SUMMARY

Chitin is the insoluble polymer of the amino-sugar N-acetylglucosamine (NAG) and in marine environments large amounts are produced by Crustacea. Present knowledge of the microbial degradation of polysaccharides in anoxic marine environments is scant, especially with respect to the structure of the communities involved and the relationships among the species present in such mixed populations. The study has focussed on the interactive relationships between the chitin-hydrolyzing bacteria and so-called secondary populations, consisting of saccharolytic species only capable of utilizing small solubilized fragments of chitin (NAG-oligomers).

The microbial community that has been the subject for study was enriched from anoxic estuarine sediment with chitin as the sole carbon and energy source. In this community the chitin was mineralized almost to completion with CO_2, H_2O and ammonia as the major end products. The chitinolytic mixed population consisted of three physiologically discernable groups of microorganisms: (1) strictly anaerobic chitin-degrading bacteria belonging to the genus *Clostridium* (primary organisms); (2) facultatively anaerobic non-chitinolytic bacteria capable of fermenting NAG and NAG-oligomers (secondary organisms); (3) sulfate-reducing bacteria relying for their growth on products formed in the fermentation of chitin such as fatty acids, alcohols and H_2 (Chapter II).

The primary bacteria were highly specialized organisms only capable of fermenting chitin and NAG-oligomers. In the fermentation of chitin small amounts of NAG (50-250 μM) accumulated in the culture fluid. Accumulation of chitobiose (NAG_2) was observed when the concentration of yeast extract in the medium was very low. Compared to strictly anaerobic cellulolytic bacteria with a similar limited substrate versatility, the growth rate of the primary organisms was low (Chapter III).

When the primary species were cocultured with a secondary non-hydrolytic bacterium (strain HA 8.1) the rate of chitinolysis was substantially accelerated. In a more detailed study with one of the isolated primary species, designated as *Clostridium sp.* strain 9.1, the kind of the secondary bacterium applied was demonstrated to be of minor importance: arbitrarily chosen saccharolytic species such as *Escherichia coli, Klebsiella aerogenes* and *Clostridium acetobutylicum* were as effective in enhancing chitin degradation as secondary bacteria isolated from the original chitinolytic community. In contrast to the generally accepted explanation for the often observed enhancement of polysaccharide degradation in mixed culture fermentations, the acceleration of chitinolysis in the presence of secondary populations was not due to the removal of potentially inhibitory sugars formed in the hydrolysis of the substrate. This was substantiated by the unaffected course of chitinolysis observed in pure cultures supplemented with NAG or NAG-oligomers, and by the absence of an unequivocal relation between the degree of enhancement and the ability of a secondary bacterium to ferment NAG(-oligomers) (Chapters V and VI).

Secondary species capable of utilizing NAG-oligomers (strain HA 8.1 and *K. aerogenes*) succeeded in consuming 50-70% of the liberated sugars. Nevertheless the rate of chitinolysis was not very different from that recorded in the presence of NAG-fermenting species consuming only 10-15% of the hydrolysis products (*E. coli* and *C. acetobutylicum*). Apparently, the chitinolytic activity expressed by *Clostridium sp.* strain 9.1 was regulated by a mechanism capable of compensating for the substantial reductions in biomass formation when the bacterium was growing in coculture with NAG-oligomer-fermenting species. In chapter VII it was argued that this property might represent an essential element in the organism's strategy in the competition for chitin with other chitinolytic species.

Strong enhancements of chitin fermentation were also observed in pure cultures of strain 9.1 supplemented with spent media or cell-free extracts of secondary bacteria, and in cocultures with bacteria not capable at all of consuming sugars (i.e. sulfate reducers utilizing ethanol and H_2). These observations indicated a dependence of the primary species on growth factors released by the secondary populations. In addition to low molecular factors, probably including the vitamin folic acid, the transfer of a thermostable high molecular component (MW > 1500) could also be demonstrated in the cocultures (Chapters V and VI). Based on these results and a critical evaluation of the literature on mixed culture fermentations of cellulose, it was concluded that at ecologically relevant polymer concentrations cross-feeding interactions probably play a more prominent role in polysaccharide degradation than the alleviation of feedback inhibition by consumption of sugars (Chapter VI).

A clue to the identity of the thermostable factor was provided by previous observations on the growth characteristics of *Clostridium sp.* strain 9.1. In pure culture the addition of a strong reducing reagent (dithionite, sulfite or titanium-III$^+$) resulted in a marked enhancement of chitin fermentation. The transient accumulation of chitobiose in such cultures suggested that the chitinolytic enzyme system of the primary bacterium was affected by these reductants (Chapter III). Experiments with specific thiol-binding/-oxidizing reagents indicated the presence of essential SH-groups in the chitinolytic and the chitobiose-uptake system of strain 9.1, thus providing circumstantial evidence for the involvement of susceptible thiol groups in the observed stimulatory effect of strong reductants (Chapter IV). Based on these data the thermostable redox-active protein thioredoxin was selected as a candidate for the unknown stimulatory high molecular factor and tested in pure cultures of strain 9.1. Indeed, thioredoxin turned out to be very effective in accelerating the fermentation of chitin. When the enzyme was treated with iodine-acetate in order to block the reactive dithiol group of the catalytic centre, its stimulatory activity was reduced by 50-70% (Chapter V).

Microbes capable of degrading chitin anaerobically were also isolated from the hind gut of the flatfish *Pleuronectes platessa* (L.) (plaice). A facultatively anaerobic bacterium, designated as strain AW.D2, was studied in more detail and seemed superior to *Clostridium sp.* strain 9.1 in the degradation of chitin. In pure cultures of strain AW.D2 chitinolysis proceeded much faster, and in coculture with secondary bacteria most, if not all of the hydrolysis products formed, were consumed by the gut endosymbiont itself. A direct competition for chitin was always lost by the chitinolytic *Clostridium*. However, in the presence of the NAG-oligomer-fermenting bacterium strain HA 8.1, the endosymbiontic bacterium was out competed. This result was most surprising since strain HA 8.1 consumed 60 to 70% of the hydrolysis products formed when cocultured with strain 9.1, and the rate of chitinolysis in such mixed cultures was always lower than the one recorded in pure cultures of strain AW.D2. Apparently the strain AW.D2 chitinase did not succeed in occupying the best sites for substrate hydrolysis whilst the sugars released by the hydrolytic activity of the *Clostridium* were not sufficiently accessible to the endosymbiontic bacterium (Chapter VII).

The isolation in pure culture of the acetate-oxydizing sulfate-reducing bacteria present in the chitinolytic community proved to be impossible by conventional techniques because of a dependence of the organisms on not yet identified growth factors. In order to provide the essential factors, a rapidly autolysing lactate-oxidizing sulfate reducer was applied as a co-organism in the ultimate isolation procedure. Based on its relatively rapid growth on acetate, the dependence on growth factors, its morphology and the intracellular formation of polyglucose the isolate was classified as a new species in the genus *Desulfobacter*: *Desulfobacter splendidus* sp. nov. (Chapter VIII).
