THE ROLE OF ENTERIC BACTERIA IN THE ABALONE, *HALIOTIS MIDAE*.

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ABSTRACT

The role of bacteria in the digestive tract of the abalone *Haliotis midae* was examined to determine whether bacteria aid hydrolysis of polysaccharides present in seaweeds which farmed abalone consume. The enteric bacteria were enumerated using culturable and total (DAPI) counts. The numbers of culturable bacteria fell between $3.5x10^5$ and $2.3x10^8$ cfu/g wet weight tissue. The DAPI counts were between $1.6x10^9$ and $5.1x10^9$cells per gram of tissue. The numbers of bacteria differed between the crop, stomach and intestine. Electron microscopy showed that bacteria were present on the food and gut wall. No specialised structures, to aid adhesion of bacteria, were apparent on the gut wall. The isolated bacteria were identified to genus level using standard biochemical and morphological tests. The common genera identified were *Vibrio, Alcaligenes, Flavobacteria, Pseudomonas* and *Aeromonas*. The bacterial communities in each gut region varied, suggesting that both resident and transient bacterial populations are present in *H. midae*. *Alcaligenes* occurred mainly in the crop, while *Vibrio* species were predominant in the stomach and intestine.

The bacterial isolates were tested for their ability to hydrolyse the polysaccharides alginate, laminarin, CMC, carrageenan and agarose. Bacteria able to utilise these polysaccharides belonged to the genera *Flavobacteria, Pseudomonas, Vibrio, Alcaligenes, Bacillus* and *Enterobacteria.* Many of the isolated bacteria were capable of utilising two or three of the substrates tested.

Quantitative polysaccharase assays using the reducing sugar assays of Nelson (1944) and Somogyi (1952) and Gardner *et al.* (1988) were employed. These assays showed that bacteria from the crop exhibited the greatest degree of CMC and alginate hydrolysis. Bacteria from

the intestine exhibited the greatest carrageenan and agarose hydrolysis. The endogenous enzymes produced by *H. midae* were examined using extracts of the hepatopancreas as it was found to be bacteria free. It was found that abalone synthesize a CMCase, laminarinase, alginase, carrageenanase and agarase. However, the synthesis of these enzymes was related to the diet of the abalone. Abalone fed *Ecklonia* (which contains relatively high concentrations of alginate and laminarin) exhibited significantly higher alginase and laminarinase activity than abalone fed *Gracilaria*. Similarly, abalone fed *Gracilaria* (which contains relatively high proportions of carrageenan and agar) produced significantly higher carrageenanase and agarase activity. Furthermore, these enzyme activities were found to be similar to those extracted from gnotobiotic abalone (obtained using the antibiotics chloramphenicol (250μg/ml), ampicillin (600μg/ml) and cefotaxime (250μg/ml)), indicating that polysaccharide synthesis occurs in the hepatopancreas of *H. midae*. Polysaccharase assays on gnotobiotic abalone were compared to assays on untreated abalone. Bacteria were found to significantly enhance the polysaccharase activity of alginase, laminarinase and agarase.

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CHAPTER 1

INTRODUCTION

Abalone are a highly priced food in many regions of the world. The abalone fishery in South Africa, which began in 1949 and extended from Cape Columbine to Quoin point, near Cape Agulhas (Tarr, 1992), utilises a single species, *Haliotis midae*. Prior to 1984, the total catch of the fishery (about 600 MT per year) was exported to the East but, since then 10% is reserved for local consumption. In 1992 the export value of abalone was about R16 million (Tarr, 1992).

The South African fishery began to decline in the 1960's due to overfishing and poor fisheries management (Britz, 1990). Similarly, the global abalone fishery declined by 35% from 1975 to 1989 (Britz, 1990) which resulted in an increase in the price of abalone as fisheries could not meet the market demand. Typically, when a fishery declines, the economic viability of farming the species is enhanced (Britz, 1990).

The culture of abalone began in Japan in the 1960's with the aim of restocking the wild stock (Britz, 1990). South Africans were reluctant to enter into the culture of abalone because *H. midae* is a slow grower, taking 8 years to reach maturity and 12 years to reach the legal, harvestable size. Interest in the culture of South African abalone began when Genade, Hirst and Smit (1988) showed that *H. midae* could be successfully spawned in captivity. Coupled to this was the development of a market for "cocktail" (50mm shell length) abalone in the East and the demonstration that growth rates of *H. midae* were faster in captivity than in wild populations (Cook, 1990). Britz (1994) reported that cultured abalone are rapidly becoming respected as a reliable source of abalone all year round and cultured abalone can demand very high prices on the international market.

One problem that still faced aspirant abalone farmers in South Africa was the identification of an optimal diet for farmed abalone. The seaweeds *Plocamium, Laminaria* and *Ecklonia* are preferred by wild abalone (Barkai and Griffiths, 1987), but these seaweeds may not be the best feeds in culture (Simons, 1990) as *Ecklonia* and *Laminaria* are low in protein, containing 7.8 and 9.1% respectively (Simons, 1990). Therefore, there is little nitrogen available to the abalone. Stepto (1993) showed that *H. midae* achieve faster growth rates on a diet of *Porphyra* but this seaweed may not be sufficiently abundant as a food for abalone farms. Even with the introduction of artificial foods *Ecklonia* would be the most cost effective food.

Branch and Branch (1981) noted that bacteria were present on kelp fronds, and possibly these bacteria precondition the kelp prior to consumption by a host. If this is so, then it is possible that *Ecklonia* could become a more nutritive food source. Recent research has focused on rotation diets using seaweeds and artificial feeds (Simpson, 1994). One area of research that has been neglected is possible strategies that could increase enzyme activity in the gut. An increase in enzyme activity would enhance digestion and could result in faster growth rates.

Abalone enzymes have been studied in the past (McLean, 1970; Knauer, Britz and Hecht, in press), but not with the view to isolate enzymes to improve digestive efficiency or to quantify a bacterial component of the enzyme activity. Previous studies have shown that abalone produce enzymes that break down the polysaccharides found in kelp species which abalone consume (Mclean, 1970; Knauer *et al.*, in press). Knauer *et al.* (in press) reported that the activity of specific enzymes in juvenile *H. midae* changed with a change in diet. This study led to an interesting idea that synthesis of enzymes could be related to diet. However,

as bacterial enzymes were not considered, it is possible that the change in enzyme activity reported by Knauer *et al.* (in press) could have been the result of changes in bacterial enzymes associated with the food. Therefore, a study which examines the influence of bacteria in the abalone digestive system could provide information which would assist the culture of abalone, especially if bacteria are implicated in polysaccharide digestion.

Previous studies on marine invertebrates have shown that enteric microflora can enhance the growth rate of certain species (De Ridder, Jangoux and De Vos, 1985; Bonar, Weiner and Colwell, 1986; Vitalis, Spence and Carefoot, 1988; Douillet, 1993). Ward and Cummins (1979) reported that the growth rate of the chironomid, *Paratenolipes albimonus* was directly related to the number of microbes in the gut. A number of studies on oysters have also shown the benefit of microbes in the gut. Larvae of the oyster *Crassostrea virginica* showed increased survival with the presence of bacteria in the gut (Bonar *et al.*, 1986), and specific bacteria, designated CA2, enhanced the survival and growth of larvae of the oyster *Crassostrea gigas* (Douillet, 1993). Specific bacteriolytic enzymes in the crystalline style of commercial mussels are capable of lysing the majority of free living bacteria in the water column (Seiderer, Newell and Cook, 1982; Davis, 1985). Other studies have shown that bacteria supply cellulases to digest cellulose present in the diet of these invertebrates (Martinez, 1982; Musgrove, 1988). Dempsey and Kitting (1987) reported that bacteria associated with the shrimp *Penaeus aztecus* assist digestion of the seagrass *Thalassia*. All these studies are examples of how enteric bacteria have influenced the digestive capabilities and growth rate of the host invertebrates. As reported above, it has been suggested that bacteria could help to predigest kelp, and as kelp is a major dietary component of abalone, it is possible that enteric bacteria could enhance the growth rate of *H. midae* by enhancing

the digestive capability of the animal.

Vitalis *et al.* (1988) state that bacteria are generally important in invertebrates feeding on a diet which is not optimally nutritious or which contains indigestible components like cellulose. The diet of *H. midae* may not be optimally nutritious as the percentage of protein in the diet is low. Therefore, it is likely that bacteria may be beneficial to the nutrition of the abalone either in their ability to degrade recalcitrant polysaccharides or by themselves being utilised as a food source.

The role of bacteria in invertebrate digestive systems is quite complex. There are four major symbiotic relationships that could exist between bacteria and invertebrate hosts. These are: commensalism, mutualism, predation and parasitism (Schlegel, 1990). Each will be discussed in turn.

Commensalism suggests that enteric bacteria in the digestive system play no role in the digestive processes of the host animal. This argument has been supported by a number of studies (Payne, Thorpe and Donaldson, 1972; Monk, 1977; Morton, 1978; Seiderer and Newell, 1988). Harris (1993) found that bacteria associated with the hind gut of the prawn *Callianassa craussi* had no effect on digestion in these prawns. Similarly, Barlocher, Newell and Arsuffi (1989) showed that the periwinkle *Littorina* did not require fungal enzymes to digest food. These studies have shown that although bacteria are present in the gut, the invertebrate digests its food. Therefore, the bacteria are simply hitching a ride through the hosts digestive tract.

The most common form of predation between invertebrates and microbes is the actual digestion of bacteria by the host. Bacteria are utilised as a carbon source in the polychaete *Nereis* (Cammen, 1980; Hanson and Tenore, 1981) and as a nitrogen source in many other invertebrates (Lesser and Walker, 1977; Hanson and Tenore, 1981; Martin and Kukor, 1984; Vitalis *et al.*, 1988; Barlocher *et al.*, 1989).

In a mutualistic association, bacteria could precondition food, complement the invertebrate enzymes or fix nitrogen from which the host would benefit. In return, the bacteria acquire residence in a nutrient rich environment. Barlocher *et al.* (1982) showed that fungal enzymes help digest leaves that the shrimp *Gammarus* consumes. Musgrove (1988) reported that microbial enzymes aid digestion of food in fresh water crayfish. Bacteria isolated from the gut of echinoids can physically degrade seaweeds eaten by the sea urchin (Prim and Lawrence, 1975). Perhaps the most elegant study reporting the activity of bacteria in invertebrate digestive systems, was the study by Fong and Mann (1980) on the role of bacteria in the sea urchin *Stronglyocentrotus droebachiensis*. They reported that the microflora metabolise radiolabelled glucose, producing amino acids that were incorporated into the sea urchin tissue. This study suggested that microbial protein may have more nutritive value to invertebrates than kelp.

Guerinot and Patriquin (1981) showed that bacterial nitrogen fixation, in the sea urchin *Stronglyocentrotus droebachiensis*, accounted for 8 to 15% of the urchin's daily nitrogen requirements. Similarly, bacteria in the shipworm, *Teredo malleous,* doubled the cellular nitrogen available to the host (Carpenter and Culliney, 1975). Therefore, the growth of these species is dependant on bacteria colonizing the gut.

The fourth type of symbiosis is parasitism. This usually occurs in the form of pathogenesis or utilisation of invertebrate nutrients by resident microflora to the extent that the host suffers. Parasitism would decrease the growth rate of the host. Some *Vibrio* species have been identified as pathogenic in shellfish (Elston and Lockwood, 1983; Cameron, Garland, Lewis and Machin, 1988; Adams 1991). *Vibrio alginolyticus* is pathogenic in the abalone *Haliotis rufescens* (Elston and Lockwood, 1983).

The majority of studies on marine invertebrate, microbe-host relations, have focused on arthropods (Harris, 1993) and bivalves. The bivalves studied include the clam, *Donax gouldi* (Beeson and Johnson, 1967), oysters (Crosby and Peele, 1987; Douillet, 1993), *Scrobiculana* (Payne and Thorpe, 1993) and mussels (Seiderer *et al.*, 1982; Davis, 1985; Seiderer, Newell, Schultz, Robb, Turley, 1987). The studies on the sea hare *Aplysia* (Vitalis *et al.*, 1988) and the periwinkle *Littorina* (Barlocher *et al.* 1989) are two of the few studies which reported on microbes in gastropod digestive systems.

The present study examined the role of bacteria in the hydrolysis of complex polysaccharides present in the gut of *H. midae*. The study quantified the numbers of bacteria in the digestive system to elucidate whether there are sufficient bacteria in *H. midae* to aid digestion. Bacteria isolated from the digestive tract were tested for their ability to degrade polysaccharides present in seaweeds consumed by abalone. Thereafter, bacterial hydrolysis of seaweed polysaccharides in the abalone gut was quantified *in situ*. The answers to these studies would make it possible to determine whether bacteria could be utilised in the diet of commercial abalone to enhance the growth rate of this animal.

CHAPTER 2

CHARACTERISATION OF ENTERIC BACTERIA IN THE ABALONE, *HALIOTIS MIDAE*

2.1 INTRODUCTION

Studies which attempt to elucidate the role of bacteria in a host animal need to determine whether enteric bacteria are present in sufficient quantities to influence the host's digestion. If the numbers of bacteria are similar to those isolated from the ocean it could be an indication that the bacteria in the gut are simply acquired from the ocean and are passing through with the food. Normally the isolated bacteria are identified to genus, in order to determine whether the "right" bacteria are present. In general, enteric bacteria isolated from marine invertebrates include: *Vibrio, Alcaligenes, Pseudomonas* and *Flavobacteria* (Unkles, 1977; Dempsey and Kitting, 1987; Harris, Seiderer and Lucas, 1991). The answers to these two questions could indicate whether it is worthwhile to pursue the study under investigation.

Most studies on marine invertebrates have involved enumerating and identifying the enteric bacteria present in these invertebrate digestive systems (Colwell and Liston, 1960; Unkles, 1977; Fong and Mann, 1980; Deming and Colwell, 1982; Garland, Nash and McMeekin, 1982; Vitalis *et al.*, 1988; Harris, 1993). Some studies have investigated the difference in the number and genera of bacteria in the gut compared to those associated with the food or the surrounding water (Payne *et al.*, 1972; Garland *et al.*, 1982; Dempsey and Kitting, 1987; El-Shanshoury, Mona, Shoukr and El-Bossery, 1994). These studies have shown that the numbers of bacteria in the digestive tract are greater than those in the water or associated with the food in the environment. This is not surprising as the gut presents a nutrient rich medium to the bacteria whereas the oceans are oligotrophic. In general, the quantity of bacteria enumerated from the gut of marine invertebrates were similar. These numbers frequently fell between $1x10^5$ and $1x10^9$ bacteria per unit measured (Colwell and Liston, 1960; Unkles, 1977; Fong and Mann, 1980; Deming and Colwell, 1982; Vitalis *et al.*,

1988). Unfortunately, the units used in different studies have varied which makes it difficult to make more direct comparisons.

The genera of bacteria isolated from the gut are generally similar to those associated with the food that the host consumes (Dempsey and Kitting, 1987; Payne and Thorpe, 1993; El-Shanshoury *et al.*, 1994) suggesting that marine invertebrates obtain enteric bacterial communities from the food which they consume. Studies on juvenile *Haliotis ruber* show that the bacteria isolated from this species were similar to those on crustose red algae which the abalone consume (Garland *et al.*, 1982). The bacteria *Moraxella* constituted 66% of the community on the alga and 71% of the community in 13 week old abalone.

Aside from enumerating and identifying bacteria, electron microscopy has frequently been employed to determine whether enteric bacteria are associated with structures on the gut wall of the invertebrate studied (Harris *et al.*, 1991; Harris, 1993). Harris (1993) suggested that bacteria associated with villi in the gut are more likely to be resident in the gut and hence beneficial to the host. However, the lack of these structures cannot rule out the possibility that enteric bacteria are mutualistic.

The aim of the present study is to elucidate the role of bacteria in the gut of the abalone *H. midae*. The role of bacteria in abalone has never been studied, making it necessary to begin with the basics in understanding microbe-host relations. Therefore, it is necessary to start by enumerating and identifying the bacteria present in the gut of *H. midae*. This chapter examines these two questions in the context of whether the enteric bacteria are present in

numbers which could influence the nutrition of the host and whether the "right" bacteria are present to have any influence.

2.2 MATERIALS AND METHODS

The abalone used in this study were obtained from an abalone hatchery at the Sea Fisheries Research Institute, Sea Point, Cape Town. The animals were maintained under conditions of controlled temperature (18°C, ± 0.5°C) and fed a diet of the kelp, *Ecklonia maxima*.

2.2.1 Isolation of bacteria

The abalone were dissected aseptically within 2 hours of collection. Three regions of the digestive tract were excised, namely: the crop, stomach and intestine. Initially the oesophagus was excised, but initial bacterial counts yielded low numbers, so it was not considered in this study. Figure 2.1 is a schematic representation of the abalone digestive tract and the portions excised for further analysis.

2.2.2 Scanning electron microscopy

Scanning electron microscopy was used to detect the presence of bacteria in different regions of the gut and whether or not bacteria are associated with food and/or the gut wall. The gut of *H. midae* has very thin walls which made it difficult to remove the entire gut. As a result the gut was removed in sections shown in Figure 2.1.

A piece of the mantle was excised to act as a control, in order to determine whether contamination of gut fluids had occurred and whether the surface bacteria had been adequately removed. The samples of tissue were placed into sterile bottles for 12 hours with the fixative 2.5% glutaraldehyde. Subsequently, the samples were removed and washed in 0.1M phosphate buffer, pH 6.7 (Cross, 1987). The phosphate buffer contained KH_2PO_4 (13.6g/l) and $Na_2HPO_4.2H_2O$ (35.8g/l) mixed at the ratio 2 : 8 final volume. Samples were

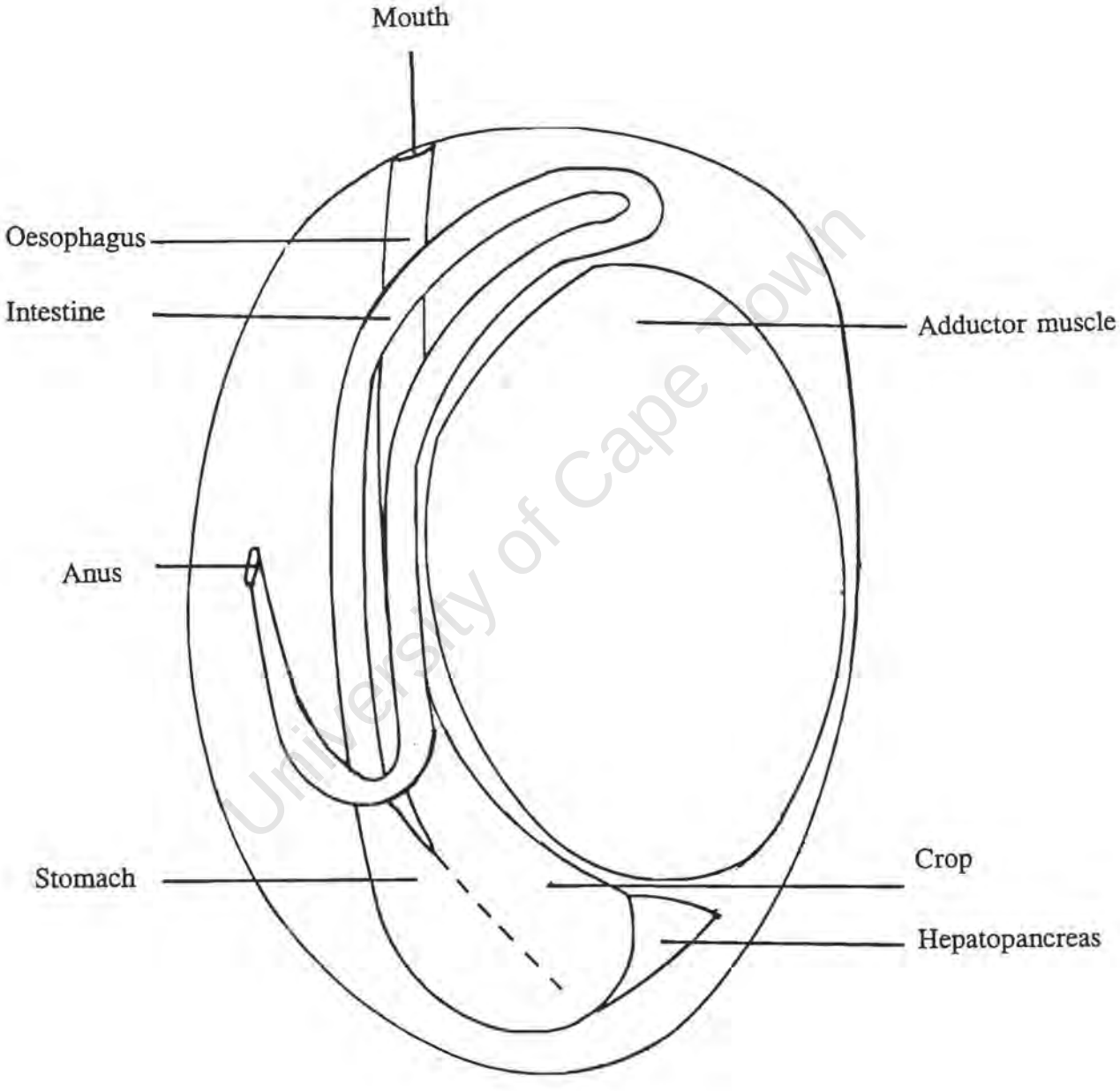

Figure 2.1. Schematic representation of the gut of *H. midae* and the regions of gut excised in this study.

dehydrated in an alcohol series for 10 minutes at each concentration (30, 50, 70, 80, 90, 100% x2). The samples were then critically point dried, mounted onto stubs and coated with 20μm of gold-palladium using a Pelaron 200 sputter coater. Samples were examined using a Cambridge S-200 Scanning Electron Microscope.

2.2.3 Culturable bacterial counts

Samples of abalone gut were weighed and homogenised with 5ml sterile salts buffer (Appendix 3.2) in autoclaved glass homogenisers. The salts buffer was used to prepare 10-fold serial dilutions of the homogenate. An aliquot (200μl) of each dilution was inoculated onto Difco marine agar (no.2216) and estuarine salts agar (Appendix 3.1). The cultures were incubated at 20°C under aerobic or anaerobic conditions. Colonies appearing on the plates were enumerated after 48 hours and normalised to number of colony forming units (CFU) per gram wet weight tissue.

2.2.4 Total bacterial cell counts

The total number of bacteria in each region of the gut was determined by epifluorescence microscopy using the DNA stain 4'6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980) using the method of Harris (1993). Samples of each region of the gut were excised, weighed and fixed in 5 ml 4% formalin (in 0.2 μm filtered seawater). Samples were kept in the dark at 4°C until required. Glassware was washed with 10% HCl throughout. When required, samples were homogenised using acid washed glass homogenisers, diluted 1:2 with 0.1M tetrasodiumpyrophosphate (deflocculant) and incubated at 4°C in the dark for 15 minutes. The tetrasodiumpyrophosphate is used to separate the bacterial cells from organic material in order to achieve a homogenous mixture. Thereafter, the samples were sonicated

on ice for exactly 5 minutes in the dark using an Elma, Transsonic T460. Sonication disperses the cells and bacteria evenly, thereby preventing masking of the bacteria by organic matter. Dilutions of the samples were prepared using 0.2μm filtered seawater. The dilutions used were: 1:1000, 1:1500, 1:2000. The dilution required was determined empirically as a function of the number of bacteria which could be accurately enumerated in one field under the microscope. The samples were vortexed to distribute the cells evenly. DAPI stain was made to a concentration of 1mg/ml and added to 5ml of each sample to a final concentration of 5μg per ml. Two millilitres of each sample was filtered onto 25mm 0.2μm Nucleopore polycarbonate filters using a glass 25mm filter apparatus. The filters were prestained for 48 hours with 1% Irgalon black. Prior to filtration the filters were wetted with 1% photoflow (Paterson anti-static wetting agent) to ensure an even dispersion of cells onto the filters. The filters (with the sample) were mounted onto glass microscope slides with non-luminescent oil. Samples were examined using an epi-fluorescent microscope (magnification 1000x) and the bacteria within the field of view were counted. Twenty random fields were counted for each sample. A Nikon Diaphot inverted compound microscope with a mercury light source, Nikon Neolar 11/1.30 oil objective and Nikon DM 400 filter block were used. The microscope counts were standardised to number of bacteria per gram wet weight tissue, using the following formula:

$$N = \frac{(\text{number of bacteria counted} \times \text{area of filter})}{(\text{area of field of view})} \times \text{mass in g}$$

Where number of bacteria counted is the average from 20 fields.

The effects of variability between animals was reduced by examining the digestive tract from 10 abalone.

2.2.5 Identification of enteric bacteria

Bacteria isolated on marine agar and estuarine salt agar (as described in section 2.2.3) were subjected to standard morphological and biochemical tests for Gram negative and Gram positive bacteria. The tests used were: the Gram stain, the oxidase test, the catalase test, the hanging drop test for motility, the Oxidative-Fermentive (O-F) test, the Voges-Proskauer test, the gelatinase test and growth on thiosulphate-citrate-bile-sucrose (TCBS) agar (Smibert and Krieg, 1994). The bacteria were identified to genus level.

2.3 RESULTS

Scanning electron microscopy (SEM) indicated that bacteria occur throughout the abalone gut. Rod shaped bacteria (approximately 2μm in length) were associated with food particles lining the intestinal wall (Figure 2.2). Bacteria in the stomach were observed to occur on food particles and on the gut wall (Figure 2.3). Since large portions of the gut wall were covered with mucous, it was difficult to detect bacteria throughout the digestive tract (Figure 2.4) or detect any structures to aid adhesion of bacterial populations. Consequently, enumeration of bacteria from electron micrographs was not possible. Coccoid cells were identified amongst the food in the three regions of gut (Figure 2.5).

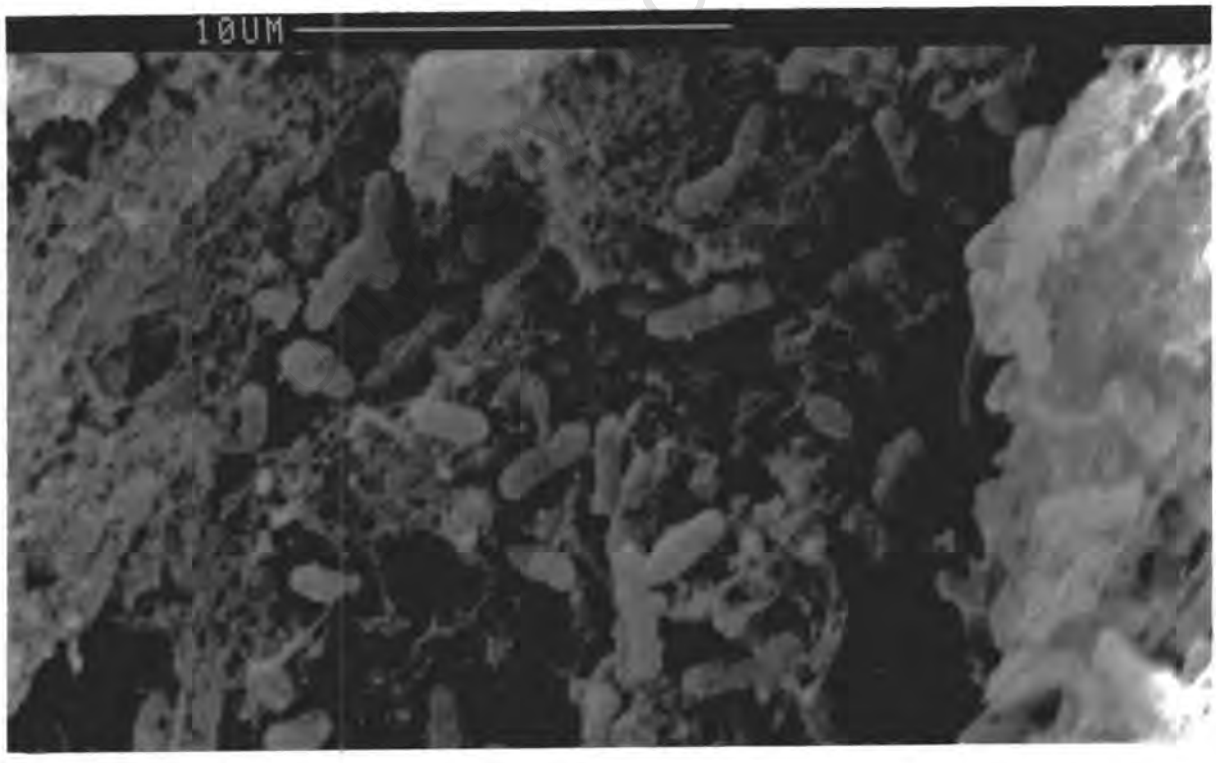

Figure 2.2. Intestine of *H. midae* depicting rod shaped bacteria on the food. (Mag. 3.88 KX)

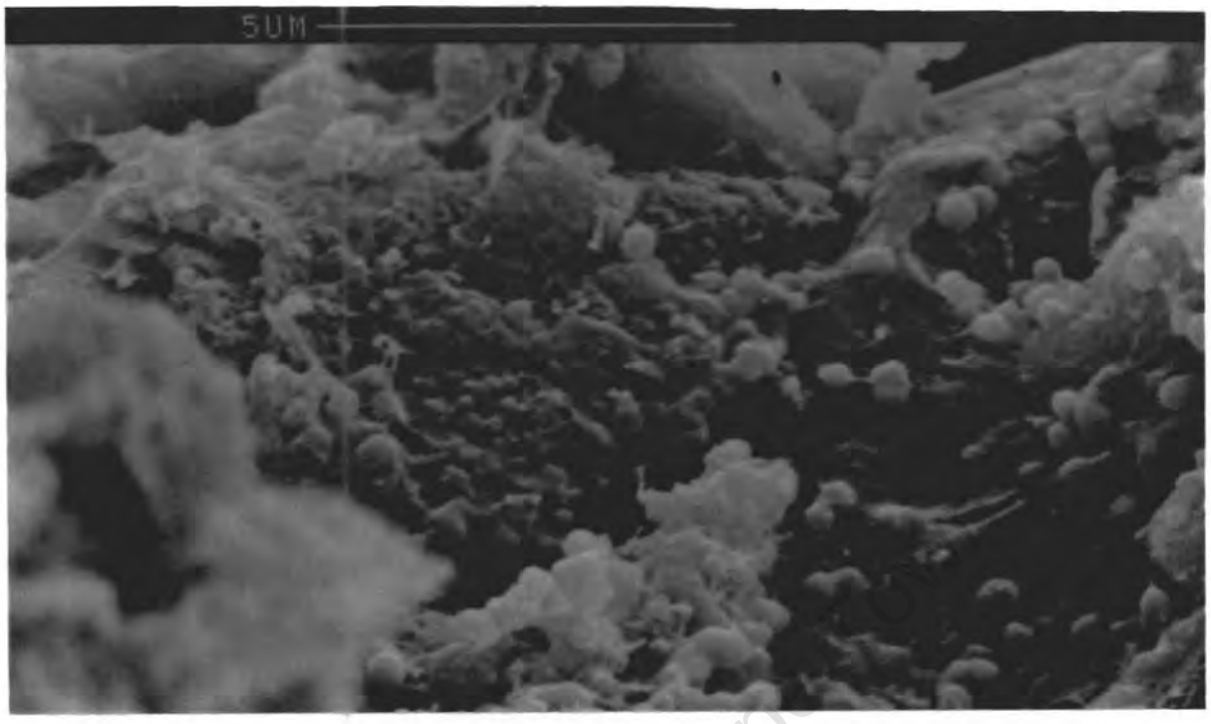

Figure 2.3. Cocci bacteria on the gut wall of the stomach. (Mag.7.45 KX)

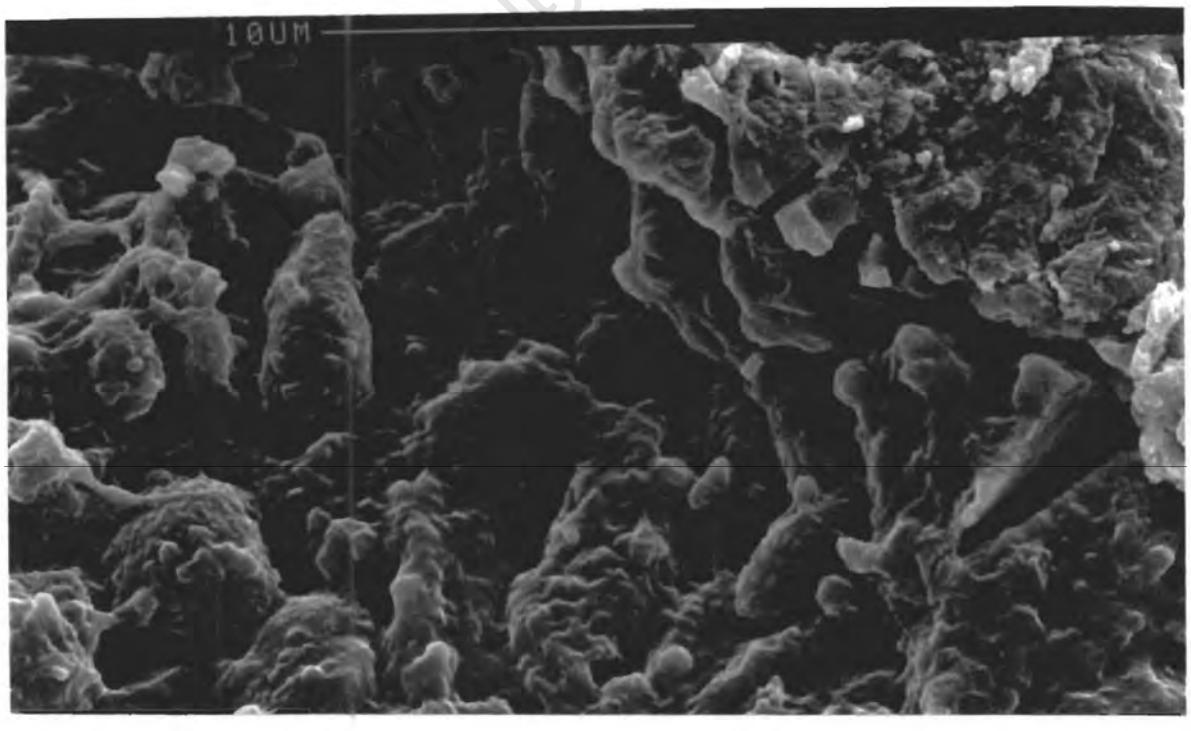

Figure 2.4. Mucous in the gut, covered many areas of the digestive tract. (Mag. 3.36 KX)

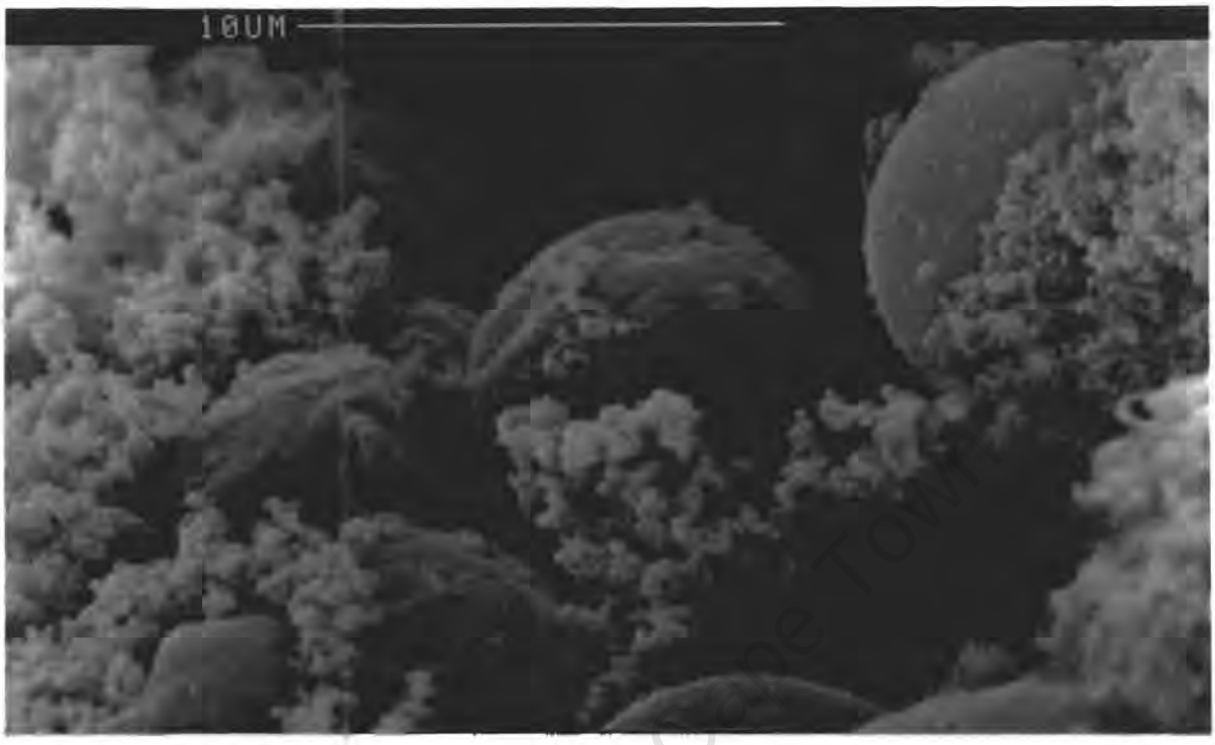

Figure 2.5. Coccoid bodies observed in the food in the stomach. (Mag.4.38 KX)

The results in Table 2.1 indicate that the growth media were selective in that not all the gut bacteria were culturable. This result is reflected by the large discrepancy in bacterial numbers between the culturable and total cell counts. The numbers of bacteria in the crop were less than those from the stomach and intestine, for both enumeration methods (Table 2.1). The numbers of bacteria in the intestine were the greatest. DAPI counts of stomach fluids yielded values of 5.35×10^8 cells/ml. This single result indicates that large numbers of bacteria are associated with the food (this count is from one animal only and must be treated as preliminary). The result does correlate with the micrographs where bacteria were seen on the food.

Table 2.1. Number of viable and total bacteria associated with each region of the digestive system. Values represent the average count.

Gut Region	Viable bacteria[1]		Total bacteria[2]	
	count (n)	SE	count (n)	SE
Crop	$3.5x10^5$ (4)	$9.0x10^4$	$1.6x10^9$ (10)	$9.6x10^8$
Stomach	$5.5x10^7$ (4)	$2.9x10^7$	$3.3x10^9$ (12)	$8.1x10^8$
Intestine	$2.3x10^8$ (4)	$1.1x10^8$	$5.1x10^9$ (10)	$2.4x10^8$

[1] expressed as number of CFU per gram wet weight tissue

[2] expressed as the number of bacterial cells per gram of wet weight tissue

SE is the standard error value for each count.

The culturable bacterial isolates, obtained from various regions of the digestive system of *H. midae*, were assigned to various genera on the basis of a variety of physio/chemical tests (Appendix 1). Although similar bacterial communities occurred throughout the abalone gut, the relative proportions of the bacterial genera comprising these enteric communities differed in the crop, stomach and intestine (Table 2.2). A large percentage of the microbial community in the crop belonged to the genus *Alcaligenes*, while *Vibrio* species constituted a large portion of the microbial community colonising the stomach and intestine of *H. midae*. The diversity of bacteria in the intestine was greater than in either of the other two regions. In general the most commonly isolated bacterial strains were: *Vibrio, Alcaligenes, Flavobacteria* and *Pseudomonas*. Two genera in the crop and stomach comprise 66 and 68% of the bacterial community present in each of those regions, respectively. Three genera of bacteria comprise 61% of the community in the intestine (Data calculated from Table 2.2).

Table 2.2. Genera of v.able bacteria isolated from the abalone digestive system.

Crop	Stomach	Intestine
Alcaligenes (44%)[a]	*Vibrio* (40%)	*Vibrio* (33%)
Pseudomonas (22%)	*Pseudomonas* (28%)	*Enterobacteria* (16%)
Micrococcus (11%)	*Flavobacteria* (16%)	*Alcaligenes* (12%)
Flavobacteria (11%)	*Alcaligenes* (4%)	*Bacillus* (12%)
Enterobacteria (11%)	*Aerococcus* (4%)	*Pseudomonas* (9%)
	Aeromonas (4%)	*Flavobacteria* (7%)
	Micrococcus (4%)	*Aeromonas* (5%)
		Moraxella (2%)
		Unidentified (2%)
		Chromobacteria (2%)

[a] The percentage values indicate the proportion of each bacterial genus comprising the bacterial community within each of the three regions of the digestive system.

2.4 DISCUSSION

The gut wall of *H. midae* had no apparent structures to aid adhesion of bacteria which could be due to the mucous which covered large areas of the gut wall. Sweijd (1990) and Garland *et al.* (1982) reported a similar finding. Sweijd (1990) used amyl acetate to remove the mucous to look at the underlying structures. A similar approach could be employed for a more detailed analysis of the gut wall of *H. midae*. However, it is not imperative that structures are present on the gut wall as Harris (1993) showed that although crustaceans did not have villi for attachment, the bacteria were resident.

The electron micrographs showed that there were large coccoid bodies present in the abalone gut. It is uncertain what these bodies are, but Seiderer and Newell (1988) suggested that these bodies are associated with mucilage strands. Harris *et al.* (1991) also reported the presence of these coccoid bodies but could not suggest a function or origin of these bodies. The size and shape of the bodies seen in *H. midae* are similar to those observed in the gut of fish (Andlid, 1995) which were identified as yeast cells. Therefore, the cells in *H. midae* could be yeast but identification tests need to be done to be certain.

The number of viable bacteria isolated from the abalone gut was less than the total number of bacteria in the gut. This is to be expected unless a greater variety of test media are used to isolate the bacteria. Harris (1993) reported that about 10% of enteric bacteria are isolated on nutrient media. Nokugama (pers. comm.) has suggested that at least 10 different media are required to isolate more than 10% of resident bacterial population. Two different media were used in this study and over 90% of the isolated bacteria grew on the marine agar. A

greater range of media was not required for this study because it was only necessary to isolate bacteria capable of hydrolysing the polysaccharides present in the sea weeds which cultured abalone consume.

The numbers of bacteria isolated from the crop were lower than those from the stomach and intestine. This observation is supported by previous studies where the hindgut was found to harbour the greatest numbers of bacteria (Plante, Jumars and Baross, 1990; Harris *et al.*, 1991). Plante *et al.* (1990) predicted that the hindgut of invertebrates was more likely to harbour large numbers of bacteria as it is the region in which absorption of food occurs.

The numbers of bacteria in the abalone gut were between $1x10^5$ and $1x10^8$ cfu per gram of abalone gut, similar to those reported in other marine invertebrates (Colwell and Liston, 1960; Unkles, 1977; Fong and Mann, 1980; Deming and Colwell, 1982; Vitalis *et al.*, 1988). The numbers of bacteria in abyssal holothurians were between $4x10^8$ and $19x10^8$ bacteria per gram of gut, and these bacteria transformed organic matter in sediments into a form that can be ingested by the holothurians (Deming and Colwell, 1982). The gut of the sea urchin, *Stronglyocentrotus droebachiensus* is colonised by between $2x10^5$ and $6x10^9$ bacteria per ml of tissue. These bacteria were reported to synthesize essential amino acids which were subsequently made available to the sea urchin (Fong and Mann, 1980). An average of $43x10^5$ bacteria per ml gut fluid occurred in the sea hare *Aplysia juliana* (Vitalis *et al.*, 1988). Unkles (1977) reported counts of bacterial numbers between $1.2x10^6$ and $6.3x10^7$ cfu per 3cm of echinoid gut. As seen above, the units used to enumerate bacteria have not been standardised and include units such as "region of gut" and "ml of gut fluid".

I suggest that future studies should standardise the unit used to cfu or total bacteria per gram of tissue. This would make direct comparisons between studies more meaningful. Although direct comparisons with the present study are not possible, the similarity between the present results and previous results cannot be ignored.

The bacterial counts obtained in this study were subject to variation similar to that reported in previous studies (Unkles, 1977; Fong and Mann, 1980; Crosby and Peele, 1987; Dempsey, Kitting and Rosson, 1989). Dempsey *et al.* (1989) tried to determine the cause of such variation by examining the effects of a change in substratum, locality, water temperature, size and depth of penaeid shrimps. None of these factors were found to cause the variation in bacterial numbers. Finally, it was suggested that perhaps the variation could be due to moulting of shrimps (Dempsey *et al.*, 1989). This argument could not be used for abalone because they do not moult. However, the individual variation between abalone is large, perhaps indicating a need for larger sample sizes. Harris (1993) postulated that studies with large variations in bacterial numbers are most likely a result of transient bacterial populations, which constantly change. Resident bacterial populations may be more stable.

The bacteria identified from the abalone gut showed interesting trends. The genera of bacteria isolated from the crop were present in both the stomach and intestine. Specific strains of bacteria occurred either in the stomach or intestine. These bacteria could possibly be resident bacterial populations as they are not present in either of the other gut regions. Harris *et al.* (1991) suggested that resident bacterial populations may be involved in the host animal's nutrition. It is difficult to accurately discern between resident and transient bacterial populations and further studies would be required to be certain that both types of populations

are present in the gut of *H. midae*.

The genera of bacteria isolated from the abalone gut were similar to genera isolated from other marine invertebrates and include the genera *Pseudomonas* and *Vibrio* (Unkles 1977, Harris *et al.* 1991; Colwell and Liston 1960; Dempsey and Kitting 1987), although *Flavobacteria, Alcaligenes* and *Chromobacteria* have also been identified (Dempsey and Kitting, 1987; Dempsey *et al.*, 1989). Two thirds of the communities isolated from each gut region of the abalone consisted of two or three genera. This result is in accordance with previous studies (Dempsey *et al.*, 1989). Perhaps this observation indicates that these dominant genera are more suited to the habitat of a particular gut region. It would be interesting to determine what makes a specific gut region more suitable to some bacteria and not others.

The information that is gained from identifying enteric bacteria has a number of implications. Firstly, because the genera isolated from abalone are similar to those from other marine invertebrates, it is possible that enteric bacteria in abalone have a similar effect to those from previous studies on marine invertebrates. Secondly, if further tests on the bacteria of *H. midae* show that specific bacteria are involved in digestion, then those bacteria could be used in further experiments to analyze the interaction between bacteria and host. Douillet (1993) showed that a specific strain of bacteria was responsible for digestion in oyster larvae.

In summary, this chapter has shown that bacteria isolated from the digestive system of *H. midae* could play a role in seaweed digestion in *H. midae*. This possibility is likely since the

abalone digestive tract is colonised by large numbers of bacteria and the bacterial genera isolated from the gut have been shown to influence digestion in other marine invertebrates. The following chapters will directly address whether the isolated bacteria synthesize polysaccharolytic enzymes capable of influencing the nutrition of *H. midae*.

CHAPTER 3

POLYSACCHAROLYTIC ACTIVITY OF BACTERIA ISOLATED FROM THE ABALONE DIGESTIVE TRACT.

3.1 INTRODUCTION

A wide variety of organic nutrients are available to bacteria in the marine environment. These are in the form of dissolved organic matter (DOM) and particulate organic matter (POM). The latter can be of macrophyte origin. Substrates available to bacteria from kelp include proteins, fats, sugar, polyols, alginates and laminarin (Newell *et al.*, 1980 in Davis 1985). Red seaweeds contain large amounts of agar or carrageenan. Bacteria have been isolated that produce polysaccharolytic enzymes which enable them to utilise these substrates.

Bacteria capable of hydrolysing the substrate alginate have been extensively investigated (Doubet and Quatrano, 1984; Hansen, Doubet and Ram, 1984; Muramatsu and Sogyi, 1990; Gacesa, 1992), probably because of the commercial importance of alginate. In 1990, 27 000 tonnes of alginate was produced in the United States which grossed US$ 230 million (Boyen, Kloareg, Polne-Fuller and Gibor, 1990). Alginate lyase is also of commercial importance and studies which identify bacteria capable of producing alginate lyases could have commercial applications in the future.

Laminarinases have been described and isolated from many sources including: bacteria, yeasts and fungi (Chesters and Bull, 1963). Laycock (1974) identified bacteria growing on kelp fronds which could degrade laminarin and alginate. More recently, Davis (1985), reported that bacteria associated with the fronds of kelp growing along the West coast of South Africa could hydrolyse these complex polysaccharides. These discoveries are relevant to this study as abalone consume kelp species colonised by bacteria, and thus, are possibly colonised by these polysaccharolytic bacteria. Furthermore, it is possible that these bacteria continue polysaccharide degradation in the abalone digestive system and hence, make

monasaccharides and disaccharides available to abalone.

Previous studies on invertebrates have shown that enteric bacteria are capable of utilising the complex polysaccharides described above (Payne *et al.*, 1972; Prim and Lawrence, 1975; Musgrove, 1988; Vitalis *et al.*, 1988). The role of bacterial cellulases has been studied extensively. There are a number of studies that suggest that bacterial enzymes hydrolyse cellulose in the invertebrate host's gut (Quatrano and Caldwell, 1978; Dempsey and Kitting, 1987; El-Shanshoury *et al.*, 1994) whilst others have reported opposite findings (Payne *et al.*, 1972; Prim and Lawrence, 1975; Vitalis *et al.*, 1988; Harris *et al.*, 1991; Harris, 1993). This issue has been topical as it is generally accepted that eukaryotes cannot produce cellulases and that microbial cellulases are necessary to degrade cellulose. Further studies are needed to clarify this issue.

It is well accepted that enteric bacteria do assist the degradation of laminarin, alginate and carrageenan and other substrates. Bacterial alginases have been isolated from mollusca, echinoderms (Boyen *et al.*, 1990), and crustaceans (Harris *et al.*, 1991; Harris, 1993). These enzymes have been shown to increase the digestive efficiency of their invertebrate hosts (El-Shanshoury *et al.*, 1994).

Although a number of studies report that abalone synthesize polysaccharolytic enzymes (Ostgaard, Knutsen, Dyrset and Aasen, 1993; Knauer *et al.*, in press) it is unknown whether bacterial enzymes contribute to this activity. This chapter examines the enzyme activity of the bacteria isolated and identified in Chapter 2. The substrates agar, carrageenan, alginate, laminarin and carboxymethylcellulose were used to test this hypothesis, as these substrates

are present in the diet of the cultured abalone. If the bacteria are able to degrade the substrates tested, then it may be possible that these bacteria are degrading these substrates in the abalone gut and if so, then the abalone could benefit from the association with the bacteria.

3.2 METHODS

3.2.1 Bacterial utilisation of algal polysaccharides.

The bacteria isolated from the crop, stomach and intestine of abalone were propagated on synthetic media to test their ability to degrade carrageenan, carboxymethylcellulose (CMC), agarose, laminarin and alginic acid. The time of incubation varied according to how well the bacteria grew on each of the plate media.

Culture media for the identification of alginate utilising bacteria consisted of (wt/vol) peptone and yeast extract 0.1%, agar 2%, alginic acid 2.5% and potassium phosphate 0.03% in filtered sea water (Whatmans no. 1 filter paper).The bacterial cultures were incubated at 22°C for 5 days. The identification of polysaccharolytic bacterial strains was according to the protocol of Teather and Wood (1982). The petri dishes were flooded with 0.1% Congo red for 15 minutes and subsequently scored for zones of hydrolysis surrounding the bacterial colonies.

Abalone enteric bacteria capable of degrading laminarin were identified following growth on media containing (wt/vol) peptone, yeast extract and laminarin (Sigma L-9634) 0.1% and agar 1.5% in filtered sea water. The bacteria were incubated for 3 days at 22°C. Plates were flooded with 0.1% Congo red for 15 minutes and the zones of hydrolysis noted.

Cellulolytic bacteria were identified on media containing CMC prepared according to Davis (1992). Following incubation at 22°C for 2 days, the cultures were initially flooded with 0.1% Congo red for 15 minutes and subsequently with 0.1% HCl in order to visualise hydrolytic zones around cellulolytic bacteria.

Agarolytic bacteria were isolated by growth on culture media consisting of solution A ((wt/vol) 0.25% peptone, 0.1% yeast extract mixed with 750ml filtered sea water) and solution B ((wt/vol) 2% agarose in 250ml distilled water) which were sterilised separately prior to mixing. The bacterial cultures were incubated at 22°C for 3 days. Plates were flooded with Gran's iodine and incubated at 4°C for 15 minutes. Zones around the bacterial isolates denoted agarolytic bacteria.

Culture media and assay procedure for carrageenan hydrolysis followed the protocol for agarose, except that 3% carrageenan was substituted for 2% agarose in solution B.

3.2.2 Enzyme Assays

Bacterial strains which exhibited large zones of hydrolysis on the plate media, were investigated further using quantitative enzyme assays.

Bacterial cells were grown in minimal media supplemented with either agarose, CMC, carrageenan, laminarin or alginate as a sole carbon source. The basic minimal media consisted of (wt/vol): 0.1 % yeast extract, 0.1% peptone and the appropriate polysaccharide substrate in filtered (Whatmans no. 1 filter paper) sea water. The final concentrations (wt/vol) of the polysaccharides added to the media were as follows: 1% alginate, 1% CMC, 0.2% laminarin, 0.1% agarose and 0.1% carrageenan. The alginate, CMC, agarose and carrageenan bacterial cultures were incubated at 22°C for 12 hours with agitation. The laminarin cultures were incubated at 22°C for 16 hours. Subsequently, 500μl of the CMC, agarose and carrageenan bacterial cultures were transferred into 4.5ml of fresh minimal media, containing the appropriate polysaccharide, for a further 12 hours. Cells were

harvested by centrifugation at 12 096 (x g). The cells for the alginate, laminarin and CMC assays were centrifuged for 15 minutes, while the other two media for 10 minutes. The centrifugation time period for the alginate, CMC and laminarin bacterial cultures was longer as these cultures did not form pellets within 10 minutes. The supernatant was collected in order to assay for the presence of extracelluar bacterial polysaccharases, and the pelleted bacteria resuspended in either 5 ml 100 mM potassium phosphate buffer, pH 6.9 (Nelson-Somogyi assay) or 5 ml 20 mM PIPES buffer, pH 6.8 (ferricyanide reducing sugar assay). The cells were disrupted using a French press.

Polysaccharase activity was determined by assaying the amount of reducing sugar produced following the hydrolysis of a particular polysaccharide substrate over a specific time period. The polysaccharide substrates were assayed at the following concentrations (wt/vol): 0.4% carrageenan, 0.4% agarose, 1% CMC, 0.4% laminarin and 1% alginate. Alginate lyase, laminarinase and CMCase were assayed according to the method of Nelson (1944) and Somogyi (1952). Agarase and carrageenanase activities were determined according to the ferricyanide reducing sugar method of Gardner *et al.* (1987).

Time zero tubes for each assay were prepared by adding the stop reagent simultaneously with the enzyme extract. The time zero tubes took into account any reducing sugar formed before the experiment. *Escherichia coli* was used as a negative control for the Nelson-Somogyi assay in order to be certain that the assay was working as predicted.

The amount of reducing sugar formed was obtained by reading the light absorbance at 660 nm (Nelson-Somogyi assay) and 420nm (ferricyanide reducing sugar assay) using a Beckman

spectrophotometer. The protein concentration of each sample was determined by the method of Bradford (1976). Enzyme activities were expressed as mg reducing sugar generated/ mg protein/ hour, where the amount of reducing sugar liberated from each of the polysaccharide substrates was determined from standard curves constructed by plotting light absorbance against known concentrations of the appropriate sugar.

3.3 RESULTS

The culturable bacterial strains isolated from *H. midae* were screened for their ability to hydrolyse a variety of polysaccharides which occur in seaweeds (Table 3.1). The results for each strain of bacteria are presented in Appendix 2 and the data summarised in Table 3.1. *Moraxella, Chromobacteria, Micrococcus* and *Aerococcus* species were unable to utilise the five polysaccharides tested. *Flavobacteria, Pseudomonas, Vibrio* and *Alcaligenes* species were the most common strains capable of hydrolysing the algal polysaccharides in each region of the digestive system. The crop, stomach and intestine contained bacteria capable of utilising each of the five polysaccharides tested. Sixty eight percent of the isolated bacteria utilised two or three of the substrates tested on plate media (Figure 3.1). Almost the same number of bacteria utilised none, one, four or five of the substrates (data calculated from Appendix 2).

Table 3.1. The genera of isolated bacteria capable of utilising seaweed polysaccharides.

SUBSTRATE	CROP	STOMACH	INTESTINE
Cellulose	*Flavobacteria* *Alcaligenes* *Pseudomonas*	*Flavobacteria* *Pseudomonas* *Aeromonas* *Vibrio*	*Flavobacteria* *Pseudomonas* *Aeromonas* *Vibrio* *Alcaligenes* *Bacillus* Unidentified
Laminarin	*Alcaligenes* *Pseudomonas*	*Flavobacteria* *Pseudomonas* *Vibrio*	*Flavobacteria* *Enterobacteria* *Alcaligenes* *Vibrio* *Bacillus*
Agarose	Unidentified	*Vibrio* *Pseudomonas*	*Vibrio* *Alcaligenes* *Bacillus* Unidentified
Alginate	*Pseudomonas*	*Vibrio* *Pseudomonas* *Flavobacteria*	*Enterobacteria* *Bacillus* *Vibrio* Unidentified
Carrageenan	*Flavobacteria* *Alcaligenes*	*Vibrio* *Pseudomonas*	*Vibrio* *Alcaligenes* *Bacillus* *Pseudomonas* *Flavobacteria*

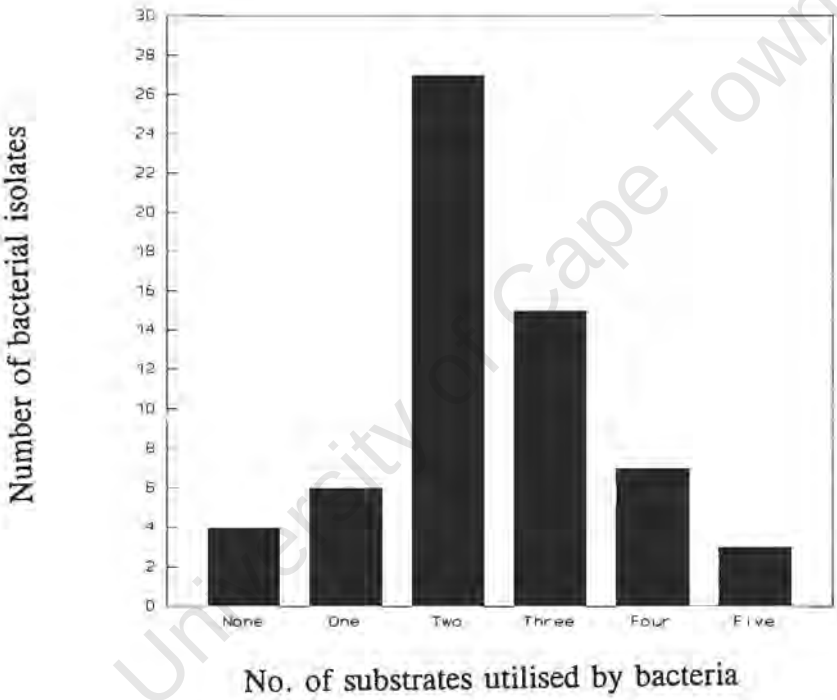

Figure 3.1. The number of substrates utilised by each bacterial strain.

The results of the bacterial polysaccharase assays indicated that between 70 and 90 % of activity detected was extracelluar (data for the cell extract reducing sugar assays not shown as many of the values were zero). From Table 3.2 it is apparent that *Vibrio* (13 strains) were the predominant polysaccharolytic bacteria. In particular, *Pseudomonas* strain (C4), isolated from the crop, exhibited the highest alginate lyase activity (Table 3.2). This strain also yielded the highest laminarinase and CMCase activity. The bacteria which could utilise alginate were *Pseudomonas, Flavobacteria, Bacillus* and *Vibrio* (Table 3.2). *Pseudomonas, Vibrio, Flavobacteria* and *Alcaligenes* exhibited CMCase, agarase, carrageenanase and laminarinase activity (Table 3.2). The *Alcaligenes* strain S4 produced the highest agarolytic activity of the bacterial strains tested. The carrageenanolytic activity of strain S4 was comparable to that of the other intestinal bacteria able to hydrolyse this glucan polymer (Table 3.2). Although the number and diversity of bacteria is greater towards the latter region of the abalone digestive system (Table 2.1, Table 2.2), the distribution of extracelluar bacterial polysaccharases did not follow the same trend. Bacterial alginate lyase and CMCase activity was maximal in bacteria isolated from the abalone crop, while laminarinase activity was equivalent in bacteria colonising the crop and intestine (Figure 3.2). Bacterial agarase and carrageenanase activity was most pronounced in bacterial strains isolated from the intestine of *H. midae* (Figure 3.2).

Table 3.2. Extracelluar enzyme activity and substrate specificity of the polysaccharases of the enteric bacteria isolated from the digestive system of *H. midae*.

Gut Region	Bacterial Strain	Alginate[1] a	Laminarin[2] a	CMC[2] a	Agarose[3] a	Carrageenan[2] a
Crop	*Pseudomonas* C4	152.3 4.7	11.7 1.4	67.4 11.9		
	Flavobacteria Z2			1.8 1.7		
	Alcaligenes C3		5.9 2.0			24.3 4.7
Stomach	*Vibrio* Y8				7.0 1.3	13.8 0.8
	Flavobacteria Y2	4.1 2.4		4.0 1.8		
	Pseudomonas Y7			5.9 3.0		
	Pseudomonas Y5	3.0 1.8	2.9 1.9		2.4 0.4	29.2 1.7
	Vibrio S1		2.8 2.1			
	Flavobacteria S2	5.6 2.1	2.8 1.5			
	Vibrio X6	8.8 2.1				
Intestine	*Vibrio* S2			2.6 2.2		
	Pseudomonas S7			8.0 2.6		
	Moraxella S3			6.3 2.6		
	Unidentified J2	4.9 2.1		5.1 1.8		
	Vibrio J4	6.1 1.2	5.3 0.2			
	Vibrio J3	2.8 1.8	4.8 1.9			
	Pseudomonas I3	3.9 1.7	4.2 1.7			
	Alcaligenes U6		3.1 0.9			
	Vibrio J1	10.6 0.5			8.8 0.6	
	Vibrio R5	2.9 1.7				
	Bacillus T4	1.8 1.1				
	Vibrio I4				5.9 1.0	
	Vibrio I1					24.8 3.1
	Alcaligenes S4				98.9 13.1	25.8 1.0
	Vibrio U5				5.0 1.0	31.1 0.8
	Vibrio U4				4.8 1.2	26.6 4.8
	Vibrio S5				2.9 0.5	
	Flavobacteria S6					23.3 3.9

a Standard error;[1] mg galacturonic acid / mg protein / hour; [2] mg glucose / mg protein / hour; [3] mg galactose / mg protein / hour.

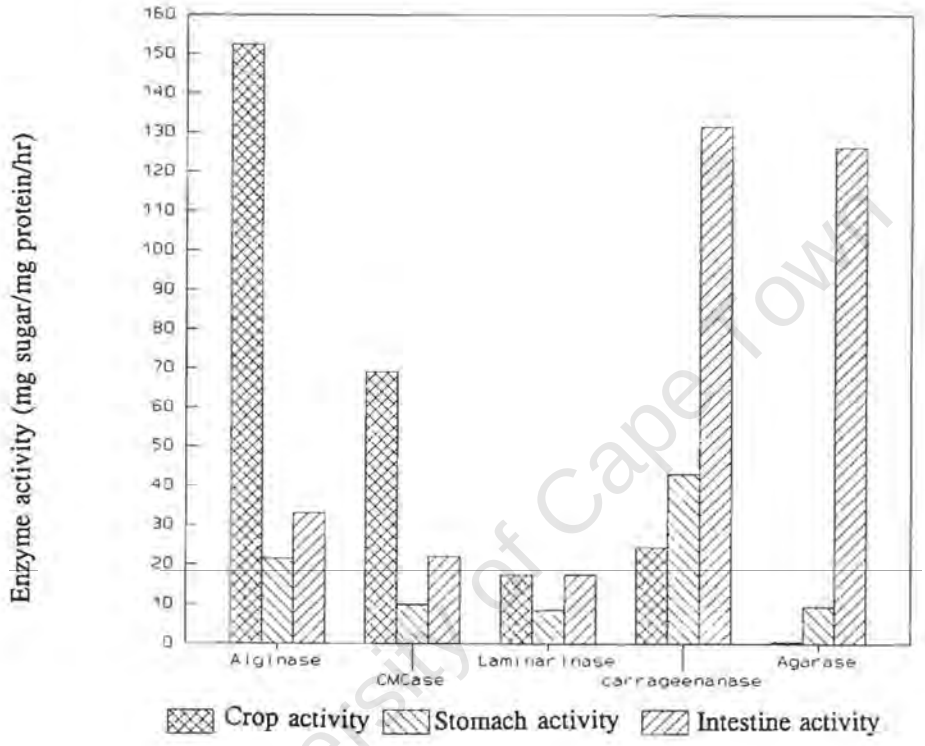

Figure 3.2. The summed polysaccharolytic activity of the bacterial strains in the crop, stomach and intestine of *H. midae*.

3.4 DISCUSSION

Chapter 2 showed that enteric bacteria are present in the abalone digestive system and that there are potentially sufficient bacteria to influence polysaccharide digestion. However, information regarding the number and types of bacteria which occur in *H. midae* is not sufficient evidence that bacteria are metabolically active in the abalone gut. This chapter examined whether the enteric bacterial strains isolated from *H. midae* are capable of hydrolysing the complex polysaccharides which comprise the cell wall of kelp and other sea weeds.

Data presented in this study demonstrated that the gut bacteria of *H. midae* were able to hydrolyse agarose, carrageenan, CMC, laminarin and alginic acid. In fact, approximately two thirds of the isolated bacteria, inoculated onto polysaccharide media, could utilise two or three of the substrates tested. Previous studies on the hydrolysis of polysaccharides in marine invertebrates have also demonstrated that enteric bacteria utilise these substrates (Prim and Lawrence, 1975; Vitalis *et al.*, 1988), in addition to starch, lipids and gelatin (Dempsey and Kitting, 1987; Harris *et al.*, 1991; Harris, 1993). These studies also reported that the enteric bacteria could utilise two or three of the polysaccharides tested. The present results, in conjunction with past results, indicate that enteric bacteria can produce more than one type of polysaccharase.

The role of bacterial cellulase in marine invertebrates has been studied extensively. Previous studies results present conflicting evidence regarding the action of this enzyme. Studies have reflected that although cellulolytic bacteria are present on kelp (Laycock, 1974; Davis, 1985) they are inactive in the digestive tracts of a number of marine animals. Bacterial cellulases

have been reported to be inactive in echinoids (Dempsey and Kitting, 1987), prawns (Harris *et al.*, 1991; Harris, 1993), *Aplysia* (Vitalis *et al.*, 1988) and *Scrobicularia* (Payne *et al.*, 1972). These studies indicated that enteric bacteria were most likely non-symbiotic in invertebrate guts. Harris *et al.* (1991) warn that the influence of bacterial cellulase cannot be discounted, as some studies have shown these enzymes to function in invertebrate systems. The present study has shown that bacterial isolates of the genera *Flavobacteria, Alcaligenes, Pseudomonas* and *Vibrio* are able to utilise CMC. Similarly, Dempsey and Kitting (1987) reported that *Flavobacteria* and *Alcaligenes,* isolated from the gut of shrimps, possess cellulases while, *Pseudomonas* species from a marine woodboring isopod hydrolyse cellulose (El-Shanshoury *et al.*, 1994). Based on the present data and the results of past studies, it appears that certain strains of marine bacteria may be important in cellulose hydrolysis in marine invertebrate guts. However, further studies on more marine invertebrates are required to validate this hypothesis.

Bacterial alginases have been extensively studied (for review see, Gacesa, 1992), partly due to the economic importance of these enzymes. Alginolytic bacteria isolated from *H. midae* include: *Pseudomonas, Bacillus, Vibrio* and *Flavobacteria* species. These results are in agreement with previous studies where the following marine bacteria were also found to produce an alginase: *Pseudomonas* (Muramatsu and Sogyi, 1990), *Bacillus cicrulans* (Larsen, Hooen and Ostgaard, 1993), *Klebsiella pneumoniae* (Ostgaard *et al.*, 1993) and *Pseudomonas, Vibrio, Flavobacteria* (Laycock, 1974). In particular, many alginase producing bacteria have been isolated from kelp fronds of the genera *Laminaria* and *Macrocystis* (Laycock, 1974; Quatrana and Caldwell, 1978; Doubet and Quatrana, 1984). Presumably, the South African kelp species *Laminaria* and *Ecklonia* harbour similar bacteria and these are

ingested when the abalone consumes these seaweeds. Therefore, it is not surprising that alginolytic bacteria were isolated from the gut of *H. midae*.

It is uncertain whether bacterial alginases degrade the guluronate or mannuronate blocks of alginate. In general, bacteria are reported to degrade guluronate blocks and eukaryotes mannuronate blocks (Ostgaard and Larsen, 1993). This difference in substrate specificity could have important implications for alginate digestion in the abalone gut. The alginase enzyme of abalone has previously been shown to degrade mannuronate blocks. If the isolated bacteria are shown to degrade guluronate blocks, then possibly the two enzymes could function synergistically to degrade alginate in the gut.

Agarolytic bacteria have been isolated from a number of marine sources; for example, a tar ball (Nokugama, Coury, Polne-Fuller, Gibor and Harikashi, 1993) and the gut of mullet (Mountfort, Rainey, Burghardt and Stockebrandt, 1993). Although few agarolytic bacteria have been isolated from marine invertebrate digestive tracts approximately half of the bacteria isolated from *H. midae* were capable of hydrolysing agarose. Perhaps the differences reported between previous studies and the current study can be related to the diet of the invertebrate studied. As mentioned previously, earlier studies largely examined enteric bacteria associated with bivalves, which are filter feeders that would not acquire agarose in the diet. Agar forms a large proportion of the diet of abalone as it is present in the seaweed *Gracilaria*, and therefore, it is likely that the abalone would acquire agarolytic bacteria when the *Gracilaria* is consumed.

The enzyme assays performed on the isolated bacteria showed that most of the enzyme

activity occurred in the culture supernatant. This means that the bacterial enzymes are predominantly extracelluar and would be secreted into the lumen of the abalone gut. Extracellular bacterial enzymes are reported for a number of marine bacteria (Quatrana and Caldwell, 1978; Muramatsu and Sogyi, 1990; Gacesa, 1992; Ostgaard *et al.*, 1993) and enteric bacteria (El-Shanshoury *et al.*, 1994).

The assays showed that different bacterial strains had vastly different enzyme activities. One bacterium, designated *Pseudomonas* C4, from the crop had alginase activity 15 times greater than any of the other isolated alginolytic bacteria and CMC activity three times greater than other isolated cellulolytic bacteria. The agarolytic bacterium designated S4 had agarolytic activity ten times greater than any other agarolytic bacteria. It would be impossible, at this stage, to determine whether these "super bugs" are predominantly responsible for the hydrolysis of alginate, CMC or agarose in the abalone gut. None the less, this discovery is exciting as it may be possible to use these "super bugs" in experiments to artificially enhance the digestive processes of *H. midae*.

The data presented in this study showed that bacteria in different gut regions produced greater quantities of a specific polysaccharase than bacteria in other regions. The total alginolytic and CMCase activity from the crop was higher than that in the other two regions due to the activity of the bacterium C4. The bacteria isolated from the intestine exhibited the highest agarase and carrageenanase activity. The greater carrageenanase activity is possibly due to the larger number of carrageenanolytic bacteria isolated from the intestine. The elevated agarolytic activity was due to the polysaccharase activity of the bacterium *Alcaligenes* S4. One possible reason for these differences could be the presence of resident

bacteria in each of these regions. However, this hypothesis needs to be examined further.

In summary, this chapter has produced a number of significant findings. The results of the enzyme assays showed that the abalone enteric bacteria can utilise the polysaccharides agarose, carrageenan, CMC, laminarin and alginate, present in the seaweeds which abalone consume. The majority of these bacteria can produce more than one type of polysaccharase, which appears to be a common phenomenon of bacterial polysaccharases. This study also showed that marine invertebrates do harbour agarolytic bacteria. The author suggests that the presence of these agarolytic bacteria may be related to the diet of the host. Certain strains of the isolated bacteria had extremely high polysaccharase activity which could be exciting for further studies. The bacterial polysaccharase enzymes were found to be extracelluar and would be secreted in the abalone gut. However, this does not mean that the bacteria actually supply these enzymes for the benefit of the host. The following chapter examines whether the bacterial polysaccharase activity does benefit the nutrition of *H. midae*.

CHAPTER 4

POLYSACCHAROLYTIC ACTIVITY OF *HALIOTIS MIDAE* DIGESTIVE ENZYMES.

4.1 INTRODUCTION

The previous chapters showed that enteric bacteria associated with the digestive tract of *H. midae* produce polysaccharases which would be secreted into the lumen of the abalone gut. However, that does not mean that bacterial enzymes are actively influencing the digestive processes in abalone. The abalone could synthesize their own polysaccharases which would make the presence of the enteric bacteria redundant.

A number of studies have documented polysaccharolytic activity in bivalves (Jacober, Rice and Rand, 1980; Seiderer *et al.*, 1982; Davis, 1985; Seiderer and Newell, 1988; Jamieson and Wardlow, 1989), crustaceans (Friesen, Mann and Novitsky, 1985; Mc Conville, Ikeda, Bacic and Clarke, 1986; Fang and Lee, 1992) and echinoderms (Hultin and Wanntorp, 1966; Elyakova, Shevchenko and Avaeva, 1981; Sweijd, 1990). Previous studies on marine invertebrates have documented the following polysaccharolytic enzymes: alginase, laminarinase, cellulase, chitinase, lipase, amylase, carrageenanase, protease and agarases (Elyakova, 1972; Monk, 1977; Elyakova *et al.*, 1981; Gomez-Pinchetti and Garcia-Reina, 1993; Harris, 1993; Payne and Thorpe, 1993).

Polysaccharolytic enzymes of gastropods are less documented although most of the studies on gastropods have examined abalone enzymes (Boyen *et al.* 1990, Gomez-Pinchetti and Garcia-Reina, 1993). The interest in abalone polysaccharases is fairly recent and is linked to the commercial importance of the animal. Studies which have documented abalone enzymes fall into two categories, namely: Abalone alginases isolated for producing protoplasts from kelp (Boyen *et al.*, 1990; Gomez-Pinchetti and Garcia-Reina, 1993; Larsen

et al., 1993; Ostgaard and Larsen, 1993; Ostgaard, Stokke and Larsen, 1994) and abalone polysaccharolytic enzymes which influence the digestive processes of the animal (Knauer *et al.*, in press). However, these studies have not reported on the activity of the enzymes agarase, carrageenanase or laminarinase, which is surprising because these polysaccharides are constituents of the food fed to cultured abalone. The cell walls of phaeophytae (brown algae eg.*Ecklonia* and *Laminaria*) contain algin, laminarin and cellulose (Day, 1969). The Rhodophyta (red algae eg. *Gracilaria* and *Plocamium*) consist mostly of carrageenan and agar (Kloareg and Quatrano, 1988), although other polysaccharides may be extracted (Day, 1969). Abalone farmers use both types of seaweeds in the diet of the cultured abalone and, as Fang and Lee (1992) reported, it is important to understand the digestive physiology of a cultured species as this information can be used to enhance the digestion of a particular diet.

A better understanding of the digestive enzymes present in abalone would enable scientists to produce an optimal food for abalone to digest. Although growth rates on diets give an indication of the suitability of a diet, the growth rates could be further improved by supplying a diet aimed at the digestive enzymes present (Fang and Lee, 1992). Knauer *et al.* (in press) have already shown that enzymes extracted from juvenile abalone are related to diet.

This chapter investigated the endogenous polysaccharase activity of *H. midae*, fed either *E. maxima* and *Gracilaria*, to determine whether endogenous enzyme activity was related to diet. Experiments were designed to produce gnotobiotic abalone which were then used to compare the polysaccharolytic activity of abalone with enteric microflora to those without. The implications of these results are discussed.

4.2 METHODS

4.2.1 Production of gnotobiotic abalone

Abalone were treated with antibiotics to remove microorganisms from the gut. In order to avoid using antibiotics at a concentration harmful to abalone, homogenates of abalone gut were inoculated onto bacterial culture media supplemented with antibiotics at various concentrations. The antibiotics screened were streptomycin, chloramphenicol, penicillin, kanamycin, tetracycline, ampicillin and cefotaxime.

Design of antibiotic experiments:

Abalone (3.5cm shell length) were placed in sterile one litre plastic beakers containing 500 ml autoclaved sea water which was aerated by pumping sterile air through an autoclaved airstone placed at the bottom of the water column. The air entering the water was sterilised by filtration through a 0.2 μm acetate filter inserted in a plastic, autoclaved air line. The sea water was amended with the following antibiotics (final concentration): ampicillin (600μg/ml), chloramphenicol (250μg/ml) and cefotaxime (250μg/ml).

Prior to the start of the experiments, the animals were starved for 3 days to ensure they would eat the artificial food supplied during the experiment. To test this, the artificial food was dyed with red food colouring and fed to the animals. After 3 days the animals were dissected and the gut contents analyzed for uptake of the food colouring.

Figure 4.1. Apparatus employed to generate gnotobiotic *H. midae*.

The apparatus, for the antibiotic treatment, was assembled as depicted in Figure 4.1, in a temperature controlled room at 18°C. The abalone used for the treatment were washed with sterile water to remove surface bacteria and placed in beakers. Only one animal was used per beaker because more than one animal per 500 ml of water resulted in water purity problems.

The antibiotic cocktail was administered to the water every 12 hours over a 72 hour period. Food was added once a day in the morning. The animals were kept in constant darkness as abalone prefer to feed in the dark.

A control experiment, to ascertain whether any difference existed in the enzyme activity between gnotobiotic and normal abalone, was conducted on abalone not treated with the antibiotic cocktail.

4.2.2 Removal of the digestive tract from untreated and gnotobiotic abalone for polysaccharase assays.

Following the 72 hour antibiotic treatment, the animals were dissected aseptically on ice. The digestive tract of individual animals was excised and homogenised separately in 10ml of 100 mM potassium phosphate buffer pH 6.9, using autoclaved glass homogenisers. Tenfold serial dilutions of homogenized digestive tract from gnotobiotic abalone were inoculated onto Difco marine agar and incubated at 22°C for 2 days to confirm the absence of bacteria. The remainder of the homogenate was clarified by centrifugation at 1 089 (x g) for 15 minutes and the supernatant retained for enzyme assays. The homogenate was kept on ice during the experiment to reduce protease activity.

4.2.3 Removal of the hepatopancreas for polysaccharase assays.

Polysaccharases produced by *H. midae* were identified by assaying hepatopancreas extracts with various polysaccharide substrates. Animals fed *E. maxima* were collected from the abalone hatchery at the Sea Fisheries Research Institute, Cape Town, South Africa. Animals fed primarily *G. verrucose* were obtained from Marine Growers, an abalone farm in Port

Elizabeth, Eastern Cape.

The hepatopancreas was removed from abalone by aseptic dissection to avoid contamination with gut fluids containing bacteria from other regions of the digestive tract. The identical region of hepatopancreas was excised from each animal to avoid the possibility that different regions produce different enzymes. The gut region excised is depicted in Figure 2.1.

Each hepatopancreas was homogenised in 10 ml sterile 100 mM potassium phosphate buffer, pH 6.9 using an autoclaved glass homogeniser. Diluted samples of homogenised hepatopancreas were inoculated onto marine agar and incubated at 22°C for 3 days to determine whether bacteria were present in the hepatopancreas. Due to the selectivity of synthetic bacterial culture media, DAPI epifluorescence microscopy (described in section 2.2.4.) and scanning electron microscopy (SEM) was performed on extracts of abalone hepatopancreas to establish unequivocally whether the digestive gland was sterile. The preparation of the samples for SEM was adapted from the protocol of Eisenman and Alfert (1982). The samples were coated with 60μm gold palladium and observed with a Cambridge S200 scanning electron microscope. The hepatopancreas homogenate was centrifuged at 1 089 (x g) for 15 minutes to remove cell debris and the supernatant was collected for the polysaccharase assays.

Hydrolysis of the following polysaccharides were assayed according to the method of Nelson (1944) and Somogyi (1952),: alginate, laminarin and cellulose. Agarose and carrageenan digestion were assayed according to the method of Gardner *et al.* (1987). Protocols for the assays are described in section 3.2.2. All assays were performed for a 60 minute period.

4.2.4. Statistical analysis

The comparable rate of substrate utilisation between gnotobiotic and normal abalone, and between kelp fed and *Gracilaria* fed abalone, was analyzed using the students T test and the Mann-Whitney U test. The test used depended on the value of the F statistic to determine whether variances were similar (Zar, 1984).

4.3 RESULTS

The initial tests, to determine the concentration of antibiotics required to remove bacteria from gut homogenates, indicated that streptomycin, kanamycin, tetracycline and penicillin had little effect on the growth of enteric bacteria. Concentrations of 20μg/ml chloramphenicol and 150μg/ml of cefotaxime were found to inhibit the growth of bacteria on marine agar. However, this concentration of antibiotics was insufficient to reduce the numbers of bacteria colonizing the abalone gut when applied to live abalone. Further tests, involving progressively higher concentrations of chloramphenicol and cefotaxime, together with the addition of ampicillin, were conducted with live abalone. An antibiotic cocktail containing 600 ug/ml ampicillin, 250 ug/ml chloramphenicol and 250 ug/ml cefotaxime was found to render 3.5 cm abalone gnotobiotic, without any noticeable toxic side effects.

Reducing sugar assays on the digestive tracts removed from gnotobiotic abalone demonstrated the presence of enzymes which hydrolysed CMC, alginate, carrageenan and laminarin (Figure 4.2). Similarly, CMCase, alginate lyase and laminarinase activity occurred in the hepatopancreas of *Ecklonia* fed abalone (Figure 4.3). Agarolytic and carrageenanolytic enzyme activity was not detected in either the digestive tract of gnotobiotic abalone (Figure 4.2) or the hepatopancreas of abalone fed *Ecklonia* (Figure 4.3). High carrageenanase and agarase activity, and decreased levels of CMCase, alginate lyase and laminarinase activity occurred in the hepatopancreas of abalone fed a diet consisting primarily of *G. verrucosa*.

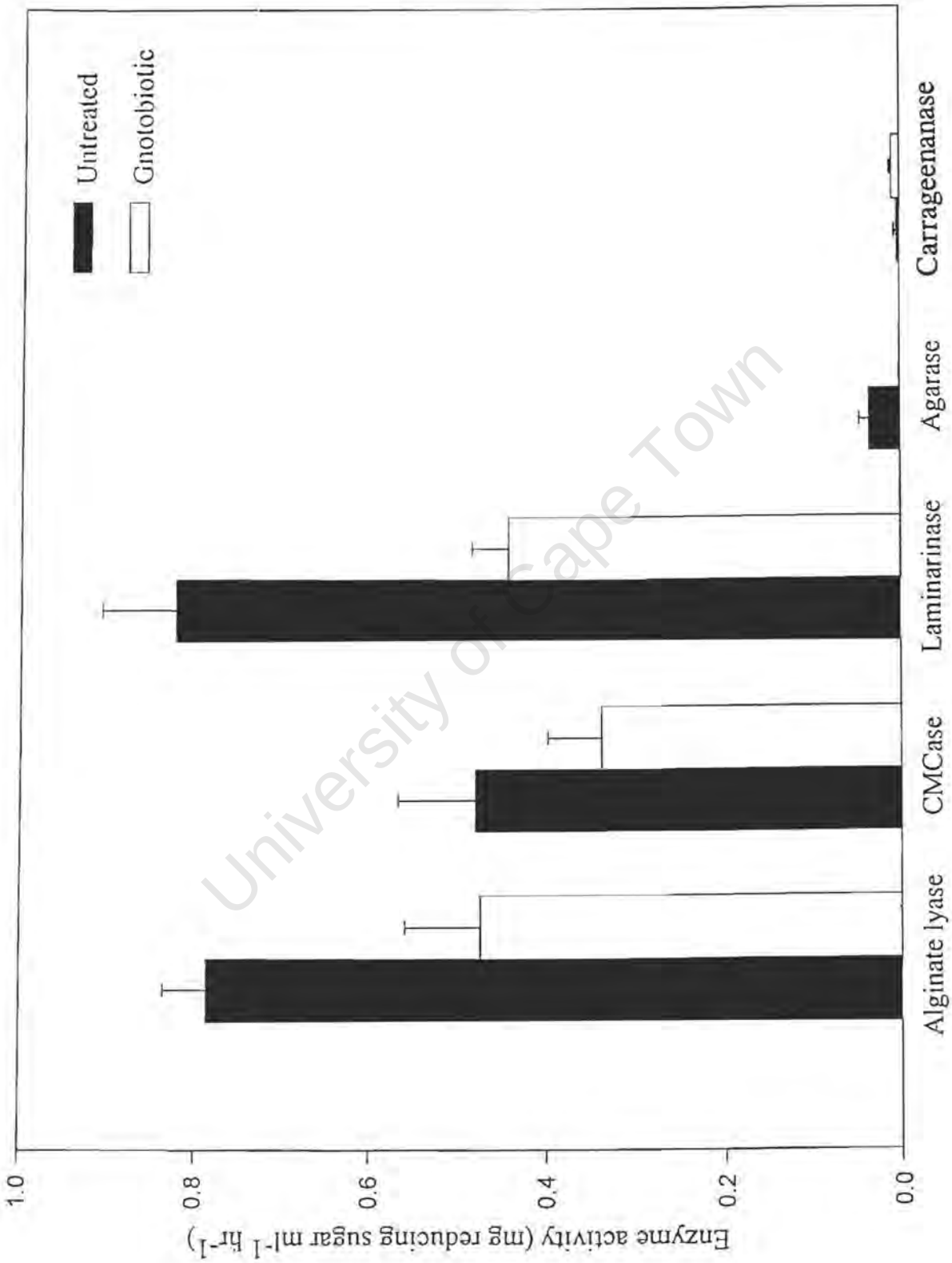

Figure 4.2. Polysaccharase activity from the hepatopancreas of gnotobiotic and untreated abalone. Standard error bars are present on the graph.

The statistical comparison of abalone fed on the two diets showed that there was no significant difference between the activities of the enzymes laminarinase ($p > 0.05$, $T = 1.37$) and CMCase ($p > 0.05$, $T = -1.61$) produced by either group of animals, whereas there was a significant difference in the activity of the enzymes alginase ($p > 0.05$, $Z = 2.895$), carrageenanase ($P > 0.05$, $Z = 2.689$) and agarase ($p > 0.05$, $Z = 2.816$).

Bacteria were not detected in the hepatopancreas by either SEM (Figure 4.4 and 4.5) or DAPI epiflourescent microscopy. No bacteria grew on the marine agar inoculated with hepatopancreas homogenate.

The alginase ($p>0.05$, $T=3.12$), laminarinase ($p>0.05$, $T=4.28$) and agarase activity was significantly higher in untreated abalone (ie, those with bacteria), than for gnotobiotic abalone (Figure 4.2). There was no significant difference in the polysaccharolytic activity between treatments for the enzymes cellulase ($p>0.05$, $T= -1.34$) and carrageenanase ($p>0.05$, $T= -1.11$).

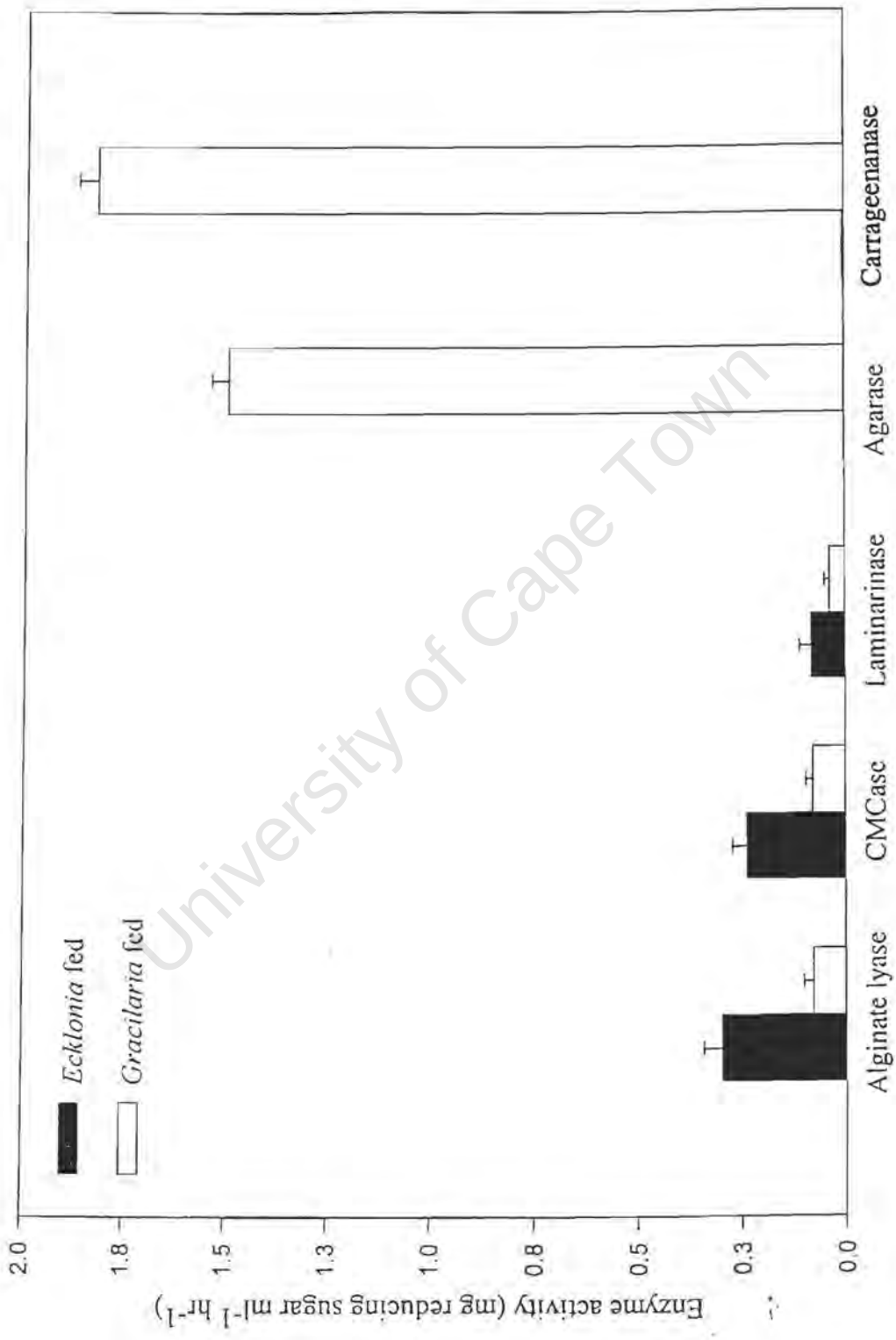

Figure 4.3. Polysaccharase activity from abalone fed on a diet of either *Ecklonia* or *Gracilaria*. The standard error bars are present on the graph.

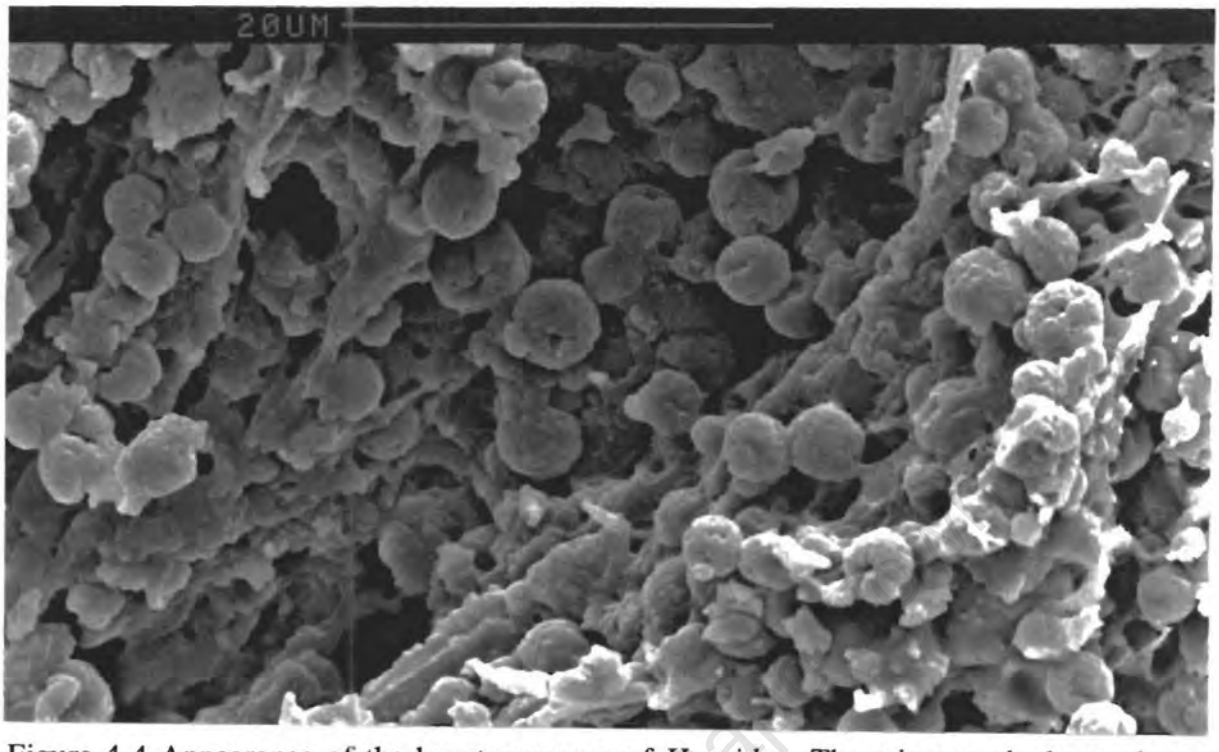

Figure 4.4. Appearance of the hepatopancreas of *H. midae*. The micrograph shows that no bacteria are present. (Mag. 3.55 KX)

Figure 4.5. The structure of the hepatopancreas can be clearly seen. It is composed of the observed coccoid bodies. Once again, no bacteria are apparent. (Mag. 1.84 KX)

4.4 DISCUSSION

The enzyme assays performed on the hepatopancreas of abalone indicated that abalone endogenous enzymes vary with respect to diet. The abalone fed kelp had significantly greater alginase activity than the animals fed *Gracilaria*, whereas animals fed *Gracilaria* had significantly higher carrageenanase and agarase activity. *Ecklonia* has a higher proportion of alginate in the cell wall (Day, 1969) than *Gracilaria*, which could explain why animals fed on *Ecklonia* yielded a higher alginase activity. Similarly, *Gracilaria* has a greater proportion of carrageenan and agarose in the cell wall (Kloareg and Quatrano, 1988). Carrageenan and agar galactans occur in the cell walls of certain Rhodophyceae. The former polymer consists of alternating α-1,3- and ß-1,4-linked D-galactopyranose units, while the latter polymer is comprised of alternating α-1,3-linked ß-D-galactose and ß-1,4-linked anhydro α-L-galactose units (Craigie and Leigh, 1978; McCandless, 1985). Thus, carrageenan and agarose are closely related polymers (Barbeyron *et al.*, 1994). Since these polymers are structurally so similar, it may be possible that the *H. midae* carrageenanase is induced by the presence of agarose.

Dietary preferences have been reported to influence the enzyme activity of a number of marine invertebrates and a number of theories have been put forward to explain these differences in enzyme activities. Harris, Samain, Moal, Martin-Jezequel and Poulet (1986) found a difference in the enzyme activity of adult *Calanus helgolandicus* fed diets with different proportions of starch. They suggested that there may be a compensatory mechanism between digestive enzymes and the substrate ingested. In another study, Knauer *et al.* (in press) found that juvenile *H. midae* fed on diatoms and artificial food exhibited different

lipase and amylase activities. A study on two species of mussels showed that, although the two species had a similar array of enzymes, the specific activities were different (Seiderer *et al.*, 1982) due to different metabolic requirements of the two species. Finally, the digestive enzymes of the shrimp *Penaeous monodon* changed between nauplius and adult and this was attributed to a change in diet (Fang and Lee, 1992). Although these studies reported a difference in the production of polysaccharases with diet, none of these studies have proved that the enzymes were endogenous. The present study has proved unequivocally that the hepatopancreas of *H. midae* does not harbour bacteria and the detected polysaccharase activity is endogenous. Therefore, this study is the first to prove that endogenous polysaccharase activity is related to diet. However, this does not mean that bacterial polysaccharases do not vary with diet as these enzymes may also be related to diet.

The assays of the hepatopancreas of abalone indicate that abalone produce a cellulase which is unusual because it is uncertain whether eukaryotes do produce a true cellulase. Cellulase activity has been documented for mollusca, crustaceans, and echinoderms (Hultin and Wanntorp, 1966; Elyakova, 1972; Payne *et al.*, 1972; Monk, 1977; Gianfreda *et al.*, 1979; Jacober *et al.*, 1980; Elyakova *et al.*, 1981; Friesen *et al.*, 1985; Fielding, Harris, Lucas and Cook, 1986; Brock, 1989; Gomez-Pinchetti and Garcia-Reina, 1993). In particular, cellulases have been documented for a number of *Haliotis* spp. (Gianfrieda *et al.*, 1979; Elyakova *et al.*, 1981; Boyen *et al.*, 1990; Gomez-Pinchetti and Garcia-Reina, 1993). However, none of the previous studies indicated whether the cellulase was of microbial origin.

A few studies on marine invertebrates have attempted to show that the invertebrate studied

possess a cellulase, but few have succeeded. These studies did not perform adequate tests to determine whether the detected cellulase was of microbial origin (Payne *et al.*, 1972; Brock, 1989). Monk (1977) presumed that the cellulase detected in *Gammarus pulex* was endogenous as the bacteria isolated from the gut fluids were not cellulolytic. However, the study would only have isolated transient bacteria and the resident bacteria may have produced the cellulase detected. Friesen *et al.* (1985) reported that bacteria in mysids do not have cellulases. However, the study was based on circumstantial evidence as the study did not test for cellulase activity of the bacteria present.

Since the hepatopancreas of *H. midae* was free of bacteria, this is the first study to demonstrate that abalone possess a cellulase. This cellulase is most likely a poly-ß-glucanase, as this enzyme is known to hydrolyse CMC (Payne *et al.*, 1972). The presence of the other two cellulases, required to hydrolyse cellulose, remains to be confirmed.

Enzyme assays on gnotobiotic abalone yielded results similar to those obtained from the hepatopancreas of abalone fed *Ecklonia*. This suggests that the polysaccharolytic activity of abalone originates in the hepatopancreas.

Antibiotics were administered to obtain gnotobiotic abalone. A number of previous studies have used antibiotics to decrease the bacteria in the gut of marine invertebrates (Wainwright and Mann, 1982; Crosby and Peele, 1987; Vitalis *et al.*, 1988), but the antibiotics used in these studies decreased the bacteria by an order of magnitude. In the present study the number of culturable bacteria was reduced to zero by the antibiotic treatment. It is not

imperative to rid the gut of bacteria, but the numbers must be decreased sufficiently to observe the role bacteria play with respect to polysaccharolytic enzyme activity in the gut. Previous authors have also noted that antibiotics may increase the bacteria in the gut, by changing the resident bacterial populations (Yetka and Wiebe, 1974; Manahan, Davis and Stephens, 1982). Therefore it is important to initially screen a number of antibiotics to determine which are the best to use. The antibiotics used in this study were chloramphenicol, ampicillin and cefotaxime.

The assay results from the normal and gnotobiotic abalone show that although abalone synthesize an alginase, agarase and laminarinase, bacterial polysaccharases increase the activity of these enzymes in the gut. Therefore it is possible that bacteria hydrolyse part of the substrate and abalone another part. It has been shown previously that abalone alginases hydrolyse the mannuronate blocks of alginate and bacteria hydrolyse the guluronate blocks (Larsen *et al.*, 1993; Ostgaard and Larsen, 1993; Ostgaard *et al.*, 1994).

In summary, this chapter developed a number of important ideas. *H. midae* was found to possess the polysaccharases cellulase, alginase, laminarinase, carrageenanase and agarase. The activity of these enzymes is related to the composition of the diet, as the enzymes produced on a diet of kelp or *Gracilaria* varied. These enzymes are endogenous as the hepatopancreas was found to be bacteria free. These enzymes are synthesized by the hepatopancreas as the polysaccharolytic activity from kelp fed and gnotobiotic abalone was similar. Most importantly, this chapter showed that microbial enzymes **do** increase the polysaccharolytic activity of alginase, laminarinase and agarase in the gut of *H. midae*.

CHAPTER 5

CONCLUSIONS

This final chapter contains a summary of the main findings of this thesis and discusses the implications of this study with respect to the commercial abalone industry. The aim of the thesis was to try and gain a better understanding of the role of bacteria in the digestive process of the abalone, *H. midae*. As outlined in the introduction, this was a difficult question to answer as there are many facets involved in microbe/invertebrate relationships. The facet that was chosen for this study, was the role that microorganisms play in the hydrolysis of complex polysaccharides ingested by abalone. Pertinent questions asked were: Are enteric bacteria present in the digestive tract of *H. midae* and in what numbers? Are there specialised structures in the digestive tract which help bacteria adhere to the gut wall? What genera of bacteria are present? What role do enteric bacteria play in the digestion of complex polysaccharides ingested by abalone? Each of these questions was addressed in this project.

Bacteria were isolated from the crop, stomach and intestine of abalone. The numbers of bacteria obtained were similar to those obtained from studies on other marine invertebrates. This finding was encouraging as it suggested that bacteria were present in quantities which could influence digestion in the abalone.

Bacteria isolated on marine agar were identified to genus level. The bacterial community in the gut was almost identical to that identified from other marine invertebrates. *Alcaligenes* was the dominant genus isolated from the crop. *Vibrio* was found to be the dominant genus in both the stomach and intestine.

The results of electron microscopy were disappointing as mucous in the gut obscured large

regions, preventing detailed studies of the gut wall. Furthermore, it was anticipated that bacteria would be associated with specialised structures on the gut wall. No such structures were observed. Therefore, the only pertinent information gained was verification that bacteria were present on the food and gut wall of *H. midae*.

Chapter 3 showed that the isolated bacteria were capable of hydrolysing the 5 polysaccharide substrates tested. The enzyme assays showed that usually over 90% of bacterial enzymes were extracelluar, and therefore, would be excreted into the abalone gut. A few of the isolated bacteria exhibited exceptional activity for each of the 5 polysaccharides. Although results demonstrated the ability of the isolated bacteria to hydrolyse the polysaccharides, this did not prove that bacteria hydrolyse these polysaccharides in abalone.

Chapter 4 examined the role of bacterial hydrolysis of these polysaccharides in the abalone. The hepatopancreas was examined to quantify abalone endogenous enzymes as it was found to be bacteria free. The results of the digestive enzyme assays on the hepatopancreas showed that abalone secrete a carrageenanase, laminarinase, alginase, agarase and a cellulase. The assays on the hepatopancreas also showed that abalone fed different diets produced different polysaccharase enzymes.

Finally, and perhaps most importantly, this study showed that enteric bacteria in abalone contribute significantly to laminarin, agar and alginate hydrolysis. It appeared that bacteria also produce the only detectable agarase in *H. midae* fed on a diet of *Ecklonia*.

A number of conclusions can be drawn from these studies. Firstly, few previous studies have

investigated or reported enteric microflora in marine gastropods. Thus, this study is one of a few to document enzyme activity from enteric bacteria in a marine gastropod. Further studies on enteric bacteria in gastropods are required to determine whether the results obtained from this study are applicable to all gastropods or only to abalone.

The assays on gnotobiotic abalone proved that bacteria do enhance the hydrolysis of alginate, agar and laminarin, but not cellulose or carrageenan. However, cellulose is a complex substrate and perhaps if the hydrolysis of powdered cellulose had been investigated, more definite results would have been obtained. The substrate CMC does not require a true cellulase for hydrolysis, and therefore, the present study has not proved or disproved whether bacteria aid hydrolysis of cellulose in abalone. Eukaryotes have never been found to possess a true cellulase, although prokaryotes have. Therefore, possibly bacteria secrete a true cellulase to completely degrade cellulose and abalone only partially hydrolyse the substrate. Additional studies are required to examine this hypothesis further.

In the same way, bacteria and abalone probably function together to degrade alginate. It is suggested that abalone degrade the mannuronate blocks and bacteria the guluronate blocks of alginate. Although substrate specificity was not examined in the present study, these results do support these findings because, although gnotobiotic abalone degraded alginate, the hydrolysis was improved when bacteria were present.

The substrates used in this study were selected as these substrates are constituents of the seaweeds which abalone consume. Therefore, if the composition of a diet is known, specific bacteria could be selected from those already isolated and used to assist hydrolysis of that

food. For example, if an artificial diet had a high alginate content, then *Pseudomonas* (C4) could be inoculated into abalone to enhance alginate hydrolysis. Furthermore, the activity of this bacterium could be enhanced, through genetic engineering, and reinoculated into *H. midae* to increase digestion further. These experiments could lead to an improvement of the growth rate of farmed *H. midae*. An improved growth rate would obviously benefit abalone mariculture as the turnover of abalone would be increased.

Aside from showing that bacteria increase the hydrolysis of laminarin and alginate, this study showed that endogenous digestive enzyme activity was related to diet. This difference could either be due to endogenous differences in enzymes from animals on different diets or the enzymes could be induced by the composition of the diet; ie. a diet high in alginate could induce alginase activity. Enzyme induction is a common phenomenon for bacterial enzymes, but is less well understood in eukaryotes. The reason for the observed difference should be determined as new methods to manipulate abalone enzymes could become apparent. Further studies on the activity of enzymes in relation to diet should be pursued in order to obtain a better understanding of how enzymes change with respect to diet.

Aside from the suggestions for further study discussed above, experiments on gnotobiotic abalone could yield valuable information about microbe/invertebrate relations. Comparison of growth rates between gnotobiotic and normal abalone could show how important bacteria are under field conditions. Growth trials could be conducted under a range of conditions to determine whether bacteria are obtained from ingested food or the surrounding sea water. These experiments would be possible as this study developed a method to obtain gnotobiotic abalone.

The primary objective of this thesis was to determine whether bacteria could be used to improve the digestion of *H. midae*. The study has shown that hydrolysis of polysaccharides present in *Ecklonia* and *Gracilaria,* is increased by the microbial flora in the abalone gut. Furthermore, techniques developed in this thesis could be used to gain further information about the digestive physiology of this species. Further information in this field could serve to increase the growth rates of abalone either by choosing a diet more suitable to the endogenous abalone enzymes, and/or adding bacterial enzymes to further increase polysaccharide digestion in abalone. The present study could lead to a new and improved method of culturing abalone which could increase the growth rate of farmed abalone and decrease the cost of production.

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APPENDIX 1

Identification of bacteria isolated from the digestive tract of *H. midae*.

Strain no.	Gram stain	shape	catalase	oxidase	motility	o-f Test	Genus
CROP	**BACTERIAL**	**ISOLATES**					
C1	-	rod	+	-	+	F	*Enterobacteria*
C2	-	rod	+	-	-	F	*Aeromonas*
C3	-	rod	+	+	+	n/u	*Alcaligenes*
C4	-	rod	+	+	+	O	*Pseudomonas*
B5	+	cocci	+	-	-	O	*Micrococcus*
Z2	-	rod	+	+	-	O	*Flavobacteria*
Z4	-	rod	+	+	+	n/u	*Alcaligenes*
Z5	-	rod	+	+	+	n/u	*Alcaligenes*
Z6	-	rod	+	+	+	n/u	*Alcaligenes*
A1	-	rod	+	-	-	F	*Enterobacteria*
A2	-	rod	+	-	-	F	*Enterobacteria*
A3	-	rod	+	+	+	n/u	*Alcaligenes*
A5	+	cocci	+	-	-	O	*Chromobacteria*
A7	-	rod	+	+	+	n/u	*Alcaligenes*
STOMACH	**BACTERIAL**	**ISOLATES**					
B1	-	rod	+	+	+	n/u	*Alcaligenes*
B2	+	cocci	+	-	-	F	*Aerococcus*
B3	+	cocci	+	-	-	O	*Micrococcus*
Y1	-	rod	+	+	+	F	*Aeromonas*
Y2	-	rod	+	+	-	O	*Flavobacteria*
Y4	-	rod	+	+	-	O	*Flavobacteria*
Y5	-	rod	+	+	+	O	*Pseudomonas*
Y6	-	rod	+	+	+	O	*Pseudomonas*
Y7	-	rod	+	+	+	O	*Pseudomonas*
Y8	-	rod	+	+	+	F	*Vibrio*
X1	-	rod	+	+	+	F	*Vibrio*
X2	-	rod	+	+	+	F	*Vibrio*
X3	-	rod	+	+	+	F	*Vibrio*
X4	-	rod	+	+	-	O	*Flavobacteria*
X6	-	rod	+	+	+	F	*Vibrio*
X8	-	rod	+	+	+	F	*Vibrio*
W1	-	rod	+	+	+	O	*Pseuodomonas*
W4	-	rod	+	+	+	O	*Pseudomonas*
W6	-	rod	+	+	+	F	*Vibrio*
W7	-	rod	+	+	+	O	*Pseudomonas*
W8	-	rod	+	+	+	F	*Vibrio*
S1	-	rod	+	+	+	F	*Vibrio*
S2	-	rod	+	+	+	O	*Pseudomonas*
S3	-	rod	+	+	+	F	*Vibrio*
S4	-	rod	+	+	+	O	*Pseudomonas*

Strain no.	Gram stain	shape	catalase	oxidase	motility	o-f Test	Genus
INTESTINAL	**BACTERIAL**	**ISOLATES**					
R1	-	rod	+	+	+	O	*Pseudomonas*
R2	-	rod	+	+	+	n/u	*Alcaligenes*
R3	-	rod	+	+	+	n/u	*Alcaligenes*
R5	-	rod	+	+	+	F	*Vibrio*
R6	-	rod	+	+	-	O	*Flavobacteria*
R7	-	rod	+	+	+	F	*Aeromonas*
R8	-	rod	+	+	-	o	*Flavobacteria*
S1	-	rod	+	+	+	F	*Aeromonas*
S2	-	rod	+	+	+	F	*Vibrio*
S3	-	rod	+	+	-	n/u	*Moraxella*
S4	-	rod	+	+	+	n/u	*Alcaligenes*
S5	-	rod	+	+	+	F	*Vibrio*
S6	-	rod	+	+	-	O	*Flavobacteria*
S7	-	rod	+	+	+	O	*Pseudomonas*
S8	-	rod	+	-	-	F	*Enterobacteria*
I1	-	rod	+	+	+	F	*Vibrio*
I2	-	rod	+	+	+	O	*Pseudomonas*
I3	-	rod	+	+	+	O	*Pseudomonas*
I4	-	rod	+	+	+	F	*Vibrio*
J1	-	rod	+	+	+	F	*Vibrio*
J2	-	rod	-	-	-	n/u	unidentified
J3	-	rod	+	+	+	F	*Vibrio*
J4	-	rod	+	+	+	F	*Vibrio*
T2	-	rod	+	+	+	F	*Vibrio*
T4	+	rod	+	+	+	F	*Bacillus*
T5	+	rod	+	+	+	F	*Bacillus*
T6	+	rod	+	+	+	F	*Bacillus*
T7	+	rod	+	+	+	F	*Bacillus*
T8	+	rod	+	+	+	F	*Bacillus*
B7	-	rod	+	-	+	F	*Enterobacteria*
U2	-	rod	+	+	+	F	*Vibrio*
U3	-	rod	+	+	+	n/u	*Alcaligenes*
U4	-	rod	+	+	+	F	*Vibrio*
U5	-	rod	+	+	+	F	*Vibrio*
U6	-	rod	+	+	+	n/u	*Alcaligenes*
U7	-	rod	+	+	+	F	*Vibrio*
U8	-	rod	+	+	+	F	*Vibrio*
V6	+	rod	+	+	+	n/u	unidentified
V7	-	rod	+	-	+	F	*Enterobacteria*
V8	-	rod	+	+	+	n/u	*Alcaligenes*

n/u - bacteria cannot utilise glucose.
Vibrio and *Aeromonas* were distinguished apart by growth on TCBS agar.
Flavobacteria identified by gelatinase activity.
Micrococcus and *Aerococcus* were identified by their positive reaction to the Voges-Proskauer test.

APPENDIX 2

Polysaccharide degradation by the culturable enteric bacteria of *H. midae*.

Gut Region	Strain	Genus	CMC	Agar	Carrageenan	Alginate	Laminarin
Crop	Z2	*Flavobacteria*	+++	+++	+	-	-
	Z4	*Alcaligenes*	-	-	-	-	-
	Z6	*Alcaligenes*	-	-	-	-	-
	C2	*Aeromonas*	+	-	+	-	+
	C3	*Alcaligenes*	+	-	-	-	-
	C4	*Pseudomonas*	++++	-	-	+++	++
Stomach	Y1	*Aeromonas*	+	-	+	-	-
	Y2	*Flavobacteria*	++	+	+++	-	-
	Y4	*Flavobacteria*	-	++++	++++	-	-
	Y5	*Pseudomonas*	+	+++	+++	++	++
	Y6	*Pseudomonas*	-	+++	+	-	-
	Y7	*Pseudomonas*	++	-	+	-	-
	Y8	*Vibrio*	+	+++	+++	+	-
	X1	*Vibrio*	-	+	-	+	-
	X3	*Vibrio*	+	++	++	+	+
	X4	*Flavobacteria*	+	+	+	-	-
	X6	*Vibrio*	-	+	+	-	-
	W1	*Pseudomonas*	-	+	-	-	-
	W4	*Pseudomonas*	+	++	-	-	-
	W6	*Vibrio*	-	++	-	+	-
	W7	*Pseudomonas*	-	+++	+	-	-
	W8	*Vibrio*	-	+	-	-	-
	S1	*Vibrio*	+	++++	+	-	++
	S2	*Flavobacteria*	+	+	+	+	++

Gut Region	Strain	Genus	CMC	Agar	Carrageenan	Alginate	Laminarin
Intestine	R1	*Pseudomonas*	-	+	++	-	-
	R2	*Alcaligenes*	-	-	-	-	-
	R3	*Alcaligenes*	-	+	+	+	-
	R5	*Vibrio*	+	+	++	++	-
	R6	*Flavobacteria*	-	+	+	-	-
	R7	*Aeromonas*	+	+	-	+	-
	R8	*Flavobacteria*	-	-	++	+	-
	S1	*Aeromonas*	+	+	++	-	-
	S2	*Vibrio*	++	-	-	+	-
	S3	*Moraxella*	++	-	+++	-	-
	S4	*Alcaligenes*	+	++++	+++	-	-
	S5	*Vibrio*	+	+++	-	-	-
	S6	*Flavobacteria*	+	-	++	-	+
	S7	*Pseudomonas*	++	+	++	-	-
	S8	*Enterobacteria*	-	-	+	+	+
	I1	*Vibrio*	-	++++	+++	+	-
	I2	*Pseudomonas*	-	++	+	-	-
	I3	*Pseudomonas*	-	+	++	-	-
	I4	*Vibrio*	-	+++	+	-	-
	U2	*Vibrio*	-	+	+	-	-
	U4	*Vibrio*	-	++++	+++	+	+
	U5	*Vibrio*	-	++++	++	-	-
	U6	*Alcaligenes*	-	+	-	-	+
	U7	*Vibrio*	-	-	-	-	+
	U8	*Vibrio*	-	-	++	-	-
	V6	Unidentified	-	+	-	-	-
	V7	*Enterobacteria*	-	+	-	-	+
	V8	*Alcaligenes*	-	-	-	-	-
	T2	*Vibrio*	-	+	++	-	-
	T4	*Bacillus*	+	-	-	++	-
	T5	*Bacillus*	-	++	+	+	+
	T6	*Bacillus*	-	+	++	-	-
	T7	*Bacillus*	-	+	+	+	-
	T8	*Bacillus*	-	++	+	-	+
	J1	*Vibrio*	++	+++	-	++	-
	J2	Unidentified	+++	+	++	++	-
	J3	*Vibrio*	-	-	-	++	++
	J4	*Vibrio*	+	++	-	++	++

- refers to no activity on plate media

+, ++, +++, ++++ refer to increasing zones of hydrolysis on the plate media.

APPENDIX 3

3.1 Estuarine salt media.

Estuarine salts

30g	NaCl
2.3g	$MgCl.6H_2O$
0.3g	KCl
1l	distilled water

Basal medium

2g	glucose
5g	cassamino acids
1g	yeast extract
20g	agar
1l	estuarine salts

3.2 Sterile salts buffer

21.6g	NaCl
0.7g	KCl
6.3g	$MgSO_4.7H_2O$
4.7g	$MgCl_2.6H_2O$
1.2g	Tris
1l	distilled water

pH 7.2

3.3 Nelson Somogyi reducing sugar assay

Somogyi reagent:

4g	$CuSO_4.5H_2O$
24g	anhydrous Na_2CO_3
16g	Rochelle salt
180g	anhydrous Na_2SO_4

Dissolved in distilled water and made up to one litre.

Nelson reagent:

15g	ammonium molybdate dissolved in 450ml water
21ml	concentrated sulphuric acid (H2SO4)
3g	$Na_2HAsO_4.7H_2O$ (sodium arsenate) dissolved in 25ml distilled water.

Incubate at 37C for 24 hours.
Cover in tin foil, light sensitive.

Stock sugars

1g	sugar per litre of saturated benzoic acid.

3.4 Ferricyanide reducing sugar assay

Mineral 1

6g/l K_2HPO_4

Mineral 2

12g NaCl
12g $(NH_4)SO_4$
6g K_2HPO_4
1.2g $CaCl.2H_2O$
2.5g $MgSO_4.7H_2O$

20mM PIPES buffer

0.6g PIPES buffer
5ml mineral 1
5ml mineral 2
100ml distilled water
pH to 6 and autoclave

Substrate

0.4g agarose or carrageenan
100ml 20mM PIPES buffer

Stock reducing sugar solution

5% glucose or galactose made in distilled water
Filter sterilise.

Stop reagent

1g $Na_2HPO_4.7H_2O$
1g NaOH
100ml distilled water
pH to 12.5

Colour reagent

0.12g $K_3(Fe[CN]_6)$
0.22g NaOH
100ml distilled water
pH to 11.8
Cover in tin foil, light sensitive

3.5 Bradford protein assay

Bovine serum albumin:

5mg BSA in 10ml distilled water. Check concentration by reading the A280, which should be 0.33.

NACl:

0.88g NaCl
100ml distilled water

Comassie brilliant blue:

In a 1 litre volumetric flask:
100mg Comassie brilliant blue G-250
50ml 95% ethanol
100ml 85% phosphoric acid.
Bring to volume with distilled water.
Filter through Whatmans no. 1 filter paper.
Store at 4oC.