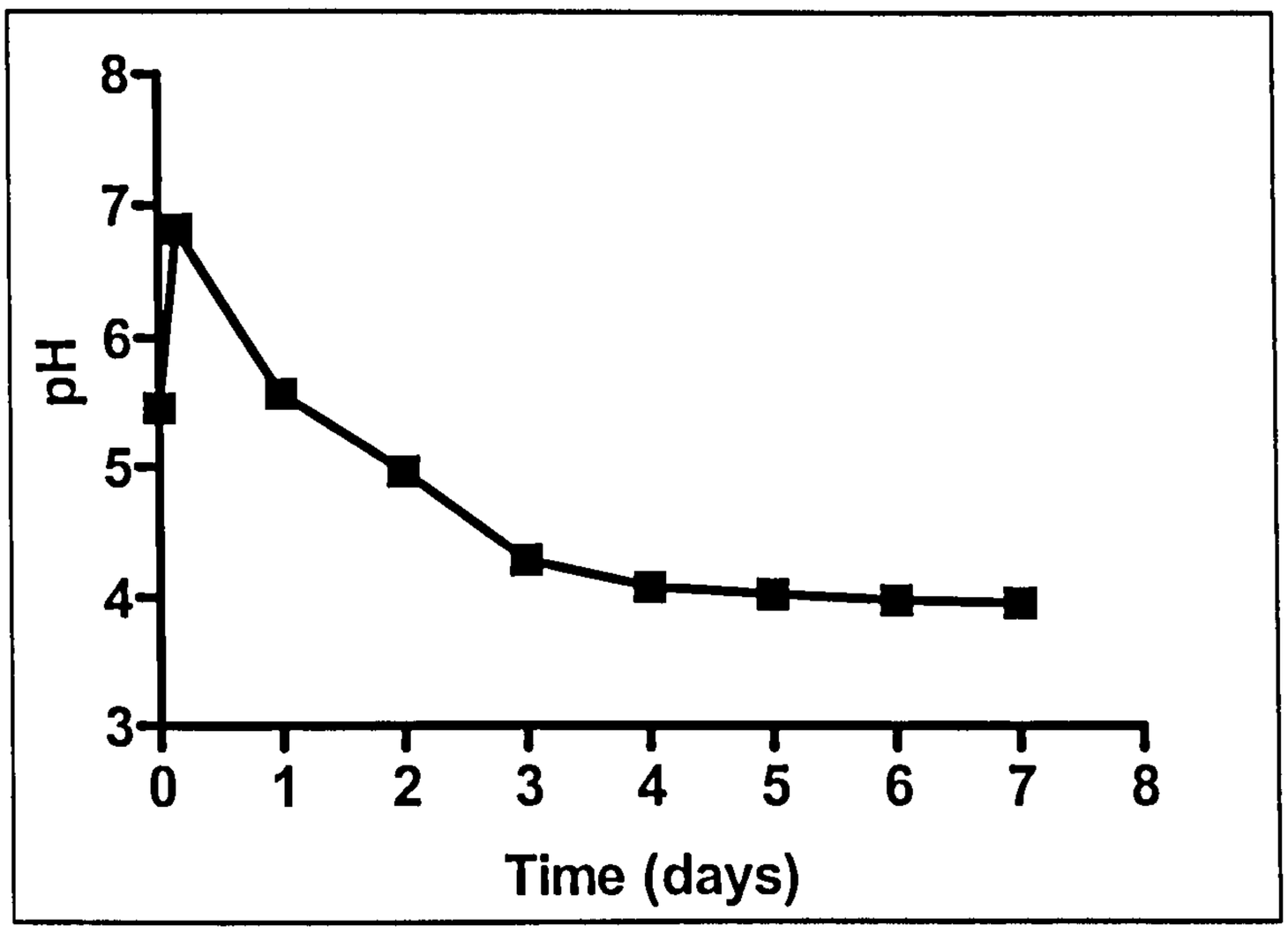


Fig. 5.43 *Effect of time on the pH of the lactic acid fermentation culture broth.* The results shown are from a single experiment.

5.3.3.6 Effect of Repeating the Lactic Acid Fermentation

A 1 litre flask was set up under the conditions shown in Table 5.7. The resultant sample, after washing and drying, was subjected to a second fermentation treatment under the same conditions. During the first treatment 267.84 ± 31.17mg lactic acid/g dry shell were produced (Fig. 5.44). Much lower levels of lactic acid were produced in the second treatment (51.12 ± 7.44mg/g dry shell) (Fig. 5.44).

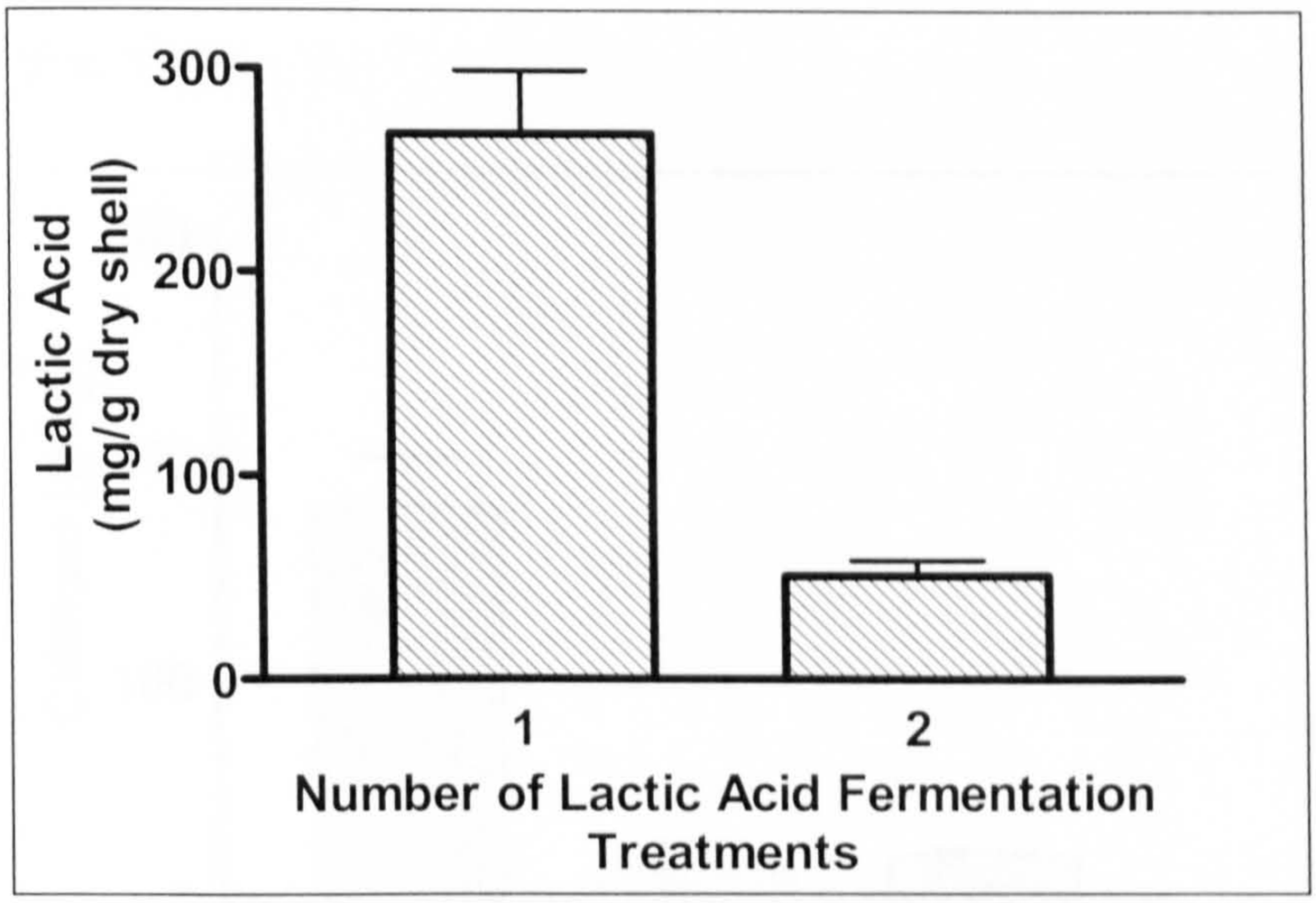


Fig. 5.44 *Effect of number of fermentation treatments on lactic acid production.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The small amount of lactic acid produced in the second treatment had no significant affect on the ash content of the shell. The level of ash after two treatments remained similar to that after one treatment (Fig. 5.45).

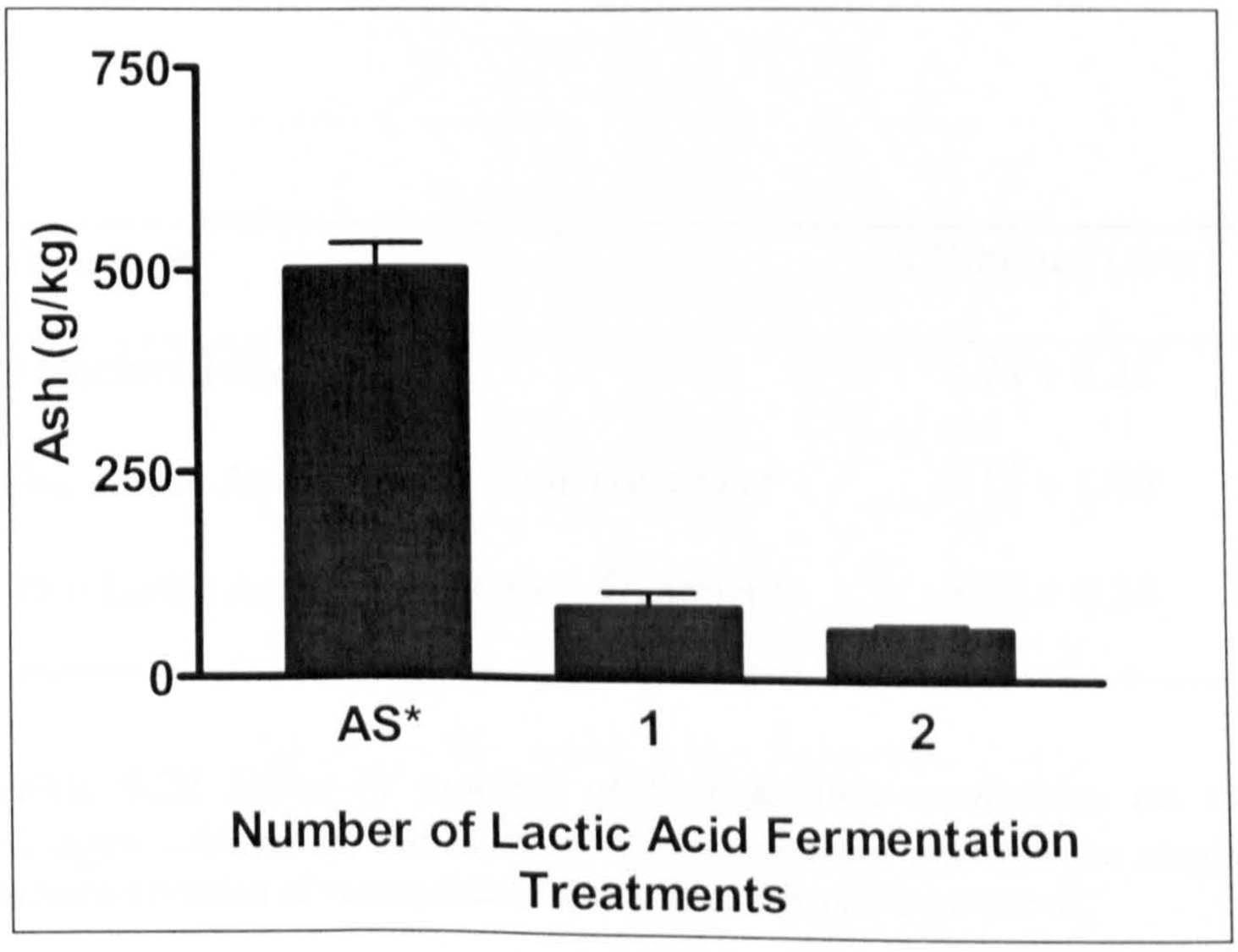


Fig. 5.45 *Effect of number of lactic acid fermentation treatments on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Likewise, the calcium content of the material was not reduced by repeating the fermentation (Fig. 5.46).

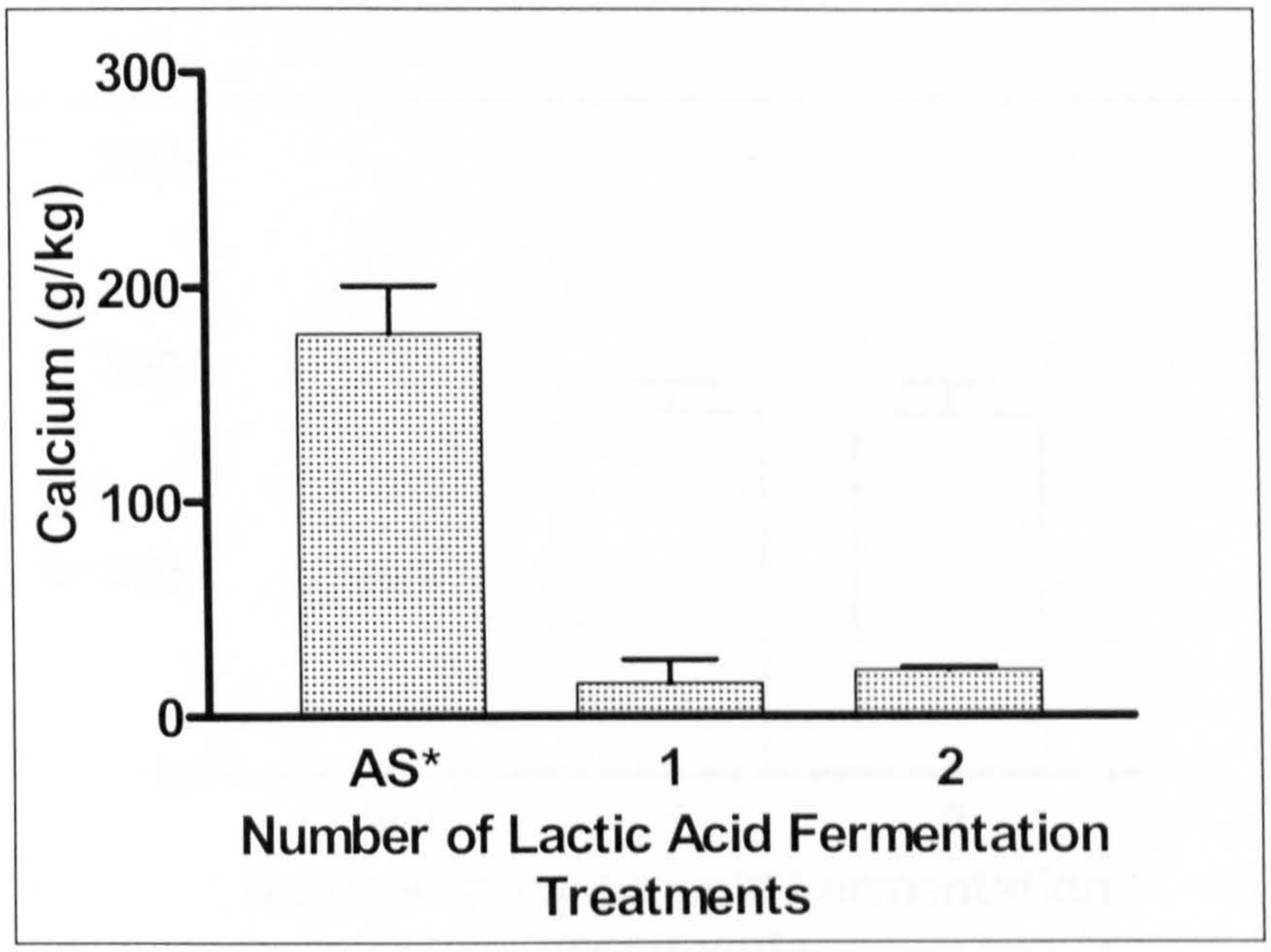


Fig. 5.46 *Effect of number of lactic acid fermentation treatments on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

A second fermentation treatment did not alter the nitrogen content of the product obtained (Table 5.22).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
One Lactic Acid Fermentation Treatment	9.11 ± 1.08
Two Lactic Acid Fermentation Treatments	8.95 ± 0.26

Table 5.22 *Effect of number of fermentation treatments on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Chitin and protein levels were not significantly affected by repeating the fermentation treatment (Figs. 5.47 and 5.48).

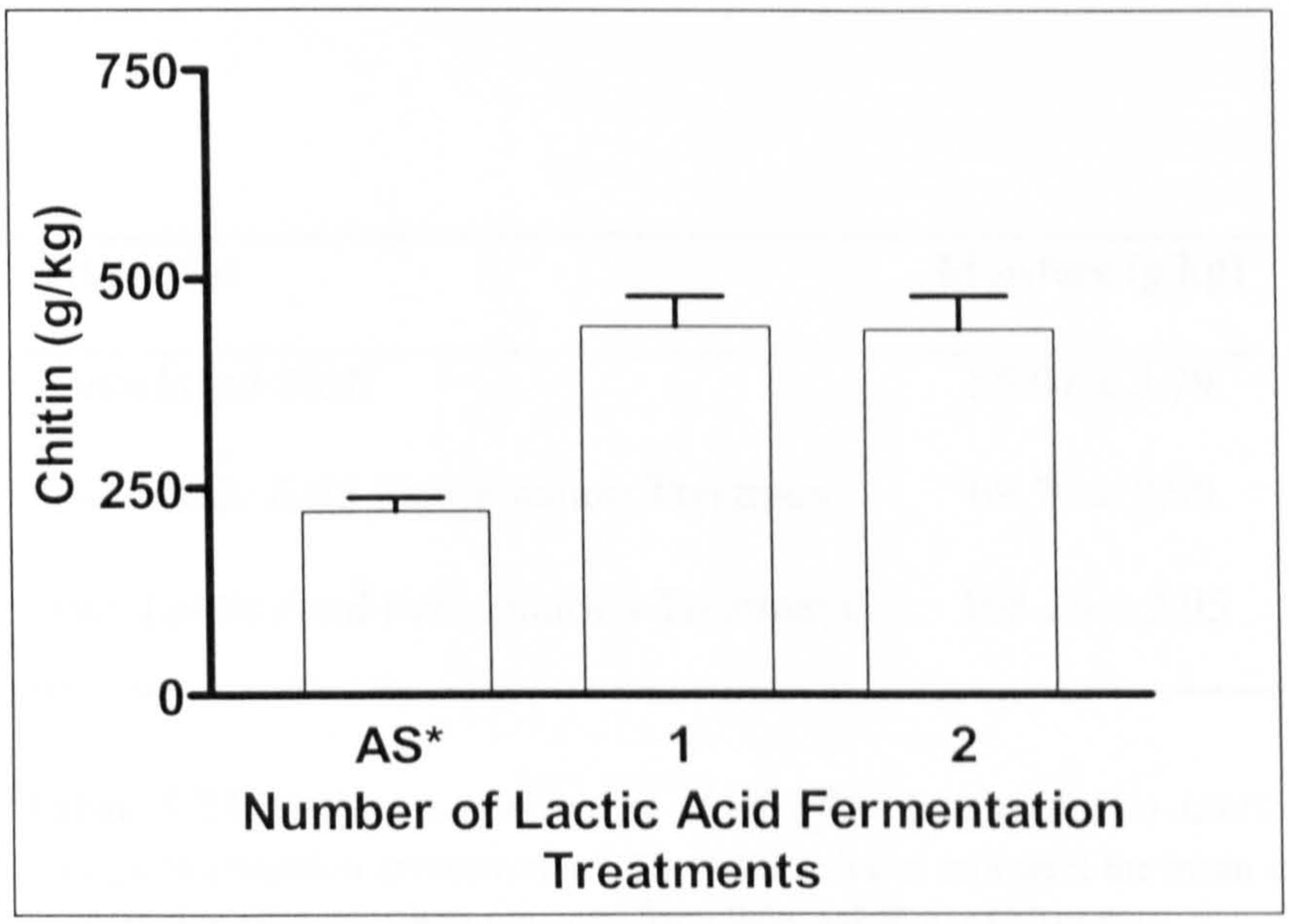


Fig. 5.47 *Effect of number of lactic acid fermentation treatments on the chitin content of shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (initial starting material)

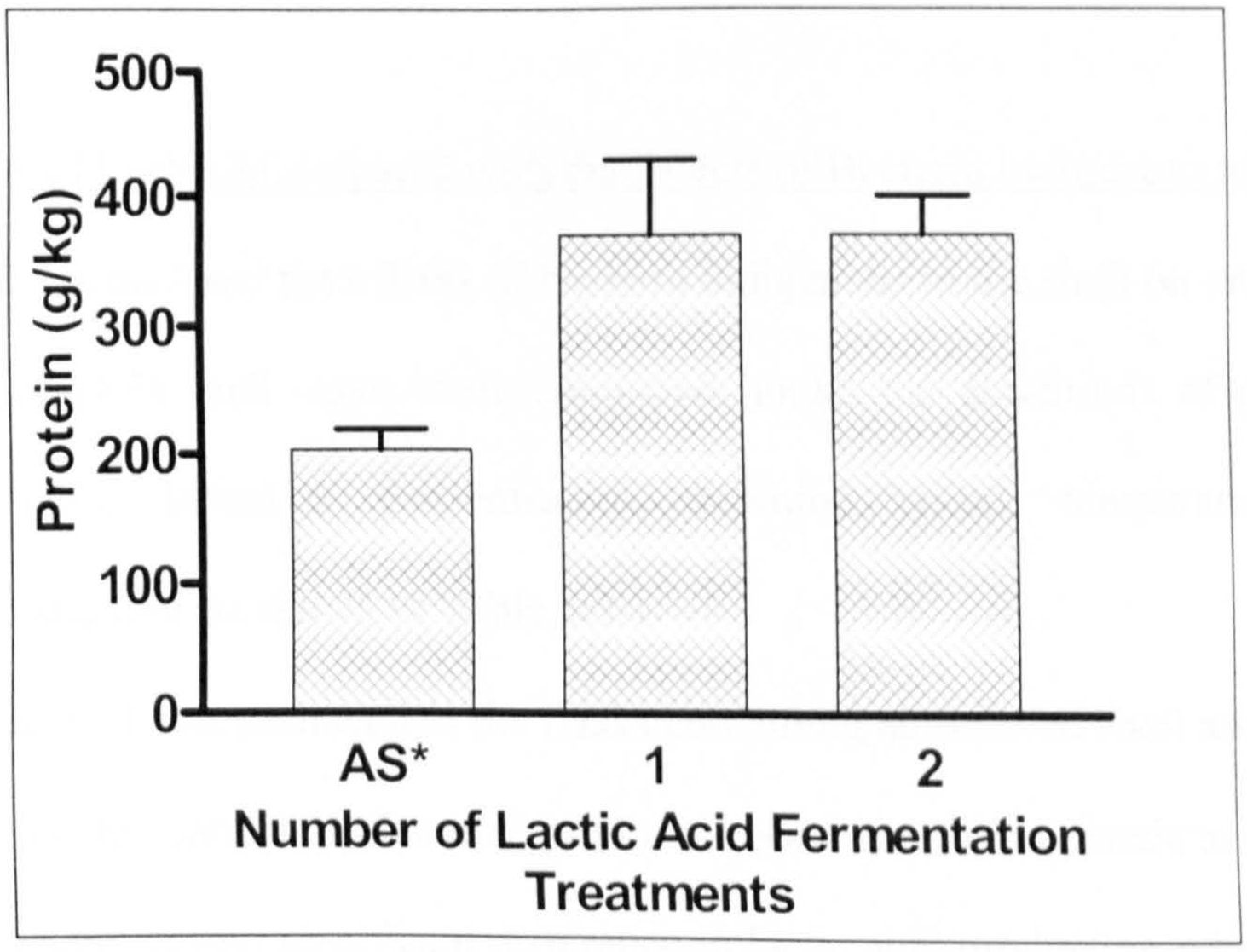


Fig. 5.48 *Effect of number of fermentation treatments on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell (original starting material)

Moisture content varied from 7.0% (w/w) after one fermentation treatment to 10.5% (w/w) after two treatments (Table 5.23). When these moisture contents were taken into account the relative amounts of ash, calcium, chitin and protein in the degraded samples did not change.

Treatment	Moisture (g/kg)
Autoclaved Shell	55.07 ± 5.79
One Lactic Acid Fermentation Treatment	69.70 ± 0.00
Two Lactic Acid Fermentation Treatments	105.26 ± 2.95

Table 5.23 *Moisture content of shell after one and two lactic acid fermentation treatments.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Overall, subjecting the shell to two consecutive lactic acid fermentation treatments, as opposed to one treatment, did not assist in the isolation of chitin from the shell.

5.3.3.7 Effects of Lactosil Compared with the Effects of Bacteria Indigenous to the Shell

The effects of Lactosil and the effects of bacteria indigenous to the shell on untreated (US) and autoclaved (AS) shell waste were compared, under the conditions of particle size, solid:liquid ratio, inoculant concentration, agitation speed, temperature, glucose concentration and time as shown in Table 5.7.

No lactic acid was produced in the flasks containing autoclaved shell and no Lactosil whereas the systems containing Lactosil produced more than 200mg lactic acid/g dry shell (Fig. 5.49). There was no significant difference in lactic acid production when autoclaved shell and 15% (v/v) Lactosil was replaced with untreated shell. The flasks containing untreated shell and glucose but no inoculant produced, on average, similar amounts of

lactic acid to flasks containing inoculant. However, more variation between the triplicate flasks was noted when the inoculant was not used.

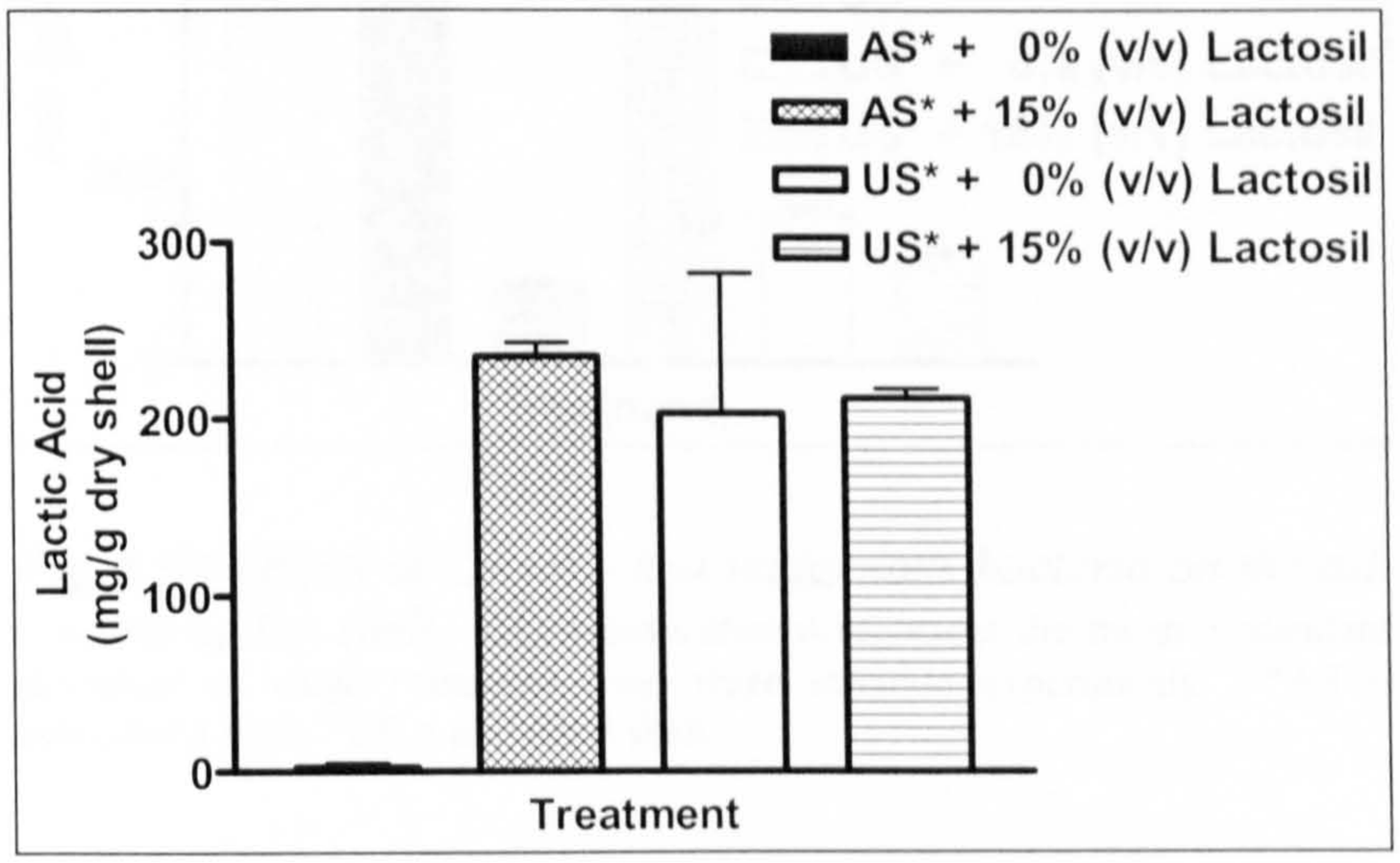


Fig. 5.49 *Effects of Lactosil and indigenous bacteria on the production of lactic acid.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

Ash content in samples containing autoclaved shell and no added inoculant varied little from the initial starting material, of autoclaved shell (Fig. 5.50). However, ash content in samples containing untreated shell and no added inoculant was significantly lower than its starting material, untreated shell ($p<0.001$).

When 15% (v/v) Lactosil was added to autoclaved shell the ash content dropped from 501.90 ± 31.27g/kg in autoclaved shell to 104.77 ± 1.42g/kg. When 15% (v/v) Lactosil was added to untreated shell, ash content dropped from 465.58 ± 8.59g/kg in untreated shell to 138.44 ± 7.30g/kg. Untreated shell plus 15% (v/v) Lactosil had a significantly lower ash content than untreated shell plus 0% Lactosil (v/v) ($p<0.01$).

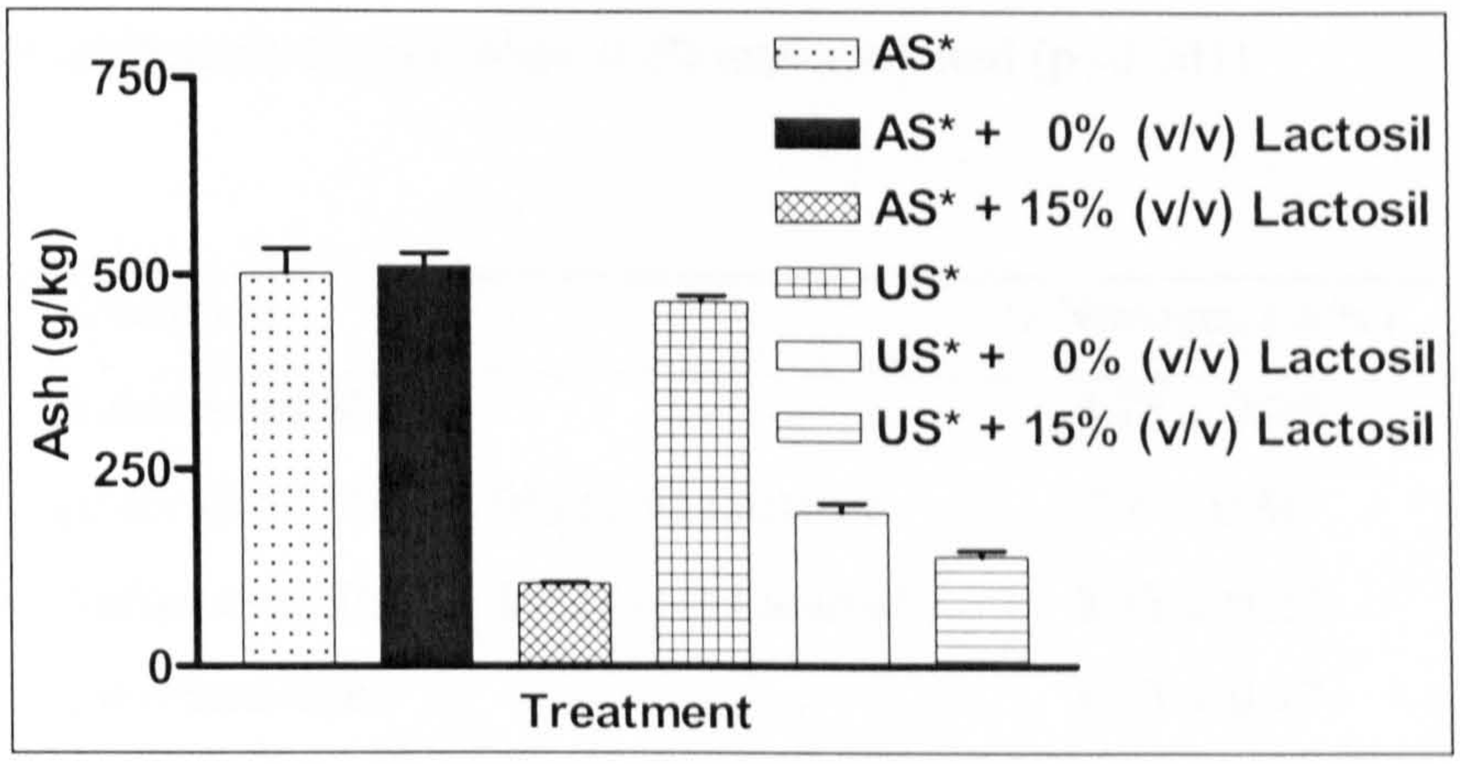


Fig. 5.50 *Effects of Lactosil and indigenous bacteria on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

Calcium content of the different samples followed the same pattern as the ash content (Fig. 5.51).

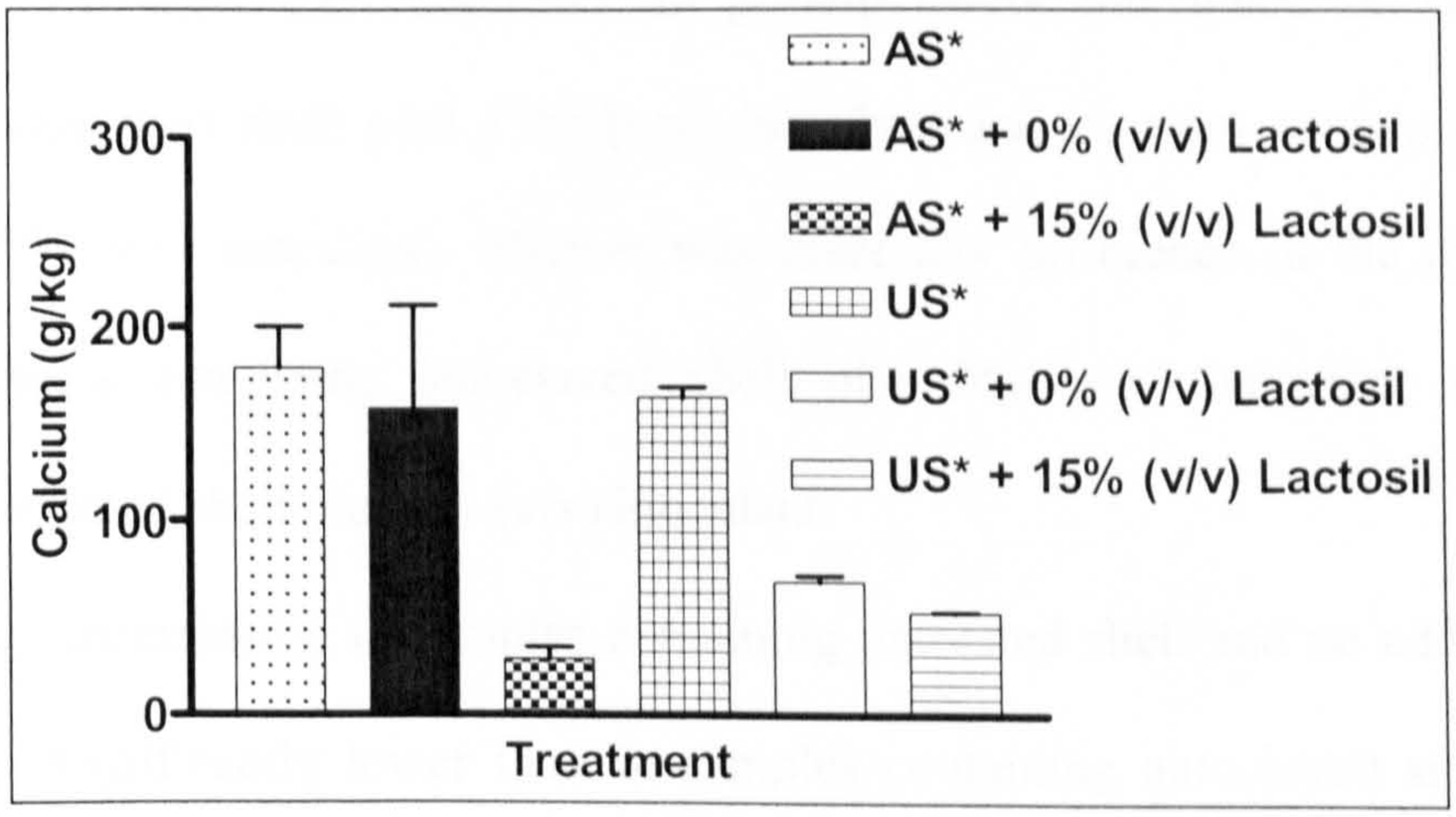


Fig. 5.51 *Effects of Lactosil and indigenous bacteria on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

Figures for percentage nitrogen are shown in Table 5.24. In the presence of 15% (v/v) Lactosil, nitrogen levels did not vary irrespective of whether the shell was autoclaved or

not. However, when Lactosil was not included in the system (0% v/v Lactosil), percentage nitrogen was significantly higher when shell was untreated ($p<0.001$).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
Autoclaved Shell + 0% (v/v) Lactosil	3.6 ± 0.56
Autoclaved Shell + 15% (v/v) Lactosil	8.13 ± 0.27
Untreated Shell	4.13 ± 0.42
Untreated Shell + 0% (v/v) Lactosil	6.33 ± 0.13
Untreated Shell + 15% (v/v) Lactosil	7.54 ± 0.47

Table 5.24 *Effects of Lactosil and indigenous bacteria on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Addition of Lactosil to autoclaved shell or untreated shell caused an apparent increase in protein levels (Fig. 5.52). This was due to the reduction in ash caused by the inoculant (Fig. 5.50). No significant difference in protein levels was noted between samples containing autoclaved shell plus 15% (v/v) inoculant and samples containing untreated shell plus 15% (v/v) inoculant. Neither was there any difference in the protein levels between samples containing autoclaved shell plus 0% (v/v) inoculant and samples containing untreated shell plus 0% (v/v) inoculant.

Protein concentration in samples containing untreated shell and no added inoculant was however significantly lower than in samples containing autoclaved shell and 15% (v/v) inoculant ($p<0.001$) and samples containing untreated shell and 15% (v/v) inoculant ($p<0.01$).

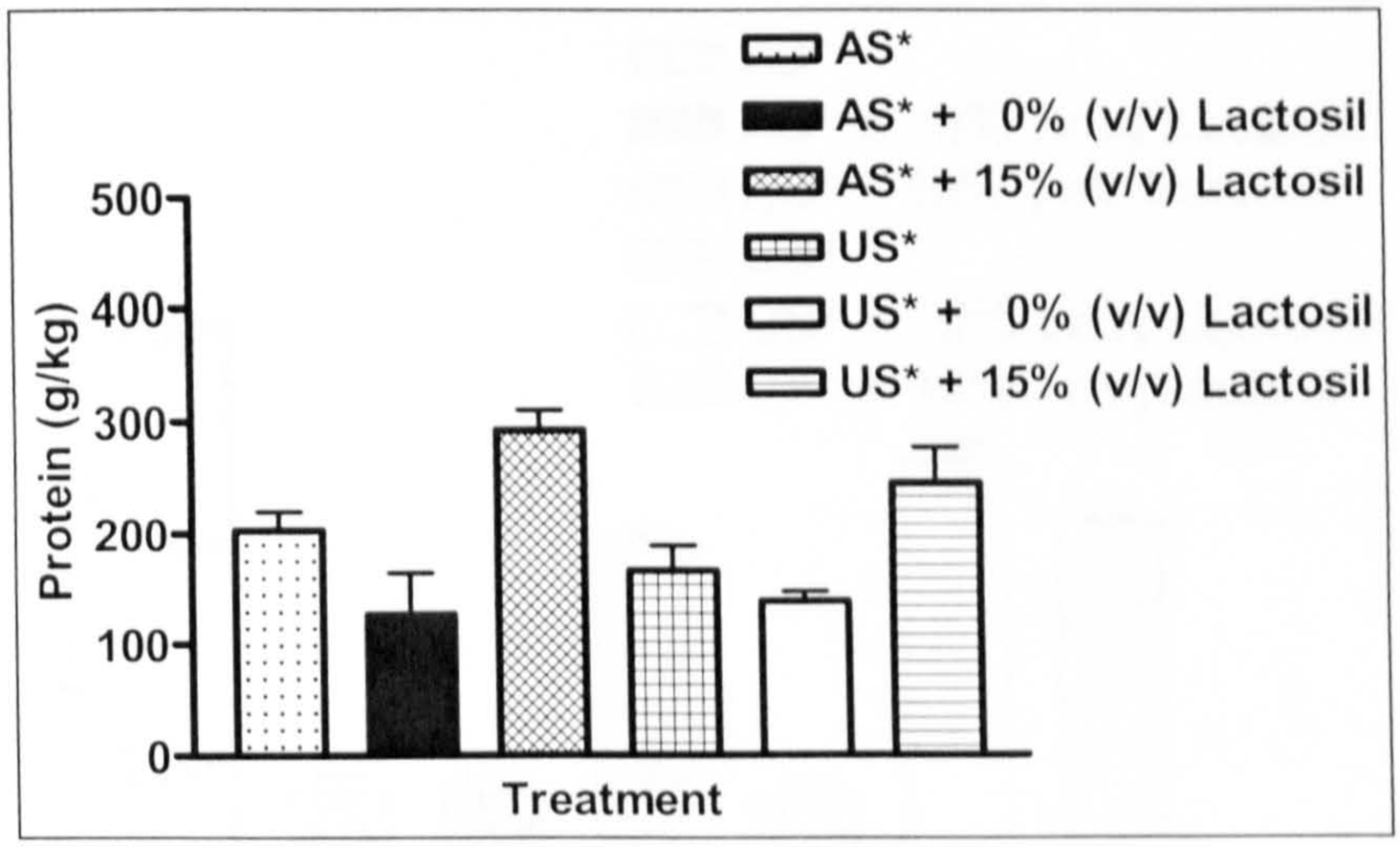


Fig. 5.52 *Effects of Lactosil and indigenous bacteria on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

The highest chitin level was noted in samples containing untreated shell and 0% (v/v) inoculant (596.67 ± 18.93g/kg) (Fig. 5.53). It was significantly higher than the chitin content of samples containing autoclaved shell plus 15% (v/v) Lactosil (501.67 ± 11.55g/kg) ($p<0.001$) and the chitin content of samples containing untreated shell plus 15% (v/v) Lactosil (523.33 ± 5.77g/kg) ($p<0.001$).

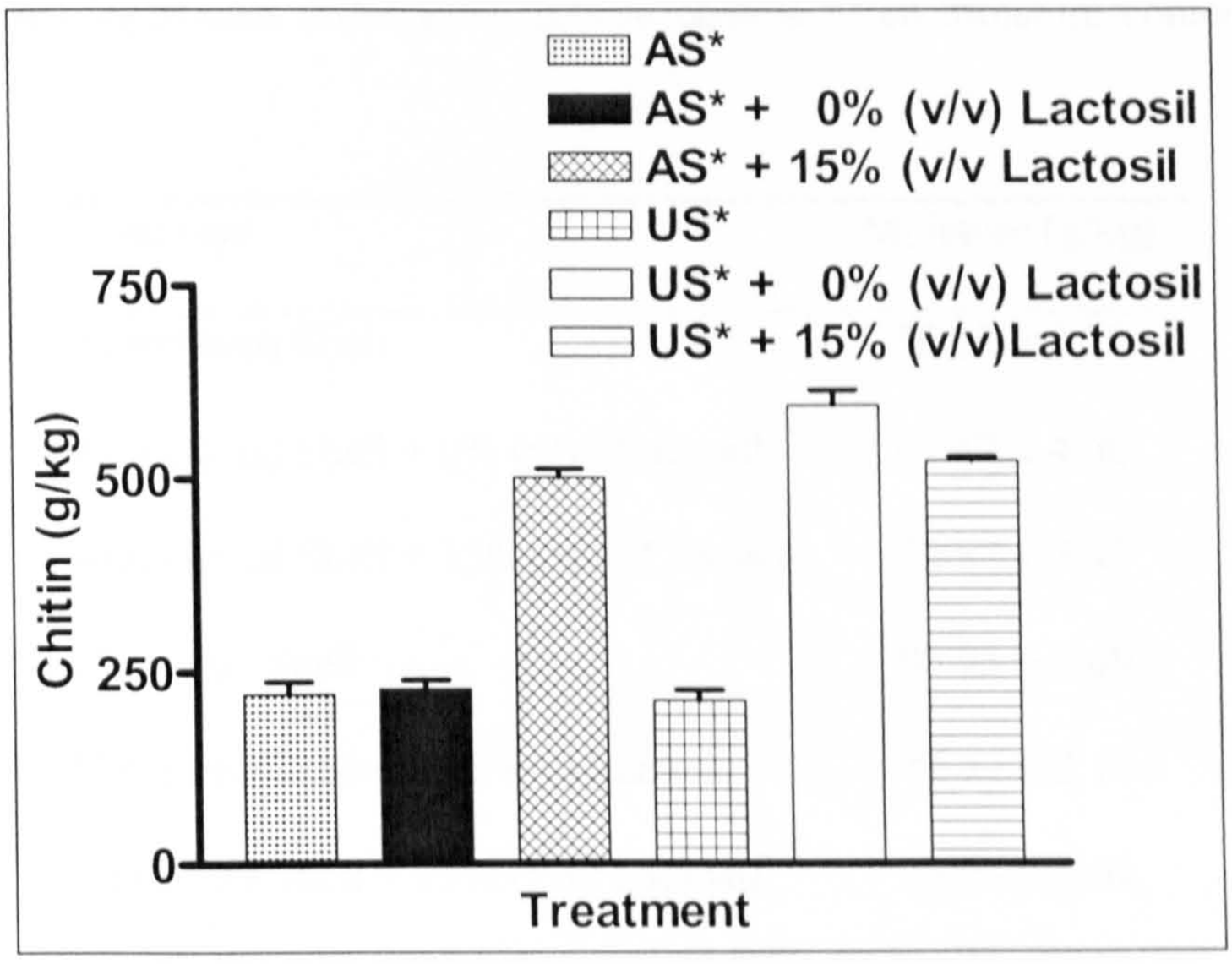


Fig. 5.53 *Effects of Lactosil and indigenous bacteria on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

Untreated shell with no added inoculant showed the largest loss in yield at 65.31 ± 1.35% (w/w) compared with 56.65 ± 0.06% (w/w) for autoclaved shell plus 15% (v/v) inoculant and 58.68 ± 0.47% (w/w) for untreated shell plus 15% (v/v) inoculant (Fig. 5.54).

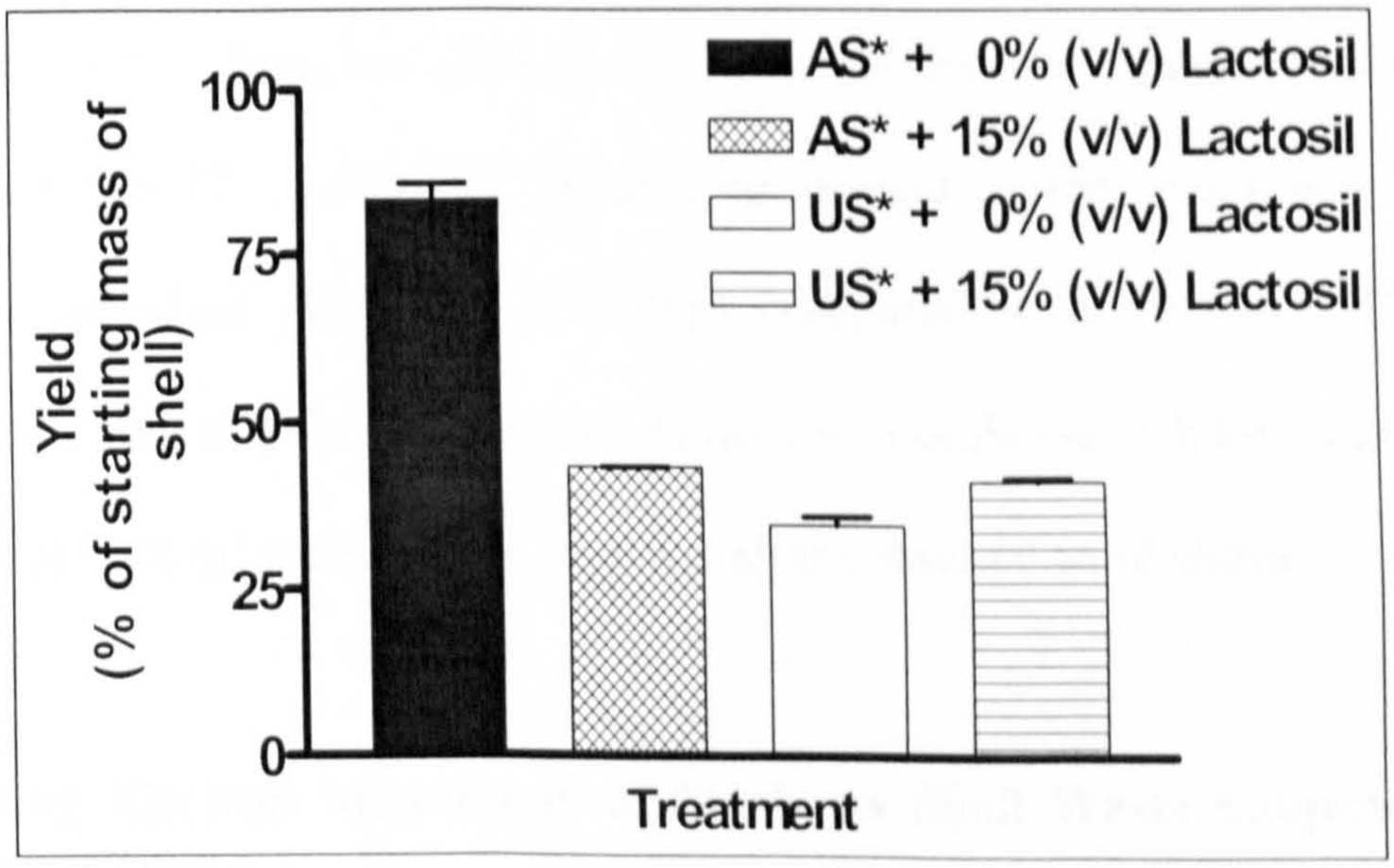


Fig. 5.54 *Effects of Lactosil and indigenous bacteria on percentage yield.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments. *AS = autoclaved shell, *US = untreated shell

Moisture content of the samples varied between 3.9% (w/w) and 9.7% (w/w) (Table 5.25) but the reported significant differences did not change when moisture content was taken into account.

Treatment	Moisture (g/kg)
Autoclaved Shell	55.07 ± 5.79
Autoclaved Shell + 0% (v/v) Lactosil	67.47 ± 4.60
Autoclaved Shell + 15% (v/v) Lactosil	62.87 ± 3.47
Untreated Shell	96.95 ± 7.09
Untreated Shell + 0% (v/v) Lactosil	42.47 ± 2.77
Untreated Shell + 15% (v/v) Lactosil	39.03 ± 1.01

Table 5.25 *Moisture content of shell after lactic acid fermentation with (a) Lactosil inoculant and (b) indigenous bacteria.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments

To summarise, a system containing untreated shell with no added inoculant provided a product with higher levels of chitin (596.67 ± 18.93g/kg) than achieved with autoclaved shell and 15% (v/v) inoculant (501.67 ± 11.55g/kg). Ash content was also higher for untreated shell and no inoculant (194.23 ± 11.55g/kg) than for autoclaved shell and 15% (v/v) inoculant (104.77 ± 1.42g/kg). In contrast, protein content was lower for untreated shell and no inoculant (138.10 ± 8.50g/kg) compared with 291.78 ± 17.50g/kg for autoclaved shell and 15% (v/v) inoculant. However, protein and ash levels after treatment with or without Lactosil were still too high to call the residual solid chitin.

5.3.4 Scanning Electron Microscopy of *Nephrops* Shell Waste Subjected to Lactic Acid Fermentation.

Bacterial colonies were visible on shell that had been subjected to lactic acid fermentation for one day (Fig. 5.55). Many of these bacteria were rod shaped.

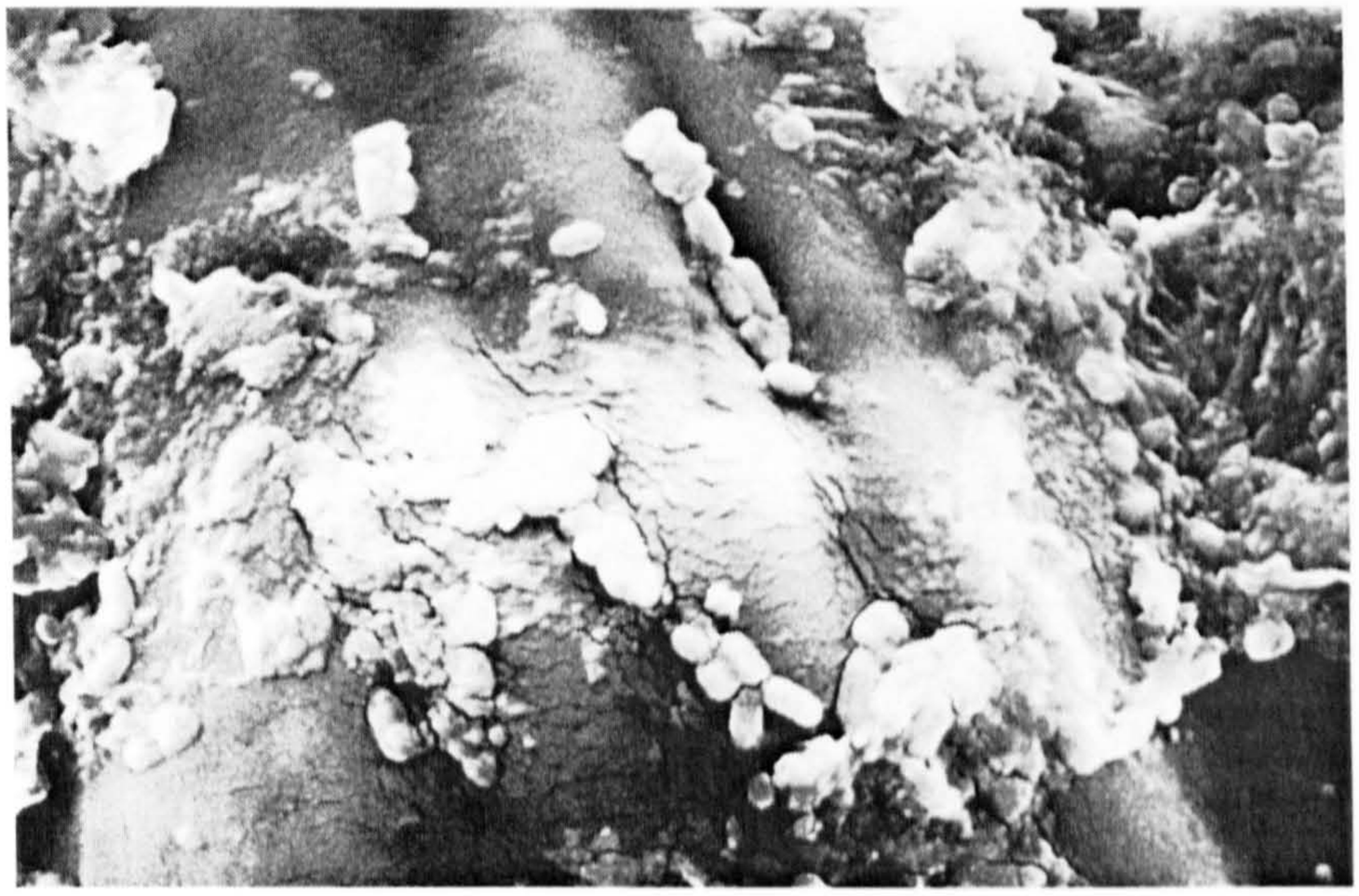


Fig. 5.55 *Scanning electron micrograph of Nephrops shell waste subjected to lactic acid fermentation for 1 day* (x2500).

The final washed residue is shown in Figs. 5.56 and 5.57. Similarities can be drawn between this microbially treated shell and the chemically extracted chitin pictured in Fig. 4.7 despite the ash and protein contaminants remaining after microbial treatment.

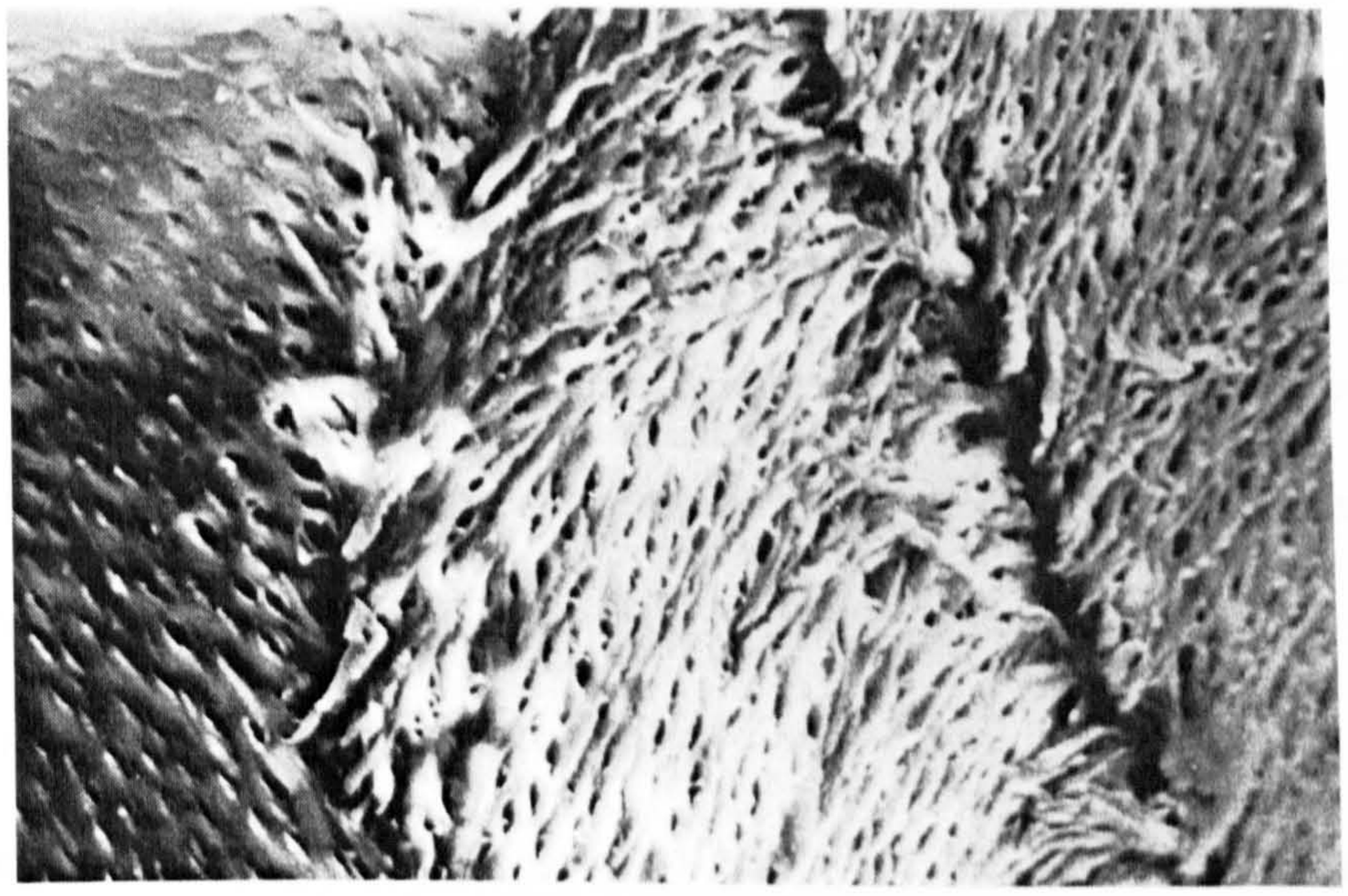


Fig. 5.56 *Scanning electron micrograph of Nephrops shell waste subjected to lactic acid fermentation for 7 days. Final washed residue* (x1250).

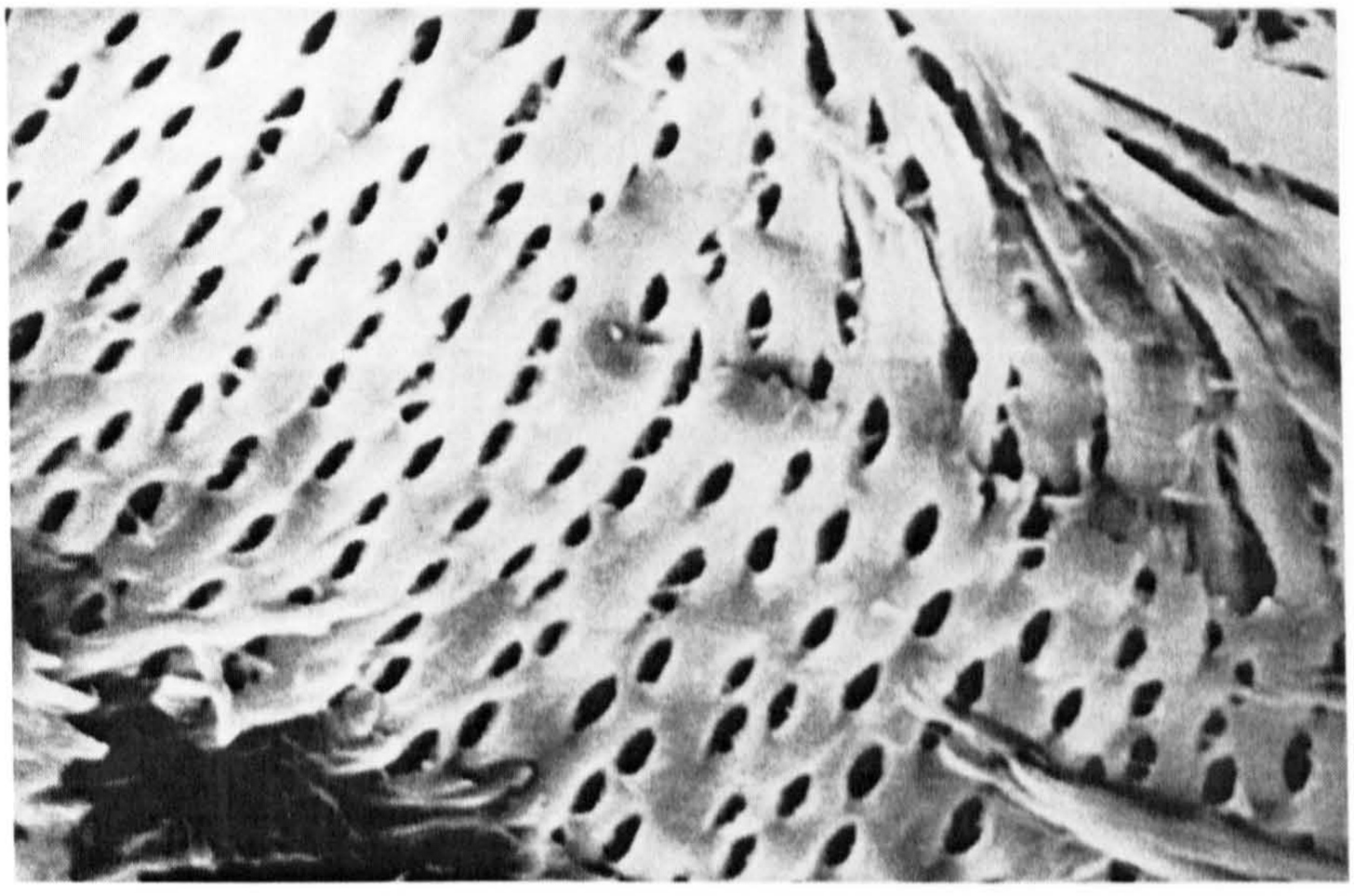


Fig. 5.57 *Scanning electron micrograph of Nephrops shell waste subjected to lactic acid fermentation for 7 days. Final washed residue* (x2500).

5.3.5 Assessment of Lactic Acid Fermentation Culture Broth with Respect to Calcium Lactate, Lactic Acid and Glucose

After lactic acid fermentation the solid material, which contained chitin, was removed from the system by filtration. This left a yellow milky broth that had a sweet smelling cheesy odour. If left to settle the broth separated into three layers - an uppermost oily layer, a middle milky layer and a bottom layer containing fine sediment.

The broth was centrifuged and the supernatant used for lactic acid determinations - results shown in section 5.3.2 - 5.3.3. The broth contained lactic acid concentrations of more than 200mg/g dry shell. The concentration of glucose remaining, after lactic acid fermentation, was also measured in a broth supernatant sample. The broth contained 68.9g glucose per litre of broth - equivalent to 6.89% (w/v).

An aliquot of the whole broth was refrigerated at 4°C. After refrigeration a white crystalline substance separated out from the liquid broth. FTIR analysis of this material, after washing and drying, revealed a profile identical to that of commercial calcium lactate (Figs. 5.58 and 5.59).

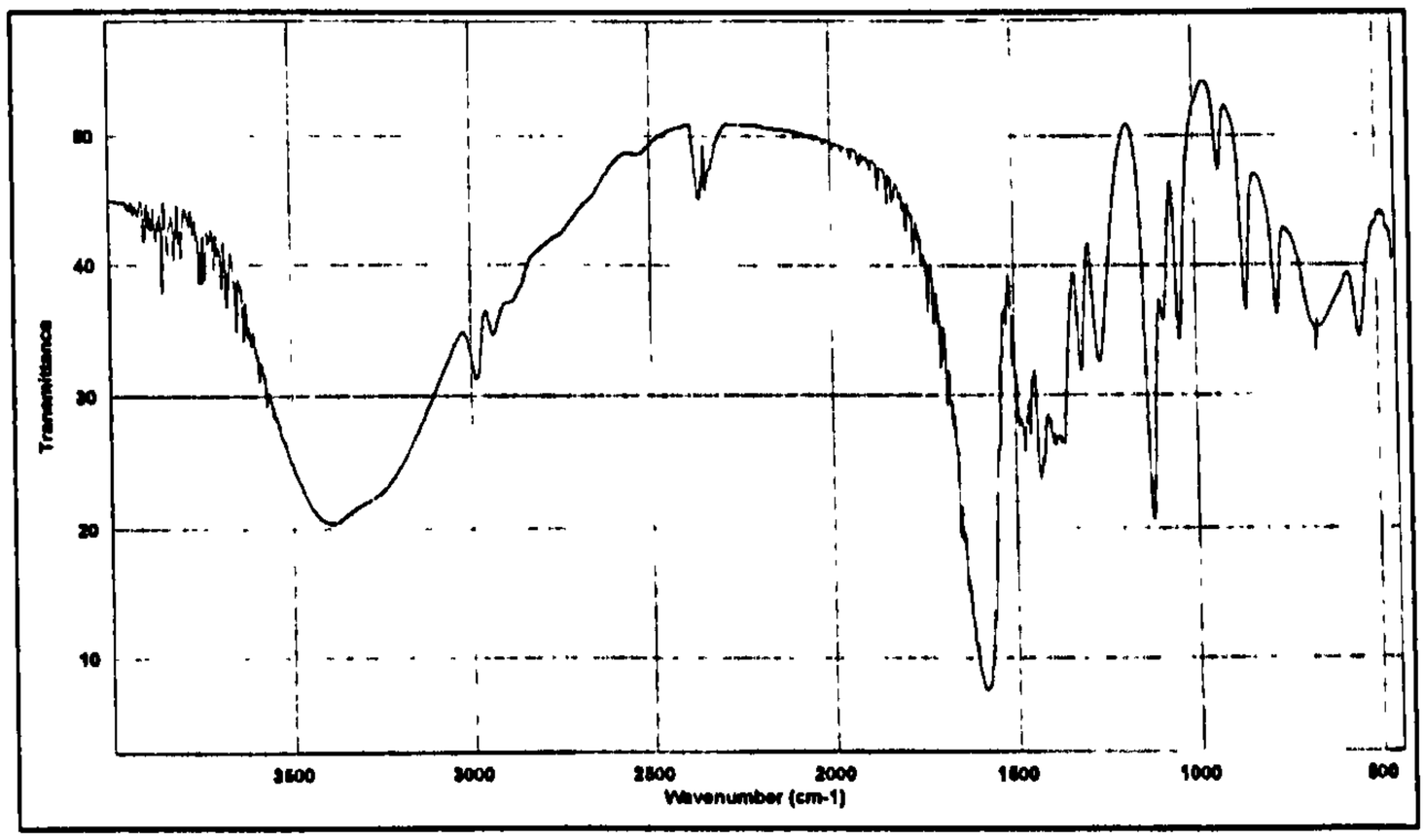


Fig. 5.58 *FTIR of culture broth material.*

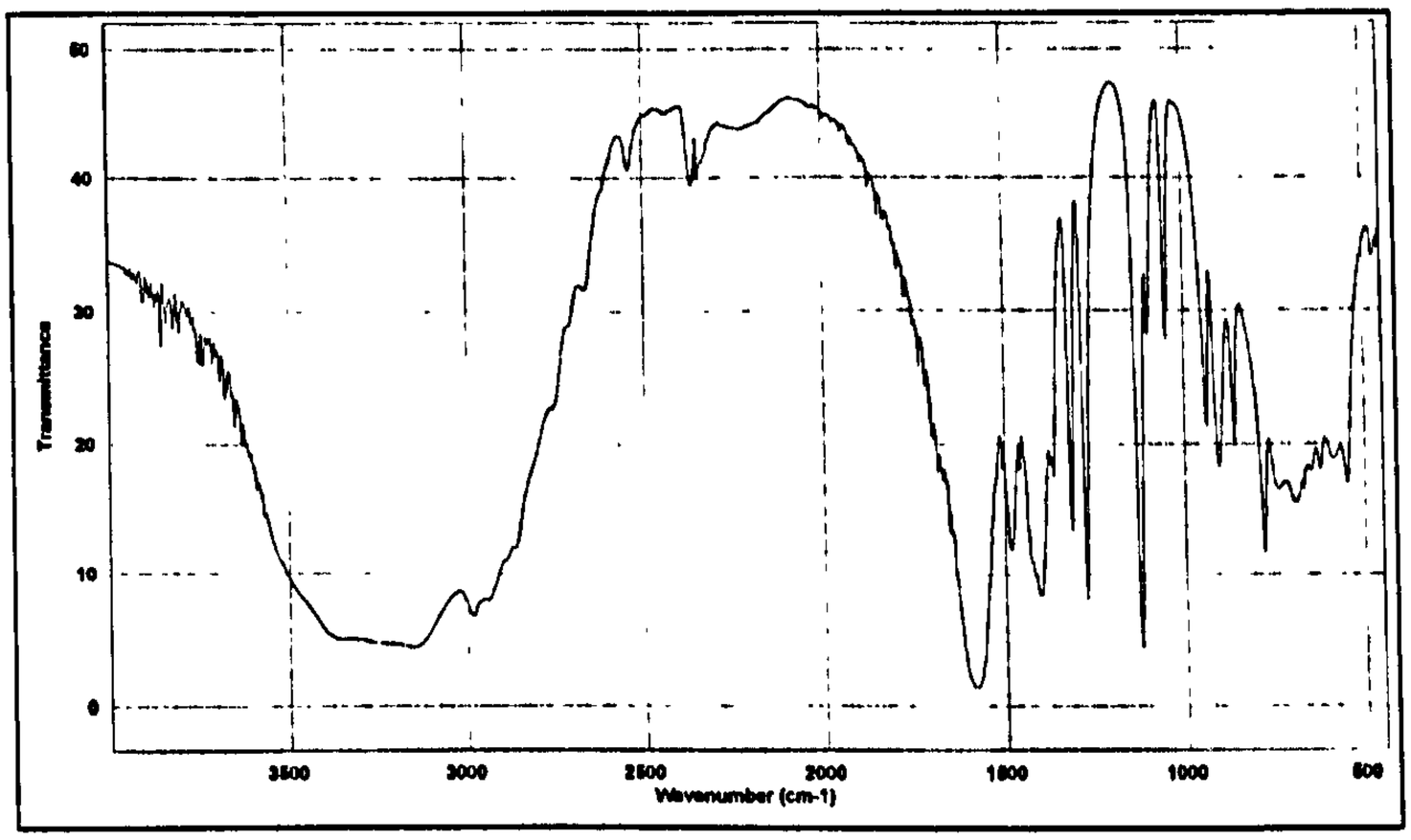


Fig. 5.59 *FTIR of commercial calcium lactate.*

It is known that soluble protein, fatty acids and pigments are present in culture broth, after fermentation of crustacean shell waste, but these were not assessed within the scope of this project.

5.4 DISCUSSION

Fermentation of fish waste using lactic acid bacteria has been used to produce a silage suitable for use in animal feeds (Faid *et al.*, 1994), salmon diets (Jackson *et al.*, 1984) and food products for human consumption (Owens and Mendoza, 1985). During the ensilation process, the fish waste gradually liquefies due to the presence of tissue degrading enzymes naturally present in the fish (Raa and Gildberg, 1982). Lactic acid bacteria that are indigenous to the waste or added separately, act on a carbohydrate source to produce lactic acid, which decreases the pH and acts to preserve the nutrient value of the ensiled material (Raa and Gildberg, 1982).

Lactic acid fermentation of proteinaceous shellfish waste occurs in a similar manner. Generally, a lactic acid inoculant and a carbohydrate source are added to minced or ground shellfish waste in a solid-state or semi-solid-state fermentation system (Hall and De Silva, 1992; Zakaria *et al.*, 1996; Rao and Stevens, 1997). The proteolytic enzymes indigenous to the shell act autolytically to liquefy the protein element of the waste. The lactic acid produced acts to preserve nutrients but also solubilises the calcium carbonate portion of the shell. A liquid silage and a chitin containing sediment are the end result.

The *Nephrops* waste employed in this project lacked the proteinaceous heads usually available and therefore solubilisation of the shell protein by indigenous enzymes was unlikely. Solid-state fermentation of this waste, prior to the present study, had resulted in inadequate removal of ash (Bustos, 1996) and was not therefore attempted. Another important difference between this study and others was that in this case the waste used was dried whilst still fresh and ground up to appropriate particle sizes. Usually the waste is minced and fermented as soon as possible after collection (Fagbenro and Bello-Olusoji, 1997) or is stored frozen and then thawed as required (Hall and De Silva, 1992; Rao and Stevens, 1997).

5.4.1 Characterisation of Inoculant

The species of bacteria contained in the Lactosil inoculant that was used for the fermentation were already known - *Lactobacillus plantarum*, *Enterococcus faecium* and *Pediococcus acidilactici*. Growth of the inoculant was observed on different types of agar but only Gram positive cocci were visible under the conditions used. *Enterococcus faecium* is a Gram-positive bacterium and was most likely the coccoid bacterium observed. It is possible that the initial growth period of 24 hours in MRS broth before plating unto agar plates was not long enough for the *L. plantarum* and the *P. acidilactici* to initiate growth. In experiments using Lactosil to degrade *Nephrops* shell waste (section 5.3.4) rod shaped bacteria (probably *L. plantarum*) were seen in scanning electron micrographs of waste that had been subjected to lactic acid fermentation by Lactosil inoculant for 24 hours (Fig. 5.55). In these experiments, the inoculant was cultured in MRS broth for 48 hours before addition to the fermentation system in contrast to the 24-hour period used for inoculant characterisation.

5.4.2 Experimental Set-up of the Microbial Treatment of Shell

Initial experiments for the microbial degradation of prawn shell were set up in 100ml bottles using previously optimised parameters - 30°C, 1:15 solid to liquid ratio and 15% (w/v) glucose (Bustos, 1996). Fermentation systems containing Lactosil and those without Lactosil produced similar amounts of lactic acid (Fig. 5.4). Lactic acid bacteria such as *Lactobacillus paracasei, Pediococcus* species and *Lactobacillus* species have been isolated from shellfish waste (Guerrero-Legarreta *et al.*, 1996). *Lactobacillus plantarum* and coccoid species have been isolated from *Nephrops norvegicus* shell waste (Hall *et al.*, 1994). Therefore, presumably lactic acid bacteria indigenous to the *Nephrops* shell were activated under the fermentation conditions utilised and acted in a similar manner to the bacteria contained in the Lactosil inoculant. Autoclaving the shell waste eliminated these

bacteria and hence stopped the production of lactic acid in the absence of Lactosil inoculant (0% v/v) (Fig. 5.9). It is possible that the added inoculant showed dominance over the indigenous bacteria as found by Shirai *et al.*, (2001) but it was decided, nevertheless, to eliminate the indigenous bacteria so that effects due to Lactosil alone could be determined. Since autoclaving did not have any significant effect on the chemical composition of the shell waste (section 5.3.2.2) the shell used in later experiments was autoclaved so that bacterial numbers and the bacterial species involved in the fermentation process could be controlled.

5.4.3 Optimisation of Parameters for Lactic Acid Fermentation of *Nephrops* Shell Waste

Lactic acid production increased as the concentration of Lactosil was raised from 0% (v/v) to 8% (v/v) (Fig. 5.9). In a similar study employing the same waste product, glucose concentration and temperature (Bustos, 1996), increasing the inoculant concentration showed no effect except to increase the speed of lactic acid production. However, in that study the shell waste had not been autoclaved and therefore, indigenous bacteria, as well as bacteria in the inoculant, contributed to the production of lactic acid. Indigenous bacteria have been shown to lead to the production of similar amounts of lactic acid as produced under the action of 15% (v/v) Lactosil (Fig. 5.49).

Another difference, which could have affected lactic acid production, was the specific inoculant used. Bustos (1996) employed a commercial inoculant called Stabisil. Stabisil contained the same bacterial strains as Lactosil but the strains were not in the same proportion to each other as in Lactosil. Zakaria *et al.*, (1998) required 10% (v/w) of an inoculant (*Lactobacillus paracasei* strain A3) containing 10^8cfu/ml to achieve satisfactory acidification of cold-water prawn waste in a semi solid-state fermentation system, as did Guerrero-Legarreta *et al.*, (1996). This level of inoculant approximated to 10^7cfu/g shell waste whereas the 8% (v/v) Lactosil used here approximated to 10^{10}cfu/g shell waste. The

higher numbers of bacteria required in the present study may have been due to the lack of indigenous bacteria in the autoclaved shell.

At 8% (v/v) Lactosil, ash levels had been reduced from 50.2% (w/w) in autoclaved shell to 11.6% (w/w). Further increase in Lactosil concentration had no effect on the ash content (Fig. 5.10) or on the calcium content (Fig. 5.11) of the shell waste. However, increasing the inoculant concentration from 8 to 15% (v/v) caused a further significant increase in the chitin content of the final product (Fig. 5.12). At this level of inoculant a pH of 4.0 was obtained (Fig. 5.43) and 239.27mg lactic acid/g dry shell were produced (Fig. 5.9). This equated with 1.6% (w/v) total titratable acid (TTA). Zakaria *et al.*, (1998) achieved similar amounts of lactic acid (1.2% w/v TTA) in a semi solid-state fermentation system but the pH reached was not as low (pH 5).

Protein concentration fell when no Lactosil (0% v/v inoculant) was included in the system (Fig. 5.13). This was due to solubilisation of protein rather than microbial degradation (Fig. 5.15). This same protein loss probably occurred in all the samples mixed with glucose at 30°C whether Lactosil was present or not. Some studies advocate supplementing the lactic acid fermentation by using 0.1% (w/v) NaOH to wash the shell to remove protein remaining after fermentation and 4% (w/v) HCl to remove remaining minerals (Hall and Reid, 1995; Rao and Stevens, 1997) but adding these steps here would entail adding a chemical treatment to a biotechnological process. No washing step was introduced at this stage so that later experiments could be compared with the ones already carried out.

At 4% (v/v) Lactosil the protein concentration of the waste appeared to almost double (Fig. 5.13). This apparent increase was due to a large reduction in the level of ash in the same sample. Protein levels did start to fall at a Lactosil concentration of 8% (v/v). However, no significant difference in protein levels was seen between 8 and 15% (v/v) inoculant. Variability in the protein results was high compared to results for the other solid

components. Protein results were not actual measurements but instead were obtained by a calculation which took into account the chitin content and the nitrogen content of the shell. Therefore, a combination of normal experimental error in both the chitin and nitrogen determinations will have contributed to the variation observed in the protein results.

The optimum temperature for the growth of lactic acid bacteria is approximately 30°C so the majority of the degradation experiments were carried out at this temperature. However, since *E. faecium* grow within the range 10 to 45°C, *L. plantarum* between 15 and 40°C and the optimum for *P. acidilactici* is 40°C (Sneath *et al.*, 1986) fermentation at 30°C and 40°C was compared. Production of lactic acid, which was significantly higher at 30°C than at 40°C (Fig. 5.18), resulted in a lower ash content at 30°C (Fig. 5.19). In contrast, protein content was lower at 40°C than at 30°C (Fig. 5.22).

It is possible that the lower level of protein at 40°C was caused by a greater solubilisation of protein at 40°C than 30°C, as opposed to the proteolytic action of the bacteria being greater at the higher temperature. Cold water at pH 7.0 for 48 hours is known to remove soluble, unbound protein from crab (*Cancer pagurus*) (Hackman and Goldberg, 1958) and Fig. 5.15 shows that washing shell waste at a high temperature removed more protein from shell than washing at a lower temperature. In subsequent experiments a temperature of 30°C was maintained because ash content in the solid product was lower at this temperature and lactic acid fermentation was being used primarily to remove ash. Thirty degrees Celsius is the temperature most often used for similar fermentations (Guerrero-Legarreta *et al.*, 1996; Kungsuwan *et al.*, 1996; Shirai *et al.*, 1997; Zakaria *et al.*, 1998).

This project focused on the isolation of chitin, from the shell waste, but protein is another valuable component of the shell. If the protein is to be extracted it is important to keep the fermentation temperature as low as possible so that the protein is not denatured.

The optimum agitation speed tested for lactic acid production was 200rpm (Fig. 5.24). More ash was removed at 200rpm than at 100 or 150rpm (Fig. 5.25) due to the higher level of lactic acid produced at 200rpm. Stirring does not significantly help the growth of a pure culture of lactic acid bacteria (Rao and Stevens, 1997). The bacteria are reportedly sensitive to shear and therefore intermittent mixing is advocated (Hall and Reid, 1995). Some form of mixing is essential, however, because shellfish biowaste deteriorates in a lactic acid fermentation system that is not mixed (Rao and Stevens, 1997). The better mixing achieved at 200rpm evidently provided conditions under which the bacteria present could convert more glucose to lactic acid, and caused the lactic acid to come into contact with more shell particles than at 100 or 150rpm allowing greater levels of ash to be dissolved. The introduction of air by mixing was not detrimental to the microorganisms since lactic acid bacteria are microacrophilic i.e. they can tolerate oxygen.

A readily available carbohydrate source is an essential requirement for the production of maximum levels of lactic acid. A glucose concentration of 15% (w/v) in the present study was found to be optimal for lactic acid production (Fig. 5.30). Increasing the concentration further, to 20% (w/v), did not affect the amount of lactic acid produced (Fig. 5.30). Ash content fell significantly when more than 5% (w/v) glucose was added to the system with maximal ash removal occurring at 10% (w/v) glucose (Fig. 5.31). Despite the ash reduction noted at 5% (w/v) glucose lactic acid content had not yet started to rise (Fig. 5.30). pH levels however had started to fall (Fig. 5.32). A very small change in lactic acid can cause a large change in pH therefore an increase in lactic acid production, undetectable by the methods used, must have occurred. Increasing the lactic acid concentration beyond 150mg/g dry shell did not aid in the removal of ash from the shell. Therefore, approximately 10 - 14% (w/w) of the ash present could not be removed by lactic acid treatment alone. Bustos (1996) suggested that some of the calcium and phosphate in the shell exists intimately bound to other components of the shell and in areas not easily

accessible to acid attack. This would explain why 100% of the ash was not removed from the waste at high levels of lactic acid.

Unlike this study, where water content was high, the moisture content in semi solid-state fermentation systems is unable to support high concentrations of glucose added at the start of fermentation (Guerrero-Legarreta *et al.*, 1996). A concentration of 10% (w/w) glucose (equivalent to 0.1g glucose/g wet shell waste) has been used successfully for the lactic acid fermentation of whole *Nephrops* waste (Zakaria *et al.*, 1998). However, the lowest pH reached was pH 5 and this rose to pH 6 after forty-eight hours of fermentation. The rise in pH was due to neutralisation of the lactic acid by calcium carbonate with the formation of calcium lactate crystals. Attempts are being made to operate a 'draw and fill' method where glucose additions are made during the course of the fermentation to overcome this problem (Zakaria *et al.*, 1998). In the present study, where much higher levels of glucose were incorporated (15% w/v glucose which is equivalent to 2.25g glucose/g dry shell waste), pH levels remained low (pH 4) for the duration of the fermentation (Fig. 5.43).

Protein levels increased at glucose concentrations greater than 5% (w/v) i.e. as ash was removed from the shell, protein levels showed an apparent increase (Fig. 5.35). Lactic acid bacteria contain proteolytic systems (Law and Kolstad, 1983) and it had been hoped that at low levels of glucose the bacteria would use the shell protein as a carbon source but this did not happen. Protein levels in shell treated with 0% and 1% (w/v) glucose were seen to be lower than protein levels in autoclaved shell however this was probably due to solubilisation of adventitious protein in the glucose solution and not due to microbial action. The protein autolysis seen in similar studies using 'head on' *Nephrops* waste is most likely due to the action of proteolytic enzymes present in the proteinaceous heads (Zakaria *et al.*, 1998; Kungsuwan *et al.*, 1996). In subsequent experiments 15% (w/v) glucose was used because this maximised the production of lactic acid. However, a

10% (w/v) concentration of glucose would have been enough since maximal ash reduction was achieved at this concentration.

Fermentation was studied on two occasions over a period of 14 days. The standard deviation between lactic acid measurements from experiments carried out on separate occasions was large for the time course experiments (Fig. 5.37), however, the standard deviation in ash results was not so great (Fig. 5.38) showing that large fluctuations in lactic acid cause small ash changes. Maximal lactic acid production occurred by day 5 (Fig 5.37). Beyond day 3 little change in ash appeared to occur. However, the variation in ash results was large on day 3 and day 4 may possibly have shown a lower ash content. On day 4 the maximum production of lactic acid, when standard deviation values were taken into account, was 150mg/g dry shell. Similar amounts of lactic acid were produced at 10% (w/v) glucose concentration (Fig. 5.30) - the glucose concentration that provided the optimum reduction in ash (Fig. 5.31). This suggests that even if the levels of lactic acid had been increased further the levels of ash would not have fallen more. During the natural moult in another decapod crustacean, the shore crab, *Carcinus maenas*, digestion of the chitin-protein complex appears to occur before the calcium carbonate portion of the shell is dissolved (Compère et al., 1998). Hydrochloric acid used for the chemical isolation of chitin from shell waste acts to degrade protein and chitin as well as dissolving the calcium carbonate. Hydrogen ions present in the moulting fluid may act in a similar manner. It might, therefore, be necessary to dissolve the protein component of the shell before all the calcium carbonate can be removed.

Beyond day 3 no further significant differences were seen in chitin levels (Fig. 5.40), protein levels (Fig. 5.41), or in percentage yield (Fig. 5.42). These results indicated that fermentation could be stopped at day 3. Nevertheless, subsequent experiments were allowed to continue for 7 days so that all experimental data came from fermentations run for the same lengths of time. Zakaria *et al.*, (1998) continued fermentation for 3 days only.

After 48 hours, production of total titratable acid fell and a rise in culture broth pH was seen. This rise may have been due to insufficient levels of carbohydrate available as already discussed or to a high protein content. Proteolysis or autolysis of nitrogen compounds to more basic amines, peptides and ammonium would have caused the pH to rise.

The conditions of fermentation adopted after optimisation were 15% (v/v) Lactosil, 30°C, 200rpm and 15% (w/v) glucose concentration over a time period of 7 days. The product achieved under these conditions contained 10.5% (w/w) ash, 33.6% (w/w) protein, 48.3% (w/w) chitin and 6.2% (w/w) moisture. Slightly altered conditions using 8% (v/v) Lactosil and 10% (w/v) glucose over a 3 - 4 day period would possibly have provided an equivalent product. However, 15% (v/v) inoculant provided a slight increase in chitin content over 8% (v/v) inoculant and 15% (w/v) glucose provided maximal lactic acid production even though 10% (w/v) was enough for maximal ash removal. Seven days for length of time of fermentation was only chosen so that all results could be compared.

Since the ash and protein contents were still high, one batch of shell was subjected to two consecutive fermentation treatments to see if more ash and protein could be removed under fresh fermentation conditions. Lactic acid levels differed significantly between the two treatments (Fig. 5.44). Much lower levels were produced in the second treatment. This indicated that components essential to the growth of the bacteria were missing in the second system. Lactic acid bacteria require a source of amino acids (Sneath *et al.*, 1986). Therefore it is possible that the removal of adventitious protein, via solubilisation during the first fermentation treatment, and washing the product subsequent to fermentation, was detrimental to the bacteria, since no other readily available source of amino acids was available. This suggests that washing shell in warm or hot water before fermentation would not be beneficial for the fermentation system. It was hoped that the bacteria would utilise the shell protein as an alternative source of amino acids in the second treatment but

this did not occur. Shell protein did not decrease after the second fermentation treatment (Fig. 5.48).

Indigenous bacteria produced similar amounts of lactic acid to the Lactosil inoculant (Fig. 5.49) under the fermentation conditions used, but the reproducibility of the lactic acid results was not good with the indigenous bacteria. This underlines the desirability of using an inoculant additive for fermentation, where it is possible to estimate the number of bacteria added to the system, instead of using the bacteria indigenous to the shell, which could vary in bacterial numbers and bacterial species between runs. All values determined for total titratable acid (TTA) were reported as lactic acid for easy comparison with previous plots. However, indigenous bacteria are more likely to utilize glucose via heterofermentation and therefore it should not be assumed that all the TTA was lactic acid. Addition of inoculant to the untreated shell will possibly have caused a shift to the homofermentative pathway (Abazinge *et al.*, 1993; Shirai *et al.*, 2001).

Ash content was reduced the most in the system containing autoclaved shell and Lactosil (Fig. 5.50). However, when subjected to fermentation using indigenous bacteria and/or Lactosil, protein levels were lowest in the system containing indigenous bacteria alone (Fig. 5.52). Indigenous bacteria and Lactosil together resulted in a product with a high level of protein. It appeared that Lactosil had no effect on the degradation of protein in the shell and may have acted to inhibit protein solubilisation by indigenous bacteria.

The indigenous bacteria, when used on their own, showed the largest loss in yield (Fig. 5.54) and resulted in a product with the highest amount of chitin (Fig. 5.53). However, ash levels were lower when Lactosil was used alone (Fig. 5.50).

Overall, protein and ash levels after treatment with or without Lactosil were still too high to call the residual solid chitin. Nevertheless, the scanning electron micrographs of the final product (Figs. 5.56 and 5.57) were similar to those of chemically treated shell (Figs. 4.7 and 4.8). Few studies report the chemical composition of the final product,

preferring to analyse the liquor produced instead. The liquor was shown in this situation to contain calcium lactate, lactic acid and glucose but a full characterisation was not carried out (section 5.3.5). Zakaria *et al.*, (1998) reported 77.5% (w/w) calcium and 61.0% (w/w) protein solubilised. Bustos (1996) reported a final product, after lactic acid fermentation, containing 56.8% (w/w) chitin, 33.4% (w/w) protein, 4.5% (w/w) ash and 5.3% (w/w) others. This compares with the 50.2% (w/w) chitin, 29.2% (w/w) protein, 10.4% (w/w) ash, 6.3% (w/w) moisture and 3.9% (w/w) others, achieved in this study using autoclaved shell plus 15% (v/v) Lactosil. Shirai *et al.*, (2001) have shown that indigenous bacteria are likely to cause heterofermentation with the production of end products other than lactic acid. Therefore, since indigenous bacteria cannot be depended upon to show homogeneity in levels of lactic acid produced or in the type of fermentation i.e. homo/hetero-fermentation between different runs it was decided to continue to use the Lactosil inoculant with autoclaved shell but to investigate methods of increasing the proteolytic capability of the system. This was done in the hope that both the protein and the ash content of the product could be lowered further.

CHAPTER SIX

BIOPROCESSING OF NEPHROPS SHELL WASTE BY LACTIC ACID FERMENTATION AND PROTEOLYTIC ENZYME

6.1 INTRODUCTION

Several studies have investigated the proteolytic action of microorganisms on prawn shell. Shimahara *et al.*, (1984) used *Stenotrophomonas maltophilia* LC102 to deproteinise shell that had already been demineralised. In comparison with chemically treated shell the bacterially deproteinised shell was less denatured. Bustos (1996) compared the action of several microorganisms/combinations of microorganisms on chemically demineralised *Nephrops* shell waste but the resulting product contained more nitrogen than theoretically calculated to be in chitin, indicating residual protein. Rhishipal and Philip (1998) isolated various yeasts from water. One of these broke down protein in prawn shell but did not affect the chitin and could possibly be used to deproteinise demineralised shell.

Other studies have researched the action of proteolytic enzymes on crustacean shell waste e.g pepsin and trypsin (Broussignac, 1968); bacterial protease (Pronase-P), papain and enzyme from tuna (Takeda and Katsuura, 1964); papain (Brine and Austin, 1981b); chymotrypsin and papain (Gagné and Simpson, 1993); alcalase (Synowiecki and Al-Khateeb, 2000). In most work carried out using lactic acid fermentation of crustacean waste the waste used contained protein rich heads (Hall and De Silva, 1992; Hall and Reid, 1995; Kungsuwan *et al.*, 1996; Zakaria *et al.*, 1996; Guerrero-Legarreta *et al.*, 1996; Rao and Stevens, 1997; Zakaria *et al.*, 1998; Shirai *et al.*, 2001). These heads also contained proteolytic enzymes. Shirai *et al.*, (1997) recovered some of the indigenous proteases from lactic acid fermentation broth by ultracentrifugation and used them to deproteinise shell that had been demineralised with 1.2M HCl. The amount of soluble protein released after 48 hours compared favourably with soluble protein released by treatment with trypsin.

In another study Shirai *et al.*, (1998) subjected lactic acid fermented waste to proteolytic treatment using trypsin. More protein was removed using lactic acid fermentation followed by enzymic treatment (77.06% w/w) than when lactic acid fermentation was used alone (67.05% w/w). Elemental analysis and infrared spectrometry

showed that the chitin produced was similar to that produced using traditional chemical methods. Rao *et al.*, (1998) treated shrimp biowaste with lactic acid bacteria and a proteolytic enzyme at the same time. This combination caused 90% (w/w) deproteinisation and more than 50% (w/w) decalcification of the waste.

In the manufacture of fish sauces, by lactic acid fermentation of fish waste, proteolytic enzymes are often added to the system to accelerate the solubilisation of protein caused by indigenous enzymes. Several of the enzymes used are of plant origin, for example papain, bromelain and ficin (Mackie, 1974). Plant enzymes are available on a large scale commercially and have a wide specificity. Bromelain, a by-product of the pineapple industry (Ritonja *et al.*, 1989) has been used in the production of fish hydrolysate from mackerel (Beddows *et al.*, 1976). Stem bromelain (from pineapple stem) consists of cysteine endopeptidases. It is a glycoprotein of broad substrate specificity effective at a wide range of pH values and temperatures. It is active under weak acid conditions (Gildberg, 1993) and is therefore a suitable candidate for use in conjunction with lactic acid fermentation where the pH value falls to just under 4 pH units. This enzyme was chosen to supplement Lactosil in the degradation of *Nephrops* shell waste.

6.2 MATERIALS AND METHODS

6.2.1 Proteolytic Treatment of *Nephrops* Shell Waste

Experiments were set up as for microbial treatments except that an appropriate mass of the proteolytic enzyme bromelain (ex-Sigma, Poole, Dorset, Product Number B4882) (0 - 2.5% w/v) was added aseptically to each bottle/flask after addition of the sterile glucose and inoculant. Initial experiments were carried out at the 100ml level to avoid over use of the enzyme. The 100ml bottles were prepared as in section 5.2.2.1 using autoclaved shell waste but were fitted with bubble traps rather than screw-capped lids. Flasks (1 litre) were used in later experiments. These were prepared as in section 5.2.2.2. Tri-ammonium citrate was added in some runs at a concentration of 0.5% (w/v). The tri-ammonium citrate was dissolved in the 15% (w/v) glucose solution before filter sterilisation. All experiments were carried out in triplicate except where indicated otherwise.

6.2.2 Chemical Analyses of Solid Samples.

Ash, calcium, chitin, protein, carbon, hydrogen, nitrogen and moisture levels of solid residues were evaluated using the methods for the determination of chemical composition (section 3.2.2). The results reported for a final comparison of the different methods of chitin extraction were calculated on a dry weight basis (section 6.3.4.2 - Tables 6.12 - 6.18).

6.2.3 Amino Acid Analysis

6.2.3.1 Sample Hydrolysis

The sample (100mg) was placed in a hydrolysis tube. Boiling 6N hydrochloric acid (15ml) was added to the hydrolysis tube. The tube was evacuated, sealed and placed in a heating block that had been pre-heated to 110°C for 22 hours. The tube was removed from the block and cooled rapidly in a refrigerator or in cold water. The sample was quantitatively

washed into a 50ml volumetric flask containing 4.0ml norleucine solution (5.0μM/ml) and the volume made up to 50ml with distilled water. The contents of the volumetric flask were filtered through a sintered glass funnel (grade 3). The filtrate (5mls) was concentrated to dryness using a vacuum concentrator (Hetovac VR-1). The remainder of the filtrate was stored at 4°C.

The dried sample was dissolved in pH 2.2 loading buffer (4.0mls) and 20μl used for chromatography. Cysteine and methionine determinations were not accurate due to losses caused by the acid hydrolysis procedure.

6.2.3.2 Chromatography

Separation and quantification of amino acids was achieved on an amino acid analyser (Pharmacia Biochrom 20) using a Lithium High Resolution Programme (Pharmacia). The principle of the system used is outlined below.

The sample, containing a mixture of amino acids was loaded onto a column (high pressure PEEK column with Peltier heating/cooling system) of cation-exchange resin (Ultropac 8 cation-exchange resin, lithium form, particle size 9μm ± 0.5μm). Five lithium buffers of varying pH and ionic strength (composition - 'PCN 12', Pharmacia) were pumped through the column to separate the various amino acids. The column temperature was accurately controlled and varied as necessary to produce the required separation.

The column eluate was mixed with 'Ninhydrin' reagent and the mixture passed through a high temperature reaction coil. In the reaction coil ninhydrin reacted with the amino acids present in the eluant to form coloured compounds. The amount of coloured compound produced was directly proportional to the quantity of amino acid present in the eluant.

From the reaction coil, the eluant/ninhydrin mixture was fed to a photometer unit where the amount of each coloured compound was determined by measuring the amount of

light absorbed. The light absorption was measured at two wavelengths, 570nm and 440nm, because imino acids produce coloured compounds which absorb light with a wavelength of 440nm, whereas other amino acid coloured compounds absorb light at 570nm.

The photometer output was connected to a two-channel chart recorder, which plotted the amino acid concentrations as a series of peaks. The retention time of the peak on the chart identified the amino acid; the area under the peak indicated the quantity of the amino acid present. A calibration analysis was performed before commencing a series of analyses to produce a standard trace for comparison purposes. Peak areas were recorded and subsequent calculations performed on-line on a P.C. using Pharmacia's 'Peakmaster' software.

6.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was carried out according to the method in section 5.2.5.1.

6.2.5 Chemical Analyses of Culture Broth

All analysis of culture broth was carried out as in section 5.2.4.

6.2.6 Statistical Analysis

All statistical tests were carried out using GraphPad Prism software, version 3.0. Where three or more groups were compared one-way analysis of variance was followed by Tukey's post test. Two-way analysis of variance was used to compare three or more groups of data where the response was affected by two factors.

6.3 RESULTS

6.3.1 Determination of the Optimum Concentration of Proteolytic Enzyme Required to Deproteinise *Nephrops* Shell Waste

6.3.1.1 Effect of Bromelain Concentrations Between 0% and 2.5% (w/v)

Microbial treatment of *Nephrops* shell waste was carried out using 15% (v/v) Lactosil in conjunction with 0 - 2.5% (w/v) bromelain. The ash content of the bromelain treated product did not differ significantly from the levels of ash when bromelain was not included in the system (Fig. 6.1).

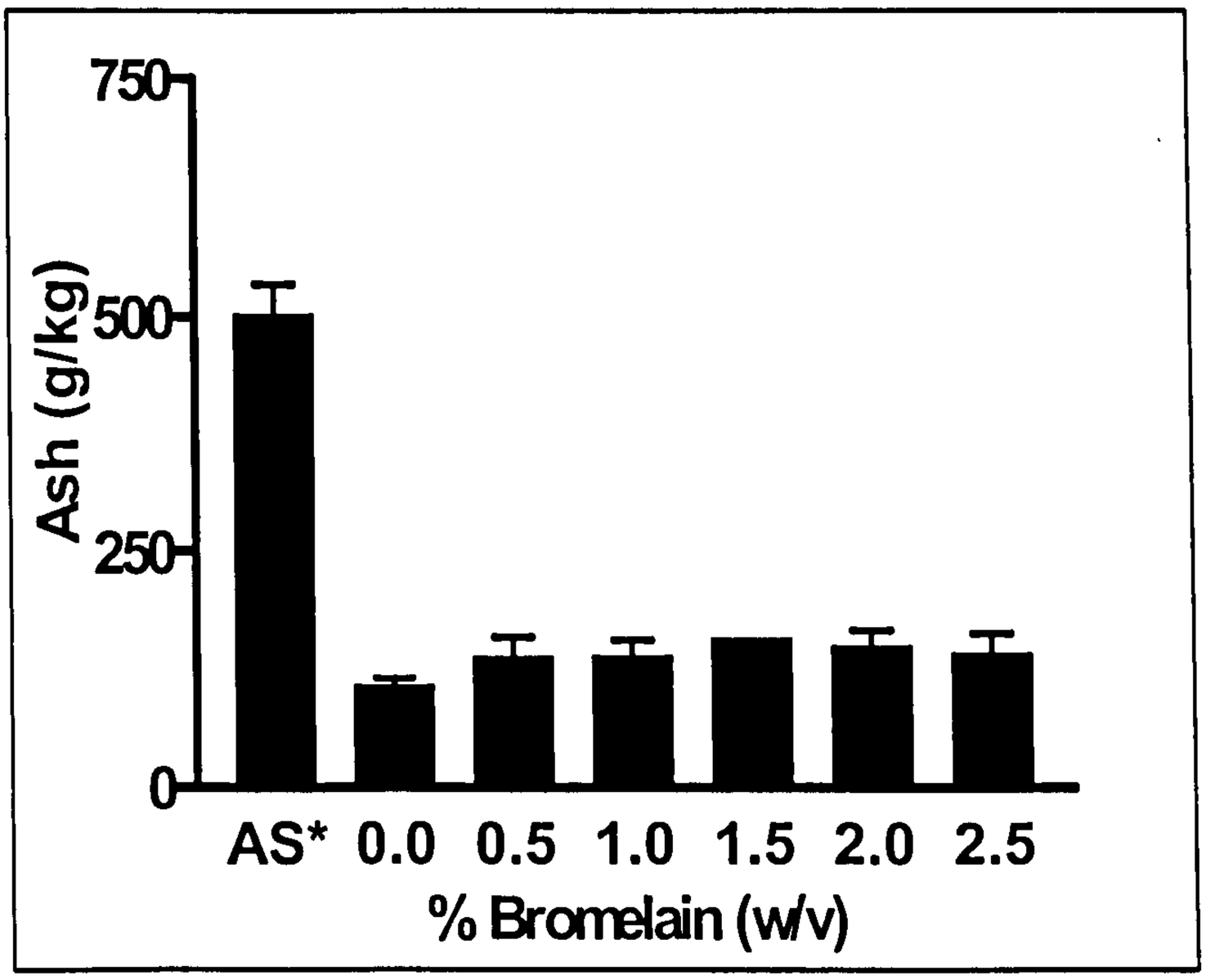


Fig. 6.1 *Effect of bromelain concentrations between 0 and 2.5% (w/v) on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Calcium content at 2.0% and 2.5% (w/v) bromelain was significantly higher than at 0% (w/v) bromelain ($p < 0.05$) (Fig. 6.2).

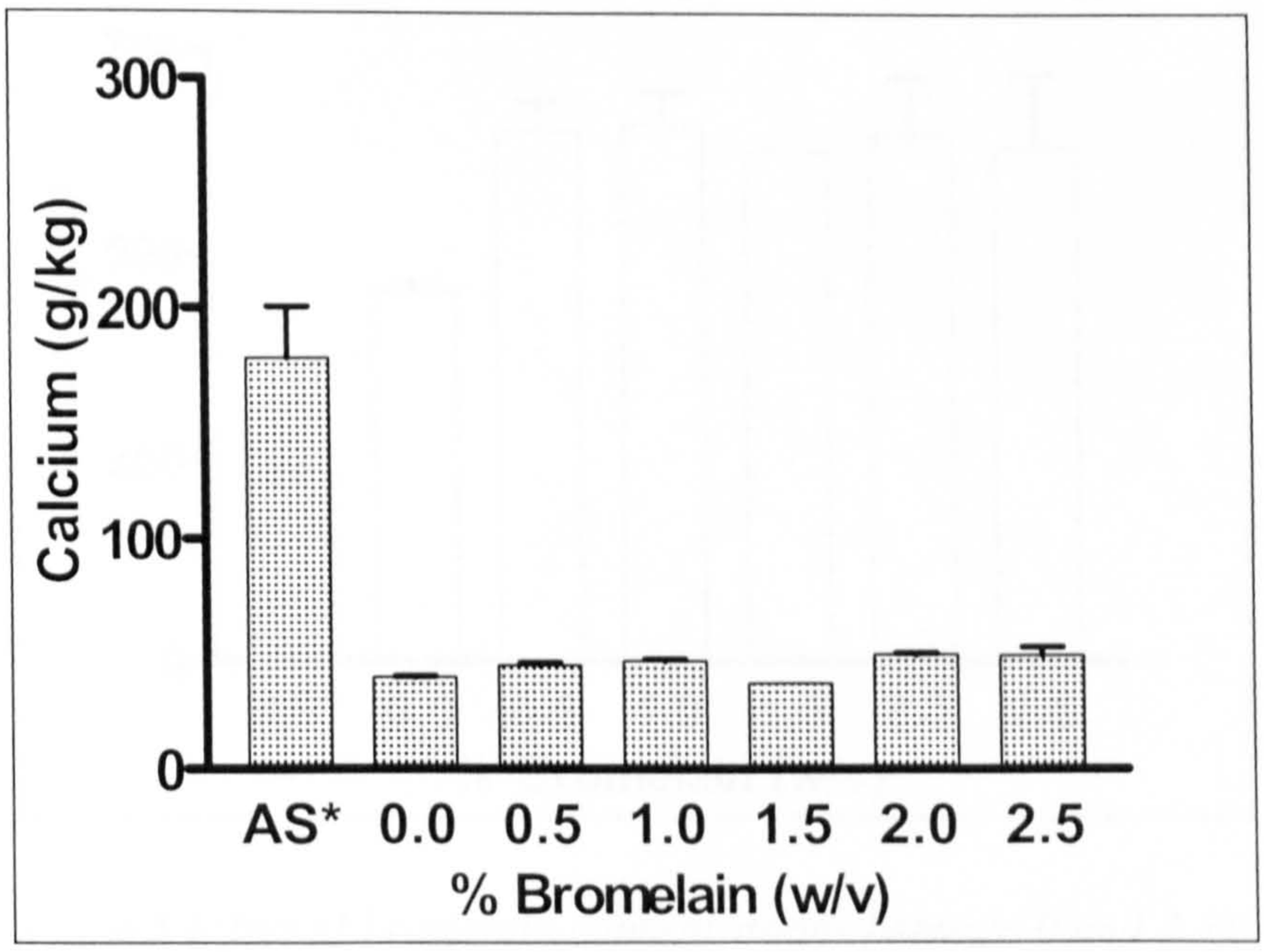


Fig. 6.2 *Effect of bromelain concentrations between 0 and 2.5% (w/v) on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

A concentration of 0.5% (w/v) bromelain caused an increase in the level of chitin in the final product when compared with a system using only Lactosil (0% w/v bromelain) ($p<0.05$) (Fig. 6.3). However, no further change in chitin was induced when the concentration of bromelain was increased to levels greater than 0.5% (w/v).

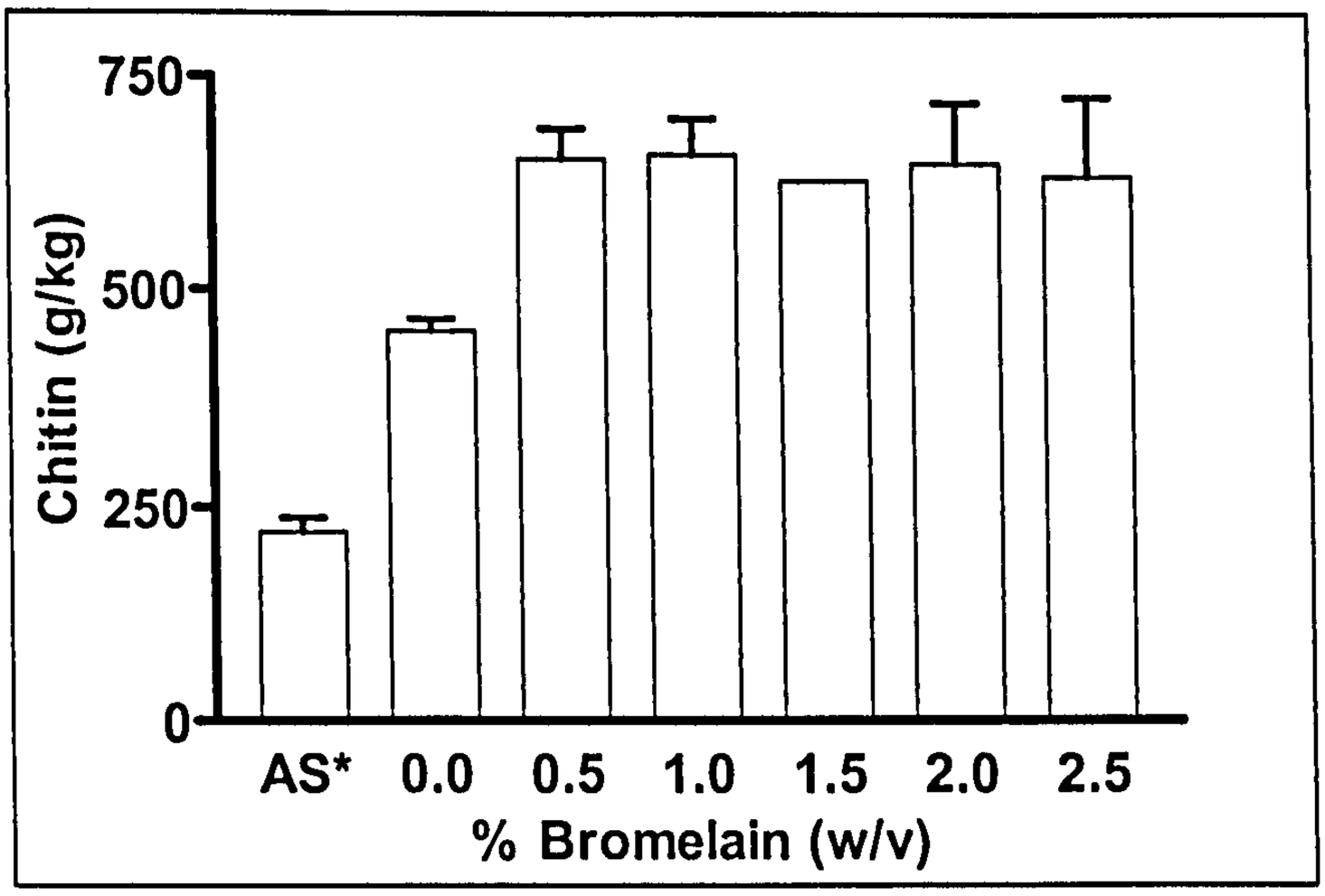


Fig. 6.3 *Effect of bromelain concentrations between 0 and 2.5% (w/v) on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

The nitrogen content of the shell was significantly reduced when concentrations of bromelain between 0.5 and 2.5% (w/v) were included in the system ($p<0.01$) (Table 6.1).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
0.0% (w/v) bromelain	8.35 ± 0.96
0.5% (w/v) bromelain	5.41 ± 0.03
1.0% (w/v) bromelain	5.31 ± 0.06
1.5% (w/v) bromelain	5.13 ± 0.00
2.0% (w/v) bromelain	5.30 ± 0.22
2.5% (w/v) bromelain	5.15 ± 0.42

Table 6.1 *Effect of bromelain concentrations between 0 and 2.5% (w/v) on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

The protein content of the shell was also lower after treatment with Lactosil in conjunction with bromelain, as opposed to Lactosil alone ($p<0.05$) (Fig. 6.4). However, increasing the bromelain concentration between 0.5 and 2.5% (w/v) led to no further change in protein levels.

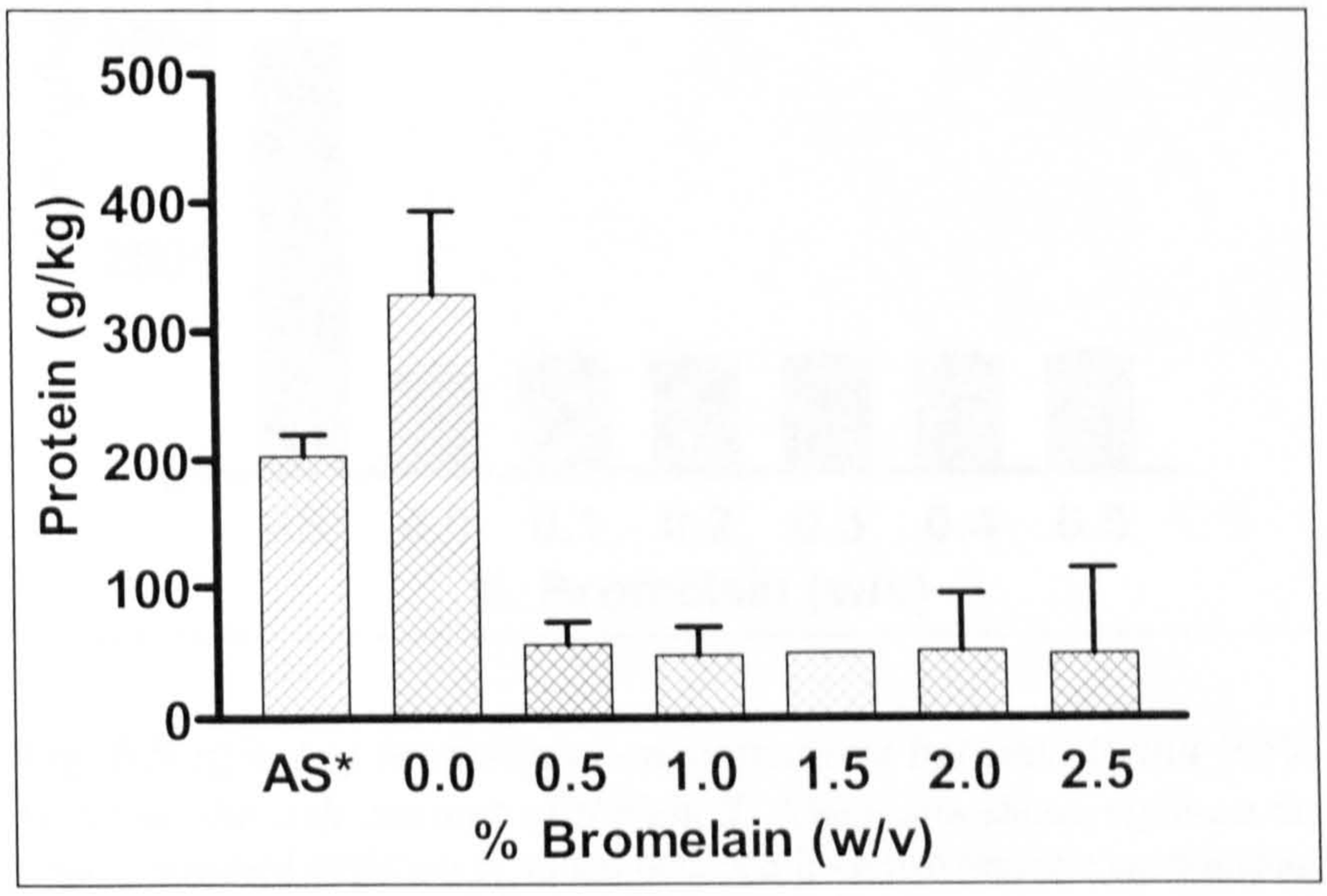


Fig. 6.4 *Effect of bromelain concentrations between 0 and 2.5% (w/v) on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

No moisture or lactic acid determinations were carried out for this experiment.

6.3.1.2 Effect of Bromelain Concentrations Between 0% and 0.5% (w/v)

The concentration of proteolytic enzyme was reduced to levels between 0 and 0.5% (w/v) because no significant difference in protein level in the shell was observed after treatment with bromelain at concentrations between 0.5 and 2.5% (w/v). Similar results were again obtained.

No significant difference was seen in ash levels (Fig. 6.5) or calcium levels (Fig. 6.6) when bromelain was added to the system.

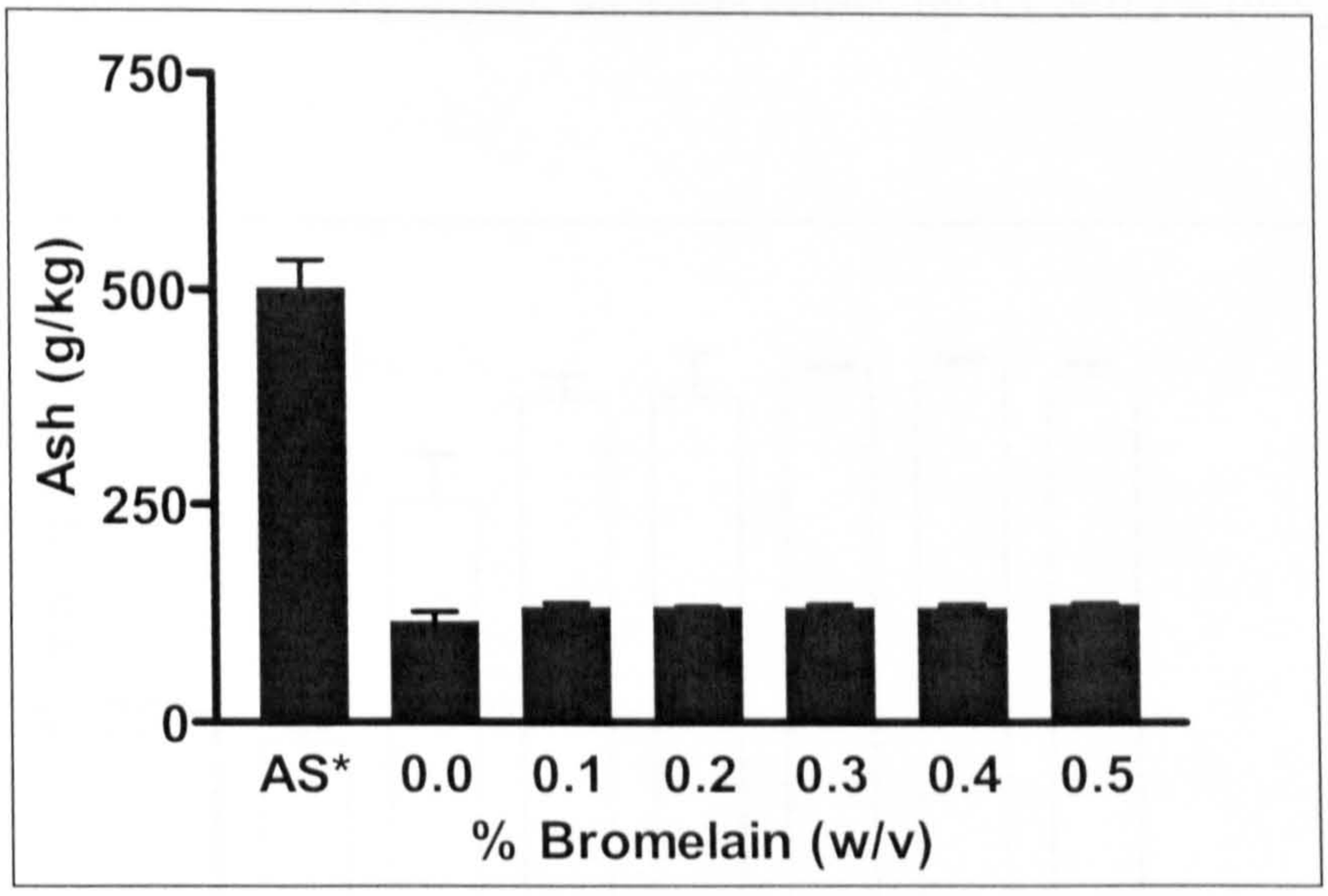


Fig. 6.5 *Effect of bromelain concentrations between 0 and 0.5% (w/v) on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

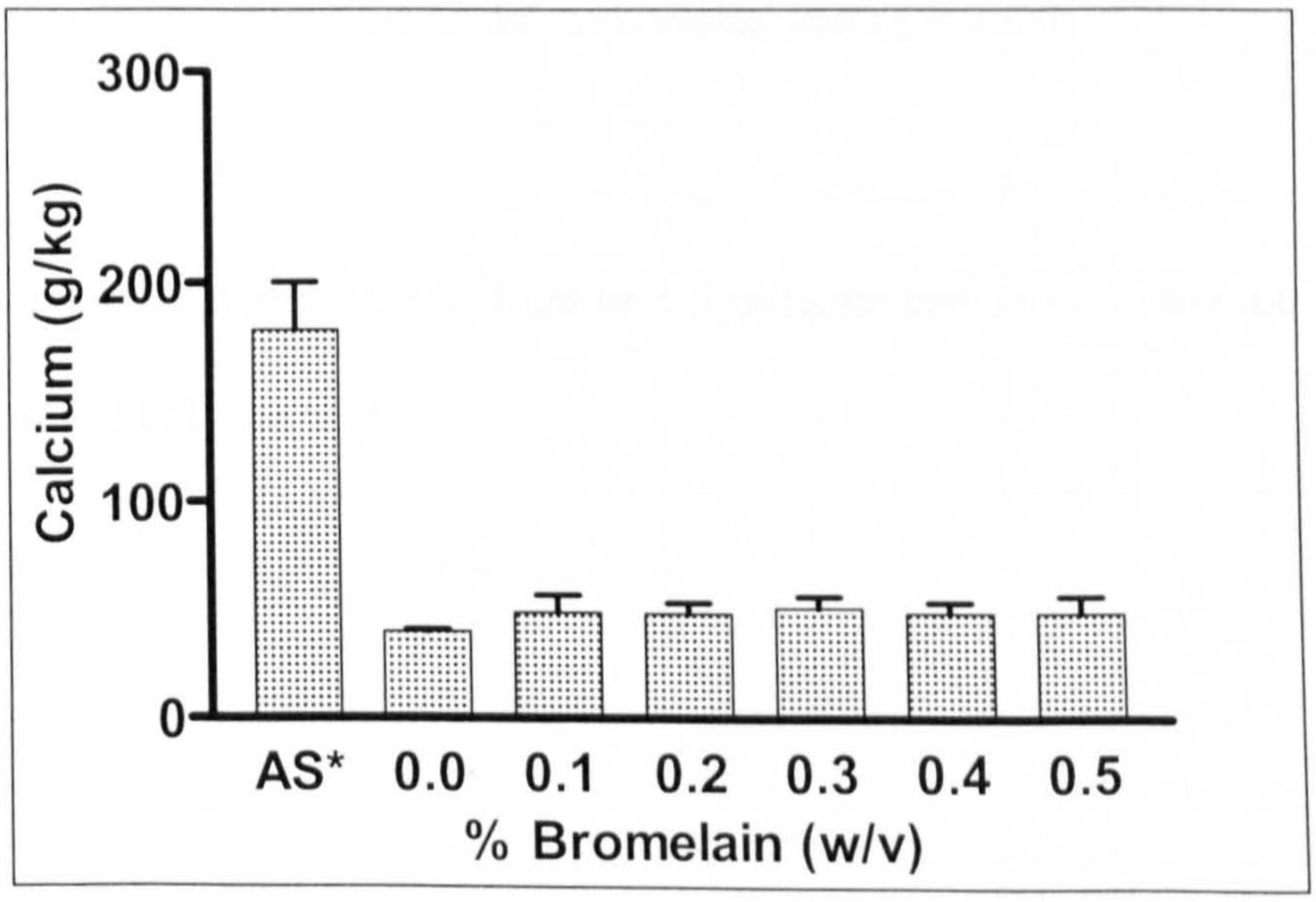


Fig. 6.6 *Effect of bromelain concentrations between 0 and 0.5% (w/v) on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Addition of 0.3 - 0.5% (w/v) bromelain to the lactic acid fermentation system caused chitin levels to increase ($p<0.05$) (Fig. 6.7). The variation in chitin determination at 0% (w/v) bromelain may have masked any significant effect caused by 0.1 or 0.2% (w/v) bromelain.

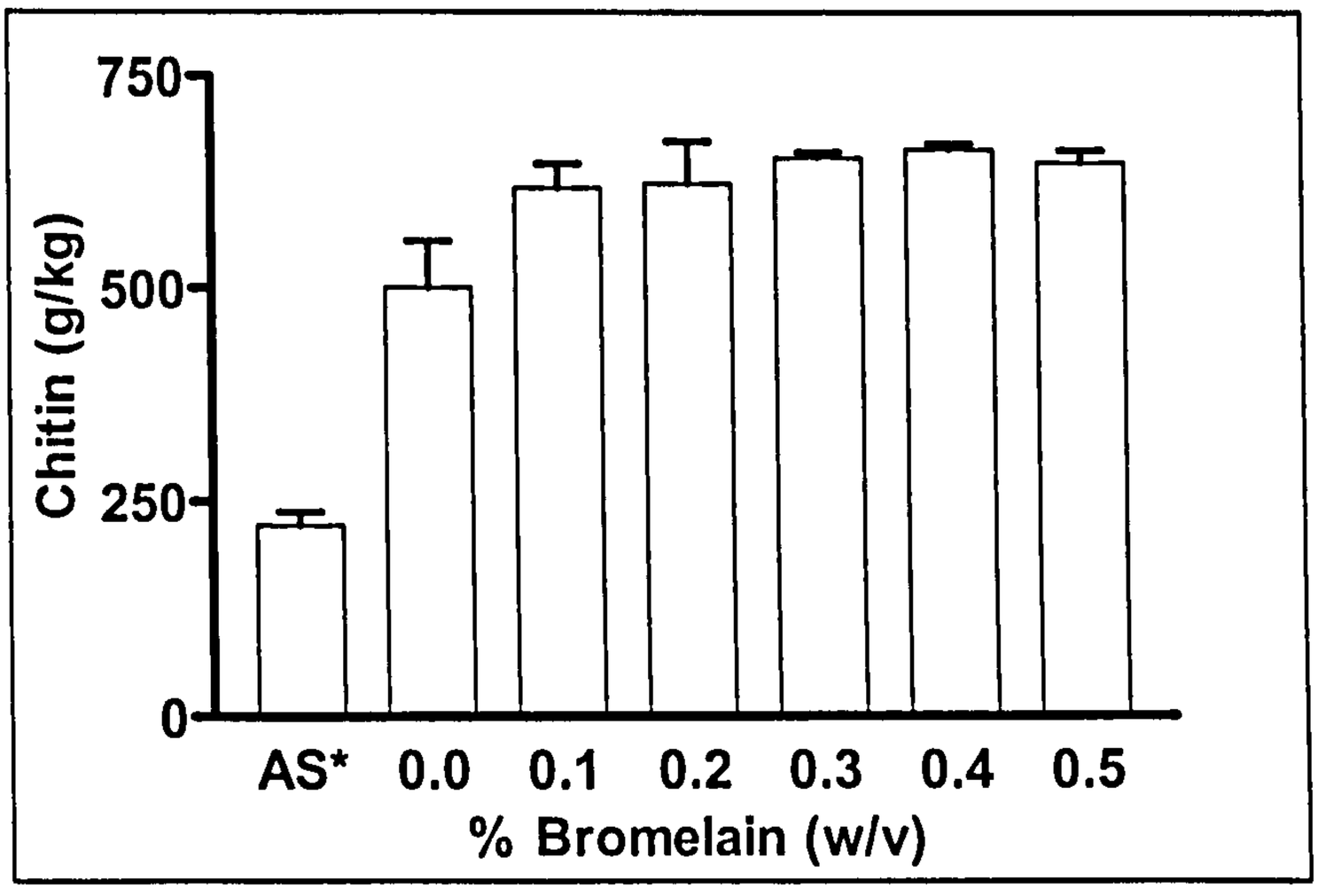


Fig. 6.7 *Effect of bromelain concentrations between 0 and 0.5% (w/v) on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

All concentrations of bromelain tested led to a significant reduction in the nitrogen content of the shell ($p<0.05$) (Table 6.2).

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
0.0% (w/v) bromelain	8.49 ± 0.38
0.1% (w/v) bromelain	5.65 ± 0.51
0.2% (w/v) bromelain	5.52 ± 0.87
0.3% (w/v) bromelain	5.77 ± 0.54
0.4% (w/v) bromelain	5.55 ± 0.33
0.5% (w/v) bromelain	6.00 ± 0.72

Table 6.2 *Effect of bromelain concentrations between 0 and 0.5% (w/v) on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Treatment with bromelain caused the protein levels to fall ($p<0.05$). However, increasing the bromelain concentration between 0.1 and 0.5% (w/v) led to no further change in protein levels (Fig. 6.8).

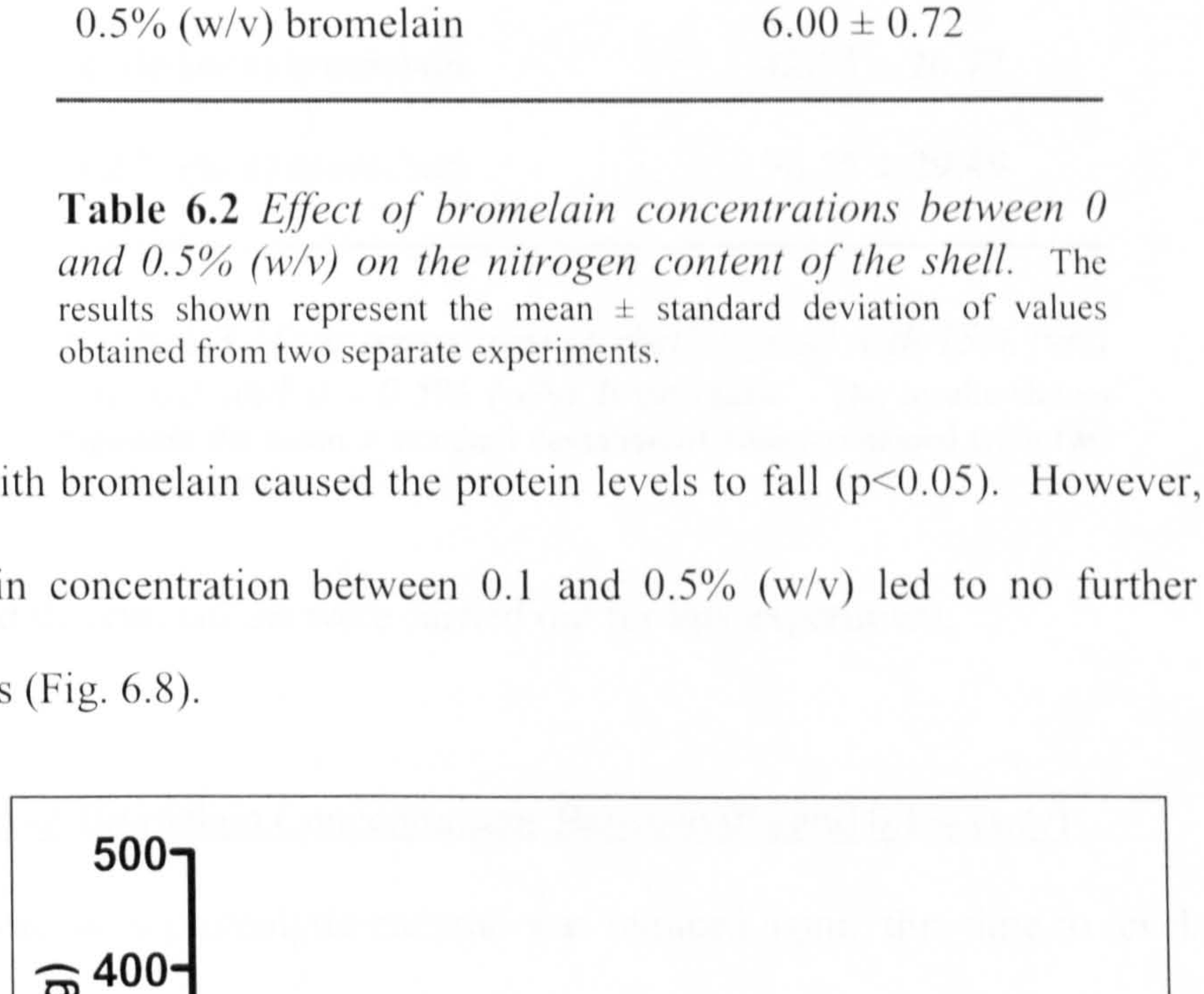


Fig. 6.8 *Effect of bromelain concentrations between 0 and 0.5% (w/v) on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

No significant difference was found between levels of moisture in samples treated with or without bromelain (Table 6.3).

Treatment	Moisture (g/kg)
Autoclaved Shell	55.07 ± 5.79
0.0% (w/v) bromelain	45.70 ± 32.39
0.1% (w/v) bromelain	37.40 ± 18.67
0.2% (w/v) bromelain	33.75 ± 17.18
0.3% (w/v) bromelain	39.30 ± 29.13
0.4% (w/v) bromelain	42.55 ± 26.77
0.5% (w/v) bromelain	36.55 ± 29.49

Table 6.3 *Moisture content of shell treated with 15% (v/v) Lactosil and 0 - 0.5% (w/v) bromelain.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

No lactic acid determinations were carried out for this experiment.

6.3.1.3 Effect of Bromelain Concentrations Between 0% and 0.1% (w/v)

The concentration of proteolytic enzyme was reduced again, this time to levels between 0 and 0.1% (w/v) since no significant difference in protein levels was observed at bromelain concentrations between 0.1 and 0.5% (w/v).

Lactic acid levels increased significantly at all levels of bromelain added ($p<0.001$) (Fig. 6.9).

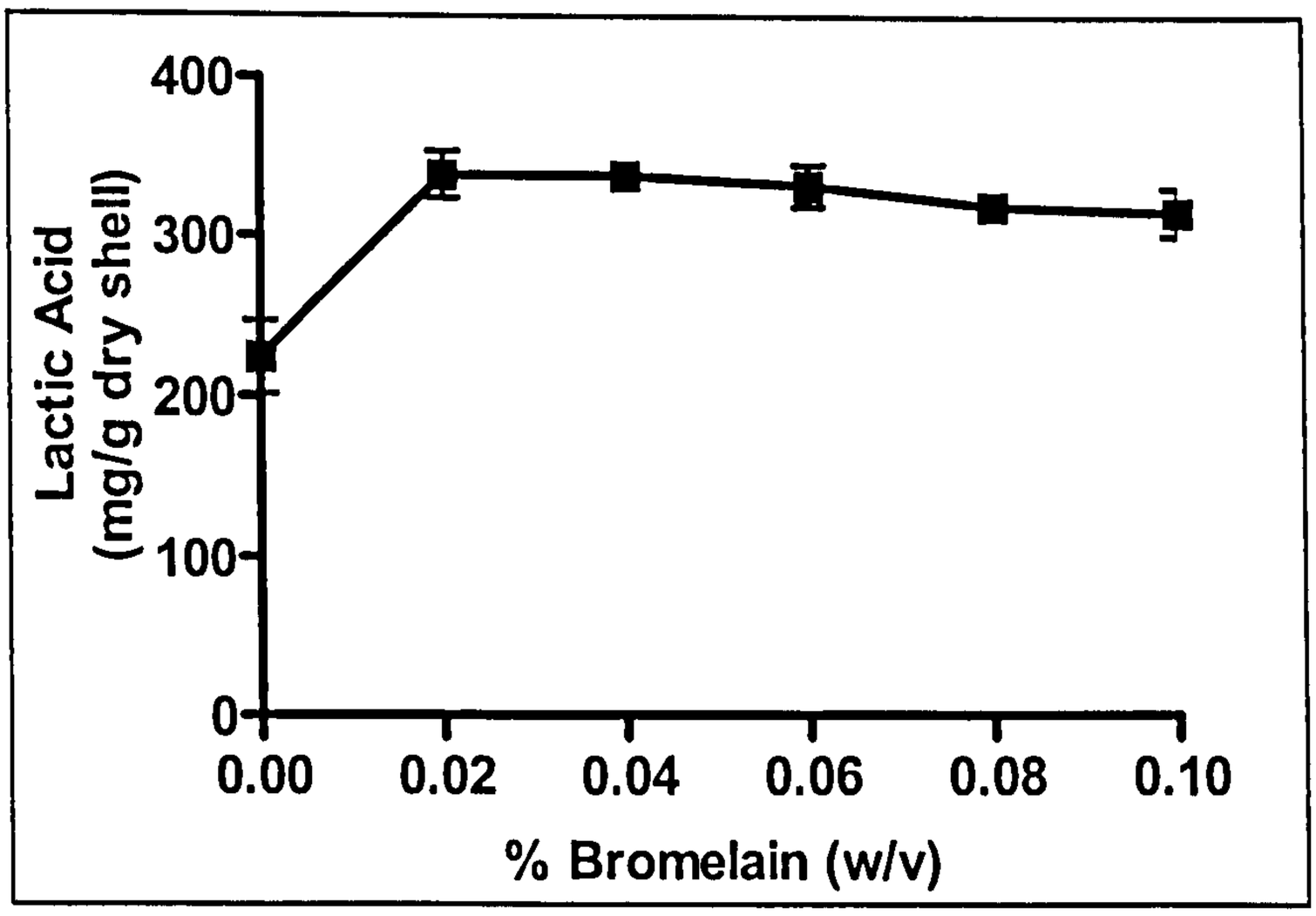


Fig. 6.9 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the production of lactic acid.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Despite this increase in lactic acid no significant change was noted in levels of ash (Fig. 6.10) or calcium (Fig. 6.11) when bromelain was added to the system.

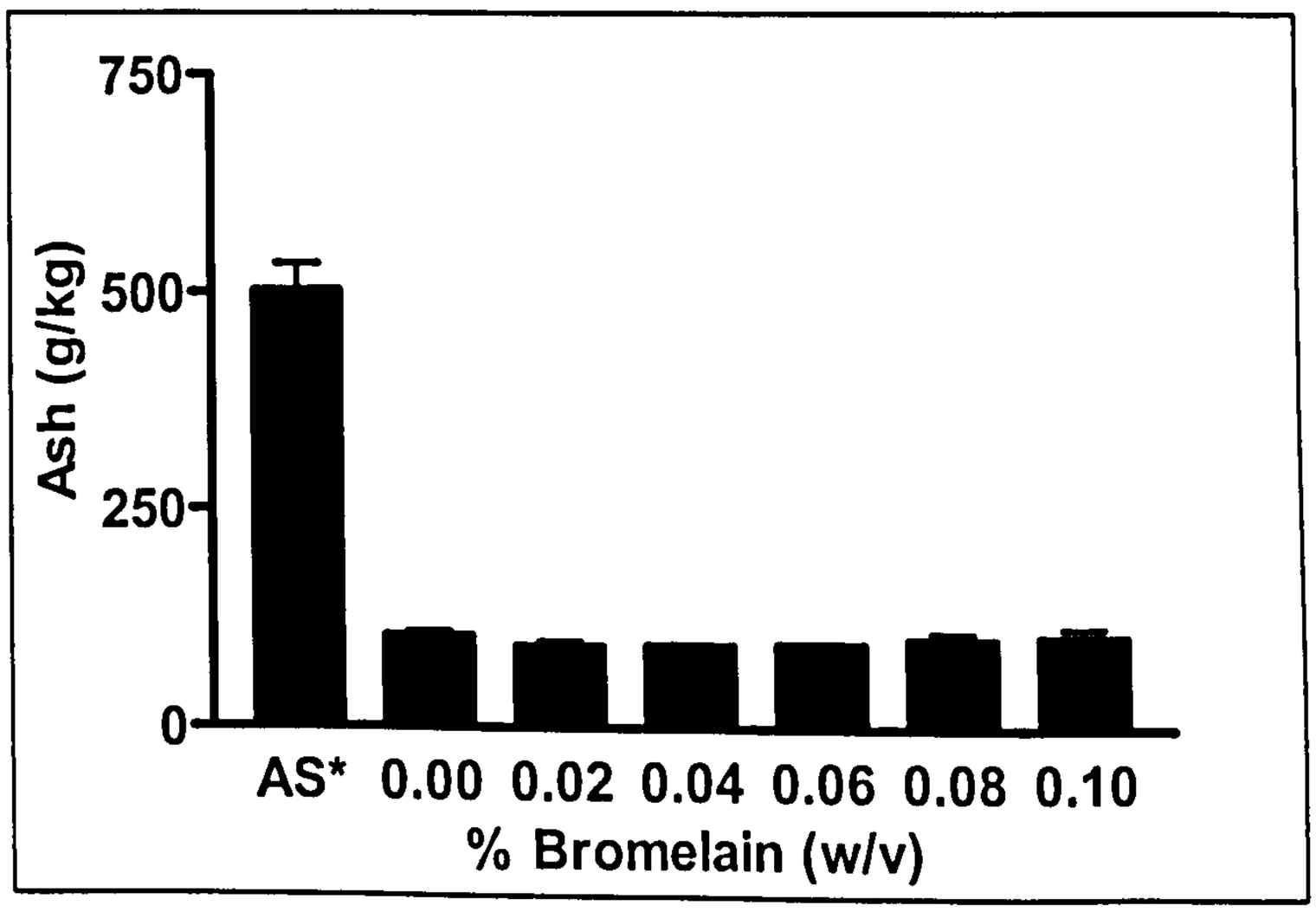


Fig. 6.10 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

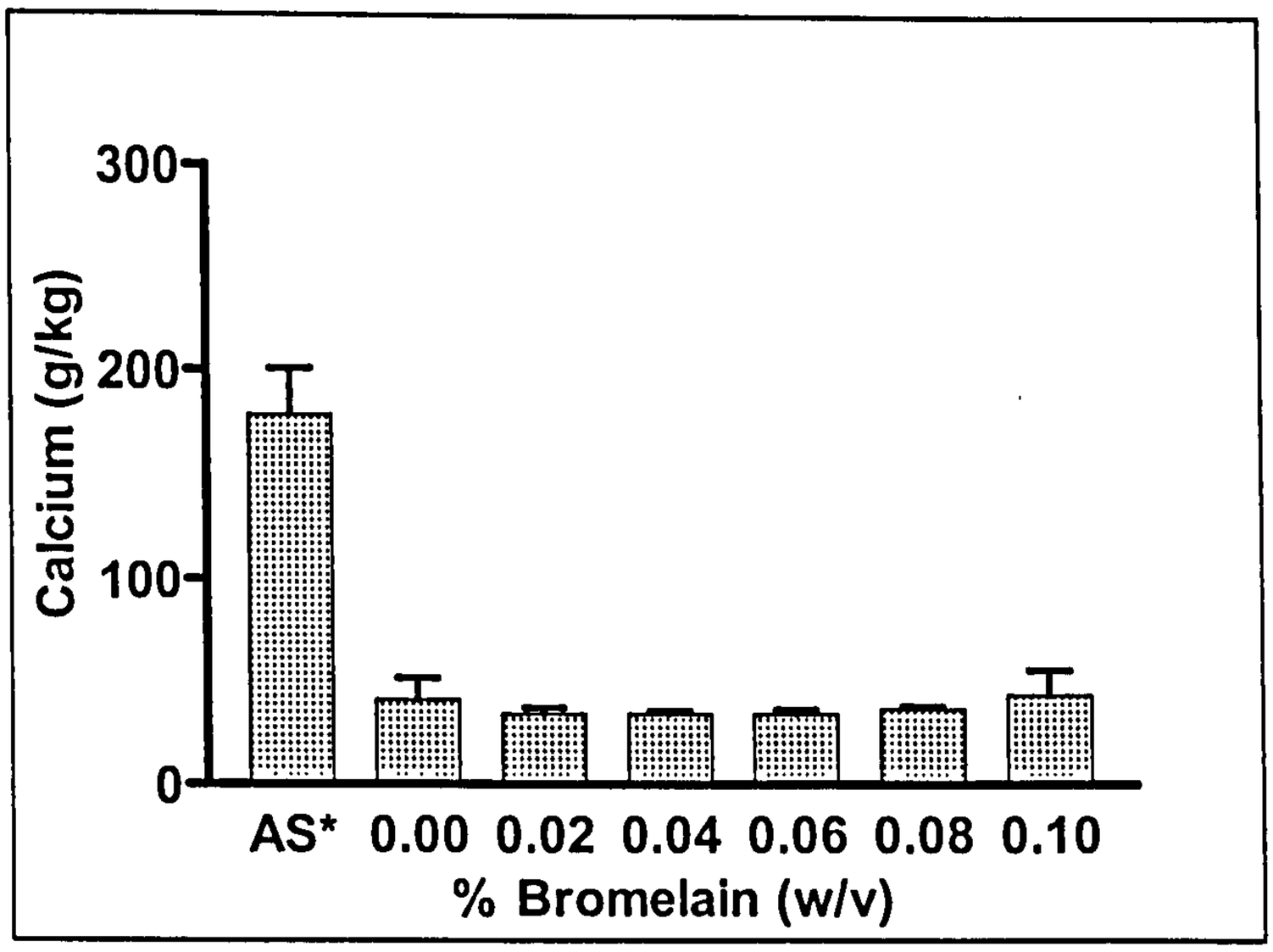


Fig. 6.11 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

Chitin levels increased significantly when bromelain at concentrations greater than 0.04% (w/v) were added to the system ($p<0.05$) (Fig. 6.12).

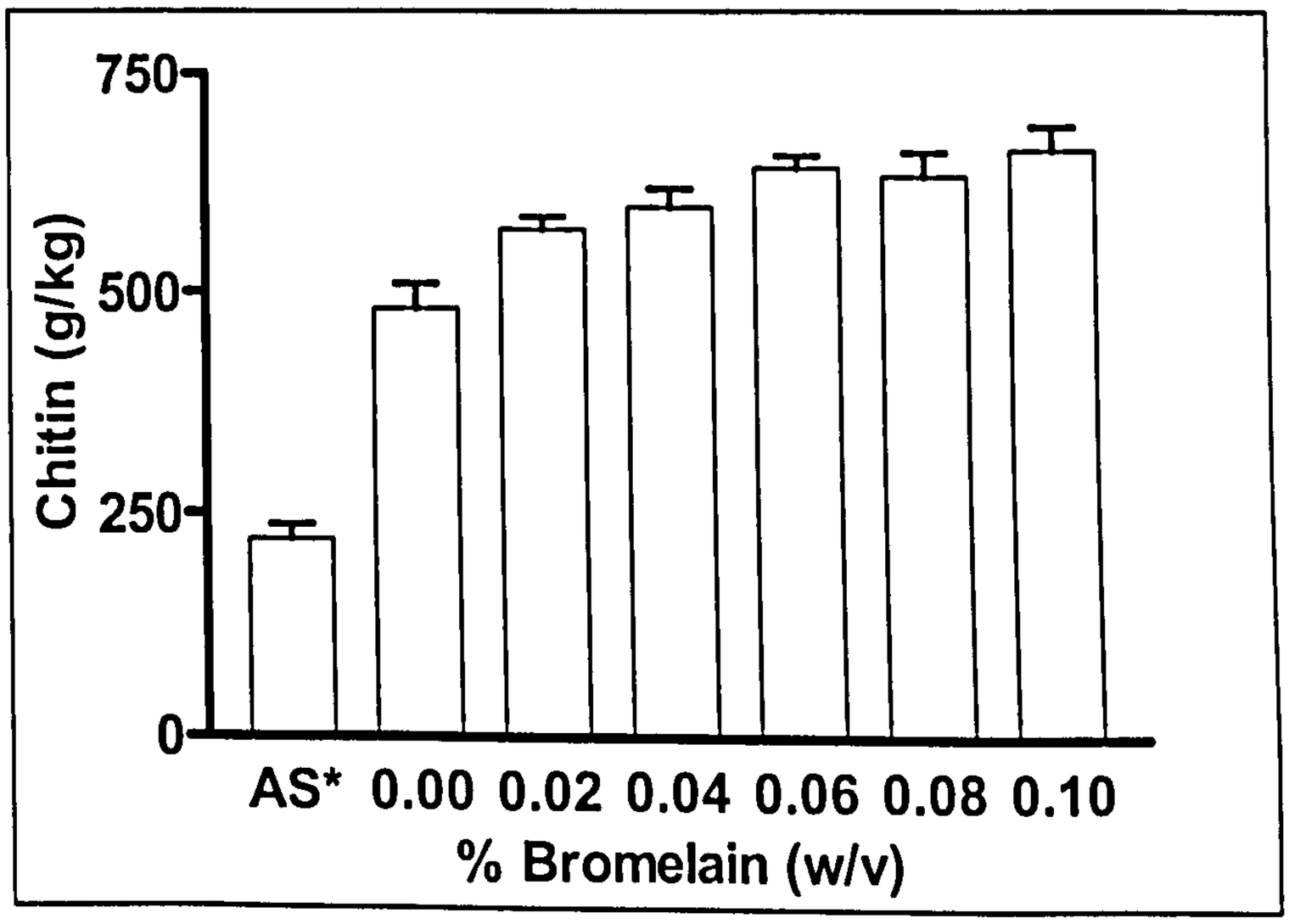


Fig. 6.12 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

No change in nitrogen levels occurred when 0.02 - 0.10% (w/v) bromelain was added to the fermentation system (Table 6.4) whereas in the previous experiment (section 6.3.1.2) 0.1% (w/v) bromelain did cause a significant decrease in nitrogen level.

Treatment	% Nitrogen (w/w)
Autoclaved Shell	4.78 ± 0.25
0.00% (w/v) bromelain	7.97 ± 0.76
0.02 % (w/v) bromelain	7.66 ± 0.16
0.04% (w/v) bromelain	6.68 ± 0.21
0.06% (w/v) bromelain	6.70 ± 0.30
0.08% (w/v) bromelain	6.70 ± 0.42
0.10% (w/v) bromelain	6.76 ± 0.91

Table 6.4 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Despite wide variation being present between duplicate protein results, 0.1% (w/v) bromelain caused a significant reduction in protein content of the final product compared with 0% (w/v) bromelain ($p<0.05$) (Fig. 6.13).

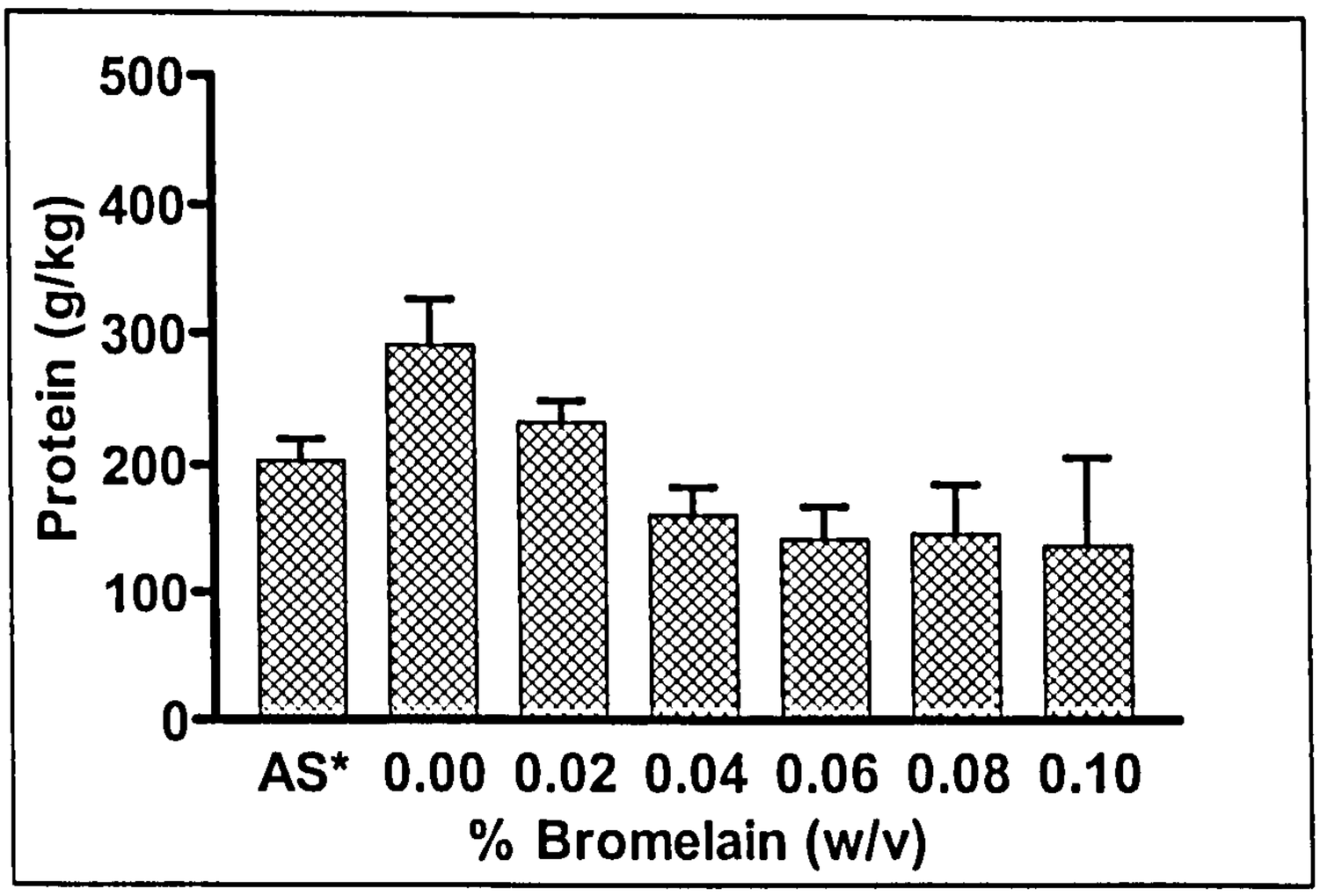


Fig. 6.13 *Effect of bromelain concentrations between 0 and 0.1% (w/v) on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments. *AS = autoclaved shell (original starting material)

No significant variation in moisture content occurred between samples treated with or without bromelain added to the lactic acid fermentation system (Table 6.5).

Treatment	Moisture Content (g/kg)
Autoclaved Shell	55.07 ± 5.79
0.00% (w/v) bromelain	68.75 ± 2.05
0.02% (w/v) bromelain	57.30 ± 7.64
0.04% (w/v) bromelain	60.85 ± 10.82
0.06% (w/v) bromelain	57.05 ± 10.96
0.08% (w/v) bromelain	62.10 ± 0.42
0.10% (w/v) bromelain	63.60 ± 3.68

Table 6.5 *Moisture content of shell treated with 15% (v/v) Lactosil and 0 - 0.1% (w/v) bromelain.* The results shown represent the mean ± standard deviation of values obtained from two separate experiments.

Due to the variation obtained in the protein determinations it was not possible to determine if concentrations of bromelain less than 0.1% (w/v) had an effect on the protein content of the shell. Therefore, it was decided to use 0.1% (w/v) bromelain in future experiments.

6.3.2 Effect of 0.1% (w/v) Bromelain in One Litre Lactic Acid Fermentation Systems

The effect of bromelain, at a concentration of 0.1% (w/v), used in conjunction with Lactosil, was examined at the 1 litre level and compared with the effects of Lactosil on its own. The experiment was carried out at 30°C and 40°C.

Lactic acid production was increased by the addition of 0.1% (w/v) bromelain at both 30°C ($p = 0.0022$) and 40°C ($p<0.0001$) (Fig. 6.14). However, the amount of lactic acid produced was lower at the higher temperature, with or without bromelain added to the lactic acid fermentation system.

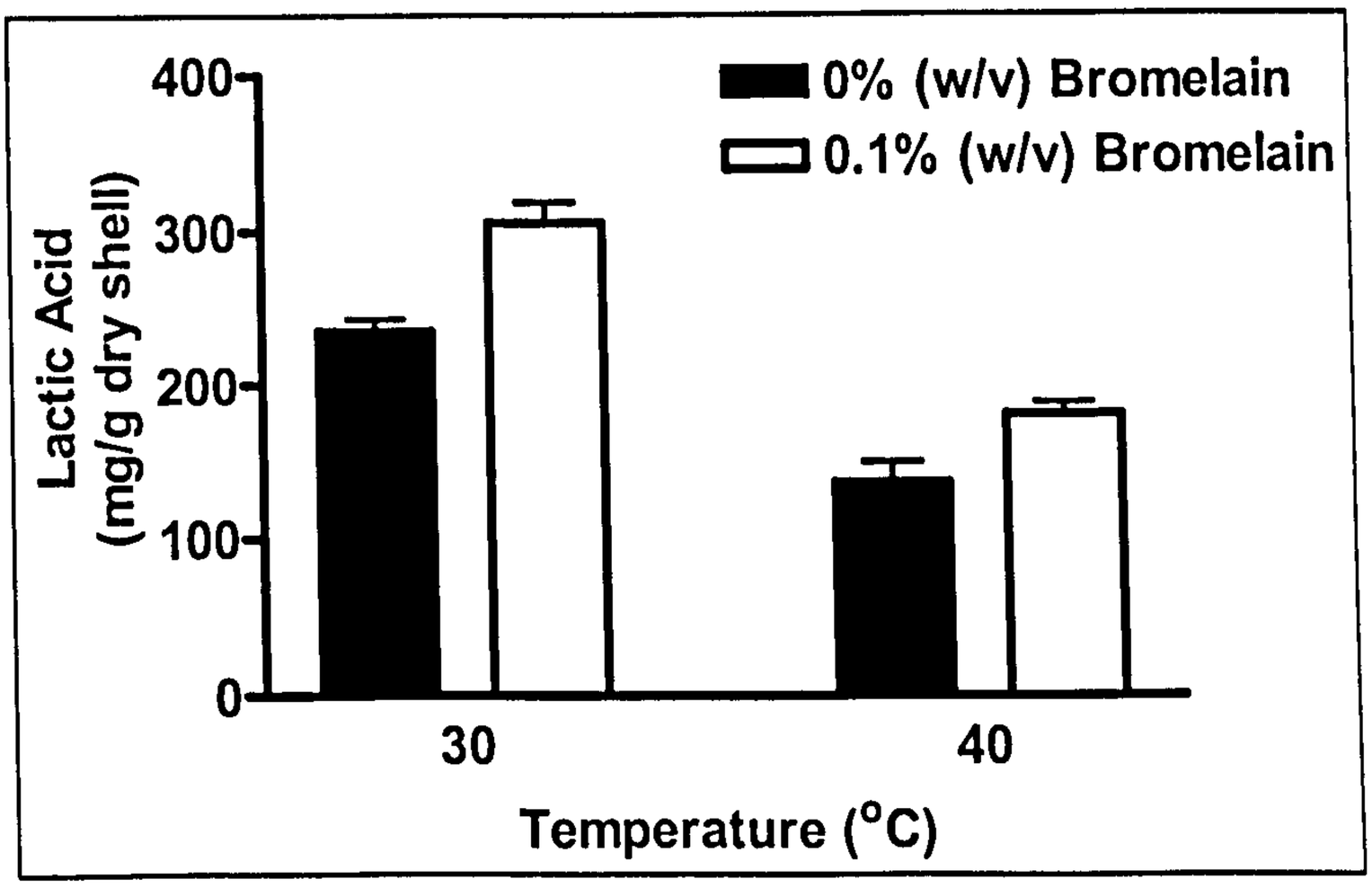


Fig. 6.14 *Effect of 0.1% (w/v) bromelain on lactic acid production, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Addition of 0.1% (w/v) bromelain caused a slight increase in ash at 30°C ($p < 0.05$) (Fig. 6.15).

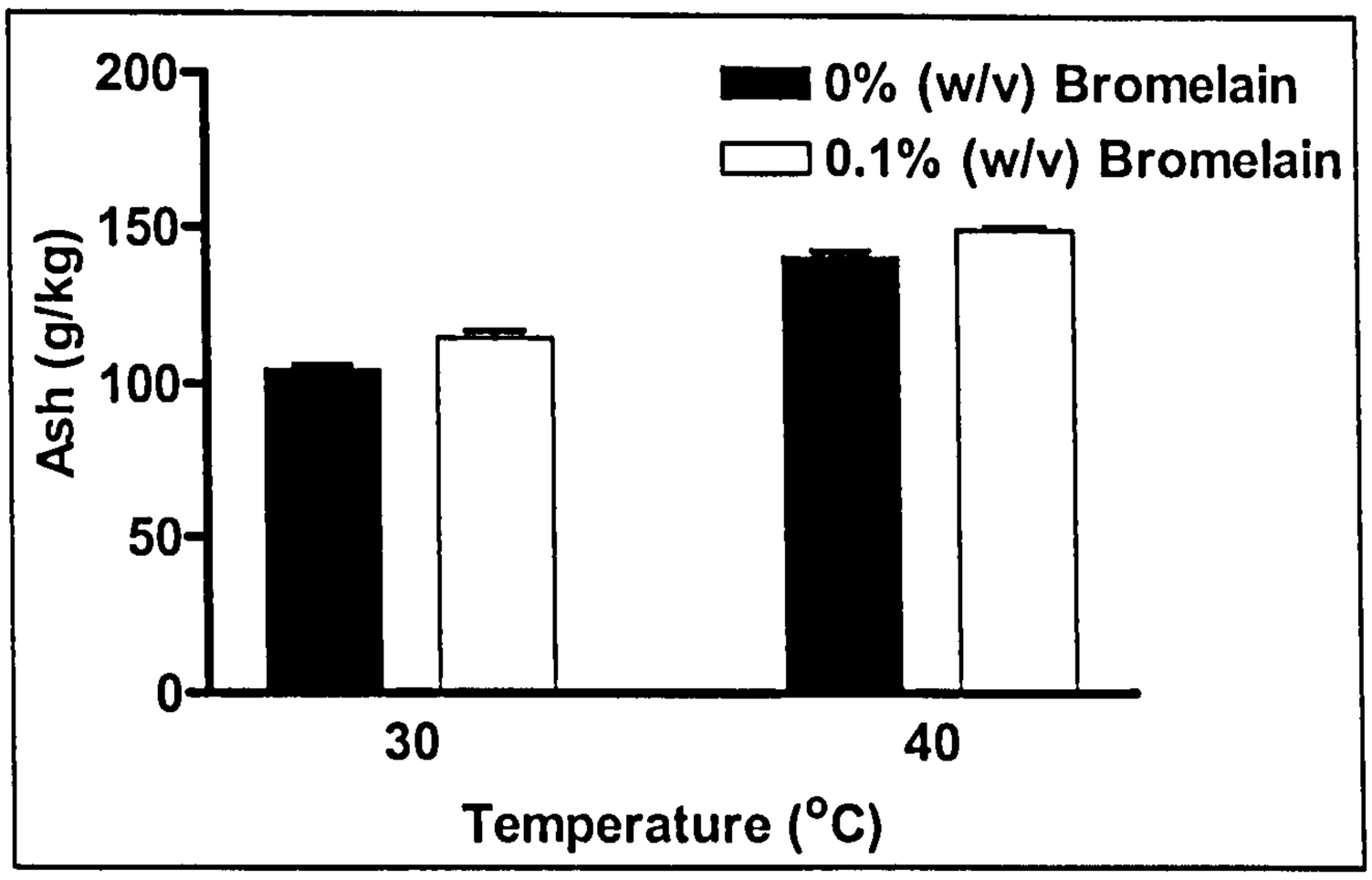


Fig. 6.15 *Effect of 0.1% (w/v) bromelain on the ash content of the shell, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Levels of calcium were not affected by the addition of bromelain at either 30°C or 40°C (Fig. 6.16).

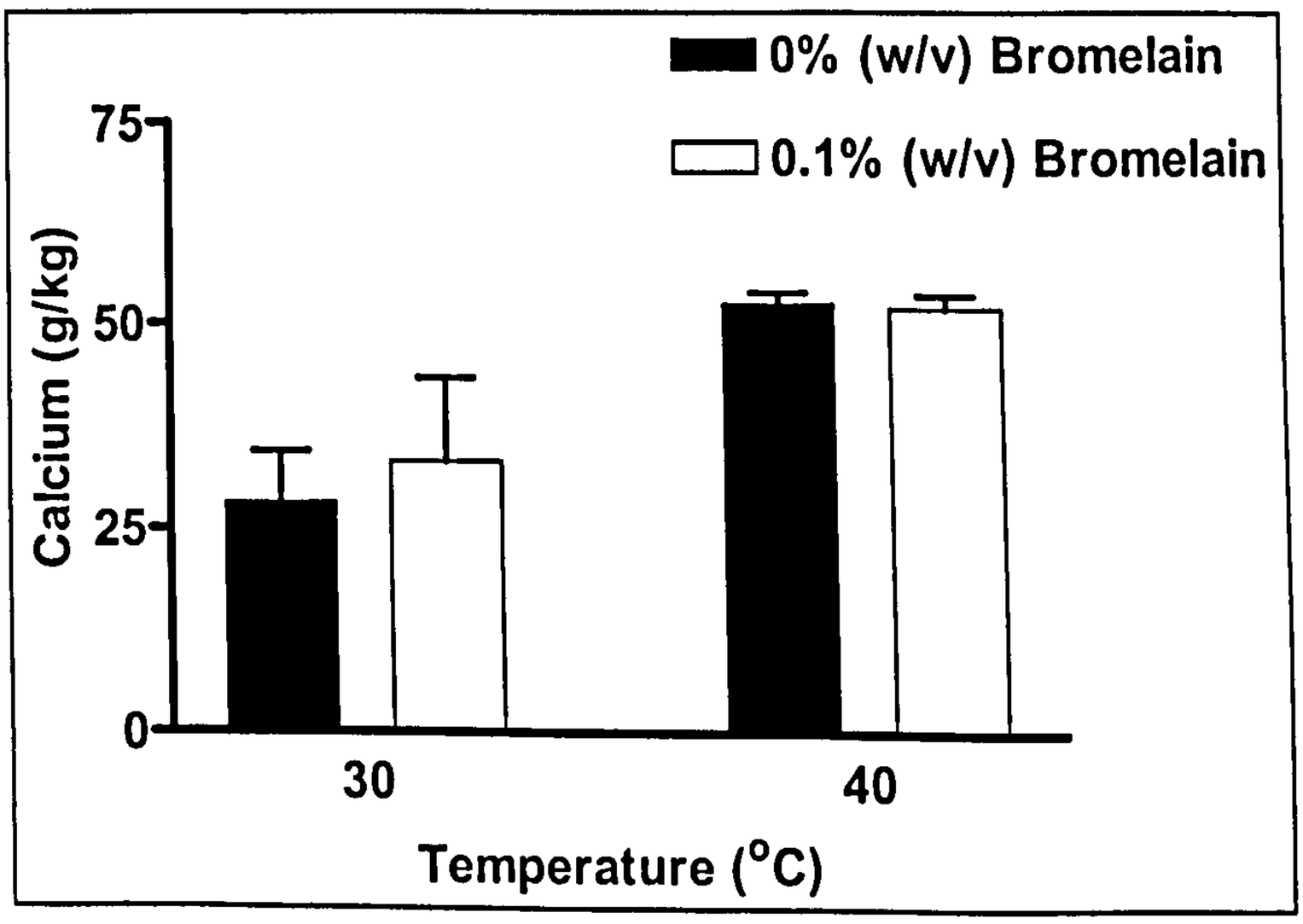


Fig. 6.16 *Effect of 0.1% (w/v) bromelain on the calcium content of the shell, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Chitin levels were increased significantly by the addition of 0.1% (w/v) bromelain at 30°C ($p<0.0001$) (Fig. 6.17). This same increase was not seen at 40°C.

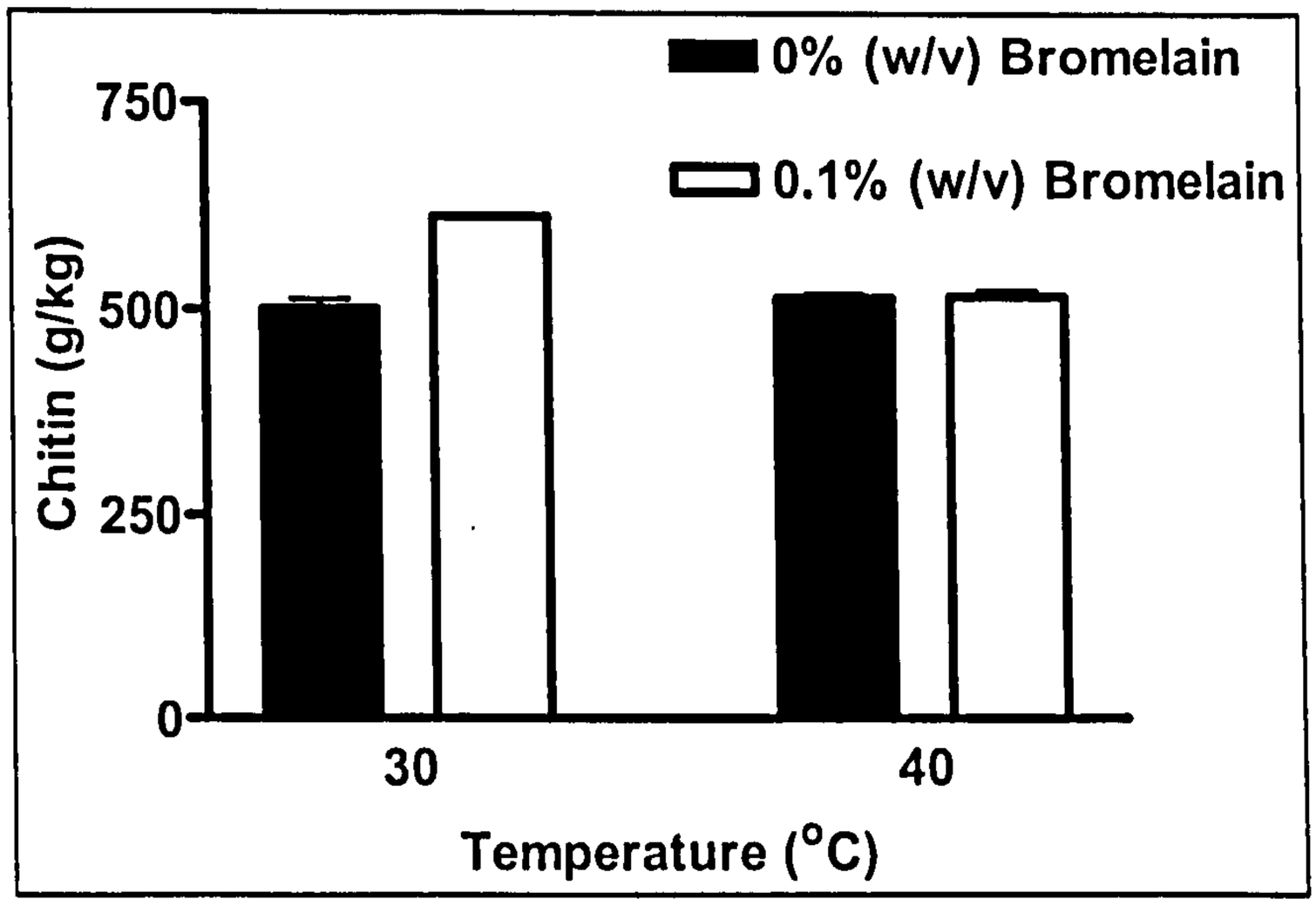


Fig. 6.17 *Effect of 0.1% (w/v) bromelain on the chitin content of the shell, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Percentage nitrogen values are shown in Table 6.6. Addition of bromelain led to lower nitrogen levels at 30°C ($p<0.001$) and at 40°C ($p<0.05$) unlike the previous experiment (Table 6.4).

Treatment	% Nitrogen (w/w)
0% (w/v) bromelain at 30°C	8.13 ± 0.27
0.1% (w/v) bromelain at 30°C	6.38 ± 0.19
0% (w/v) bromelain at 40°C	7.05 ± 0.51
0.1% (w/v) bromelain at 40°C	6.16 ± 0.26

Table 6.6 *Effect of 0.1% (w/v) bromelain on the nitrogen content of the shell, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Protein levels were reduced by the addition of 0.1% (w/v) bromelain at 30°C ($p<0.001$) and at 40°C ($p<0.05$) (Fig. 6.18). At 30°C the protein content was reduced by 15.8% (w/w). Final protein levels were similar at 30°C and at 40°C.

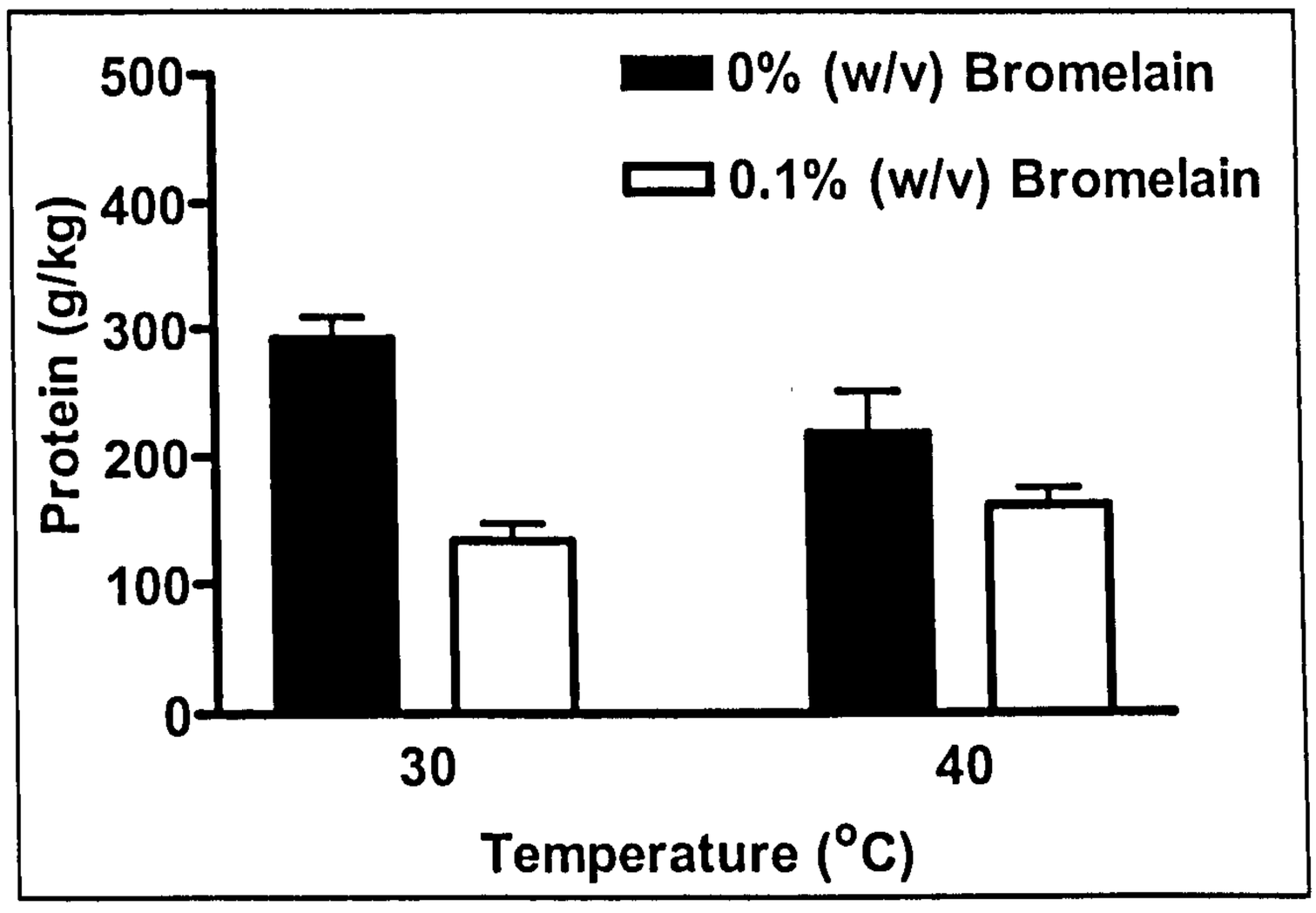


Fig. 6.18 *Effect of 0.1% (w/v) bromelain on the protein content of the shell, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

No variation in moisture levels occurred between the different samples (Table 6.7).

Treatment	Moisture (g/kg)
0.0% (w/v) bromelain at 30°C	62.87 ± 3.47
0.1% (w/v) bromelain at 30°C	62.10 ± 0.87
0.0% (w/v) bromelain at 40°C	60.70 ± 0.71
0.1% (w/v) bromelain at 40°C	59.13 ± 2.40

Table 6.7 *Moisture content of shell treated (a) with and (b) without bromelain, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The final percentage yield was similar at both 30°C and 40°C (Fig. 6.19).

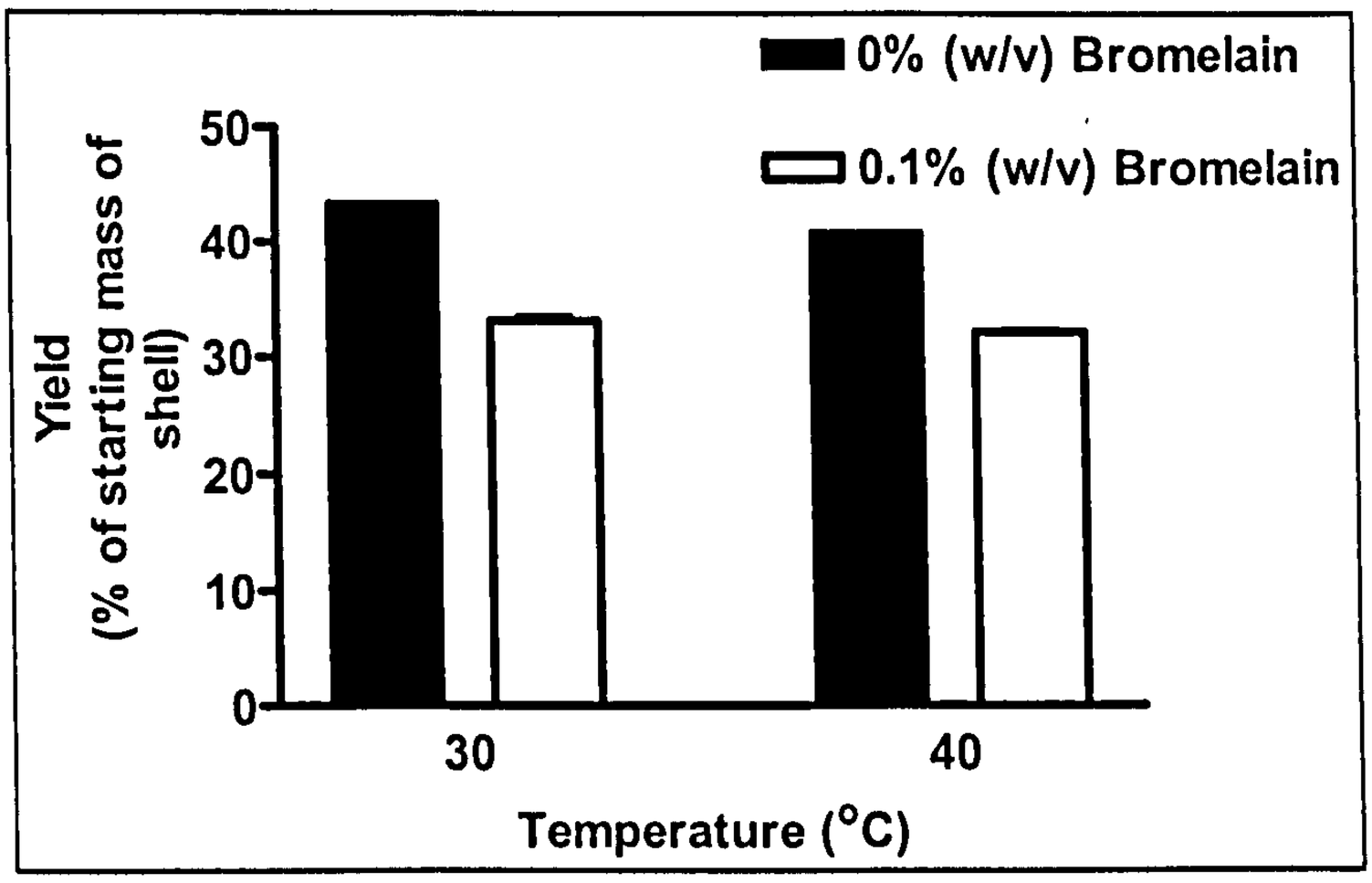


Fig. 6.19 *Effect of 0.1% (w/v) bromelain on the percentage yield, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The chemical composition of the final product obtained after addition of 0.1% (w/v) bromelain to the lactic acid fermentation system is shown in Table 6.8. The product at 30°C contained less ash and protein than the product at 40°C.

Component (g/kg)	30°C	40°C
Chitin	613.33 ± 2.89	516.67 ± 7.64
Ash	114.68 ± 2.39	149.60 ± 1.02
Protein	134.04 ± 12.95	161.98 ± 13.33
Moisture	62.10 ± 0.86	59.13 ± 2.40

Table 6.8 *The chemical composition of shell treated with 15% (v/v) Lactosil and 0.1% (w/v) bromelain, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

However, the product at 30°C still contained considerable concentrations of ash (114.68 ± 2.39g/kg) and protein (134.04 ± 12.95g/kg). Therefore, an attempt was made to alter the metabolism of the bacteria contained in the Lactosil inoculant, in the hope that the removal of more ash and protein could be induced. The lactic acid fermentation system contained no readily available source of protein for the bacteria at the start of fermentation. The growth of lactic acid bacteria depends on the presence of an adequate nitrogen supply (El Soda *et al.*, 1986) therefore it was possible that the bacteria were limited by a lack of nitrogen. Tri-ammonium citrate (0.5% w/v) was added to the system, in conjunction with bromelain at 0.1% (w/v), to provide the bacteria with an additional source of nitrogen.

6.3.3 Addition of Tri-Ammonium Citrate to the Lactic Acid Fermentation System

The addition of tri-ammonium citrate to the fermentation system led to an increase in lactic acid production at 40°C ($p<0.001$) (Fig. 6.20). This increase was not so apparent at 30°C.

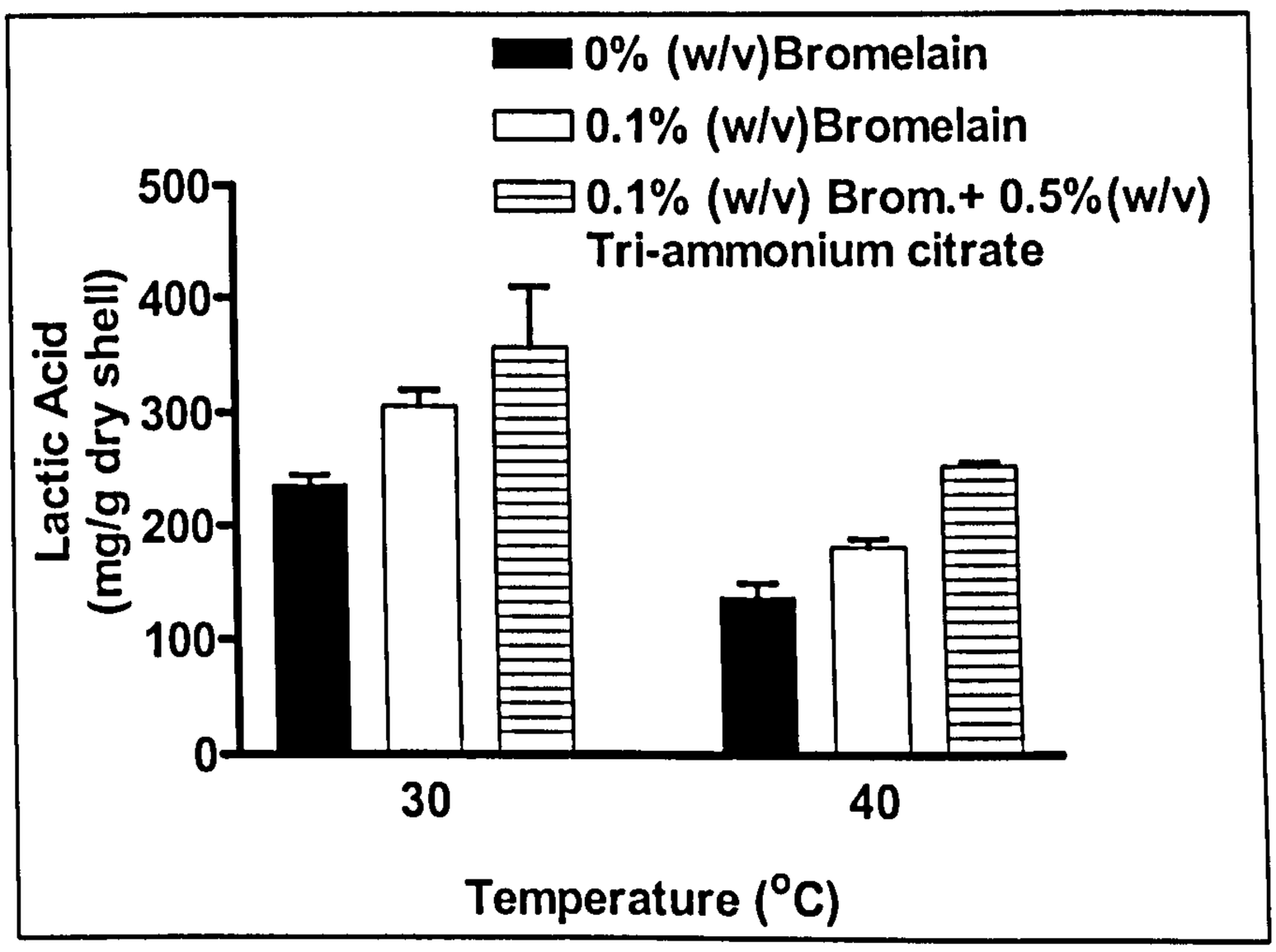


Fig. 6.20 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the fermentation system, at 30°C and 40°C, on the production of lactic acid.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

However, ash levels fell on the addition of tri-ammonium citrate at 30°C ($p<0.001$) and at 40°C ($p<0.01$) (Fig. 6.21).

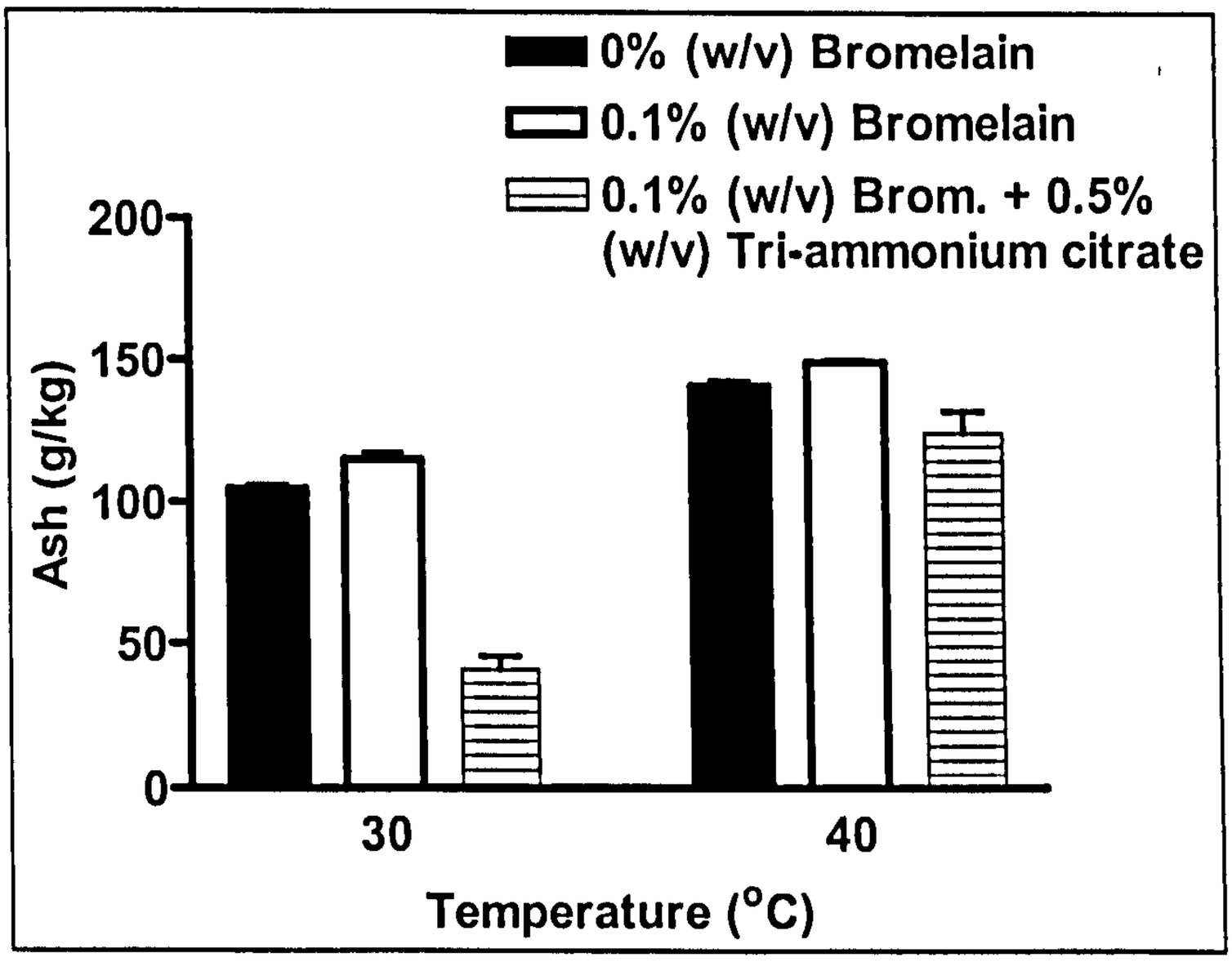


Fig. 6.21 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C, on the ash content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Calcium levels were reduced by the addition of tri-ammonium citrate at 40°C ($p<0.01$) but not at 30°C (Fig. 6.22). Variation for calcium results was wide at 30°C for samples treated with and without bromelain.

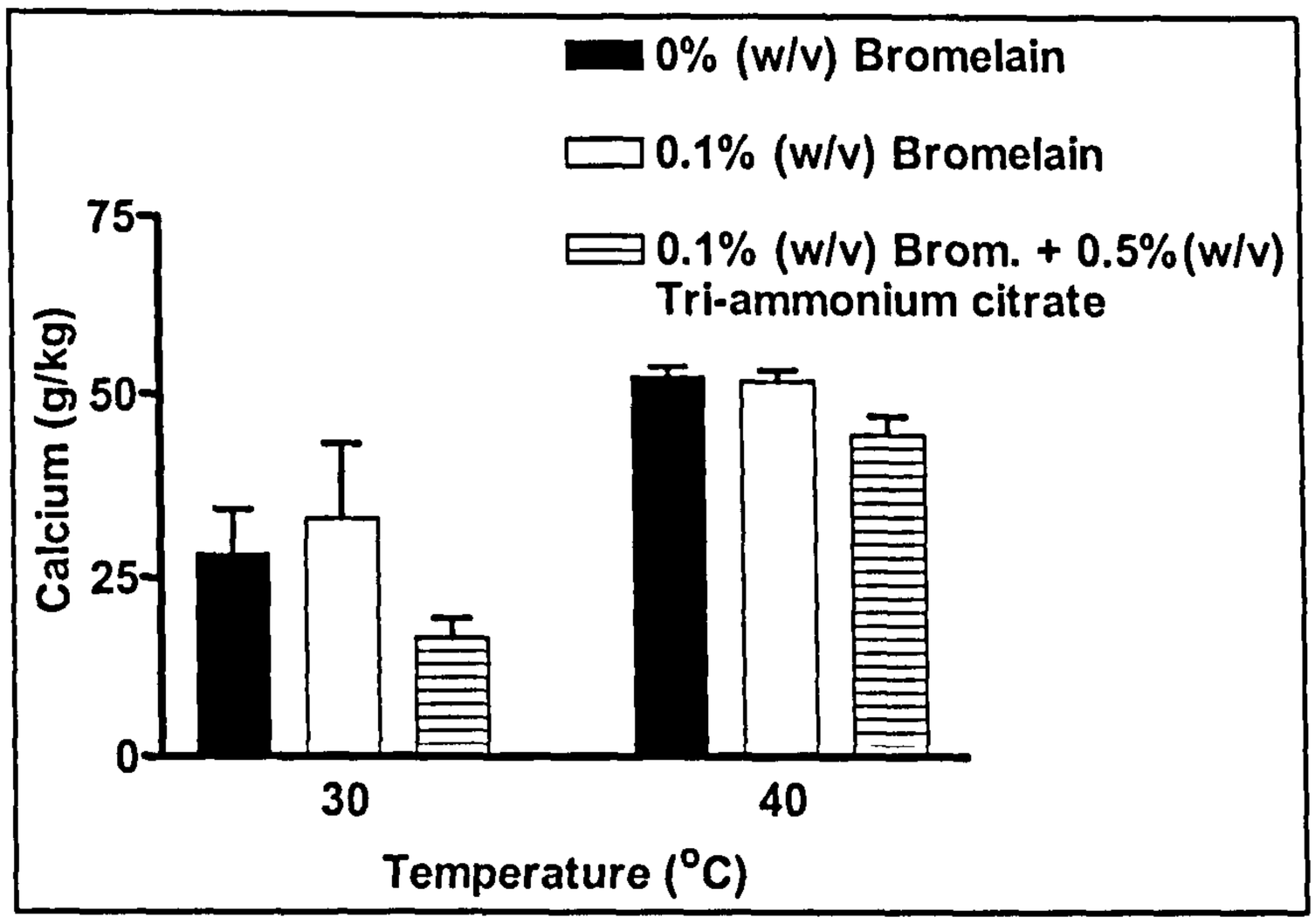


Fig. 6.22 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C, on the calcium content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Chitin content increased significantly at both 30°C and 40°C ($p<0.001$) (Fig. 6.23). The highest chitin content was found at 30°C.

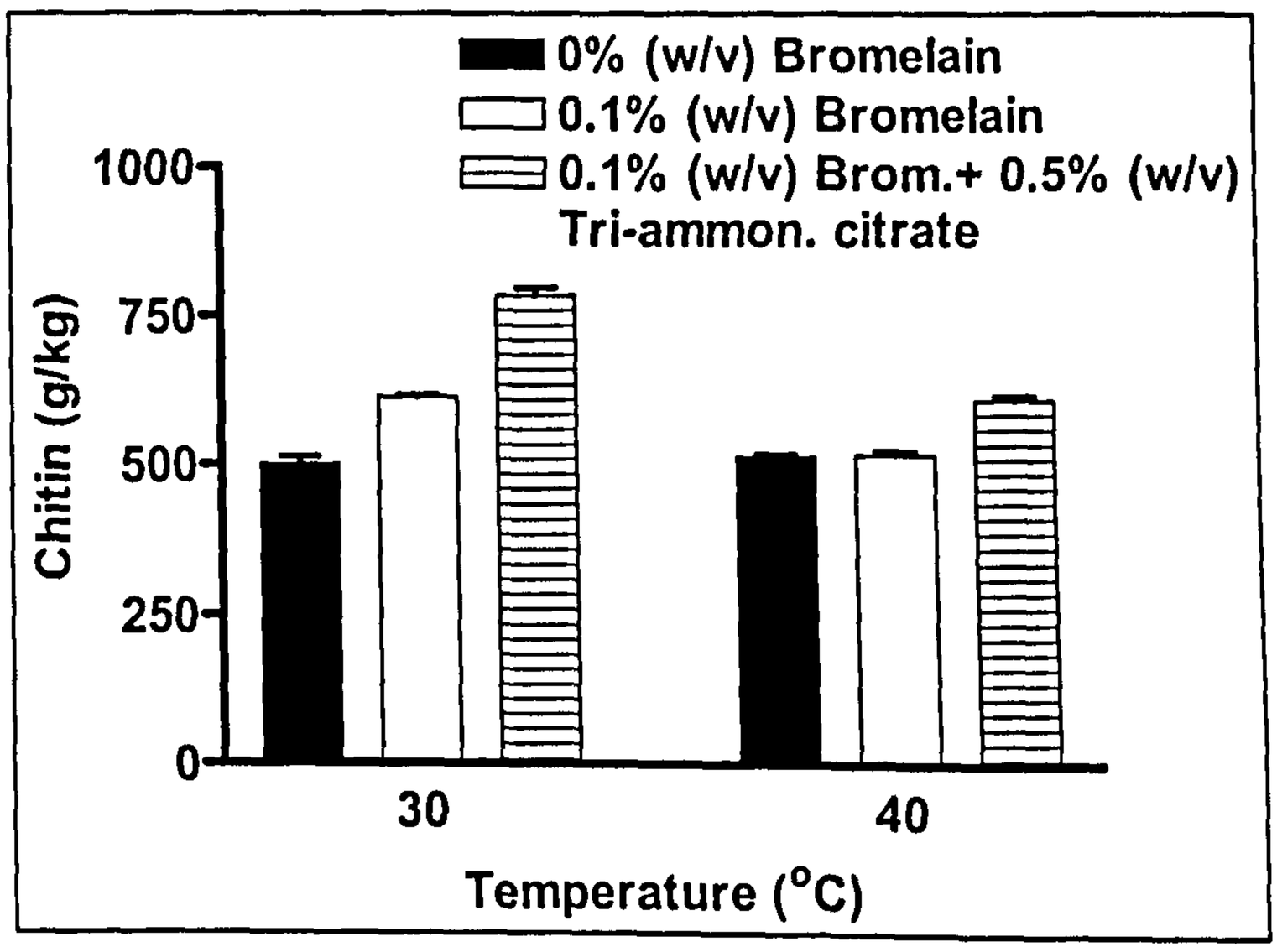


Fig. 6.23 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C, on the chitin content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The nitrogen content was not affected at 30°C or 40°C by the addition of tri-ammonium citrate (Table 6.9).

Treatment		% Nitrogen (w/w)
A	15% (v/v) Lactosil at 30°C	8.13 ± 0.27
B	A + 0.1% (w/v) bromelain at 30°C	6.38 ± 0.19
C	B + 0.5% (w/v) tri-ammonium citrate at 30°C	6.24 ± 0.43
D	15% (v/v) Lactosil at 40°C	7.05 ± 0.51
E	D + 0.1% (w/v) bromelain at 40°C	6.16 ± 0.26
F	E + 0.5% (w/v) tri-ammonium citrate at 40°C	6.49 ± 0.24

Table 6.9 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C, on the nitrogen content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

The protein content however fell significantly at 30°C ($p<0.01$) with the addition of tri-ammonium citrate (Fig. 6.24).

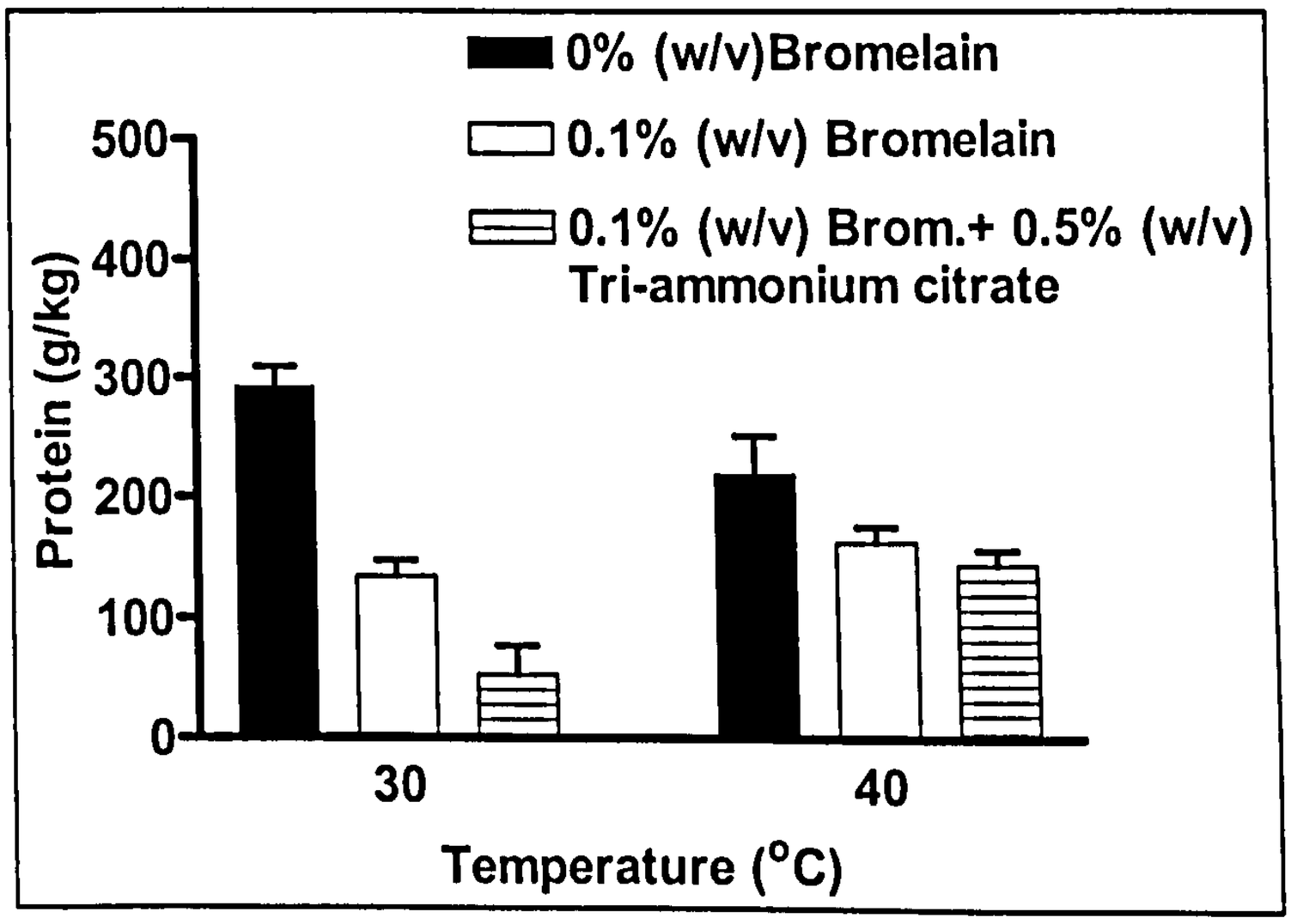


Fig. 6.24 *Effect of the addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C, on the protein content of the shell.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Moisture levels of samples treated with tri-ammonium citrate were lower than samples treated without tri-ammonium citrate (Table 6.10).

Treatment		Moisture (g/kg)
A	15% (v/v) Lactosil at 30°C	62.87 ± 3.47
B	A + 0.1% (w/v) bromelain at 30°C	62.10 ± 0.87
C	B + 0.5% (w/v) tri-ammonium citrate at 30°C	15.70 ± 1.41
D	15% (v/v) Lactosil at 40°C	60.70 ± 0.71
E	D + 0.1% (w/v) bromelain at 40°C	59.13 ± 2.40
F	E + 0.5% (w/v) tri-ammonium citrate at 40°C	37.63 ± 3.65

Table 6.10 *Moisture content of shell after addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

Recalculation of the results, with moisture content taken into account, caused the significant difference between ash content in samples treated with bromelain but with and without tri-ammonium citrate, at 40°C, to change from $p<0.01$ to $p<0.001$. No other statistically significant results were affected.

The chemical composition of the final product obtained after addition of 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammonium citrate to the lactic acid fermentation system is shown in Table 6.11. The product contained more chitin, less ash and less protein at 30°C than at 40°C.

Component (g/kg)	30°C	40°C
Chitin	783.33 ± 12.58	608.33 ± 7.64
Ash	40.85 ± 4.60	124.32 ± 7.64
Protein	52.18 ± 24.87	143.07 ± 12.22
Moisture	15.7 ± 1.41	37.63 ± 3.65
Other	107.93 ± 27.97	86.64 ± 10.44

Table 6.11 *The chemical composition of shell treated with 15% (v/v) Lactosil, 0.1% (w/v) bromelain and 0.5% (w/v) tri-ammoniun citrate, at 30°C and 40°C.* The results shown represent the mean ± standard deviation of values obtained from three separate experiments.

6.3.4 Comparison of Chemically Treated and Bioprocessed Shell Waste

6.3.4.1 Colour/Texture Analysis

The colour and texture of the final product, after extraction from the shell, was dependent on the degradative treatment used. Chemical demineralisation produced orange shell pieces, which were flaky in texture. After deproteinisation with 4% (w/v) NaOH the flakes were not so deeply coloured.

Bioprocessing treatment, with 15% (v/v) Lactosil, produced orange/brown shell pieces. Unlike the chemically demineralised shell these pieces were crumbly rather than flaky in nature. After proteolytic treatment the pieces were still crumbly but were lighter in colour than the pieces treated with Lactosil alone. A correlation was noted between an increase in concentration of proteolytic enzyme and a decrease in shell colour. Treatment with Lactosil/bromelain/tri-ammonium citrate produced a product very similar in nature to chemically treated shell - pale in colour, light and flaky in texture.

6.3.4.2 Chemical Compositional Analysis

The composition of untreated shell waste and the shell waste after chemical and microbial/ enzymatic extraction was compared (Tables 6.12 - 6.18). Ash content was lowest in the chemically treated shell - 2.95 ± 2.99g/kg (Table 6.12). Lactic acid fermentation, in conjunction with proteolytic enzyme and tri-ammonium citrate, produced a product containing 41.50 ± 4.67g/kg ash.

Treatment	Ash (g/kg)
Untreated	511.05 ± 9.94
1M HCl	7.16 ± 3.32
1MHCl/4% (w/v) NaOH	2.95 ± 2.99
15% Lactosil (v/v)	111.80 ± 1.51
15% (v/v) Lactosil/0.1% (w/v) bromelain	122.27 ± 2.55
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) tri-ammonium citrate	41.50 ± 4.67

Table 6.12 *Ash content of untreated shell waste, chemically treated shell waste and bioprocessed shell waste.* Results calculated on a dry weight basis.

Calcium levels were lowest after the chemical treatment (Table 6.13). No significant difference in calcium levels was found between shell waste treated with Lactosil and shell waste treated with both Lactosil and bromelain. However, addition of tri-ammonium citrate to the system did reduce the calcium levels.

Treatment	Calcium (g/kg)
Untreated	179.15 ± 5.88
1M HCl	1.70 ± 1.09
1MHCl/4% (w/v) NaOH	2.73 ± 2.09
15% (v/v) Lactosil	30.12 ± 6.52
15% (v/v) Lactosil/0.1% (w/v) bromelain	35.32 ± 10.83
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) tri-ammonium citrate	16.90 ± 2.84

Table 6.13 *Calcium content of untreated shell waste, chemically treated shell waste and bioprocessed shell waste.* Results calculated on a dry weight basis.

The lowest protein level was also achieved by chemical treatment of the shell waste - 0.342 ± 0.00g/kg (Table 6.14). Fermentation technology succeeded in lowering the concentration of residual protein to 53.01 ± 25.27g/kg.

Treatment	Protein (g/kg)
Untreated	181.85 ± 24.55
1M HCl	390.57 ± 29.50
1MHCl/4% (w/v) NaOH	0.342 ± 0.00
15% (v/v) Lactosil	311.36 ± 18.63
15% Lactosil (v/v)/0.1% (w/v) bromelain	142.92 ± 13.81
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) tri-ammonium citrate	53.01 ± 25.27

Table 6.14 *Protein content of untreated shell waste, chemically treated shell waste and bioprocessed shell waste.* Results calculated on a dry weight basis.

Treatment of shell by lactic acid fermentation produced a product containing 535.32 ± 12.32g/kg chitin (Table 6.15). This was very similar to the amount of chitin in chemically demineralised shell - 568.72 ± 26.52g/kg (Table 6.15). Lactic acid fermentation plus enzymatic treatment led to a final product containing predominately chitin.

Treatment	Chitin (g/kg)
Untreated	235.09 ± 17.27
1M HCl	568.72 ± 26.52
1MHCl/4% (w/v) NaOH	1015.60 ± 18.03
15% (v/v) Lactosil	535.32 ± 12.32
15% (v/v) Lactosil/0.1% (w/v) bromelain	653.94 ± 3.08
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) tri-ammonium citrate	795.83 ± 12.78

Table 6.15 *Chitin content of untreated shell waste, chemically treated shell waste and bioprocessed shell waste.* Results calculated on a dry weight basis.

6.3.4.3 Amino Acid Analysis

Amino acid analysis was carried out on individual samples after each treatment (Table 6.16). Overall, amino acid content was lowest in chemically treated shell (HCl/NaOH) and in a commercially produced chitin (Sigma, Poole, Dorset). Demineralisation by chemical treatment (HCl) or by lactic acid fermentation (LAF) produced a product containing similar total concentrations of amino acids. The use of proteolytic enzyme (LAF + bromelain) and proteolytic enzyme + tri-ammonium citrate (LAF + bromelain + TAC) reduced the amino acid content further.

Amino Acid (g/100g)	HCl	HCl/NaOH	LAF[a]	LAF + Bromelain	LAF + Bromelain + TAC[b]	Sigma Chitin
Alanine	2.23	Trace	1.84	0.51	0.41	0.219
Arginine	2.53	Trace	1.98	0.55	0.44	0.157
Aspartic	4.44	Trace	3.59	1.16	0.98	0.178
Cystine	0.22	Trace	0.11	Trace	0.09	-
Glutamic	4.88	Trace	4.00	1.15	0.89	0.301
Glycine	2.50	Trace	2.04	0.59	0.48	0.097
Histidine	1.14	Trace	0.93	0.27	0.22	0.057
Isoleucine	2.07	Trace	1.66	0.53	0.45	0.082
Leucine	2.83	Trace	2.24	0.70	0.61	0.094
Lysine	2.28	Trace	1.67	0.51	0.44	0.063
Methionine	0.13	Trace	0.08	Trace	0.04	0.002
Phenylalanine	2.20	Trace	1.81	0.57	0.43	0.114
Proline	1.90	Trace	1.68	0.53	0.37	0.160
Serine	1.98	Trace	1.59	0.49	0.39	0.042
Threonine	1.91	Trace	1.57	0.45	0.37	0.052
Tyrosine	1.67	Trace	1.37	0.43	0.35	0.035
Valine	2.45	Trace	2.04	0.64	0.53	0.161
Total	37.32	Trace	30.15	9.05	7.45	0.814
Total (dry weight basis)	39.74	Trace	32.05	9.27	7.57	ND[c]

[a]LAF = lactic acid fermentation, [b]TAC = tri-ammonium citrate, [c]ND = not determined

Table 6.16 *Amino acid content (g/100g) of chemically treated shell waste and bioprocessed shell waste.* Results shown are from individual samples analysed once. Values for cystine and methionine are inaccurate due to preparative method used.

The total protein content determined by calculation and the total protein content determined by amino acid analysis were compared (Table 6.17). Similar results were obtained by amino acid analysis and by calculation thus verifying the general trends of protein results shown thus far.

Treatment	Protein (g/kg) (by calculation)	Protein (g/kg) (by amino acid analysis)
1M HCl	364.1	397.4
1MHCl/4% (w/v) NaOH	0	0
15% (v/v) Lactosil	331.1	320.5
15% (v/v) Lactosil/0.1% (w/v) bromelain	56.0	92.7
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) tri-ammonium citrate	76.5	75.7

Table 6.17. *Comparison of residual protein values obtained by calculation and by amino acid analysis* Results shown are from single samples. Results calculated on a dry weight basis.

Discrepancies occur between Table 6.17 and Table 6.14 for protein values obtained by calculation for 15% (v/v) Lactosil/0.1% (w/v) bromelain treated shell. The result in Table 6.17 came from a single sample treated at the 100ml level whereas the result shown in Table 6.14 was the mean result from three experiments, carried out in 1 litre shake flasks.

6.3.4.4 Carbon, Hydrogen and Nitrogen Contents

A comparison of the carbon, hydrogen and nitrogen contents of untreated shell waste and chemically/microbially treated shell waste can be seen in Table 6.18. Chemically demineralised and deproteinised shell contained 6.67% nitrogen and had a N:C ratio of 0.142. Shell waste that had been bioprocessed using lactic acid fermentation plus bromelain/lactic acid fermentation plus bromelain plus tri-ammonium citrate, contained similar concentrations of nitrogen - 6.80% and 6.34%, but the N:C ratio was higher when tri-ammonium citrate was not included in the system (0.167 as opposed to 0.153). These N:C ratios fell between the ratio for chemically demineralised shell (0.211) and chemically demineralised/deproteinised shell (0.142) confirming that protein removal had occurred but was not complete. Shell subjected to lactic acid fermentation without addition of

proteolytic enzyme possessed a N:C ratio (0.200) similar to that of chemically demineralised shell (0.211) confirming that demineralisation but not deproteinisation had taken place.

Treatment	%C (w/w)	%H (w/w)	%N (w/w)	N:C ratio
Untreated	27.06 ± 3.12	4.10 ± 0.40	4.53 ± 1.04	0.167
1M HCl	48.15 ± 2.07	7.33 ± 0.18	10.18 ± 0.49	0.211
1MHCl/4% (w/v) NaOH	46.96 ± 0.33	7.11 ± 0.17	6.67 ± 0.12	0.142
15% (v/v) Lactosil	43.47 ± 0.58	6.42 ± 0.17	8.68 ± 0.28	0.200
15% (v/v) Lactosil/0.1% (w/v) bromelain	40.73 ± 1.44	6.03 ± 0.37	6.80 ± 0.21	0.167
15% (v/v) Lactosil/0.1% (w/v) bromelain/0.5% (w/v) TAC*	41.57 ± 1.53	6.08 ± 0.24	6.34 ± 0.44	0.153

*TAC = tri-ammonium citrate

Table 6.18 *Carbon, hydrogen and nitrogen content; and N:C ratio of untreated shell waste, chemically treated shell waste and bioprocessed shell waste.* Results calculated on a dry weight basis.

6.3.4.5 FTIR Analysis

FTIR analysis was carried out on bioprocessed *Nephrops* shell waste and compared with with FTIR analysis of commercially obtained chitin that had been isolated from crab shell. This qualitative method revealed no differences between the profile of commercially obtained chitin (Fig. 6.25), chitin isolated using lactic acid fermentation (Fig. 6.26) and chitin isolated using lactic acid fermentation in conjunction with a proteolytic enzyme (Fig. 6.27).

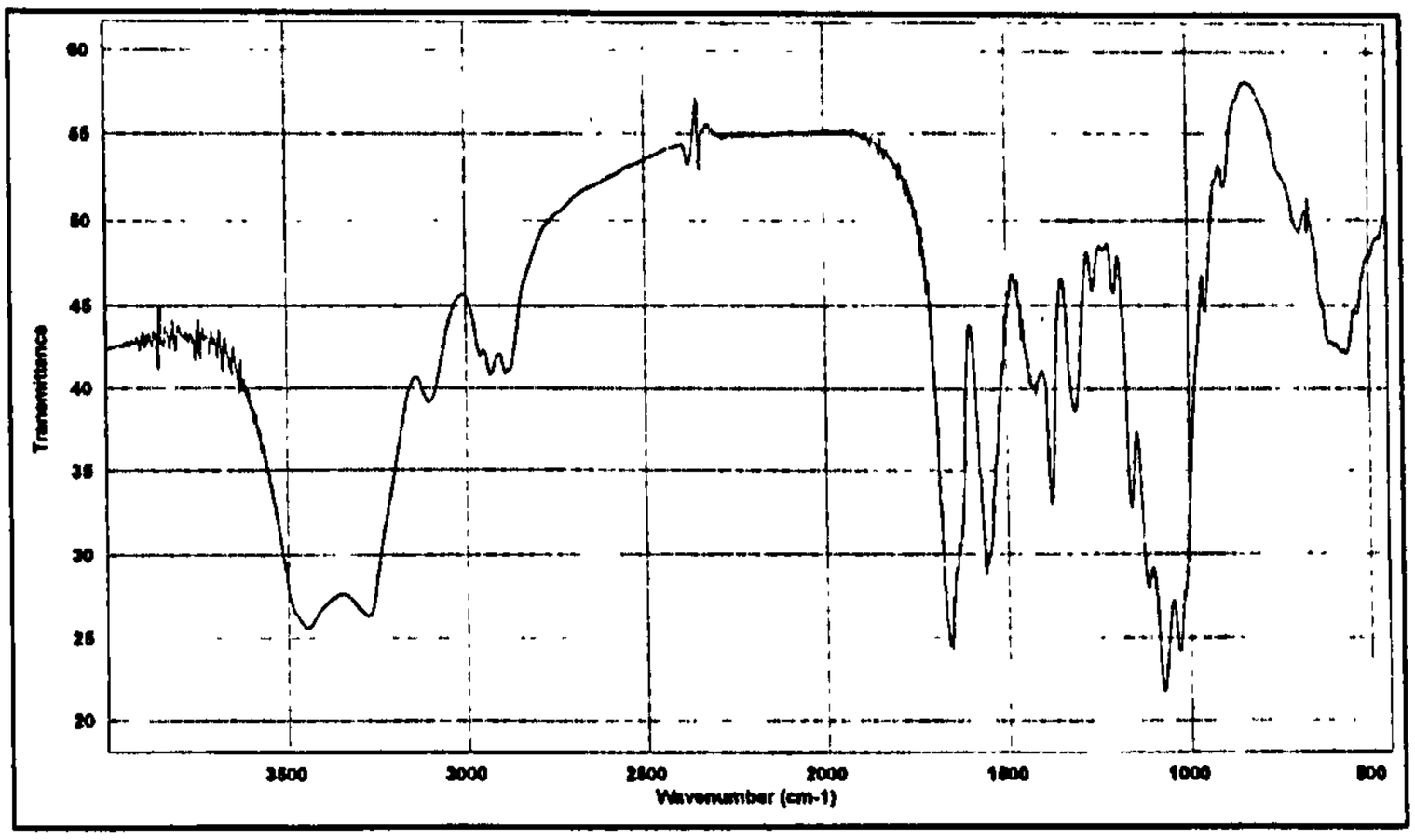


Fig. 6.25 *FTIR spectrum of commercially obtained chitin.*

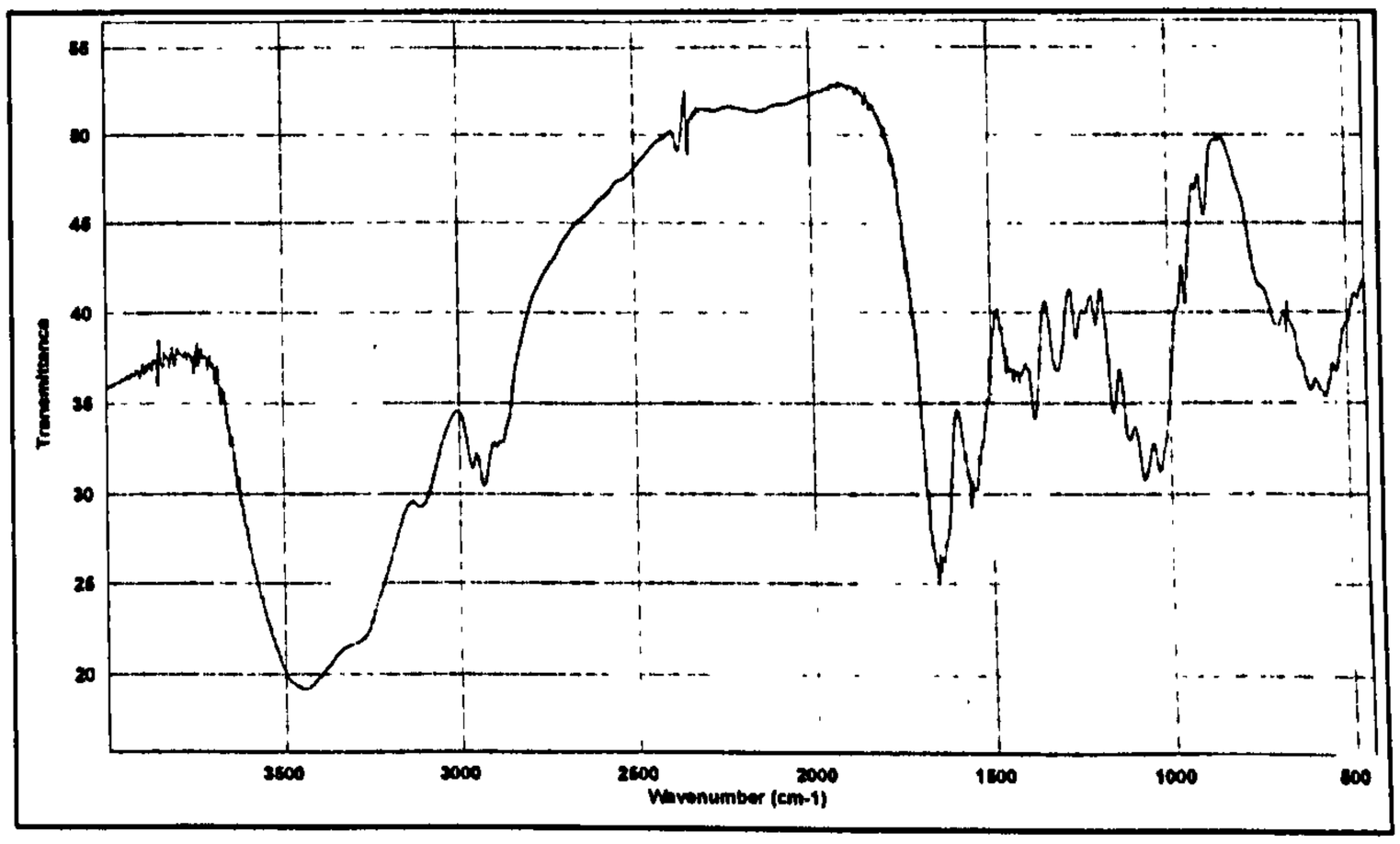


Fig. 6.26 *FTIR spectrum of Nephrops shell waste treated by lactic acid fermentation.*

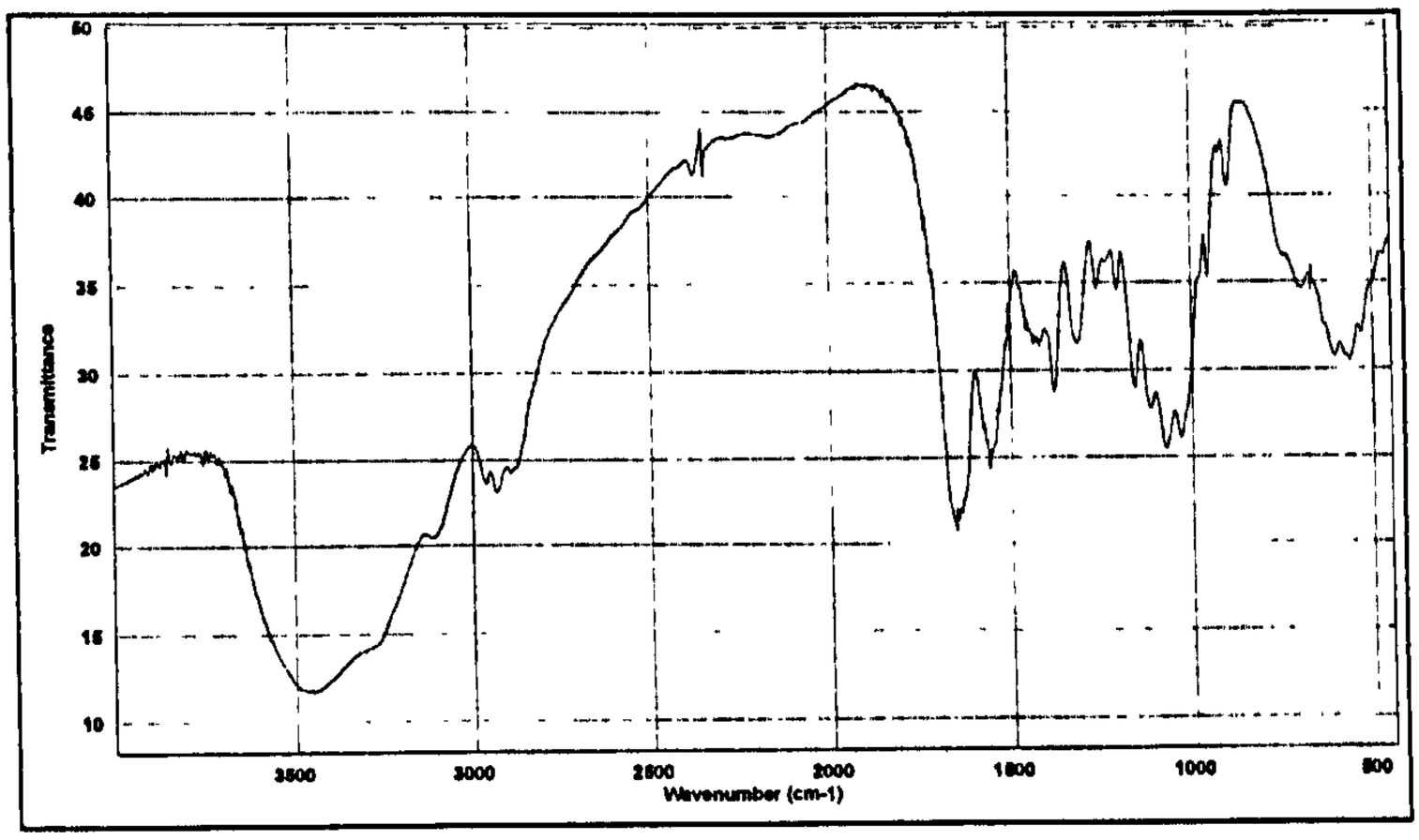


Fig. 6.27 *FTIR spectrum of Nephrops shell waste treated by lactic acid fermentation in conjunction with proteolytic enzyme.*

6.4 DISCUSSION

6.4.1 Proteolysis of *Nephrops* Shell Waste

Proteolytic enzymes can be divided into two categories - exo- and endo- peptidases. Exopeptidases remove terminal amino acids from one end of the amino acid chain by hydrolysis of the peptide bond. Endopeptidases hydrolyse peptide bonds within the molecule producing relatively large peptide chains. Endopeptidases, such as bromelain, show a wide specificity and have thus been employed for the denaturation of proteins in fish waste (Mackie, 1974). Bromelain is one of the enzymes preferred for use in the manufacture of fish sauce because it gives a favourable taste to the products (Gildberg, 1993). For the production of fish sauce the enzymes are used at temperatures of 50 - 60°C for 30 minutes (Mackie, 1974). However, bromelain has been shown to be active for the breakdown of fish waste at 33°C (Beddows and Ardeshir, 1979) and was therefore suitable for use in conjunction with the lactic acid bacteria in the fermentation system.

It was estimated, by calculation, that approximately 1% (w/v) bromelain should have been enough to deproteinise the shell waste (Fig. 6.28). In fact, maximal deproteinisation was achieved at a concentration of 0.1% (w/v) bromelain (equivalent to 1.5g enzyme/100g shell waste) (Figs. 6.8 and 6.13). Similar concentrations of bromelain have been used to break down fish waste. Beddows and Ardeshir (1979) achieved 65% (w/v) solubilisation of fish waste protein by mixing varying amounts of bromelain, up to 1.65g, with 100g fish waste.

Activity of Bromelain

One unit of bromelain will hydrolyse 1.0mg of amino nitrogen from gelatin in 20 minutes at pH 4.5 at 45°C.

1560 units / g solid

Estimation of Shell Protein in Fermentation System

Untreated shell waste contained 17% (w/w) protein.

6.67g shell waste was mixed with 100ml glucose

17% of 6.67g = 1.134g protein in 100ml reaction volume

Calculation of Bromelain Required to Hydrolyse Shell Protein

1 unit hydrolyses 1mg protein

Therefore, 1.134 g protein will be hydrolysed by 1134units

1560 units/g

1134 units = 0.72g

0.72g / 100ml reaction volume = 0.72% (w/v) Bromelain

Fig. 6.28 *Estimation of the concentration of proteolytic enzyme required to deproteinise shell waste.*

Lactic acid concentration was increased to 305.37 ± 15.13mg lactic acid/g dry shell, at 30°C, by addition of bromelain to the fermentation system compared to 235.8 ± 8.20mg/g dry shell when Lactosil was used alone (Fig. 6.14). This increase in lactic acid did not however lead to a drop in ash concentration in the final product (Fig. 6.15). A reduction in ash was not expected because increases in lactic acid concentration beyond 150mg/g dry shell in previous experiments had not caused a decrease in the ash content of the waste (Figs. 5.30 and 5.31). Ash levels instead increased slightly on addition of bromelain

compared with the effects of Lactosil on its own. This agrees with results obtained by Rao *et al.*, (1998) where a slight increase in ash content was achieved on addition of a protease (Gist brocades) to the lactic acid fermentation culture broth.

Approximately 54% of the protein in Lactosil treated shell was removed by addition of 0.1% (w/v) bromelain to the fermentation system. Protein concentration was reduced from 311.36 ± 18.63g/kg in Lactosil treated shell waste to 142.92 ± 13.81g/kg after treatment with Lactosil plus bromelain, at 30°C (Table 6.14). This caused the chitin component of the shell to increase from 535.32 ± 12.32g/kg after treatment with Lactosil to 653.94 ± 3.08g/kg after treatment with Lactosil and bromelain (Table 6.15). Addition of bromelain to the system therefore achieved the desired effect - a reduction in protein levels in the final product. Nevertheless, protein at a concentration of 142.92 ± 13.81g/kg and ash at a concentration of 122.27 ± 2.55g/kg still remained as contaminants of the chitin product.

The growth of lactic acid bacteria depends on an adequate nitrogen supply for bacterial protein synthesis (Thomas and Mills, 1981). During the production of cheese, yoghurt and sour cream these bacteria degrade α and β-casein in milk to get enough peptides to support their growth (Hebert *et al.*, 1998). At the start of the lactic acid fermentation of the *Nephrops* waste the only protein source available to the microorganisms was that contained within the organic matrix of the shell. The initial pH present at the start of the fermentation i.e. pH 6.83 (Fig. 5.43) may have caused the solubilisation of some of the adventitious shell protein because prawn shell protein is most soluble at pH values greater than pH 7 and lowest at pH 4.5 (Healy *et al.*, 1995). Nevertheless, some of the bacteria in the inoculant may not have been capable of utilising the protein present due to the low proteolytic capability of lactic acid bacteria and so an additional source of nitrogen was included in the system in the form of tri-ammonium citrate.

Tri-ammonium citrate is a component of MRS media - media that supports the growth of lactic acid bacteria. On addition of tri-ammonium citrate to the Lactosil and bromelain containing fermentation system, at 40°C, levels of lactic acid rose (Fig. 6.20). Variation in lactic acid measurements was wider at 30°C so an increase in lactic acid at this temperature cannot be ruled out.

At low pH levels some lactic acid bacteria, including *E. faecium,* can use citrate as a substrate and convert it to lactate following the equation below:

2 Citrate + ADP + Pi $\longrightarrow$ 3 Acetate + Lactate + $3CO_2$ + ATP (reviewed by McDonald *et al.*, 1991). This conversion may have caused the increase in lactic acid.

Addition of tri-ammonium citrate to the fermentation system caused a decrease in ash at 30°C and 40°C (Fig. 6.21) and a decrease in protein at 30°C (Fig. 6.24), with a corresponding increase in chitin at both temperatures (Fig. 6.23). Approximately 83% of the protein present in the lactic acid demineralised waste was solubilised, on addition of tri-ammonium citrate. Shirai *et al.*, (1998) achieved 77.06% protein removal using lactic acid fermentation followed by trypsin treatment in a system that probably included proteolytic heads. Rao *et al.*, (1998) achieved 90% deproteinisation and more than 50% decalcification when shrimp biowaste was subjected to treatment using a combination of protease and lactobacillus. Guerrero-Legarreta *et al.*, (1996) achieved removal of approximately 70% protein and 34% calcium using lactic acid fermentation. Bustos (1996) obtained 81% deproteinisation of *Nephrops* tail shell waste using proteolytic microorganisms. Therefore, the extent of deproteinisation achieved by addition of bromelain and tri-ammonium citrate to the lactic acid fermentation system was similar to that obtained in studies using proteolytic microorganisms, other proteolytic enzymes or enzymes indigenous to the waste.

The reasons behind the decreases in ash and protein contents after addition of tri-ammonium citrate were not apparent. A slight increase in pH caused by addition of

ammonium ions could have increased the activity of the bromelain thus releasing more protein from the shell. The presence of calcium binding cuticular proteins has been reported in several crustaceans (O'Brien *et al.*, 1991). Removal of these proteins may have allowed the lactic acid to come into contact with previously inaccessible shell areas and so dissolve more shell $CaCO_3$. However, pH measurements were not taken and as lactic acid content was high it is unlikely that an increase in pH affected the activity of the bromelain. Lactic acid bacterial protease expression is affected by the components of the media in which the cells are grown (Law and Kolstad, 1983). Therefore, another possible scenario is that the presence of nitrogen ions in the fermentation medium increased the proteolytic capability of the bacteria by enhancing protein synthesis. Bacterial proteolytic degradation, in conjunction with degradation caused by bromelain, may then have allowed more ash to be dissolved as surmised above.

6.4.2 Chitin Characterisation

Increasing the amount of bromelain added to the fermentation system led to a decrease in the colour of the chitin produced. This suggested that the addition of protease encouraged pigment release from the shell. Chen and Myers (1982) used a protease to disrupt the astaxanthin/protein bond prior to pigment extraction. The addition of the enzyme led to a significant increase in the amount of astaxanthin extractable by oil. Enzymatic protein removal in conjunction with lactic acid fermentation may therefore be a useful technique for the recovery of pigment at the same time as chitin.

Chitin isolates have been shown to contain different concentrations of amino acids depending on species of crustacean (Brine and Austin, 1981b). After prolonged alkaline hydrolysis or enzymatic treatment residual amino acids always remain, possibly due to the covalent linkage between chitin and protein in the crustacean shell. Hackman (1960) reported aspartyl and histidyl amino acid residues in chitin from insects, crustaceans,

cuttlefish and squid after hot alkali treatment. Brine and Austin (1981b) found that aspartic acid, serine and glycine were the principal amino acids remaining after alkaline hydrolysis of chitin isolates from several marine invertebrates. Chitin isolates from crawfish contained tyrosine as the predominant amino acid (No *et al.*, 1989). The predominant amino acid residues in the waste after lactic acid fermentation and proteolytic treatment in this study were aspartic acid and glutamic acid (Table 6.16). Bustos (1996) obtained similar results after microbial treatment with proteolytic bacteria and after chemical treatment.

The final percentage nitrogen values for shell waste enzymatically treated in one litre shake flasks at 30°C (Table 6.18) fell within the range of values that have been reported in the literature for chitin (6.14 - 8.3) (No and Myers, 1995). Low nitrogen values were obtained in two experiments, carried out in 100ml volumes (Table 6.1 and 6.2). However, contamination of chitin results in a lower nitrogen value than obtained for theoretical chitin and all the lower nitrogen values obtained here could be attributed to slightly higher residual ash contents. Shirai *et al.*, (1998) also obtained low nitrogen contents: 5.91% (w/w) for lactic acid fermented prawn shell waste and 5.31% (w/w) for prawn waste subjected to lactic acid fermentation followed by deproteinisation using the enzyme trypsin. No ash values were reported in Shirai's study but it is possible that ash removal was not complete. In contrast, shell waste treated with proteolytic microorganisms produced chitin with nitrogen contents in the range 8.31% - 9.12% (Bustos, 1996). These figures are high suggesting incomplete removal of protein.

Bustos (1996) reported that chitin samples produced microbially had higher molecular weights and lower levels of deacetylation and were therefore less degraded than chemically produced chitin. Degradation of chitin when treated with bromelain has been reported but only after the chitin was pre-treated by partial deacetylation or suspension in aqueous acetic acid for several days (Yalpani and Pantaleone, 1994). No values for

molecular weights or percentage deacetylation for enzymatically-produced shell were obtained here. Viscosity methods for the determination of the molecular weight of chitin involve the dissolution of chitin in a solvent, such as lithium chloride - dimethyl acetamide. However, due to the residual levels of ash and protein in the chitin produced it would have been difficult to dissolve and therefore molecular weight measurements were not attempted.

It is possible to estimate the percentage deacetylation of chitin isolates from N:C ratios but only when residual protein is not present (Roberts, 1992). N:C ratios for the chitin isolates in this study are shown in Table 6.18 but percentage deacetylation values were not estimated because the chitin produced by bioprocessing methods contained residual protein. It is also possible to obtain percentage deacetylation values from FTIR spectra but only when the sample analysed is completely free from moisture. The FTIR spectra in Figs. 6.25 – 6.27 showed that the microbially produced chitin contained the same transmittance spectra as commercial chitin. The spectra were also similar to those generated by Shirai for chitin isolated by chemical and fermentative methods (Shirai *et al.*, 1998). However, percentage deacetylation values could not be determined due to residual moisture levels in the samples.

CHAPTER SEVEN

PRODUCTION OF CHITOSAN FROM BIOPROCESSED SHELL WASTE FOR BIOMEDICAL APPLICATIONS

7.1 INTRODUCTION

Advancement in modern medicine has lead to the widespread and growing use of synthetic polymeric substances in the form of biomedical devices. Some of the most well known prostheses are artificial joints, prosthetic heart valves and urinary catheters but the list extends to cover a wide range of implants. Insertion of any foreign object into the body is a potential cause of infection. Therefore, for medical devices to fulfil their role in improving quality of life, any potential infection caused by these objects must be minimised.

Biomaterials are substances used in medical implants that come into contact with living cells in the extravascular and/or intravascular system. To be approved for use in a position of such close proximity to the body there are certain criteria which they must fulfil, for example they must be readily purified and sterilised, be free from leachable impurities and have appropriate mechanical and physical properties for the proposed use (reviewed by Pişkin, 1994). The living organism will respond to a medical prosthesis as a foreign body and therefore will react in an adverse manner to non-biocompatible materials. Due to their film and fibre forming properties (Rathke and Hudson, 1994) chitin derivatives are potential new materials for use in medical device manufacture.

Urinary catheters frequently give rise to problems of incompatibility. Catheters are inserted into the urethra on a short or long term basis dependent on the requirements of the patient. Bacterial adherence, either to the surface of the catheter itself or to other substances attached to the prosthesis such as metal ions or proteins, can lead to infection of the urinary tract and make removal of the device necessary (Tunney *et al.*, 1996a and b). Metal salts can also adhere to the implant leading to blockage by encrustation.

Tunney *et al.*, (1996c) have developed a model employing artificial urine for assessment of biomaterial encrustation in the urinary tract. Crystals of struvite (ammonium magnesium phosphate) and hydroxyapatite (calcium phosphate apatite) were

detected in the encrustations obtained on polyurethane samples studied. This encrustation may in part be caused by microbial colonisation. For instance, if urease-producing bacteria adhere to a device, the ammonia generated will raise the local pH. This, in turn, will encourage metal ions such as calcium and magnesium to come out of solution and form the salts named above.

Tunney *et al.*, (1996b) have used their model system to compare encrustation levels on a range of polymers used in the manufacture of urinary tract devices - polyurethane, silicone, HPU, Silitek, Percuflex. All the materials encrusted to some degree. Morris *et al.*, (1997) recommend silicone- or hydrogel- coated catheters for long-term use but further research is needed to produce polymers effective at resisting encrustation on exposure to urine. Stickler *et al.*, (1996) researched antibacterial properties of silver - coated catheters but further advances in reducing bacterial infection in catheterised patients are urgently required.

Chitosan has been shown to act as an antibacterial agent (Shigemasa and Minami, 1995). This, together with its gel and film forming capabilities, makes it an ideal candidate for use in prosthetic devices. With this in mind it was decided to convert microbially extracted chitin to chitosan, its more reactive derivative, and determine the feasibility of manufacturing urinary catheters from the chitosan.

Chitin has traditionally been converted to chitosan via the use of strong alkali at high temperatures. Ideal conditions can be optimised to produce chitosan of the desired percentage deacetylation (Chinadit *et al.*, 1998). An enzyme to convert chitin to chitosan, chitin deacetylase, has been extracted from *Mucor rouxii* (Kafetzopoulos *et al.*, 1993) and therefore it is possible to produce chitosan without the use of harsh chemicals. However, in this project the chemical method was chosen. The chitin obtained after lactic acid fermentation using Lactosil contained residual protein. The high concentrations of alkali traditionally used to chemically deacetylate chitin also remove residual protein. Therefore,

the microbially produced chitin could be converted to chitosan immediately after the lactic acid fermentation stage without resorting to the use of proteolytic enzymes and other additives to first remove protein. Indeed, in 1968 Broussignac suggested that when chitin is not the final desired product, but an intermediate step in the chitosan production process, enzymatic treatment can be readily replaced by alkali treatment at high temperatures. This indicates the advantages of knowing the final use for the chitinous product before the method of extraction/purification is determined.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of Chitosan

Chemically treated *Nephrops* shell waste, produced according to the methods in sections 4.2.1 and 4.2.2, and bioprocessed *Nephrops* shell waste, produced according to the methods in section 5.2.2.2 and Table 5.7, was deacetylated with 50% (w/v) NaOH, in a 1:20 (w/v) solid to liquid ratio, at 90°C for 1 hour. This procedure was executed four times for each sample with washing and drying of the sample, at 40°C, between treatments.

The first treatment was carried out in a one-litre reaction vessel. The vessel was heated with an electric mantle (Electromantle EM1000/CE) and stirred with a motorised stirrer (RW 20 IKA®- Labortechnik) set at 200rpm, fitted with a glass stirrer rod. The lid for the vessel was fitted with a condenser. A thermocouple, attached to a digital thermometer (Digitron), was inserted through the condenser for temperature measurement. All unused necks of the adapter remained closed. The reaction was carried out in a fume hood. After 1 hour at 90°C the temperature was allowed to drop to 60°C, before the condenser was turned off, in order to prevent evaporation. The residue was filtered, washed, dried and stored in an identical manner to the chemically demineralised and deproteinised products (sections 4.2.1 and 4.2.2).

Further deacetylation treatments, where the mass of material used was smaller, were carried out in 250ml round bottomed flasks, in a silicone oil bath, on a magnetic stirrer/hot plate. A condenser was fitted to one neck of the flask. A thermocouple, attached to a digital thermometer (Digitron), was inserted through the condenser to measure temperature. The third neck of the flask remained closed. Conditions used were a solid to liquid ratio of 1:20, at 90°C, for 1 hour. Residual sample was filtered, washed and dried as before.

7.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of chitosan samples was carried out as in section 5.2.5.1.

7.2.3 Solubility of Chitosan in Acetic Acid

After each deacetylation treatment the dried solid was tested for its solubility in 1% (v/v) acetic acid. Solubility was graded by eye on the basis that the samples were (a) almost totally insoluble, (b) partially soluble (gel like particles seen) and (c) almost totally soluble (clear solution with few undissolved particles). Soluble samples were used for the preparation of chitosan membranes (section 7.2.6).

7.2.4 C, H, N Analysis

C, H, N analysis of chitosan samples was carried out as in section 3.2.2.6.

7.2.5 Calcium Determination

Calcium determination of chitosan samples was carried out as in sections 3.2.2.2 and 3.2.2.3.

7.2.6 Preparation of Chitosan Membranes

Chitosan (1g) was dissolved in 150ml acetic acid (1% v/v) by stirring overnight on a magnetic stirrer. The chitosan solution was filtered through muslin to remove undissolved particles. The solution was then de-gassed using a water vacuum pump.

The de-gassed solution was poured onto the base of plastic petri dishes using just enough solution to cover the base of the dishes (approximately 9 - 13g). The dishes were dried overnight, at 40°C, to evaporate the acetic acid. At this stage the membranes were still water soluble. The membranes were peeled off the dishes and immersed in 4% (w/v) NaOH for 10 minutes. After washing in tap water the membranes were submerged in

deionised water for 1 day and immersed in fresh deionised water for 5 days. After 5 days the membranes were removed from the water and dried at room temperature.

7.2.7 Preparation of Polyurethane Membranes for Use as Control Samples

Polyurethane beads (3g) were mixed with 80ml chloroform (3.75% w/v), stirred with a glass rod, covered with parafilm, and left at 4°C. The mixture was swirled to mix after 24 hours. After 48 hours the solution was poured into a clean glass petri dish in the fume hood. The solution was left to dry for two and a half days with a glass funnel over the dish to aid evaporation. The membrane was peeled off the dish and stored in a polythene bag.

7.2.8 Determination of Adherence of Calcium and Magnesium Salts on Membranes in a Urinary Encrustation Tank

7.2.8.1 Apparatus and Composition of Artificial Urine

Samples of chitosan membranes and polyurethane membranes (2cm x 1cm) were suspended in a urinary tract bath for 1 week. The urinary bath was made up of a rectangular perspex box, with a loose fitting lid. The bath was placed in an incubator at 37°C and a 5% CO_2 atmosphere. The contents of the bath were stirred constantly by means of two Teflon-coated metal stirrers.

The bath contained five litres of artificial urine composed of: 2.5 litres of solution A [potassium dihydrogen ortho-phosphate (0.76% w/v), magnesium chloride hexahydrate (0.36% w/v) and urea (1.6% w/v)], 2.5 litres of solution B [calcium chloride hexahydrate (0.53% w/v) and chicken ovalbumin (0.2% w/v)] and 320ml urease [Jack bean urease type 1X (0.125% w/v)]. Each day 1 litre of solution was removed from the tank and replaced with 500ml solution A and 500ml solution B, preheated to 37°C to avoid temperature fluctuations in the vessel. Samples were suspended on coloured paper clips from a plastic grid. The grid had a hole in it for exchange of solutions.

7.2.8.2 Atomic Absorption Analysis

On removal from the tank, the samples were sonicated in 8ml acetic acid (1M) for 4 hours. Remaining solid material was removed and the acetic acid volume made up to 10ml with millipore H_2O. Solutions were filtered through a 0.45µm cellulose nitrate filter (Whatman) for atomic absorption analysis.

Calcium and magnesium contents were determined by atomic absorption spectrophotometry using a Perkin-Elmer 2380 atomic absorption spectrophotometer. Calcium standards were prepared in the range 1.0 - 50.0µg/ml using Fison's calcium solution. Magnesium standards were prepared in the range 0.5 - 10.0µg/ml using Fison's magnesium solution. All dilutions were made with millipore water. The quantity of magnesium and calcium, present on each section, was divided by the surface area of that section, to give the surface density (μgcm^{-2}) of magnesium and calcium present.

7.2.9 Tensile Strength Measurement

Tensile strength measurements were carried out on a Texture Analyser (Stable Microsystems, Model TAXT2, Surrey, England). Chitosan membranes were cut into strips measuring 5cm x 1cm. Digital callipers were used to determine the thickness of the membranes. Four readings were recorded along each strip and the mean value of the readings from eight strips determined.

Each end of a strip (1cm) was covered in a sticky pad and the sticky pads used to clamp the membrane in the analyser. All membranes that broke at the clamp whilst being stretched were discarded. The force at break and the extension at break were recorded for nine membranes. Young's Modulus was calculated as below.

$$\text{Young's Modulus} = \text{resistance to stretch}$$

$$= \frac{\text{stress}}{\text{strain}} = \frac{\text{tensile strength at break}}{\text{strain}}$$

$$\text{tensile strength at break} = \frac{\text{force at break}}{\text{cross sectional area}}$$

$$\text{strain at break} = \frac{\text{extension}}{\text{original length}}$$

7.2.10 Statistical Analysis

Statistical analysis was performed using one-way analysis of variance as in section 3.2.3.

7.3 RESULTS

7.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was carried out on chitosan prepared from bioprocessed *Nephrops* shell waste (i.e. shell waste previously subjected to lactic acid fermentation). The spectrum obtained was compared with the FTIR spectrum of a commercially obtained chitosan (medium molecular weight chitosan, ex- Fluka, Sigma, Gillingham, Dorset). Similar profiles were obtained (Figs. 7.1 and 7.2).

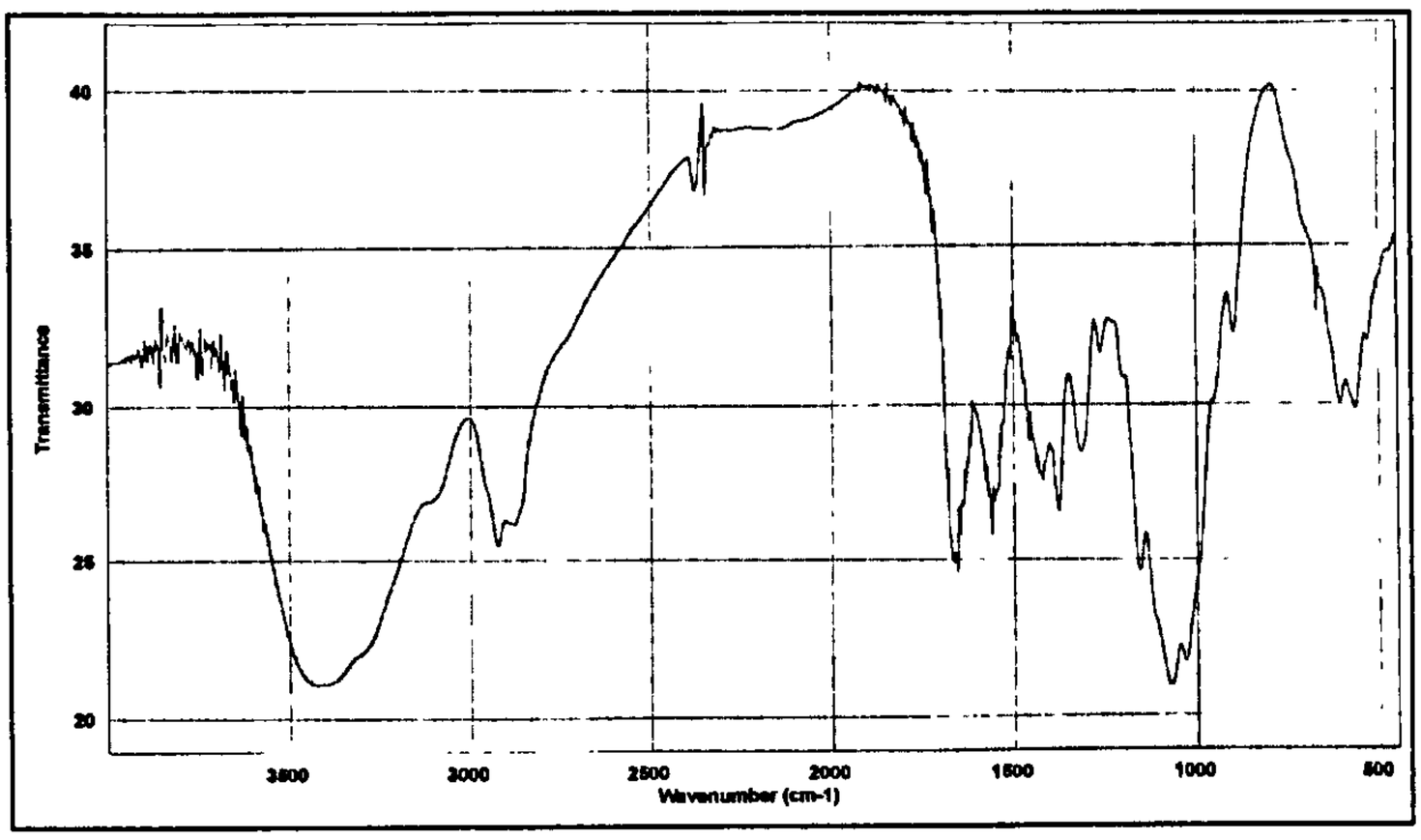


Fig. 7.1 *FTIR spectrum of chitosan prepared from bioprocessed Nephrops shell waste.*

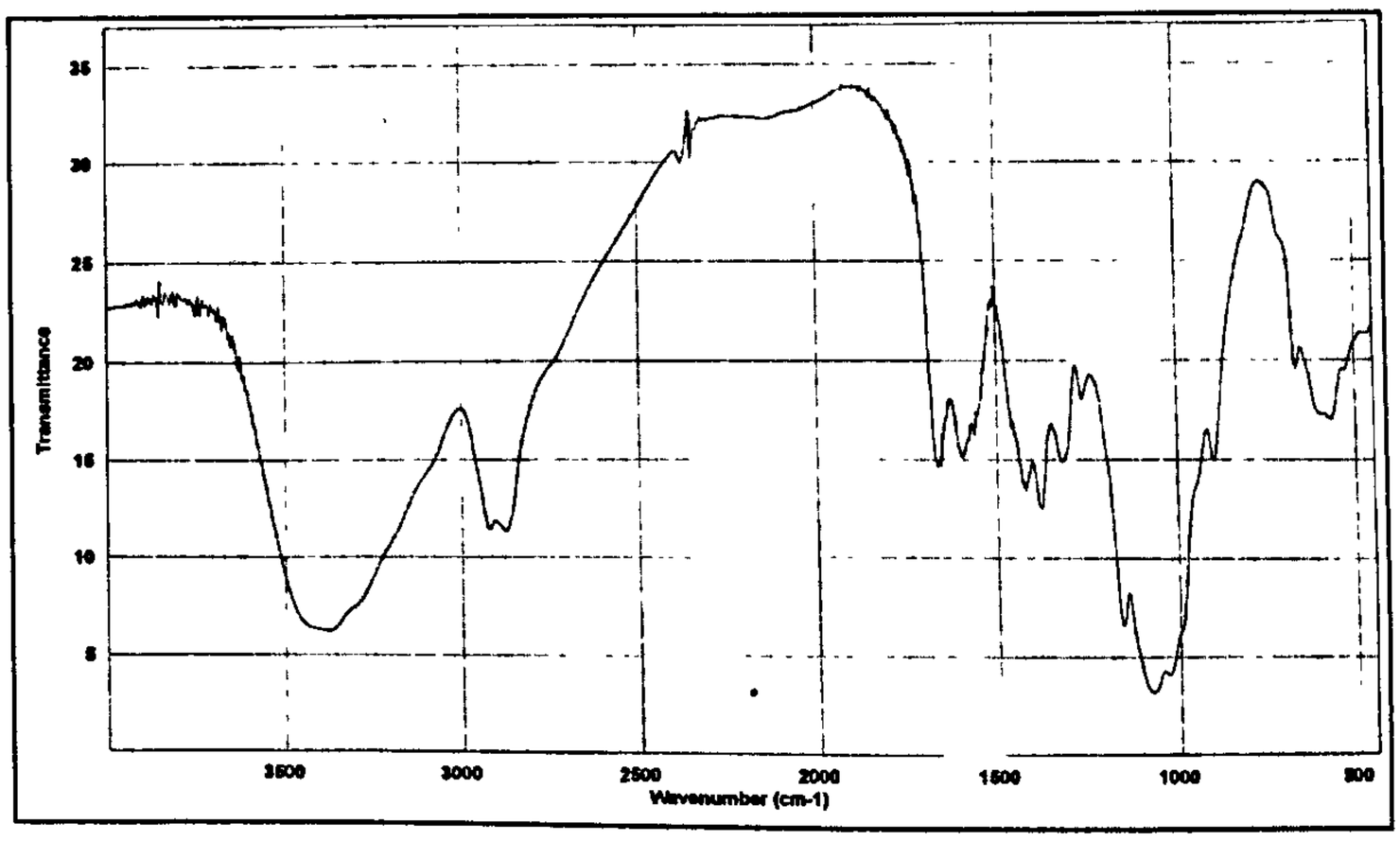


Fig. 7.2 *FTIR spectrum of commercially obtained chitosan.*

7.3.2 Carbon, Hydrogen and Nitrogen Content of Chitosan Samples

The carbon, hydrogen and nitrogen contents of a commercially obtained chitosan, and chitosan prepared from chitin that had been (a) extracted chemically, and (b) extracted using lactic acid fermentation are compared in Table 7.1. No differences in N:C ratios were noted between samples.

Chitosan Sample	Chitin Extraction Method	Number of Deacetylation Treatments	%C (w/w)	%H (w/w)	%N (w/w)	N:C Ratio
Chitosan A (commercial)	N/A	Not known	39.15	6.72	6.88	0.176
Chitosan B	Lactic Acid Fermentation	4	39.23	6.71	7.03	0.179
Chitosan C	Chemical	4	39.42	6.56	6.95	0.176

Table 7.1 *Carbon, hydrogen and nitrogen values and N:C ratio of chitosan samples.* Results shown represent single analysis of individual samples.

Intermediate C, H, N data, after each deacetylation treatment, for chitosan prepared from chitin extracted by lactic acid fermentation (Chitosan B) is shown in Table 7.2. After each stage of deacetylation the N:C ratio increased and the chitosan became more soluble in 1% (v/v) acetic acid. Chitosan membranes were prepared from chitosan that was almost fully soluble. C, H, N data for chitosan, from chemically extracted chitin (Chitosan C), was not determined after each deacetylation treatment but similar levels of solubility to those shown in Table 7.2 were observed.

Number of deacetylation treaments	%C (w/w)	%H (w/w)	%N (w/w)	N:C	Solubility in acetic acid
1	37.15	5.54	5.61	0.151	insoluble
2	38.78	6.16	6.39	0.165	partially soluble
3	39.28	5.64	6.86	0.175	partially soluble
4	39.23	6.71	7.03	0.179	almost totally soluble

Table 7.2 *Carbon, hydrogen and nitrogen data for chitosan prepared from microbially extracted chitin.* Results shown represent single analysis of individual samples.

7.3.3 Calcium Content of Chitosan Samples

The calcium contents of the commercially obtained chitosan (Chitosan A), and the chitosan prepared from chitin that had been extracted using lactic acid fermentation (Chitosan B) and extracted chemically (Chitosan C) are compared in Table 7.3. The calcium content was highest in the chitosan derived from chitin isolated by lactic acid fermentation.

Chitosan Sample	Chitin Extraction Method	Calcium Content (g/kg)
Chitosan A (commercial)	N/A	2.583
Chitosan B	Lactic Acid Fermentation	3.801
Chitosan C	Chemical	1.436

Table 7.3 *Calcium contents of chitosan samples.* Results shown represent single analysis of individual samples.

7.3.4 Determination of Build up of Calcium and Magnesium Salts on Chitosan Membranes in a Urinary Encrustation Tank

No significant difference was noted between the concentration of calcium ions adhering to the chitosan membranes and the polyurethane control membranes (Fig. 7.3). Control chitosan samples were dissolved in acetic acid and any calcium present before insertion in the encrustation bath was determined because it was already known that the chitosan samples contained calcium (Table 7.3). Calcium concentrations on the control samples amounted to less than 25μg/cm^2. Therefore approximately 20% of the calcium measured after encrustation may have been due to the calcium content of the chitosan itself and not to adherence of calcium ions from the artificial urine.

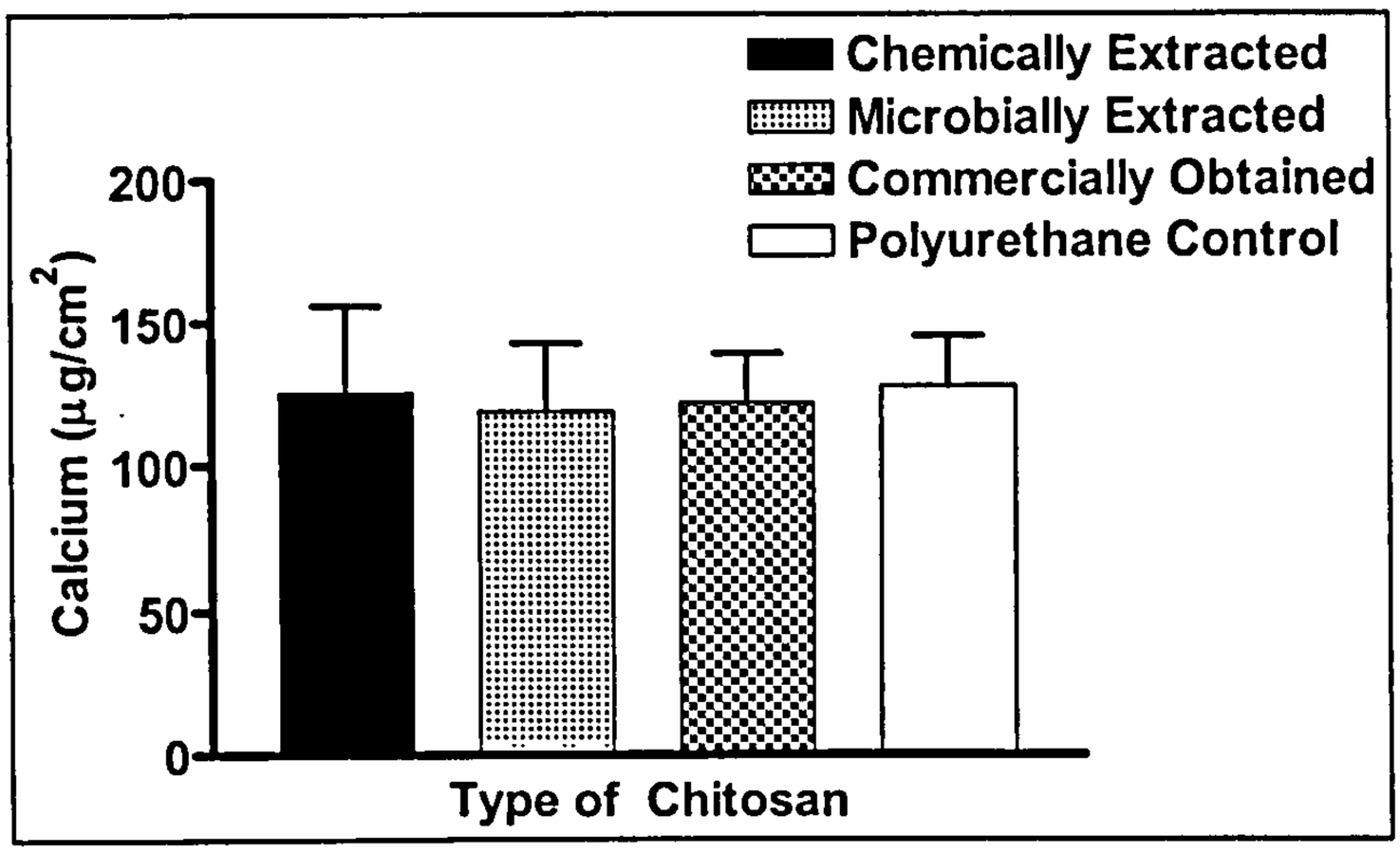


Fig. 7.3 *Adherence of calcium ions to different types of chitosan membranes in the urinary encrustation bath.* Results shown represent the mean ± standard deviation of values from five determinations..

Chitosan prepared from chitin that had been extracted using lactic acid fermentation showed significantly lower levels of adherent magnesium than either the chitosan derived from the chemically extracted shell or the polyurethane control ($p<0.05$). No difference was noted from the commercially obtained chitosan (Fig. 7.4). Magnesium determinations had not been carried out on the chitosan samples before membrane preparation. Control chitosan samples were dissolved in acetic acid and any magnesium present before insertion in the encrustation bath was determined. This amounted to less than 1.25μg/cm^2 on each of the different chitosan samples. Therefore, approximately 5% of the magnesium present on

the membranes may have been present before insertion in the urinary tract bath. If magnesium was present before encrustation tests the difference between adherence of magnesium ions to the polyurethane control samples and adherence to the chitosan samples would be wider than shown in Fig. 7.4.

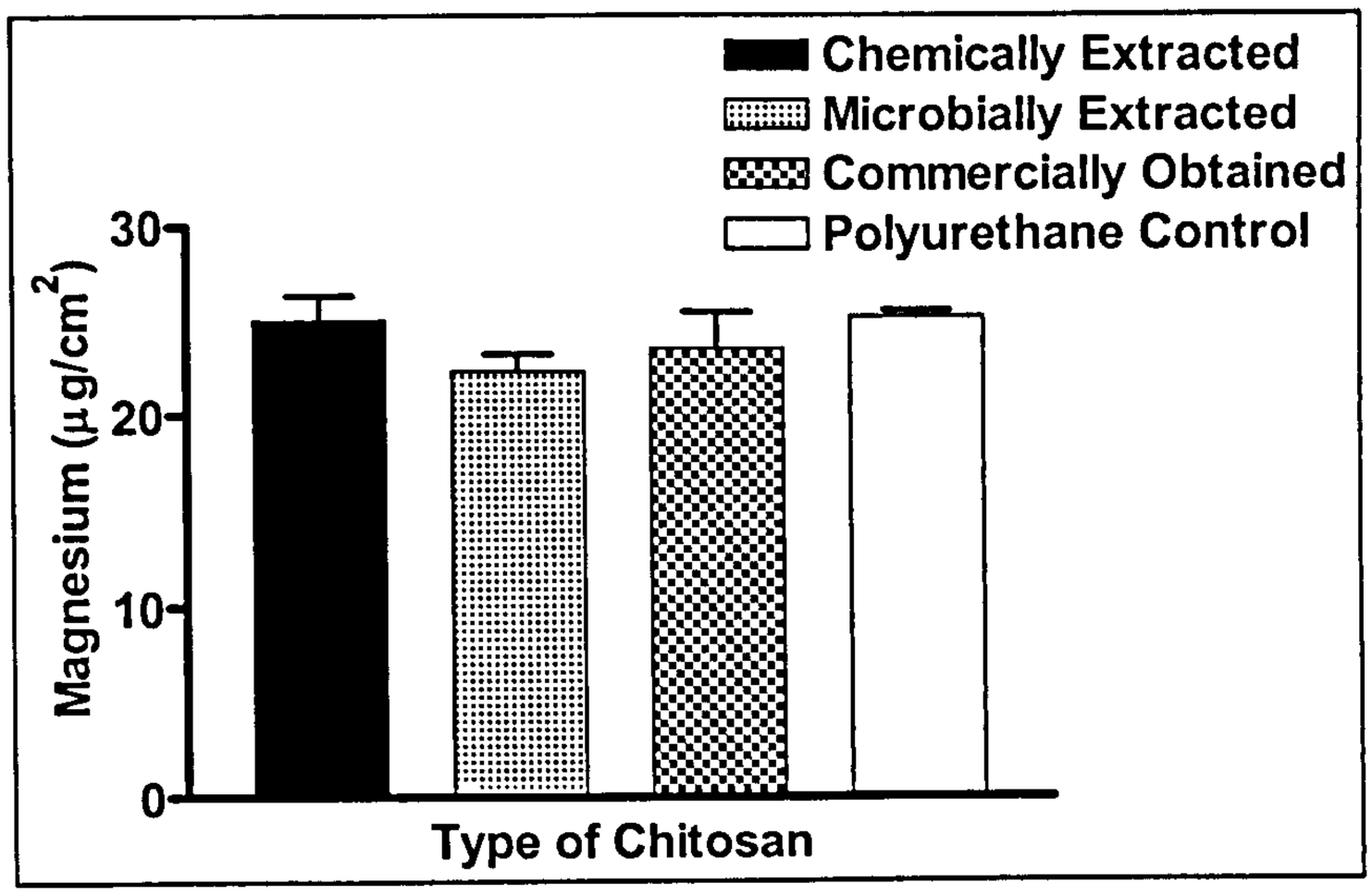


Fig. 7.4 *Adherence of magnesium ions to different types of chitosan membranes in the urinary encrustation bath.* Results shown represent the mean ± standard deviation of values from five determinations.

7.3.5 Tensile Strength Measurements

Tensile strength measurements were carried out for chitosan membranes derived from chitin extracted via lactic acid fermentation. The results obtained are shown in Table 7.4. The membranes were not brittle and showed a considerable degree of mechanical strength.

Sample	Force at Break (N)	Extension at Break (m)	CSA (m^2)	Tensile Strength at Break (Nm^{-2})	Strain at Break	Young's Modulus
1	5.086	0.00164	0.0000004	12715000	0.054666667	232591463.4
2	6.659	0.001825	0.0000004	16647500	0.060833333	273657534.2
3	8.534	0.002128	0.0000004	21335000	0.070933333	300775375.9
4	7.396	0.001888	0.0000004	18490000	0.062933333	293802966.1
5	7.699	0.00272	0.0000004	19247500	0.090666667	212288602.9
6	5.189	0.00104	0.0000004	12972500	0.034666667	374206730.8
7	7.396	0.002032	0.0000004	18490000	0.067733333	272982283.5
8	8.026	0.001745	0.0000004	20065000	0.058166667	344957020.1
9	6.01	0.00176	0.0000004	15025000	0.058666667	256107954.5
Mean		17220833.33		0.062140741		284596659.1
StDev		3079079.865		0.014845882		51257932.21
COV		17.87997018		23.89073891		18.01072872

Table 7.4 *Tensile strength measurements for chitosan membranes. The chitosan was derived from chitin extracted by lactic acid fermentation.*

7.4 DISCUSSION

Lactic acid fermentation produced a product containing approximately 50% (w/w) chitin, 30% (w/w) protein and 10% (w/w) ash (Tables 6.12, 6.14 and 6.15). Four treatments of this contaminated chitin with aqueous alkali NaOH (50% w/v), at 90°C, for one hour, were necessary to produce a chitosan product that was soluble in aqueous acetic acid (Table 7.2). An increase in temperature and/or length of time for each deacetylation step may have reduced the number of deacetylation steps required but degradation of the chitosan polymeric chain would have reduced the molecular weight of the final chitosan product (Mima *et al.*, 1983).

Neither chitin nor chitosan can be thought of as single materials with a defined composition. Chitin is not normally 100% acetylated, nor is chitosan usually 100% deacetylated. The different chitosan isolates therefore have different percentage deacetylation values. The percentage deacetylation is one of the most important characteristics of chitosan because it contributes to many chemical and physical properties of membranes produced from it, such as crystalline structure (Urbanczyk and Lipp-Symonowicz, 1994), mechanical strength (Averbach, 1977; Chen and Hua, 1996), and biodegradation properties (Tomihata and Ikada, 1997). However, the chemical and physical properties of chitosan membranes may also be altered by blending the chitosan with synthetic polymers (Qurashi *et al.*, 1992; Ratto *et al.*, 1996).

The percentage deacetylation can be determined from the nitrogen content of the chitosan, as shown in Table 7.5, provided that the chitosan contains no protein (Roberts, 1992). However, the use of the N:C ratio provides more accurate values because of the possible presence of moisture and inorganic materials in the chitosan. In this study it was assumed that the protein in the starting material had been removed by the alkaline deacetylation treatment and therefore the N:C ratio was employed to determine percentage deacetylation. The percentage N-acetylation for all the chitosan materials studied (Table

7.1) was estimated from Table 7.5 to be in the range of 20 - 40% and hence the % deacetylation was in the range of 60 - 80%. Chitosan normally dissolves in dilute aqueous acids when deacetylation is about 60% or above (Roberts, 1992).

	N-acetyl (%)					
	0	20	40	60	80	100
N content (%)	8.69	8.26	7.87	7.52	7.19	6.89
C content (%)	44.71	45.33	45.89	46.39	46.89	47.29
N:C ratio	0.194	0.182	0.171	0.162	0.153	0.146

Table 7.5 *Relationship between the % N-acetyl value of a sample of chitin or chitosan and the % N or % C content* (Roberts, 1992).

Percentage deacetylation can also be determined by FTIR analysis. The absorbance of either the amide I or amide II absorption band is measured, using the absorbance of a suitable internal reference peak to correct for sample concentration in the KBr disc (Roberts, 1997). Several different band ratios have been proposed for use in this method - A_{1550}/A_{2878} (Sannan *et al.*, 1978), A_{1655}/A_{2867} (Miya *et al.*, 1980), and A_{1655}/A_{3450} (Domszy and Roberts, 1985). However, each of these methods has limitations and the actual percentage deacetylation value achieved can depend on which band ratio is used to calculate it from the IR spectrum (Baxter *et al.*, 1992). The FTIRs shown (Figs. 7.1 and 7.2) highlight the identical profile of chitosan prepared from bioprocessed *Nephrops* shell waste and commercially obtained chitosan. However, percentage deacetylation determinations were not calculated from the FTIR spectra due to the presence of moisture in the samples, which would have led to inaccurate results.

Chitosan membranes were easily prepared once the chitosan was soluble in acetic acid. The membranes produced were soluble in acidic solutions. However, for the proposed experiments in the urinary encrustation tank this was not a problem because the

pH of the contents of the bath was alkaline. The percentage deacetylation of the chitosan is very important for the production of films, as discussed above. Highly deacetylated chitosan produced at high temperatures usually has a low molecular weight and provides brittle films, with little mechanical strength. In contrast, partially deacetylated particles do not dissolve well and produce poor films (Averbach, 1977). Deacetylation must lead to the dissolution of particles but not to the degradation of the chitosan if good films are to be produced. However, the deacetylation step is not the only stage at which the quality of the chitosan is affected. The polymer may also be degraded by shear if a rapid mixing technique is used to dissolve the chitosan (Averbach, 1977).

New polymeric materials for use in urinary catheters are continuously being sought. These materials need to withstand degradative effects, be resistant to bacterial adherence, be resistant to encrustation and fracture, be easy to insert and retrieve and cause minimal irritation to the patient (Tunney *et al.*, 1999). Chitosan is a natural biodegradable polysaccharide. However, '*in vitro*' studies showed that chitosans with a percentage deacetylation of >73% were resistant to a 4mg/ml solution of lysozyme (Tomihata and Ikada, 1997). Chitosans with lower percentage deacetylations were degraded more easily. Similar results were found in '*in vivo*' studies in rat. When the biodegradation was slow the tissue response to the chitosan was shown to be mild.

Another characteristic of chitosan that makes it a potential candidate for the manufacture of urinary catheters is its antimicrobial property. Chitosan has been shown to be detrimental to *Escherichia coli* by adhering to the bacterial surface and hence causing membrane permeability, followed by the leakage of intracellular components and death of the cells (Tsai and Su, 1999). Attempts have been made to make urethral catheters less attractive to bacteria by applying coatings of hydrophilic hydrogels or antibacterial agents such as silver (Stickler *et al.*, 1996). Alternatively, it may be possible to apply a chitosan coating to provide antibacterial properties.

One of the main problems encountered when using urinary catheters is that they become blocked by crystalline deposits of struvite (ammonium magnesium phosphate) and apatite (calcium phosphate). Therefore, potential new materials for use in the manufacture of urinary catheters must resist this encrustation. The preliminary results obtained in this study after suspension of chitosan membranes in artificial urine were very promising (Figs. 7.3 and 7.4). Encrustations of calcium and magnesium ions on the chitosan were no higher than on the polyurethane control samples. In fact, the build-up was significantly lower in the case of magnesium.

The chitosan membranes used contained significant quantities of calcium before immersion in the encrustation tank (Table 7.3) because the lactic acid fermentation procedure for the isolation of chitin left some residual ash in the chitin product. This means that the results shown (Fig. 7.3) accounted for both build up of ions due to encrustation from the artificial urine and ions on the chitosan prior to use. Actual adherence of calcium salts to chitosan produced from bioprocessed shell, due to encrustation in the urinary bath, was therefore lower than shown in Fig. 7.3 and lower than encrustation on the polyurethane control samples. With respect to encrustation, chitosan from bioprocessed shell may prove to possess properties that make it more suitable for catheter manufacture than the polymers already in use for such a purpose.

Chitosan, derived from chitin extracted by lactic acid fermentation, provided membranes with a considerable degree of mechanical strength (Table 7.4). The average percentage elongation (6.21%) was similar to that obtained by Averbach (1977) for chitosan membranes. Chang *et al.*, (1998) produced chitosan membranes in a comparable manner but the membranes were more brittle (3.65% elongation at break). The percentage deacetylation of chitosan affects the mechanical strength of membranes prepared from the chitosan (Averbach, 1977). Therefore, membranes with a desired tensile strength could be produced via carefully controlled deacetylation steps. Other properties of the chitosan

could be also altered thereby making them more suitable for use in implant manufacture. For instance sulphonation of chitosan increases its antimicrobial activity (Chen *et al.*, 1998).

While this study has shown that chitosan, produced from bioprocessed *Nephrops* shell waste, has potential for use as a coating for urinary catheters, it has also demonstrated that chitosan membranes prepared from the same chitosan possessed a substantial degree of mechanical strength indicating that the material may possibly be used in its entirety i.e. to make whole devices.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 GENERAL DISCUSSION AND CONCLUSIONS

The main aim of this project was to isolate chitin from *Nephrops norvegicus* shell waste using the biotechnological methods of lactic acid fermentation and enzymatic proteolysis. Chitin is the second most abundant naturally occurring polymer known to man, cellulose being the most abundant. Structurally it is composed of subunits of D-glucosamine and N-acetyl-D-glucosamine. These subunits are linked together via a β-(1→4) linkage. Chitin acts as a skeletal material, contributing to the hardness of arthropod exoskeletons and the walls of fungi, algae and protozoa. Its structural role shows similarities to that of cellulose in plants or collagen in animals.

Chitosan is the main derivative of chitin. It too occurs naturally, being found in the hyphal walls of fungi, but more often is produced from chitin. The distinction between chitin and chitosan is not dramatic. Chitosan is a form of chitin containing a majority of unacetylated glucosamine subunits whereas in chitin up to one in six residues may be unacetylated. The two compounds are most easily distinguished by their solubility in dilute acid. The reduced number of N-acetyl groups found in chitosan means that chitosan is more reactive than the relatively inert chitin. Therefore chitosan dissolves easily in dilute acids whereas chitin is insoluble.

Chitin and chitosan are potentially valuable commodities due to their natural abundance and the widespread applications for which they have been used. Both compounds have potential applications in industries as diverse as the food industry (Skaugrud and Sargent, 1990), the biomedical industry (Shigemasa and Minami, 1995), the water treatment industry (Kim *et al.*, 1998), and the textile and dyeing industries (McKay *et al.*, 1987). These applications stem from their abilities to adsorb proteins (Hackman, 1955), form complexes with metal ions (Muzzarelli, 1973 and 1977) and adsorb dyes (Giles *et al.*, 1958). However, despite the properties of these substances and the host of uses available very few of the applications have found their way off the pages of literature

research and into the commercial market. It is generally accepted that this under-utilisation of chitin and chitosan is due to their natural locations and a lack of environmentally friendly methods of isolation.

At present the majority of the chitin on the market is extracted from crustacean exoskeletons, such as shrimp or crab shells, and chitosan is manufactured from this source. Crustacean shell waste, i.e. waste remaining after removal of meat for human consumption, poses a problem due to its abundance - approximately 30,000 tonnes is dumped annually in the U.K. (SeaFish Industry Authority, 2001) and its perishable nature. In Northern Ireland the landings of the Dublin Bay prawn, *Nephrops norvegicus,* represent a major industry and because of current waste disposal policy new methods of disposal for the shells are sought. Making use of the waste for chitin isolation provides an alternative to disposal via landfill sites. However, due to its location within the exoskeleton, chitin isolation from the shell is not simple unless harsh, polluting chemicals are used.

The polymeric chains of N-acetylglucosamine in decapod crustacean shells studied so far are located in a microfibrillar complex with protein components of the shell (Green and Neff, 1972; Mutvei, 1974; Compère and Goffinet, 1987). Inorganic substances, mainly calcium carbonate, impregnate the chitin-protein microfibrils. The shells of *Nephrops norvegicus* have been shown in this project to follow the same pattern of arrangement as the widely studied shore crab and European clawed lobster shells (Mutvei, 1974). Isolation of chitin from crustacean shell waste is usually carried out using the long established methods of chemical degradation of the shell to leave intact chitin. In general 1M HCl is used to dissolve the inorganic portion of the shell and 4% (w/v) NaOH to dissolve the protein components (reviewed by No and Myers, 1995). After washing, the residue consists mainly of chitin with some small contamination due to ash and protein.

Whilst these methods are more than adequate for the production of chitin several drawbacks have led to research into alternative extraction methods. Of main concern,

large amounts of aqueous waste are generated. This waste can be costly to dispose of. Secondly, the harsh chemicals destroy other potentially valuable components of the shell, such as the protein. If extracted intact or with little degradation the protein could be used in animal feed production or even in human food generation. Thirdly, the chemicals used can lead to a slight denaturation of the chitin that would be best avoided.

As a consequence of these disadvantages research since the early 1990's has investigated the possible extraction of chitin and other shell components from crustacean shell waste using the technology of lactic acid fermentation (Hall and De Silva, 1992; Healy *et al.*, 1994). In this process lactic acid bacteria are provided with a carbohydrate substrate and mixed with the shell waste. Under suitable conditions of temperature, agitation speed and solid to liquid ratio lactic acid production ensues. This lactic acid prevents the growth of spoilage organisms, which may be located on the waste, and at the same time dissolves the mineral components of the crustacean shell.

In this project *Nephrops norvegicus* shell waste was mixed with glucose and Lactosil, a commercial silage inoculant containing three species of lactic acid bacteria. The waste had previously been autoclaved to eliminate bacteria indigenous to the shell. Optimisation of fermentation conditions resulted in a chitin product containing 11.1% ash and 31.1% protein. The ash contamination was higher than that found in chemically extracted shell (<1% w/w) but nevertheless was significantly lower than the ash content of the initial starting material (46.6%) indicating that lactic acid fermentation was an effective biotechnological means of shell demineralisation. However, shell deproteinisation was unaffected by lactic acid fermentation.

Several studies using lactic acid fermentation alone to extract chitin from crustacean shell waste were able to reduce the protein content substantially. Guerrero-Legarreta *et al.*, (1996) removed 70% of the protein from *Nephrops norvegicus* via ensilation with Lactobacillus or Pediococcus species of lactic acid bacteria. Shirai *et al.*, (1998) removed

67.05% protein from prawn waste (*Penaeus monodon*) by ensilation with a Lactobacillus species. Zakaria *et al.*, (1998) achieved a 61% protein reduction when *Nephrops norvegicus* waste was ensiled with *Lactobacillus paracasei*. However, the lack of protein removal in the current study was not unexpected due to the specific nature of the waste material in use. In Northern Ireland the majority of *Nephrops* landed have been decapitated and declawed at sea. Therefore the waste available consisted mainly of tail shells. In contrast, other studies in this field used waste that contained the crustacean heads (Guerrero-Legarreta *et al.*, 1996; Shirai *et al.*, 1998; Zakaria *et al.*, 1998). These heads are highly proteinaceous and contain significant quantities of proteases from the gastrointestinal tract and cathepsins from muscle tissue. Both are active at acid pH. During the course of lactic acid fermentation these enzymes therefore act to solubilise the protein component of the shell waste. The chitin isolated thus contains a lower protein content than the chitin extracted in the present study.

Tail shells of *Nephrops* were previously ensiled with a silage inoculant called Stabisil (Bustos, 1996). Stabisil contained the same bacterial species as Lactosil. After optimisation of conditions the product contained 4.5% ash and 33.4% protein, indicating that deproteinisation had not been effective in this study either. The lower ash content achieved in this instance may have been a result of bacterial populations indigenous to the shell since in Bustos' study the shell waste had not been autoclaved prior to fermentation. In an attempt to biotechnologically deproteinise *Nephrops* tail shell Bustos (1996) studied the deproteinisation of chemically demineralised tail shell using proteolytic microorganisms. The chitin product obtained using proteolytic microorganisms contained 8.45% nitrogen (Bustos, 1996). The theoretical percentage nitrogen in chitin is 6.9% so the chitin produced by Bustos was still contaminated by protein.

In the present study and in an attempt to mimic the action of the proteolytic lactic acid fermentation systems, a plant enzyme of broad specificity, active at low pH values,

was added to the lactic acid system. Used in this capacity the enzyme, bromelain, successfully reduced the protein content of the chitin from >30% to 12.2% (w/w). In addition, inclusion of a nitrogen-containing additive, tri-ammonium citrate, to supplement the glucose medium in the bromelain containing fermentation system, led to a further reduction in protein level to 5.3%. At the same time the ash level was also substantially reduced to 4.2%. The chitin produced in this case contained 6.3% nitrogen and was therefore much 'cleaner' than that produced by Bustos (1996). It was therefore concluded that protein removal was much more efficient using a proteolytic enzyme rather than proteolytic microorganisms.

Approximately 83% of the protein present in the lactic acid demineralised waste was solubilised on addition of tri-ammonium citrate and bromelain. This was higher than the 70% protein solubilisation achieved by Guerrero-Legarreta *et al.* (1996) in a system containing shrimp heads. Shirai *et al.*, (1998) achieved 77.06% protein removal using lactic acid fermentation followed by trypsin treatment in a system that probably included proteolytic heads. Rao *et al.*, (1998) achieved 90% deproteinisation when shrimp biowaste was subjected to treatment using a combination of protease and lactobacillus. Bautista *et al.*, (2001) used industrial-grade nitrogen sources to supplement a carbohydrate source of whey when isolating chitin from crayfish waste in a similar manner to the use of tri-ammonium citrate here. Protein reduction by Bautista *et al.*, (2001) (81.5%) was similar to that obtained in the present study. It was therefore concluded that the extent of deproteinisation of *Nephrops* tail shell waste achieved here was as good as that achieved for 'head containing' shell waste and better than that achieved, to date, for tail shell waste.

The chitin produced in this study, by the biotechnological methods of lactic acid fermentation, was converted to chitosan and investigated for its ability to be used in the production of urinary catheters. At present, the long-term use of these devices leads to complications partly caused by blockage due to adherence of calcium and magnesium salts

(Winn, 1998). Research is underway to find more suitable materials which do not allow this encrustation to occur (Tunney *et al.*, 1996c) and chitosan is a potential candidate.

The chitosan used was produced from the shell waste via lactic acid fermentation and chemical deacetylation of the chitin using 50% w/v NaOH at 90°C. This allowed removal of residual protein without the necessity of a proteolytic step. Allergies to *Nephrops* proteins are common (Gaddie *et al.*, 1980; McSharry *et al.*, 1994). So far, very few crustacean-shell proteins have been characterised and until they are it must be assumed that shell proteins may cause allergies in the same manner as meat proteins. In economic terms, any residual protein on the chitin causes a reduction in its market value and makes it less likely to be suitable for use in biomedical applications.

Chitosan membranes were produced and generated very promising results in the encrustation studies. Encrustation of magnesium ions from artificial urine was lower on the chitosan membranes produced from lactic acid treated shell than on the chitosan from chemically produced chitin or on the polyurethane control and it was concluded that the chitosan produced from bioprocessed shell may be more suitable, with respect to encrustation, than some of the materials already in use for catheter manufacture. The membranes also possessed a considerable degree of mechanical strength, a property that could easily be improved upon via manipulation of the chitosan.

8.2 FUTURE WORK

The chitosan produced via biotechnological methods has been shown to be suitable for use in at least one biomedical application. It therefore has to be concluded that there is no reason why bioprocessed chitin or chitosan could not be used in a whole range of applications. The need for harsh chemicals in chitin isolation has been eliminated and chitin production has therefore become a more viable process. To further enhance the viability of the process biotechnological methods must be extended to include chitosan

production. It is possible to convert chitin to chitosan under the action of chitin deacetylase, an enzyme found naturally in fungi. However, this method would necessitate the production of a cleaner chitin than produced here and therefore future work should address the viability of reducing the protein concentration of the chitin further using bioprocessing methods.

Slight alterations to the methodology used in this study may help in protein reduction. For instance, addition of the cofactor, cysteine, to the bromelain-containing fermentation system may enhance the activity of bromelain and lead to further reductions in shell protein concentrations (Beddows *et al.*, 1976; Beddows and Ardeshir, 1979). Another possibility is that the indigenous bacteria may contain a greater proteolytic capacity than the Lactosil silage inoculant and enrichment of those in the system could lead to greater deproteinisation. Alternatively, lactic acid bacteria with well-developed proteolytic systems, such as *Lactobacillus helveticus* (Sasaki *et al.*, 1995) could be used for fermentation.

Wang and Chio (1998) achieved lower rates of deproteinisation with shrimp and crab shell powder compared to untreated shrimp shell and shrimp heads using a bacterial protease and it was suggested that the Maillard reaction, associated with autoclaving and drying, rendered the shell proteins resistant to protease treatment. The shell waste in the present study was dried at 40°C and then autoclaved at 121°C so it is possible that some of the protein was rendered inactive to the action of bromelain. If non-autoclaved shell had been treated with bromelain however it would have been impossible to distinguish the effects of bromelain from those of the indigenous bacteria unless the shell was sterilised by an alternative method to autoclaving.

The meat fragments that remained attached to the tail shell segments, after removal of meat by water jet in the processing factory, may have contained proteolytic enzymes. However, the drying and autoclaving procedures used in this project will have denatured

any enzymes present. Most similar studies used the shell fresh (Hall and Reid, 1995) or stored it frozen then defrosted and minced it up before ensilation (Hall and De Silva, 1992; Guerrero-Legarreta *et al.*, 1996; Kungsuwan *et al.*, 1996). This method may be advantageous if used with tail shell waste because it would not lead to the destruction of any enzymes present, and therefore may allow greater deproteinisation of the waste.

Another important area where further work is necessary is in the utilization of the culture broth that remains after ensilation. This broth was shown to contain lactic acid, glucose and calcium lactate. It is also known to include proteins, amino acids and carotenoproteins. Each of these components is a valuable by-product that should not be discarded.

Crustacean shell silage has been shown to contain nutrients beneficial in the manufacture of fishmeal (Fagbenro and Bello-Olusoji, 1997). The protein content of the shell is a principal example. The protein generated via bioprocessing of *Nephrops* shell waste is less degraded than after the traditional chemical treatment (Bustos and Healy, 1994). Isolation of this protein from the fermentation broth has already been attempted. Kungsuwan *et al.*, (1996) dried the protein hydrolysate obtained from the lactic acid fermentation of shrimp heads and used it in the manufacture of shrimp crackers for human consumption. Synowiecki and Al-Khateeb (2000) analysed protein hydrolysate, obtained from the deproteinisation of demineralised shrimp discards with the enzyme Alcalase. The hydrolysate contained levels of amino acids that suggested it would be suitable as a source of essential amino acids in food products.

The pigment astaxanthin is of importance in salmon farming. Methods exist for the extraction of astaxanthin from crustacean shell (Chen and Meyers, 1982; Simpson and Haard, 1985). Therefore extraction from the culture broth should also be viable. The product obtained after lactic acid fermentation using Lactosil alone was orange/brown in colour indicating that the pigments were still attached to the shell. The broth obtained was

pale in colour and probably contained little astaxanthin. In contrast, the culture broth obtained when bromelain was added to the system was a red-brown coloured liquid. A correlation was also noticed between increase in concentration of proteolytic enzyme and a decrease in shell colour suggesting the release of carotenoids. Hall and Reid (1995) obtained a pink-red coloured silage due to the presence of carotenoids in an acid medium.

The lactic acid produced as a by-product of chitin isolation is another valuable by-product, which should be utilized. It can be converted to poly-(lactic acid) - a biodegradable polymer with many applications, particularly in the biomedical industry e.g. in tissue engineering (Chu *et al.*, 1999).

Calcium lactate is formed in the broth as a result of the action of lactic acid on the calcium carbonate in the shell. Storage of the broth at low temperatures causes the calcium lactate to solidify out. Calcium lactate is another commodity with many applications in different industries e.g. the food industry (Luna-Guzmán and Barrett, 2000).

8.3 MAIN CONCLUSIONS

(1) *Nephrops norvegicus* tail shell waste was ensiled using (a) lactic acid fermentation and (b) lactic acid fermentation in conjunction with the proteolytic enzyme bromelain. The chitin produced contained a lower protein concentration than that achieved previously using bioprocessing methods to extract chitin from *Nephrops* tail shell.

(2) The bioprocessed shell contained residual levels of protein that would have to be removed if the product was to be used for biomedical applications.

(3) After demineralisation via lactic acid fermentation the bioprocessed shell was successfully deproteinised and deacetylated, by a chemical method, to produce chitosan.

(4) The chitosan was shown to have immense potential for use as a coating for urinary catheters.

8.4 AREAS FOR FURTHER RESEARCH

The following major areas for further work have been highlighted:

(1) Lactic acid fermentation of non-sterile minced, fresh or frozen shell waste, as opposed to autoclaved, dried shell waste, in an attempt to encourage further deproteinisation.

(2) Use of lactic acid bacteria with more advanced proteolytic systems than those already in use, in an attempt to encourage further deproteinisation.

(3) Conversion of chitin to chitosan using biotechnological methods such as enzymatic deacetylation.

(4) Characterisation of the liquor produced from the lactic acid fermentation and application of the value-added products contained therein, such as proteins, lactic acid, calcium lactate and pigments.

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