Bioenergy from brown seaweeds

by

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To the great mystery of being alive
To all beings with whom I share this planet
To the sun, the stars, the rain, the trees and the flowers

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Preface

The practical work and writing of this thesis was carried out at the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim, from 1996 to 2000. Norsk Hydro ASA initiated the research through the NYTEK (Efficient, renewable energy technologies) program "Energy from macroalgae".

The thesis is based on the 6 research papers listed on page iii in the sequence chosen for presentation. Papers 1, 2, 3 and 5 are already published, paper 6 is in press, and paper 4 is submitted.

Chapter 1 gives a general introduction to bioenergy and seaweeds. Anaerobic degradation and microbial production of methane and ethanol are discussed, the composition of *Laminaria hyperborea* and *Ascophyllum nodosum* are presented, and possible degradation pathways of their constituents are discussed.

Chapter 2 presents an overview of the experimental approach, including a summary of the NIR method for alginate quantification developed in Appendix Paper 1.

Chapter 3 and 4 give an extensive summary of Appendix Papers 2-6. For the convenience of the reader, selected figures and tables found in the Papers are repeated in these chapters. The intent was to present the main results in comprehensive way. Appendix Papers 2 and 3 are also discussed in the thesis of Einar Moen (1997).

Chapter 5 is a summary of the main findings, discussing possible applications and economic restrictions and suggesting further studies.

For any questions about this thesis please contact me at: sveinhorn@hotmail.com

List of appendix papers

1 Svein Jarle Horn, Einar Moen and Kjetill Østgaard (1999) Direct determination of alginate content in brown algae by near infra-red (NIR) spectroscopy. J. appl. Phycol. 11:9-13.

2 Einar Moen, Svein Horn and Kjetill Østgaard (1997) Biological degradation of *Ascophyllum nodosum*. J. appl. Phycol. 9:347-357.

3 Einar Moen, Svein Horn and Kjetill Østgaard (1997) Alginate degradation during anaerobic digestion of *Laminaria hyperborea* stipes. J. appl. Phycol. 9:157-166.

4 Svein Jarle Horn and Kjetill Østgaard (2001) Alginate lyase activity and acidogenesis during fermentation of *Laminaria hyperborea*. J. appl. Phycol. 13:143-152.

5 Svein Jarle Horn, Inga Marie Aasen and Kjetill Østgaard (2000) Production of ethanol from mannitol by *Zymobacter palmae*. J. ind. Microbiol. Biotechnol. 24:51-57.

6 Svein Jarle Horn, Inga Marie Aasen and Kjetill Østgaard (2000) Ethanol production from seaweed extract. J. ind. Microbiol. Biotechnol. J. ind. Microbiol. Biotechnol. 25:249-254.

Summary

Brown seaweeds lack lignin and have a low cellulose content. Thus, seaweeds should be an easier material for biological degradation than land plants. However, seaweeds have a complex composition, and complete degradation of the material necessitates the presence of microorganisms with a broad substrate range. During anaerobic degradation of organic material, energy carriers such as methane and ethanol may be produced. This is a study of two particular species of brown seaweeds; *Laminaria hyperborea* and *Ascophyllum nodosum*, which are the most abundant Norwegian species and also the two species that are commercially harvested in Norway.

Most of the degradation studies were carried out in batch systems at pH 7 and at 35 °C. The digestion pattern of the seaweeds were studied by measuring gas production, alginate lyase activity, remaining alginate, the concentrations of uronic acids, VS, COD, mannitol, organic acids and polyphenols. NIR spectroscopy was applied as a new method for alginate quantification. Ethanol production was carried out at 30 °C at different pH, both in batch and continuous cultures. Gas production and concentrations of mannitol, laminaran, ethanol and organic acids were measured.

Methane is the end product of a mixed microbial community. However, it is the initial steps of hydrolysis and acidogenesis that are specific for the raw material. Alginate forms the major structural component of brown algae, and its degradation is catalysed by alginate lyases. Polyphenols proved to be the most important limiting factor in the biodegradation: the content of polyphenols was much higher in *A. nodosum* than *L. hyperborea*, and this led to a reduced biodegradability of *A. nodosum*. However, when the polyphenols were fixed with formaldehyde, this seaweed was also readily degraded. Manipulation of the content of polyphenols in *L. hyperborea* gave similar results. This toxic effect was probably caused by direct inhibition of the microbes, especially the methanogenic bacteria, and complexation reactions with algal material and enzymes. Generally, the guluronate content of the remaining alginate increased during biodegradation, probably due to the Ca-linked guluronate junction zones less accessible for alginate lyase. The main organic product of the acidogenesis was acetate, which was easily converted to methane. In this study, it was not attempted to optimise the methane yield.

Ethanol is an intermediate in the complete digestion of organic material and is produced by specific microbial strains. Thus, ethanol production should take place under controlled conditions to prevent contamination problems. The complex composition of seaweeds makes it a difficult substrate to ferment to ethanol by one or a few strains of microbes. In this work, laminaran and mannitol extracted from *L hyperborea* fronds were used as substrate for ethanol production. A bacterium, *Zymobacter palmae*, was able to produce ethanol from mannitol, but could not utilise laminaran. However, the yeast *Pichia angophorae* was able to produce ethanol from both substrates simultaneously. Some supply of oxygen was necessary for the fermentation of mannitol, while a too high aeration resulted in the production of organic acids.

Thus, it has been shown that both methane and ethanol can be produced from brown seaweeds. However, an optimisation of the processes will be necessary. Energy production from seaweeds will only be economic if the harvesting costs are low. It may be noted that wastes from the alginate industry may be considered a non-cost raw material for energy production.

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Abbreviations

ANAEROB	Experimental term for anaerobic degradation of *A. nodosum*
ANAFORM	Experimental term for anaerobic degradation of formaldehyde pre-treated *A. nodosum*
BSA	Bovine serum albumin
CER	CO_2 emission rate
COD	Chemical oxygen demand
ENRICHED	Experimental term used for a mixture of stipe and peel
G	Guluronic acid
GG-block	Polyguluronic acid
M	Mannuronic acid
MG-block	Heteropolymeric sequences of guluronic and mannuronic acid
MM-block	Polymannuronic acid
NIR	Near infra-red spectroscopy
OTR	Oxygen transfer rate
PEELED	Experimental term used for stipe without the phenolic outer layer
PLS	Partial least squares
TS	Total Solids
UA	Units of activity for alginate lyase
UASB	Upflow Anaerobic Sludge Blanket
VS	Volatile Solids
VFA	Volatile Fatty Acids
WHOLE	An experimental term used for regular stipe

1 Introduction

1.1 General introduction

1.1.1 The global carbon cycle

Through the process of photosynthesis, plants, algae and cyanobacteria produce organic compounds and O_2 from CO_2, H_2O and sunlight. Purple and green bacteria also use light to reduce CO_2 to biomass. In the process of aerobic respiration, the organic compounds are degraded with the release of CO_2 and energy. A similar reaction takes place under anaerobic conditions when electron acceptors such as NO_3^- or SO_4^{2-} can substitute for O_2. If no external electron acceptor is available, the degradation of organic material proceeds through the process of fermentation. This is a complex process where the final products are CH_4 and CO_2 (see Section 1.2.2). The fermentation intermediates are also the starting point for the long-term production of fossil fuels. The biogeochemical cycle of carbon is depicted in Figure 1.1.

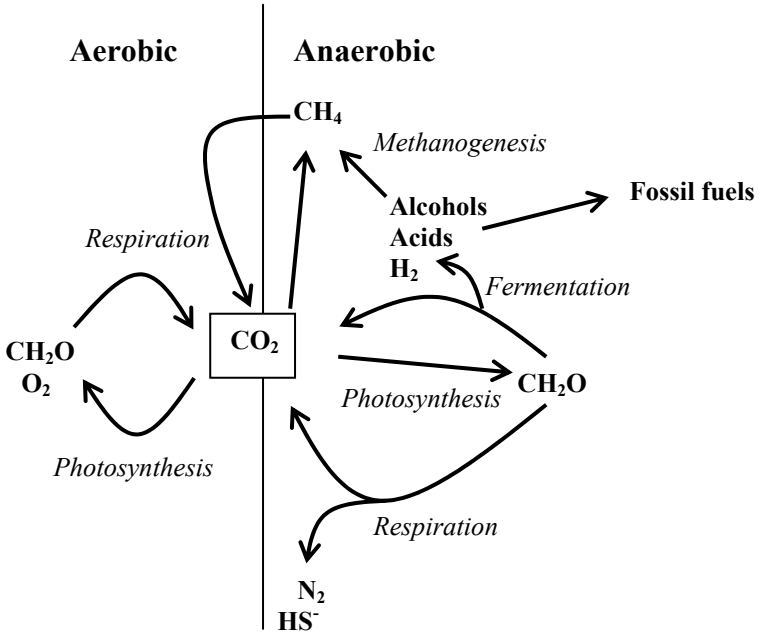

Figure 1.1 Carbon cycling in aerobic and anaerobic environments. CH_2O = organic carbon at the reduction level of glucose.

When examining this cycle it is useful to consider the different reservoirs of carbon. The atmospheric CO_2 is the most actively cycled reservoir, containing 700 billion tons of carbon. The dissolved inorganic forms of carbon in surface seawater, a total of 500 billion tons, are in direct equilibrium with the atmospheric carbon pool. The much larger carbon reservoir of 34 500 billion tons in the deep sea equilibrates only at the slow rate of vertical seawater circulation. The living biomass contains about the same amount of carbon as the atmosphere. Dead organic matter, such as humus and organic sediments, contains 3 700 billion tons of carbon. All of these can be considered actively cycled carbon reservoirs. The amount of carbon stored in fossil fuels (10 000 billion tons) and carbonaceous sedimentary rock (20 000 000 billion tons) are much higher, but their natural turnover rates are minute. The above cited numbers (Bolin, 1970) are rough estimates, but they give a feeling of the relative reservoir sizes.

The natural rates of carbon cycling in oceans and on land are close to steady state, seen as a stable concentration of CO_2 in the atmosphere. However, human activities have recently introduced changes in the carbon cycle. Atmospheric CO_2, because it is a relatively small carbon pool, has been measurably affected by human activities. The Mauna Loa Observatory at Hawai'i has recorded a steady rise of atmospheric CO_2, from 315 ppm in 1958 to 368 ppm

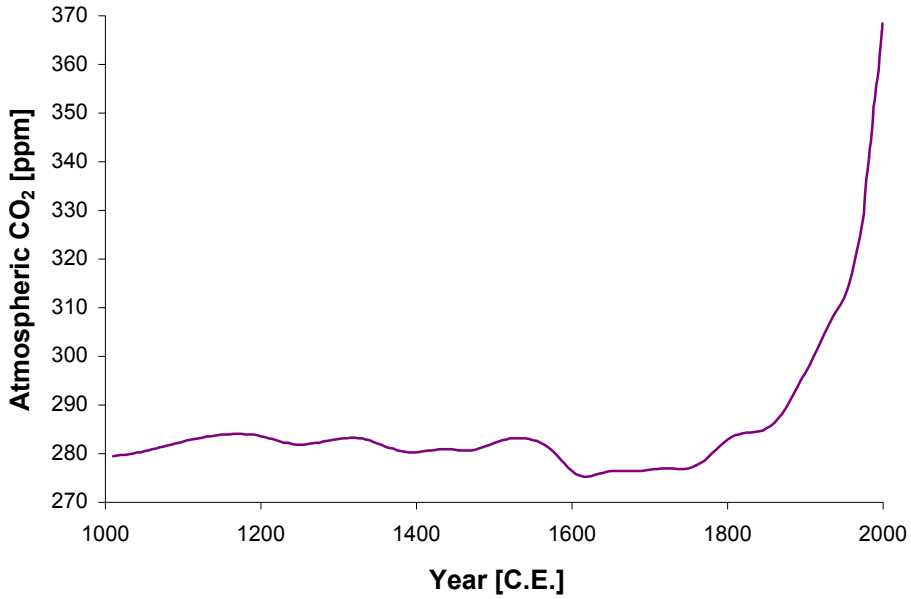

Figure 1.2 Global atmospheric CO_2 concentrations. Mauna Loa data combined with Vostok ice-core data (Etheridge et al., 1998; Keeling and Whorf, 2000).

in 1999 (Keeling and Whorf, 2000). In a 1000-year perspective, this is really a rapid increase (Etheridge et al., 1998), as shown in Figure 1.2. From 1850 to 1999 the CO_2 concentration has increased by 29 %. This increase in CO_2 levels is largely due to the burning of fossil fuels, and is today one of the most predominant environmental concerns: the rising CO_2 emissions could set off rapid global warming due to the so called "greenhouse effect" of this gas. Among the first to express this concern was Revelle and Suess:

> *"Thus human beings are now carrying out a large scale geophysical experiment of a kind that could never have happened in the past nor be reproduced in the future. Within a few centuries we are returning to the atmosphere and oceans the concentrated organic carbon stored in sedimentary rocks over hundreds of million years"* (Revelle and Suess, 1957).

This greenhouse effect is due to the fat that CO_2 and other greenhouse gases such as N_2O and CH_4 are transparent to visible light, but absorb strongly in the infrared range. Some of the visible sunlight striking Earth's surface is irradiated back as long-wavelength infrared radiation. Thus, an increase in CO_2 concentrations in the atmosphere would retain more of this radiation and probably lead to a warming trend in the climate. When we look at the global temperatures in this century, the top 7 warmest years have been in the 1990's (Jones, 2000). And the Intergovernmental Panel on Climate Change (IPCC) have concluded that *"the balance of evidence suggests a discernible human influence on climate"* (Houghton, 1996). In fact, the present day levels of greenhouse gases have been unprecedented over the last 400 000 years (Raynaud, 2000).

1.1.2 Bioenergy

Our dependency on fossil fuels for energy production is problematic, both because of the emissions of CO_2 and the depletion of a finite resource. Obviously, energy production will only be sustainable if it is based, directly or indirectly, on solar energy. Biomass is stored solar energy, and if the amount of biomass grown for energy use equalled the consumption, there would be no net build-up of CO_2 in the atmosphere. Figure 1.3 shows the 3 main processes for production of energy or fuels from biomass: direct combustion, production of

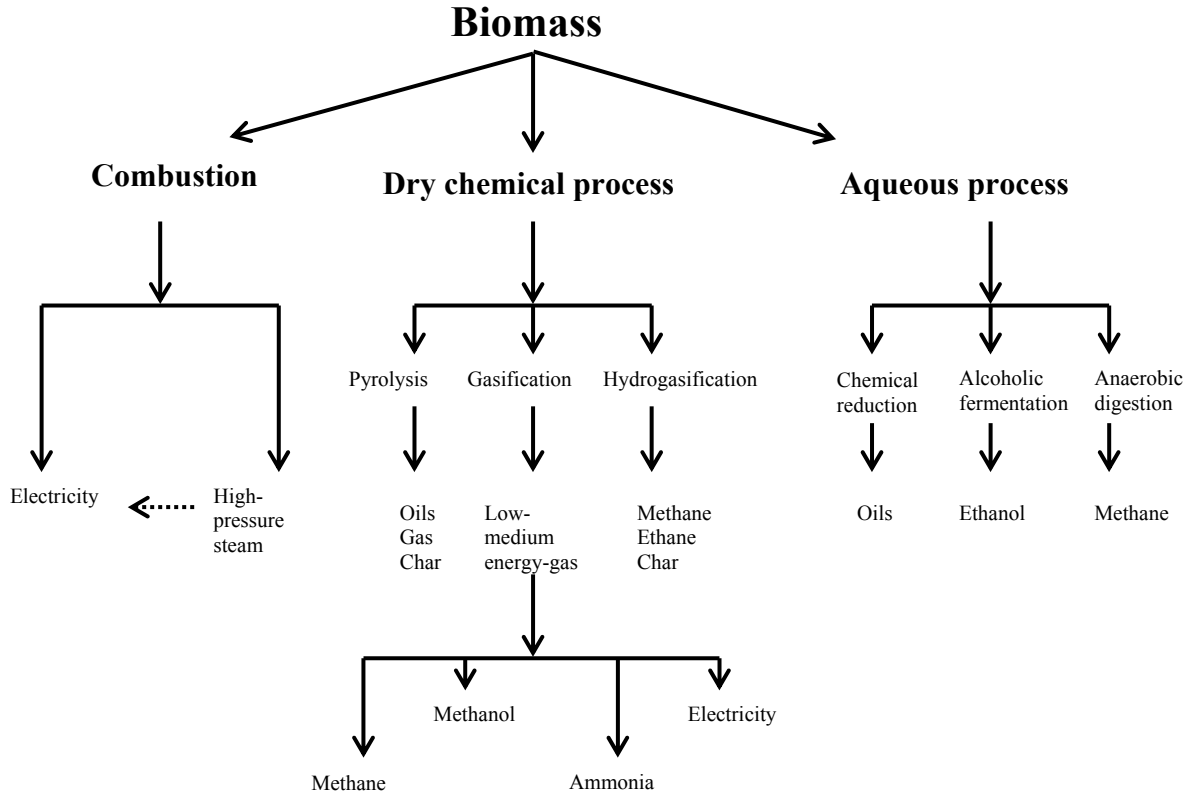

Figure 1.3 Biomass energy conversion processes and products (after Slesser and Lewis, 1979).

fuels from essentially dry biomass by chemical means, and biological production of fuels by aqueous processing.

Biomass with high water content can only produce energy by the aqueous process, owing to the high energy requirement for drying the material artificially (Slesser and Lewis, 1979). Traditionally, the burning of wood has been our most important energy resource, and even today about 15 % of the global energy production is based on biomass, mainly in developing countries (Hall and House, 1995).

Wet organic matter such as seaweeds can be degraded biologically. Under aerobic conditions, the organic compounds are converted to biomass, CO_2 and H_2O. This is the typical composting process where heat is generated and the biomass yield is about 50 % (Henze et al., 1997). Under anaerobic degradation by a mixed microbial population, the main products are biomass, CH_4 and CO_2. This process may be divided into an acidic step where the biomass

yield is 20-30 %, and a methane step where the yield is 3-4 % (Henze et al., 1997) (see Section 1.2.2). As much as 85 % of the chemical energy in the organic substrate may be preserved in the CH_4 gas (Schink, 1997). Production systems for biogas, that is a mixture of CH_4 and CO_2, are relatively simple and can operate on small as well as large scales practically anywhere, with the biogas being as versatile as natural gas (Bhatia, 1990). Both India and China have installed millions of biogas plants of various capacities (Hall and House, 1995).

Ethanol is the other principal biofuel produced by microorganisms. The process is carried out by one or a few selected microbial strains. Sugar cane is the world's largest source for microbial produced ethanol (Klass, 1998). Latin America, dominated by Brazil, is the world's largest producer region of bioethanol (Rosillio-Calle and Cortez, 1990). In 1994, 12.5 billion litres of bioethanol was consumed for fuel purposes, accounting for 48.5 % of Brazil's automobile fuel demand (Klass, 1998).

1.2 Anaerobic fermentation of complex organic matter

1.2.1 Definition

The use of the term *fermentation* may be confusing, since it has a twofold definition: (1) Substrate metabolised without exogenous electron acceptor; (2) in industrial microbiology; *any* of a wide range of processes carried out by microbes in a reactor, regardless of whether fermentative or respiratory metabolism is involved (Singleton and Sainsbury, 1993). In this work, in accordance with the common practice, the term is used according to the first definition in relation to methane production, and according to the second definition in relation to ethanol production. The absence of an exogenous electron acceptor necessarily means that the products of a fermentation, collectively, have the same oxidation state as that of the substrate. Anaerobic microorganisms play a major role in the global carbon cycle by remineralising organic matter in anaerobic environments. Fermentations are usually classified according to the main fermentation products, for instance as alcohol, lactate, acetate or methane fermentations.

1.2.2 Methane

Methane fermentation is the complete conversion of organic matter to the end products CO_2 and CH_4. It is a complex process involving many different groups of anaerobes. The process may be divided in four different main steps (Brock et al., 1994): *hydrolysis*, *acidogenesis*, *acetogenesis* and *methanogenesis*, as shown in Figure 1.4. The first step is the hydrolysis of organic polymers such as polysaccharides, lipids and proteins to their corresponding mono- and oligomers; sugars, fatty acids, short peptides and amino acids. Microbial hydrolysis is carried out by extracellular enzymes. The production of the enzymes is energy demanding, and therefore usually only happens when the environment is depleted of easily accessible carbon source alternatives. These hydrolytic organisms also take part in the next step where the products of the hydrolysis are fermented. Other organisms with no hydrolytic activity by themselves may also consume the hydrolysis products. The diverse group of microorganisms which carry out this first fermentation step are usually termed "acidogenic", due to their production of volatile fatty acids (VFA). Different alcohols may also be produced in this step (Conrad, 1999). With the VFA production follows the concomitant production of CO_2 and H_2. Reactions 1.1-1.5 in Table 1.1 show typical acidogenic products formed from glucose (Klass, 1998).

Acetate is formed through three main pathways: (1) direct fermentation of hexoses; (2) reduction of CO_2 to acetate by utilising H_2 as electron donor; (3) acetate formation from the intermediate VFAs and alcohols, by the "H_2-producing fatty-acid oxidising bacteria", commonly called the "acetogens". Bacteria that reduce CO_2 to acetate produce acetate as the sole product and are called "homoacetogens". The last acetate pathway (3) is only energetically favourable if the H_2 concentration is kept very low (reactions 1.6-1.8 in Table 1.1), which is achieved through a symbiotic partnership with H_2-consuming methanogenic *Archaea* (reaction 1.10 in Table 1.1). Names such as *Syntrophomonas* and *Syntrophobacter* indicate these bacterial genera's syntrophic relationship with methanogenic *Archaea* (Atlas and Bartha, 1998).

The methane producing methanogens represent the largest and most diverse group within the *Archaea* domain (Woese et al., 1990). They are strictly anaerobic and derive their energy from a restricted number of substrates. Hence, the methanogens rely on other microbes, both hydrolytic organisms and mixed acid fermenters. Methanogenic *Archaea* use 3 main

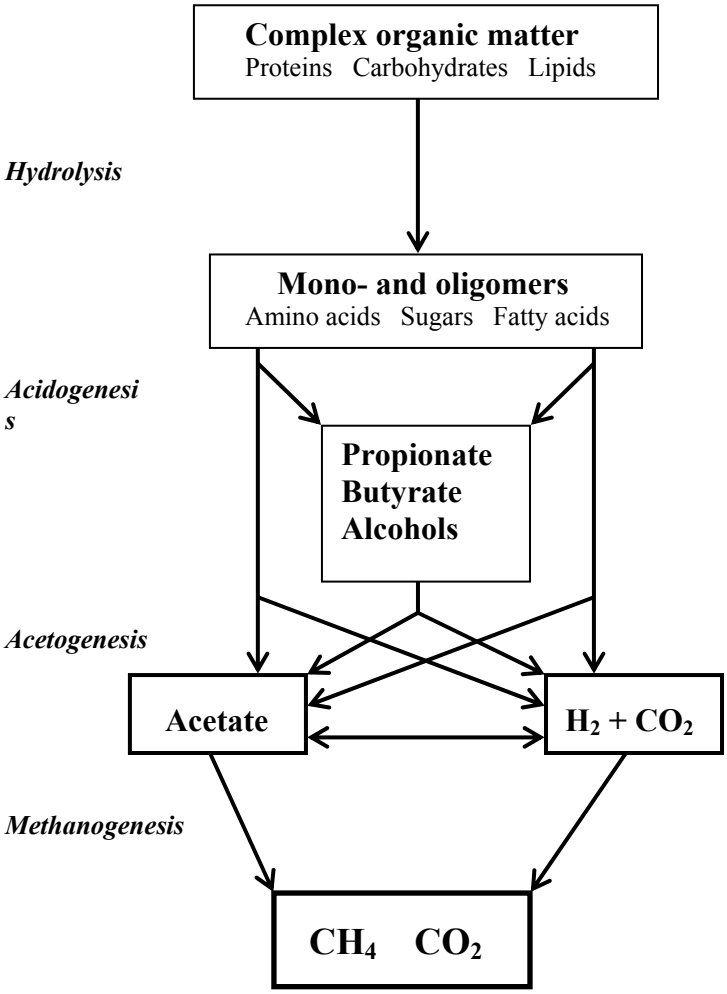

Figure 1.4 Anaerobic degradation of organic matter to CO_2 and CH_4.

pathways for methane production depending on their substrate specificity: (1) reduction of CO_2 to CH_4 using H_2 or formate as electron donor (reaction 1.10 in Table 1.1); (2) production of CH_4 and CO_2 from the methyl groups of methanol and methylamines (not shown in Figure 1.4); (3) "acetoclastic" formation of CH_4 and CO_2 from acetate carried out by a limited number of methanogens (reaction 1.9 in Table 1.1) (Deppenmeier et al., 1996).

Less than half of the Gibbs free energy content of glucose is available for the syntrophic degradation of alcohols and VFAs to CH_4 and CO_2, and this energy has to be shared between acetogens and methanogens. Only if the first fermentation step is homoacetogenic (reaction 1.2 in Table 1.1), the residual free energy is exclusively available for acetotrophic methanogenesis. Actually, there is no thermodynamic reason why homoacetogenic

degradation of carbohydrates coupled to acetotrophic methanogenesis (acetoclastic pathway) should not be one of the major pathways in anaerobic environments (Conrad, 1999). The maximum amount of H_2 relative to acetate that can be directly produced from the degradation of carbohydrates is 4 mol acetate per mol glucose (reaction 1.1 in Table 1.1), i.e. a H_2/acetate ratio of 2:1. Since 4 H_2, but only 1 acetate, are required to produce 1 CH_4 (reactions 1.9 and 1.10 in Table 1.1), the contribution of H_2 to methanogenesis can maximally be 33 % of the total CH_4 formed (Krumböck and Conrad, 1991). Indeed, this percentage is consistent with the observation that two-thirds of the methane produced in nature originates from acetate, and about one-third from the reduction of CO_2 with H_2 or formate (Ferry, 1992).

Table 1.1 Typical reactions in the fermentation of organic matter to CH_4 and CO_2 (Klass, 1998).

	Reaction	)G°´
(1.1)	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2CO_2 + 4H_2$ (acetate)	-216
(1.2)	$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+$ (acetate)	-311
(1.3)	$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COO^- + H^+ + 3CO_2 + 5H_2$ (propionate)	-192
(1.4)	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COO^- + H^+ + 2CO_2 + 2H_2$ (butyrate)	-264
(1.5)	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$ (ethanol)	-235
(1.6)	$CH_3CH_2COO^- + H^+ + 2H_2O \rightarrow CH_3COO^- + H^+ + CO_2 + 3H_2$	+72
(1.7)	$CH_3CH_2CH_2COO^- + H^+ + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$	+48
(1.8)	$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+10
(1.9)	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36
(1.10)	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-131

The numbers are standard Gibbs free energy changes at pH 7 ()G°´).

Stable methanogenesis in digesters has been obtained between 10 °C and 60 °C (Speece, 1985). Two optimum temperature ranges are reported: the mesophilic range at 30-40 °C, and the termophilic range at 50-60 °C. The acidification is strongly affected by temperature according to Arrhenius' law, but there is no general agreement in the choice of mesophilic or termophilic temperatures for the optimisation of this step (Chang et al., 1989; Perot and Amar,

1989). A suboptimal temperature may be preferable in order to avoid disturbances by the high death rate at high temperatures and because of energy requirements (Dinopoulou et al., 1988). Generally, termophilic microorganisms show higher rates of substrate utilisation and growth, but have lower growth yields compared to mesophilic microorganisms (Duran and Speece, 1997). Traditionally, anaerobic digestion of sludge has been carried out in one stage. However, in recent years, the interest in staged systems has grown significantly. The microbial ecology of acid and methane formation is very different, and a physical separation of the two processes would make it possible to optimise the environmental conditions for each group of bacteria (Chang et al., 1989). Temperature staged systems have also been considered (Duran and Speece, 1997).

The term "fermentation" denotes here the metabolisation of a substrate in the absence of any external electron acceptor (see Section 1.2.1). The oxidation of an intermediate in the fermentation pathway is balanced by equivalent reduction of others. However, an anoxic environment is not exclusively a place for fermentation. The presence of alternative electron acceptors such as Fe^{3+}, NO_3^-, S^0 and SO_4^{2-} make anaerobic respiration possible. Such respiratory microorganisms may out-compete the methanogens and prevent CH_4 formation. Especially sulphate- and sulphur-reducing bacteria tend to out-compete the methanogens, both because of their higher affinity for H_2 and acetate, and their higher growth rate. Thus, methanogensis is more extensive in freshwater and terrestrial environments than in the sulphate rich marine environments. In sulphate rich environments, the major precursors for methane are methylated substrates such as methanol and methylamines (Brock et al., 1994).

In summary, anaerobic degradation of complex biomass belongs to the field of microbial ecology. A wide range of microorganisms interacts in different ways, from competition to synergism, in the complete degradation to CH_4 and CO_2. No single microorganism is able to carry out the whole pathway.

Most natural organic materials may be degraded to biogas. However, lignin and many hydrocarbons appear to be refractory to fermentative breakdown. Lignin is a major component of wood, and coal is an organic material that ultimately is derived from wood in anaerobic habitats. Long-chain aliphatic hydrocarbons are also generally protected from decay by anaerobic conditions, and they become the major constituents of petroleum. However, it should be mentioned that biological conversion of alkanes to methane under strictly anaerobic

conditions has been observed (Zengler et al., 1999). In aerobic environments, both lignin and hydrocarbons are easily broken down biologically (Brock et al., 1994). Most of the examined land- or water growing primary biomass has good digestion characteristics or can be pretreated to promote digestion. In fact, kelp and other seaweeds that lack refractory lignocellulosic complexes are the only type of primary biomass that needs no pre-treatment prior to digestion (Gunaseelan, 1997).

1.2.3 Ethanol

While methane is the end product after a series of degradation steps involving a diverse microbial culture, a specific intermediate such as ethanol (see Table 1.1, reactions 1.5 and 1.8) is produced by specific microbial strains. The yeast *Saccharomyces cerevisiae* and the bacteria *Zymomonas mobilis* are the most widely used microorganisms for ethanol production (Dumsday et al., 1997). Generally, ethanol can be derived from any material that contains sugar. Today, raw materials used in the manufacture of ethanol by fermentation are classified as sugars, starches and cellulosic materials (Bailey and Ollis, 1986). Sugars can be converted to ethanol directly, while starches first have to be hydrolysed to fermentable sugars by the action of enzymes. Cellulose must likewise be converted to sugars before further fermentation, generally by the action of mineral acids (Bashir and Lee, 1994).

Ethanol is one of the most significant organic chemicals because of its unique combination of properties as a solvent, a fuel, a germicide, a beverage, an antifreeze and as an intermediate in the production of other chemicals. Thus, many processes for ethanol production have been carried out with a negative energy balance, since the ethanol was not intended for the fuel market. The ubiquitous nature of cellulose as the most abundant product of photosynthesis renders substances such as straw, wood, bagasse and waste paper as obvious candidates for utilisation as an ethanol source. However, these are comparatively “dry” materials and might be exploited as energy sources to a greater advantage by combustion or pyrolysis (Figure 1.3).

Generally, ethanol is formed from pyruvate in two steps: decarboxylation of pyruvate to acetaldehyde, followed by a reduction of acetaldehyde to ethanol by NADH. Thus, the two NAD^+ consumed in the glycolysis (or Entner-Doudoroff pathway) are regenerated in the production of ethanol, and there is no net oxidation-reduction in the conversion of glucose to

ethanol (Brock et al., 1994). Sugars at a different reduction level than glucose may be difficult to ferment to ethanol because of disturbances in the redox balance (see Sections 1.4.2 and 1.4.4).

The overall efficiency of microbial conversion of fermentable sugars to ethanol is quite high; usually over 90 % of the sugars are converted to ethanol (Klass, 1998).

It should be mentioned that yeasts are not truly anaerobic organisms. They grow only for some generations under these conditions. Ethanol producing bacteria, however, such as Zymomonas *mobilis*, grow very well under anaerobic conditions.

1.2.4 Hydrogen

As discussed in Section 1.2.2, many microorganisms produce hydrogen gas under anaerobic conditions. However, most studies of gas production by microbial fermentation have been concerned only with methane production. The fact that hydrogen is also produced in such processes has been largely ignored in practical applications (Roychowdhury et al., 1988). It has been observed that addition of sugars to methanogenic cultures lead to rapid termination of methanogenesis and production of a mixture of carbon dioxide and hydrogen. In nature, significant accumulation of sugars is unlikely, and may explain why fermentative production of hydrogen is not commonly observed (Roychowdhury et al., 1988).

As indicated in Section 1.2.2, methane fermentation can be separated into the acid and methane phases, where molecular hydrogen is produced at least to some extent in the acid phase. The Gibbs free energy changes for several microbial conversions of glucose to VFA are quite favourable for co-production of hydrogen (Table 1.1). Consequently, the possibility of producing molecular hydrogen as an energy product in the acid phase of methane fermentation is of interest. The problem is that if the biosynthesis of methane is the objective, any hydrogen that is withdrawn from the process reduces methane yield and the overall transfer of energy from biomass to methane (Klass, 1998).

1.2.5 Oxidation-reduction balance

During the microbial production of 2 pyruvate from glucose, 2 NAD^+ are reduced to 2 NADH. However, a cell contains only a small amount of NAD^+, and if all were converted to NADH, the oxidation of glucose would stop. In a fermentative environment, NADH is oxidised back to NAD^+ through reactions involving the reduction of pyruvate to any of a variety of fermentation products (i.e. lactate and ethanol). Microorganisms that possess the enzyme hydrogenase can maintain the electron balance by the production of H_2. This gas is produced along with other fermentation products, and primarily serves to maintain the redox balance. If hydrogen production somehow is prevented, the oxidation-reduction balance of the other fermentation products will be directed toward more reduced products. Thus, many fermentative organisms that possess hydrogenase can produce both ethanol and acetate (Brock et al., 1994).

In microorganisms, NADH is predominantly regarded as a catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes. Since catabolic and anabolic pathways share the initial reactions of sugar metabolism, NADH is also formed during the assimilation of sugars to cell material. Prokaryotes usually posses transhydrogenase, and can transfer electrons from NADH to NADPH (Gottschalk, 1987). However, yeasts lack transhydrogenase, and this necessitates the conversion of part of the sugar exclusively for the purpose of generating reducing power in the form of NADPH. This is accomplished in the oxidative steps of the hexose monophosphate pathway. Generally, the fermentation of sugars by yeasts yields two products associated with the redox balance: formation of ethanol ensures reoxidation of the NADH produced in the catabolic oxidation of glucose, whereas production of glycerol originates from the reoxidation of the excess NADH generated in the assimilation of sugars to biomass (van Dijken and Scheffers, 1986). Based on the lack of transhydrogenase, it has been concluded that pure anaerobic growth of *Saccharomyces* on mannitol is not possible. Sugar alcohols are more reduced than glucose and lead to a disturbance of the redox balance (Quain and Boulten, 1987).

1.3 Brown algae

1.3.1 Characteristics and productivity

The term algae refer to a large and diverse assembly of eukaryotic organisms that contain chlorophyll and can carry out oxygenic photosynthesis. Most algae are microscopic and unicellular, but in this assembly we also find big multicellular organisms, the seaweeds. Brown algae (*Phaeophyta*), red algae (*Rhodophyta*) and green algae (*Chlorophyta*) are the common groups of macroalgae. They are typically found along coastlines down to 50 meters, and live attached to the bottom by specialised structures called holdfasts. This sea environment is rather stable in temperature, humidity and salinity. Globally, the present utilisation of seaweeds may be divided into the consumption of algae as food in the Orient, and the industrial use of phycocolloids throughout the world.

Brown algae are divided in 9 orders, 265 genera and more than 1500 species. They absorb medium wavelength green light, which enables them to live even at 30-50 m depths, but the majority live in the intertidal belt and upper sublittoral zone. Brown algae prefer cooler water temperatures than red and green algae. Immersed in water, the seaweeds have no need for internal transport of nutrients or water. This saves energy, and many macroalgae have a very high productivity. Productivity of non-cultivated brown algae has been reported in the range 3.3 – 11.3 kg dry weight m^{-2} $year^{-1}$ (Gao and McKineley, 1993). While uses of seaweeds in the West have been based on natural beds, China has been cultivating *Laminaria japonica* since the early 1950s. Rope cultures of *L. japonica* have been reported to produce 13.1 kg dry weight m^{-2} for a 7 month growing period (Brinkhuis et al., 1987). Sugarcane, the most productive of the cultivated land plants, have in the USA a productivity from 6.1 to 9.5 kg fresh weight m^{-2} $year^{-1}$ (Nathan, 1978). Thus, brown seaweeds have a high potential for biomass production and CO_2 fixation, and may be an attractive alternative source for energy and chemicals.

1.3.2 Norwegian resources

The two species at study in this work are also the two species that are so far commercially harvested in Norway: *Laminaria hyperborea*[*] and, to a lesser degree, *Ascophyllum nodosum*[**]. Their chemical composition and seasonal changes serve here as typical examples of brown seaweeds. They also represent the two major orders of brown algae found along the Norwegian coast: Laminarales and Fucales. The standing biomass of *A. nodosum* is estimated to $1.8*10^6$ tons fresh weight, constituting about 60 % of the littoral zone Fucales. The kelp *L. hyperborea* is the most common of the sublittoral Laminarales, with a standing stock of about $10*10^6$ tons fresh weight (Baardseth, 1970). Figure 1.5 shows a characteristic cross section of the vertical distribution of brown algae along the Norwegian coast. The typical vertical zonation of the seaweeds should be noted. *A. nodosum* is found in the middle of the littoral zone, while *L. hyperborea* grows below the low-tide mark.

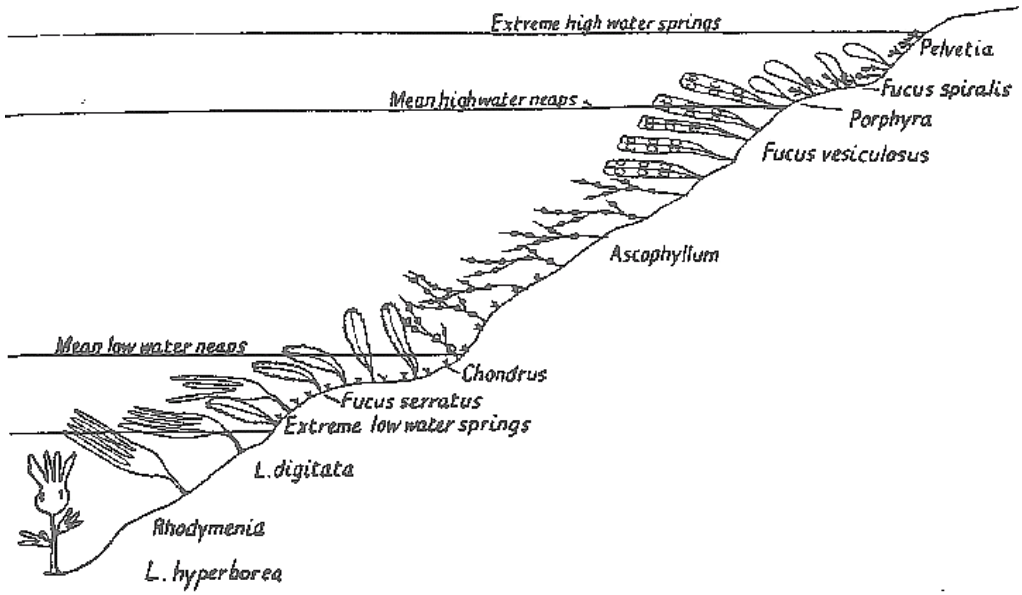

Figure 1.5 Zonation of different seaweed species at the Norwegian coast (Chapman, 1950).

[*] Laminaria: from Latin *lamina*, meaning thin layer. Hyperborea: Boreas was the ancient Greek personification of the north wind. Hyperboreans was a legendary people believed to live beyond the north wind in a land of unbroken sunshine. Here they enjoyed continuous and perfect happiness. ☺

[**] Ascophyllum: Greek, *askos* is a wine-skin, *phullon* is a leaf. Nodosum: full of swellings.

1.3.3 Physiology and chemical composition

The chemical composition of brown algae varies considerably between species, throughout the year and between habitats. Brown seaweeds exposed to seasonal changes usually accumulate mannitol and laminaran in the light season (spring to autumn), and consume these carbohydrates during growth in the dark season (Haug and Jensen, 1954).

A. nodosum is a branched, untidy looking plant (Figure 1.6). All branches have egg-shaped bladders in the body of the branch. The first bladder is formed after 3 years of growth, subsequently one bladder is formed each year. The plant is generally olive green, but the spring fruiting bodies vary with sex, being olive in the female and yellow in the male (Surrey-Gent and Morris, 1987). The chemical composition of *A. nodosum* is summarised in Table 1.2.

L. hyperborea is shown in Figure 1.7. It consists of a stipe that can become 3 meters long and 20 years old, and a frond that may be as large as 1 m^2. New growth appears at the base of the frond in early spring, and the older upper parts are eventually shed. *L. hyperborea* prefers exposed habitats and temperatures below 15 °C, and is therefore not found in the fjords. The main components of *L. hyperborea* are summarised in Table 1.3. The small seasonal variations in chemical composition of *L. hyperborea* stipe should be noted. In contrast, the composition of *L. hyperborea* fronds varies considerably throughout the year. This is caused by the seasonal alternation between accumulation and consumption of laminaran and mannitol. For autumn fronds of *L. hyperborea*, the mannitol and laminaran content may be as high as 25 % and 30 % of the dry weight, respectively (Jensen and Haug, 1956).

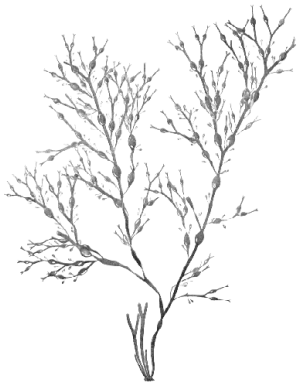

Figure 1.6 *A. nodosum*
(Printz, 1953).

Table 1.2 The chemical composition of *A. nodosum*. Water content is given as a percentage of the fresh weight. All other components are given as the percentage of the dry weight. References: (1) Jensen (1960), approximate numbers from graphs and tables; (2) Haug and Larsen (1958a, 1958b), content at different locations; (3) Ragan and Jensen (1978), seasonal variation; (4) Indergaard (1983), approximate numbers.

Component	[%]	Comments	Ref.
Water	67 - 82	Decreased with salinity and lowered during the spring	1
Ash	18 - 24	Increased from autumn to spring	1
Alginic acid	24 - 29	Fluctuations during the year	1
Laminaran	1.2 - 6.6	Increased from spring to late autumn	1
Mannitol	6.8 - 10.4	Increased from early spring to early autumn	1
Fucoidan	4 - 10		4
Other carbohydrates	10		1
Protein	4.8 - 9.8	Increased from autumn to spring	1
Fat	1.9 - 4.8	Increased from early spring to late autumn	1
Fibre (cellulose)	3.5 - 4.6	Almost constant throughout the year	1
Polyphenols	0.5 - 14	Lowered during the spring and increased greatly with salinity	2, 3
Iodine	0.06 - 0.09	Highest during the summer	1
K	2 - 3		1
Na	3 - 4		1
Ca	1 - 3		1
Mg	0.5 - 0.9		1
S	2.5 - 3.5		1
P	0.1 - 0.15		1

Figure 1.7 *L. hyperborea*
(Printz, 1953).

Table 1.3 The chemical composition of *L. hyperborea*. Water content is given as percentage of the fresh weight. All other numbers are given as the percentage of the dry weight. References: (1) Haug and Jensen (1954), average numbers from graphs; (2) Jensen and Haug (1956), average numbers from graphs; (3) Jensen (1954), numbers from one batch; (4) Haug and Larsen (1958a), numbers from one batch; (5) Ragan and Jensen (1977), relative numbers to *A. nodosum* polyphenols; (6) Larsen and Haug (1961), highest and lowest numbers from graphs; (7) Baardseth and Haug (1953), highest and lowest numbers from tables; (8) Indergaard (1983), approximate numbers; (9) Hanssen et al. (1987).

Component	Stipe [%]	Frond [%]	Ref.
Water	77 – 89		7
		84-87	Analysed
Ash	34.5 ± 2.5		1,2
		16-37	2
Alginic acid	33.4 ± 2.8		1,2
		17-34	2
Laminaran	0.68 ± 0.28		1
		0-30	2
Mannitol	5.9 ± 2.4		1,2
		4-25	2
Fucoidan	2 – 4		8
Other carbohydrates	Traces		8
Protein	8.9 ±1.6		1,2
		4-14	2
Fat	0.63 ± 0.14		1,2
Fibre (cellulose)	10.4 ± 0.8		1,2
Polyphenols	1 (Stipe)		4, 5
	0.3 (Peeled stipe)		4, 5
	5.3 (Peripheral tissue)		4, 5
Iodine	0.74 ± 0.11		1,2
K	6.3 - 11.0		3, 9
Na	1.6 - 3.0		3, 9
Ca	1.4 - 3.0		3, 9
Mg	0.6 - 0.7		3, 9
S	1.2 -1.3		9
P	0.2		9

1.3.4 Structural organisation

Brown algal cells are embedded in a three-dimensional continuous alginate network. The bulk of the alginate is located in this intercellular matrix where it may account for more than 80 % of the organic matter. While alginate is the main skeletal component in the intercellular matrix, the cell wall of brown algae consists of cellulose, alginate, fucoidan and protein (Kloareg et al., 1986). Alginate cements cells together, giving both mechanical strength and flexibility to the algal tissue (Andresen et al., 1977).

1.3.5 Alginate

Alginates are salts of alginic acid, a linear copolymer of β-1,4-D-mannuronic acid (M) and α-1,4-L-guluronic acid (G). The two uronic acids are organised in blocks of polymannuronate (M-blocks) and polyguluronate (G-block), as well as heteropolymeric sequences of both uronic acids (MG-block), see Figure 1.8.

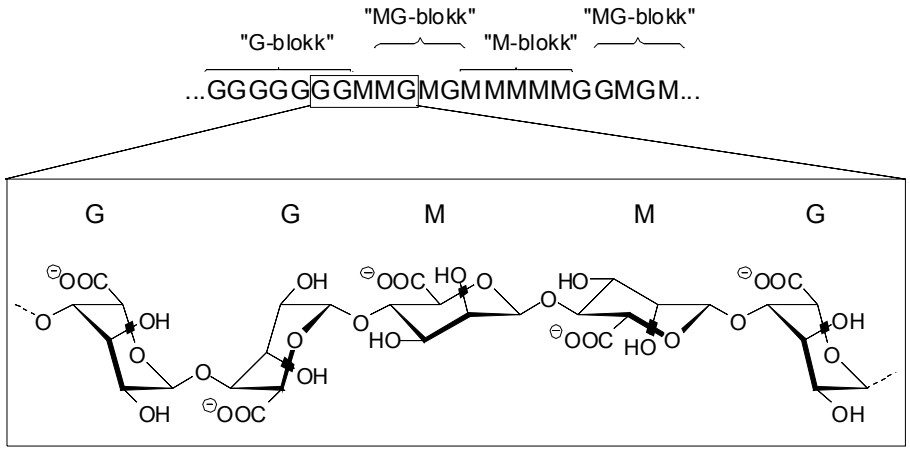

Figure 1.8 Schematically drawn alginate block structure and a segment of this showing the structure of the molecules. M=mannuronic acid; G=guluronic acid (Smidsrød and Moe, 1995).

Alginate salts of alkali metal ions are usually soluble. However, in algae, the polymers accumulate and bind divalent metal ions and form gels. These gels function as structural elements in the algae, and are formed by cooperative binding of divalent ions when the polyguluronate segment exceeds a critical length (Stokke et al., 1991). The dimerisation of guluronate residues by chelation of divalent ions, such as calcium, is shown in Figure 1.9. Alginates rich in guluronate form gels with a high mechanical rigidity, and a good stability towards competing Na-ions (Martinsen et al., 1989). In contrast, mannuronate-rich alginates form softer and more elastic gels. Because of the high selectivity of alginate for some divalent metal ions, the proportions of these ions in the algae differ radically from that of the seawater. The dominant ion in alginate junction zones of seaweed tissue is presumably calcium (Moen et al., 1997).

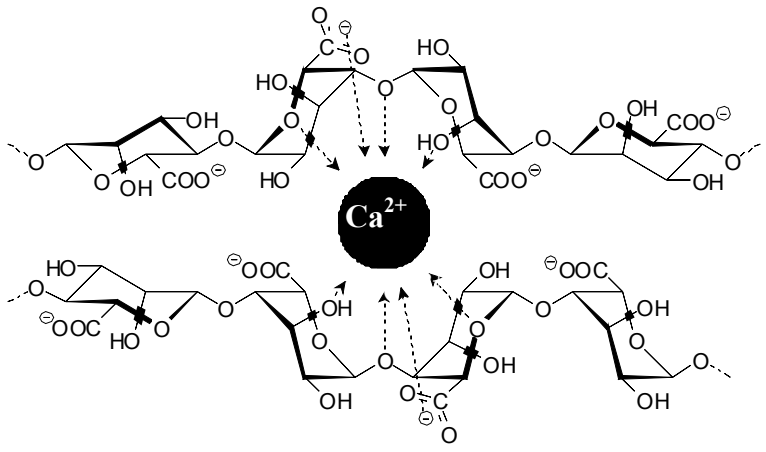

Figure 1.9 The binding of a divalent cation to adjacent dimers of guluronate residues (Smidsrød and Moe, 1995).

1.3.6 Storage carbohydrates: mannitol and laminaran

Mannitol* is the sugar alcohol corresponding to mannose. It usually constitutes less than 10 % of the dry weight in both *A. nodosum* and *L. hyperborea* stipe. In autumn fronds of *L. hyperborea*, however, the content may be as high as 25 % of the dry weight (Table 1.3).

Laminaran is a β-(1→3)-D-glucan containing about 25 glucosyl residues (Percival and Ross, 1951). However, in common with many other polysaccharides, laminaran is polydisperse, displaying some degree of structural heterogeneity. A small proportion of laminaran molecules terminate with a reducing 3-linked glucose residue and are designated G-chains, whereas the majority terminate with a non-reducing 1-linked D-mannitol residue and are designated M-chains. Some β-(1→6)-linked branching and some β-(1→6)-interchain links may occur. Generally, the side chains consist of a single glucose. The variations in the degree in branching do affect the solubility of the polysaccharide in water. Laminaran containing only β-(1→3)-linked residues is water-insoluble, while branched laminaran tend to be water soluble (Read et al., 1996).

* Mannitol: from *manna*, the mannitol containing exudation of the ash *Fraxinus ornus* and related plants. Manna also denotes the food that miraculously was supplied to the Israelites in the wilderness.

1.3.7 Other organic compounds

Algal fucoidan is mainly composed of sulphated fucose. Its molecular heterogeneity and high molecular weight has limited the structural studies of this polysaccharide (Daniel et al., 1999). Fucoidan is found in most brown algae, but is most abundant in species that grow in the intertidal zone (see Figure 1.5). When exposed to the atmosphere at low tide, many seaweeds exude a slime that provides the algae with essential protection against desiccation. This slime is a complex proteoglycan where fucoidan is one of the building units (Painter, 1983). Table 1.2 and Table 1.3 show that the intertidal *A. nodosum* usually has a higher fucoidan content than the sublittoral *L. hyperborea*.

Generally, the protein fraction of brown seaweeds is low (3-15 % of the dry weight) compared with that of green and red seaweeds (10-47 % of the dry weight). For most seaweeds, aspartic and glutamic acids constitute together a large part of the amino acid fraction (Fleurence, 1999).

While the intercellular matrix is dominated by alginate, the cell wall of brown algae also contains cellulose, fucoidan and protein. Tables 1.2 and 1.3 show that *L. hyperborea* contains about twice as much cellulose as *A. nodosum*.

Brown seaweeds also have cell vacuoles called physodes which contain polyphenols (Baardseth, 1958). The brown algae polyphenols occur as a single structural class, the phlorotannins, which are formed by the polymerisation of phloroglucinol. They are tannins in the sense that: they are soluble compounds of relative high molecular weight; and they contain sufficient phenolic hydroxyl groups to form hydrogen bonds between these groups and -N, $-NH_2$, and -OH groups in proteins and other macromolecules, giving almost undissociable complexes (Haslam, 1979; Swain, 1979). Tannins inhibit the growth of many microorganisms (Scalbert, 1991), and are especially potent inhibitors of methanogenesis (Field and Lettinga, 1987). Table 1.2 shows that the polyphenol content of *A. nodosum* maybe as high as 14 %. The polyphenol content of *L. hyperborea*, mainly found in the peripheral tissue of the stipes, is much lower (Table 1.3).

1.3.8 Alginate industry

Phycocolloids are seaweed polysaccharides that can give viscosity and gel strength to water solutions, the most important being alginate from brown algae and agar and carrageenan from red algae. The procedure for alginate extraction includes series of acidic and alkaline extractions. The process requires relatively high consumption of water and energy, and a large amount of organic compounds such as mannitol and laminaran are discharged to the environment (Fleury and Lahaye, 1993a; Fleury and Lahaye, 1993b). This represents an organic load that may cause problems in the local recipient, and stricter regulations will probably force the industry to treat this waste in the future. A selective degradation of mannitol and laminaran prior to the alginate extraction could reduce the organic load of the final discharge (Østgaard et al., 1993). Anaerobic digestion of the insoluble particle residues after alginate extraction has shown a high reduction of organic matter, biogas formation and improved settling properties of digester effluents (Carpenter et al., 1988; Kerner et al., 1991).

The total Norwegian stock of brown seaweed biomass is estimated to $15 \cdot 10^6$ tons fresh weight, with an annual production of $5 \cdot 10^6$ tons. About 50 % of the production and 20 % of the standing stock are lost annually due to erosion and shedding of fronds (Indergaard and Jensen, 1991). The annual Norwegian harvest of brown seaweeds is $180 \cdot 10^3$ tons fresh weight. Thus, only about 4 % of the annual production is harvested. Most of this is *L. hyperborea* ($160 \cdot 10^3$ tons) which is used for alginate production. Of the global annual alginate production amounting to about 30 000 tons (Onsøyen, 1996), FMC BioPolymer in Haugesund, Norway, produces 6-7000 tons. The rest of the harvest is *A. nodosum* ($20 \cdot 10^3$ tons), utilised in the production of seaweed meal and fertilisers.

1.4 Anaerobic degradation of brown algae

1.4.1 Brown algae and land plants

Terrestrial plants are primarily composed of a cellulose/hemicellulose/lignin complex embedded in an amorphous matrix of pectic substances (Roland et al., 1989; Carpita and Gibeut, 1993). They also frequently contain storage polysaccharides such as starch and fructosan, which usually are good substrates for biodegradation. However, cellulose, hemicellulose and lignin are very difficult to degrade.

In brown seaweeds, alginate is the main structural compound (Kloareg and Quatrano, 1988), while mannitol and laminaran are common storage materials. Thus, the absence of lignin and the low content of cellulose in brown algae should make them a simpler material for bioconversion than land plants. Seaweeds rich in carbohydrates and with a low content of ash and water are most favourable for biological degradation. Polyphenols (Morand et al., 1991) and salt (Ghosh et al., 1981; Moen et al., 1999) in the algae may reduce the biodegradability.

1.4.2 Alginate

Alginate can be depolymerised chemically by acid and alkali hydrolysis, by oxidative-reductive depolymerisation or enzymatically (Moen, 1997). Alginate hydrolases seem generally to be absent. Enzymatic degradation of alginate is catalysed by alginate lyases, which cleave the alginate polymer and create an unsaturated uronic acid at the new non-reducing end (Sutherland, 1995). Alginate lyases typically have an optimum around neutral pH, and may be classified as mannuronate or guluronate lyase according to their substrate specificity (Østgaard, 1993). Sources for alginate lyases include microorganisms, marine algae, marine molluscs and echinoderms (Boyen et al., 1990; Brown and Preston, 1991; Larsen et al., 1993). Microbial lyases are often detected in the culture medium, without verifying whether it is the result of directed transport of enzyme from the cells or cell lysis (Gacesa, 1992). Most of the research carried out on enzymatic alginate degradation has focused on alginate in solution (Sutherland, 1995). However, alginates in seaweed tissue, which are structurally organised in gels, are more protected against lyases. Moen and

Østgaard (1997) showed that soluble Na-alginates were consumed 6-8 times faster than Ca-alginate gels. G-rich parts of the alginate may be less accessible for lyases due to the calcium junction zones (see Section 1.3.5). Alginate associated with the cell walls, in combination with other structural elements such as cellulose (Section 1.3.4), may also be less available for enzymatic degradation.

The microbial cleavage of alginate leads to a range of oligosaccharides of different size (Haugen et al., 1989) with an unsaturated uronic acid at the non-reducing end. Most lyases are endo-acting enzymes and the major product appears to be the unsaturated triuronide (Gacesa, 1991). Further cleavage is probably catalysed by cell-bound exo-acting enzymes (Doubet and Quatrano, 1984). The ultimate product is the monosaccharide 4-deoxy-L-erythro-5-hexoseulose uronic acid, in equilibrium with its open chain form 2-keto-3-deoxy glucoaldehyde (Preiss and Ashwell, 1962a). Due to the loss of the asymmetry at C-5 both D-

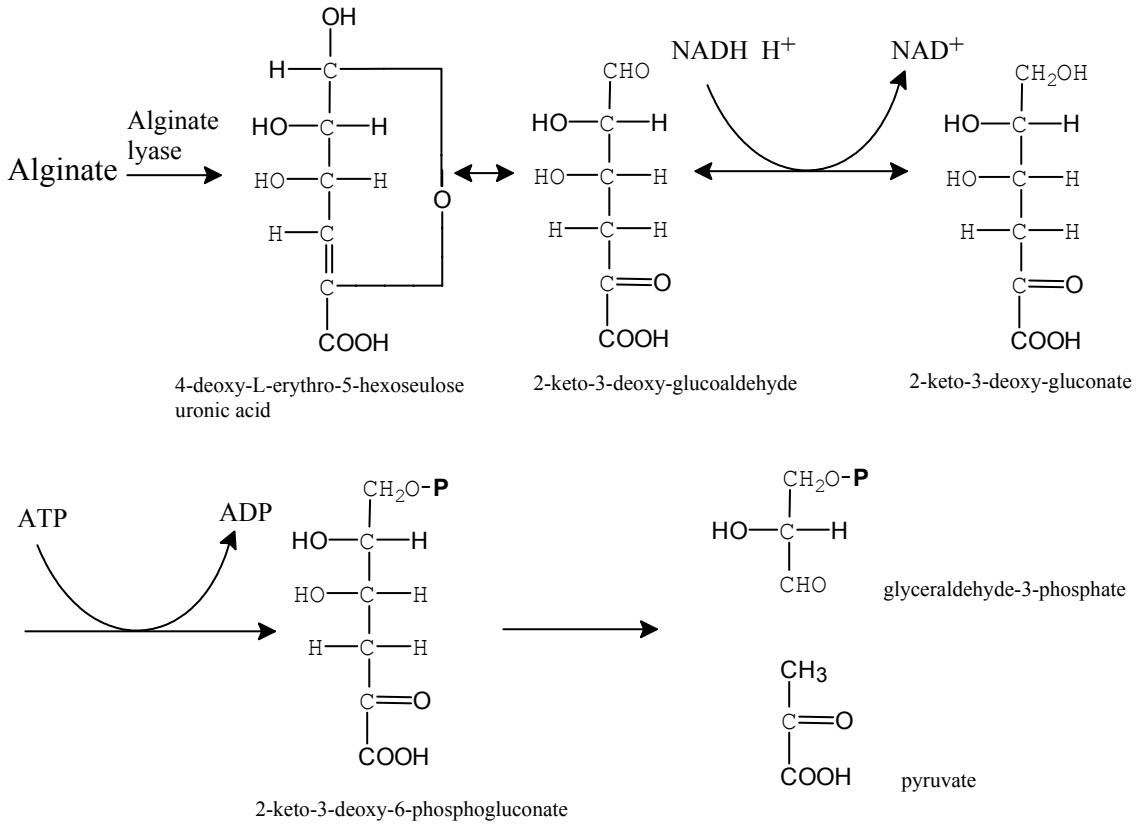

Figure 1.10 Metabolic pathway of alginate degradation (after Forro, 1987).

mannuronic and L-guluronic acid yield the same unsaturated derivative. A proposed pathway for the further intracellular metabolisation of the monosaccharide is depicted in Figure 1.10 (Preiss and Ashwell, 1962b; Forro, 1987). Investing one NADH and one ATP, the uronic acid is split into pyruvate and glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate may enter the glycolysis (Embden–Meyerhof pathway) and give rise to one NADH and two ATP under the production of pyruvate. The overall reaction is:

$$1 \text{ uronic acid} + 1 \text{ ADP} \rightarrow 2 \text{ pyruvate} + 1 \text{ ATP}$$

Thus, the redox balance is maintained and there is no need for a reduction of pyruvate to ethanol.

Three strains of *Cytophaga*, isolated from a *Macrocystis pyrifera* anaerobic degrading culture, were able to utilise alginate, mannitol and laminaran, and it was shown inducibility for all substrates. The major metabolites produced by *Cytophaga* when grown on alginate were acetate and propionate (Forro, 1987).

1.4.3 Laminaran

β-(1→3)-glucanases are relatively widespread, and many microorganisms can hydrolyse laminaran to its glucose monomer. Further breakdown follows glycolysis or the Entner-Doudorof pathway. For glycolysis the net reaction is:

$$1 \text{ glucose} + 2 \text{ ADP} + 2 \text{ NAD}^+ \rightarrow 2 \text{ pyruvate} + 2 \text{ ATP} + 2 \text{ NADH}$$

Laminaran degradation has not been widely studied under anaerobic conditions, but it does appear to be readily degraded (Chynoweth et al., 1978).

1.4.4 Mannitol

In contrast to the polysaccharides which first must be cleaved, mannitol is a soluble and readily utilisable carbohydrate. Depending upon the organism involved, mannitol is taken into the cell either by a facilitated diffusion mechanism or by an energy-dependent phosphoenolpyruvate phospho-transferase system (Dills et al., 1980). In the latter case, mannitol enters the cell as mannitol-1-phosphate, and is further converted to fructose-6-phosphate by a $NAD(P)^+$ dependent dehydrogenase. Mannitol transferred into the cell by diffusion, on the other hand, is converted to fructose via another $NAD(P)^+$ dependent dehydrogenase, and further to fructose-6-phosphate via a kinase (Forro, 1987). Fructose-6-phosphate may then enter the glycolysis or the Entner-Doudorof pathway. The net reaction for the glycolysis is:

$$1 \text{ mannitol} + 2 \text{ ADP} + 3 \text{ NAD}^+ \rightarrow 2 \text{ pyruvate} + 2 \text{ ATP} + 3 \text{ NADH}$$

Thus, compared to glucose, one extra NADH is produced, and production of ethanol will not result in a redox balance. Regeneration of all the NAD^+ then requires either oxygen (active electron transport chain), ability to produce H_2 or the presence of a transhydrogenase to convert NADH to NADPH. Thus, many microorganisms are not able to carry out strictly anaerobic fermentation of mannitol (Van Dijken and Scheffers, 1986). Both yeast and bacteria are potential organisms for ethanol production. Yeast lack transhydrogenase, and experiments done with *Saccharomyces* concluded that pure anaerobic growth on mannitol was not possible (Quain and Boulton, 1987). Thus, ethanol production from sugar alcohols by yeasts requires a supply of oxygen. This subject has been thoroughly investigated in connection with ethanol production from xylose, where metabolisation of the xylitol intermediate depends on oxygen (Hahn-Hägerdal et al., 1994). Prokaryotes, on the other hand, usually possess transhydrogenase (Gottschalk, 1986; Ingraham et al., 1983), and should be able to ferment mannitol under truly anaerobic conditions.

Forro (1987) reported three strains of *Bacteroides* that only used the mannitol fraction of *M. pyrifera* as substrate. Mannitol metabolism was inducible, and all strains produced acetate and H_2. In addition, the *Bacteroides* also produced different combinations of ethanol, formate, lactate and succinate. Presence of other substrates such as glucose did not shut of mannitol

metabolism. A *Cytophaga* strain that produced only acetate and propionate when grown on alginate or glucose also produced ethanol when grown on mannitol. The metabolic shift reflected the ability of the organism to eliminate the extra reducing equivalents found in mannitol. The same was seen for a *Lactobacillus* strain when substrate was changed from glucose to mannitol. Both strains apparently lack hydrogenase activity (Forro, 1987).

1.4.5 Other organic compounds

Anaerobic degradation of fucoidan has not been reported, nor is data available on the fate of the sulphate moiety (Forro, 1987). This is intriguing, since sulphate can be an electron acceptor under anaerobic conditions and lead to H_2S production. As mentioned in Section 1.2.2, sulphate-reducing bacteria compete with methanogenic bacteria and lower the methane yield.

Algal proteins have been reported to have a low digestibility (Michel et al., 1996). This may be due to their cellular localisation or their putative associations with cell-wall polysaccharides (Kloareg and Quatrano, 1988). The presence of soluble polyphenols in brown algae may also inhibit the degradation of proteins. The phenols form complexes with proteins, making the proteins inaccessible to biodegradation (Scalbert, 1991).

Degradation of cellulose is catalysed by cellulases, and occurs both under aerobic and anaerobic conditions (Atlas and Bartha, 1998). However, in brown algae cellulose is found in the cell wall in close association with other structural components (Section 1.3.4), which may limit the enzymatic access to cellulose. A combined enzymatic attack of alginate lyases, proteases and cellulases may be necessary to degrade the algal cell wall, as seen in the case of protoplast isolation (Butler et al., 1989). Thus, the cell walls seem to be more recalcitrant to microbial degradation than the intercellular matrix.

1.4.6 Mixed substrate utilisation

The first three steps of anaerobic degradation, hydrolysis, acidogenesis and acetogenesis (Section 1.2.2), are dependent on the substrate material. Methanogenesis may proceed

independent of the original substrate, only requiring that the preceding steps produce acetate, H_2 and CO_2. Thus, the particularities of anaerobic degradation of brown seaweeds are found prior to the methanogenic step. Brown seaweeds have a complex composition, and their degradation requires a microbial community with the ability for mixed substrate utilisation. Moreover, the capacity to degrade a specific substrate may be constitutive or inducible.

In their natural habitat, microbes generally encounter many different substrates at low concentrations. Hence, most microorganisms are well adapted to cope with low concentrations of mixed substrates, a capacity that is exploited in wastewater treatment. In contrast, microorganisms that are utilised in industrial processes often find themselves in an environment of high substrate concentrations. Under such conditions, a sequential utilisation of the substrates may take place. This phenomenon is denoted diauxy (Kuenen, 1994). The substrate that will support the fastest growth will be utilised first, often repressing induction or activity of enzyme systems required for use of other substrates. The utilisation of the second substrate may be inhibited at the level of transport into the cell, enzyme synthesis or enzyme activity. However, simultaneous utilisation of different carbon sources is also commonly observed under substrate excess conditions. Especially combinations of substrates that support medium- or low-maximum specific growth rates can be utilised simultaneously (Egli, 1995).

1.4.7 Methane production from seaweeds

Cultivation of macroalgae for production of energy by methane fermentation was first suggested in the late sixties (Neushul, 1987). From the mid-seventies until the late eighties Chynoweth and co-workers at the Institute of Gas Technology performed an extensive study of the production of methane from marine macroalgae, mainly from *Macrocystis pyrifera* (Ghosh et al., 1981; Chynoweth et al., 1987). These studies still provide the best and most relevant data for the assessment of industrial production of methane from marine macroalgae. Several other groups have also studies methane production from seaweeds (Morand et al., 1991). In general, these studies have concluded that marine algae are good feedstocks for the anaerobic digestion process, as demonstrated by high conversion efficiencies, rapid conversion rates and good process stability. Anaerobic degradation of *M. pyrifera* in

continuous culture has achieved more than 80 % of the theoretically attainable methane yield (Chynoweth, 1987). Generally, methane yields are around 0.3 L/g VS added at retention times between 10 and 20 days, but a yield as high as 0.43 L/g VS has been reported for longer retention times (Bird et al., 1990). Usually, biogas produced from macroalgae contains 50-65 % methane (Morand et al., 1991), but methane concentrations as high as 75 % have been reported for two-phase digestion of *M. pyrifera* (Chynoweth, 1987). It has also been concluded, since the theoretical methane yield of alginate is substantially lower than that of mannitol, that seaweed lots that have higher concentrations of alginate relative to mannitol can be expected to have lower methane yields (Chynoweth, 1987).

1.4.8 Ethanol production from seaweeds

The author is not familiar with any other research done on ethanol production from seaweeds. The reason may be the complex composition of seaweeds, containing several different carbohydrates. Finding a microorganism that can ferment all the different carbohydrates to ethanol is not very likely. Besides, alginate, as a major component in brown algae, may not be fermented to ethanol since the redox balance in the glycolytic oxidation of uronic acids to pyruvate is maintained (see Section 1.4.2). Hence, no excess electrons are available for the reduction of pyruvate to ethanol. Laminaran and mannitol, on the other hand, may be converted to ethanol, and these sugars can also easily be extracted from milled seaweed (Percival and McDowell, 1967).

1.5 Scope

This study is aimed at identifying the potentials and problems of microbial production of energy carriers such as methane and ethanol from brown seaweeds. When comparing the composition of seaweeds to that of land plants, two major differences should be emphasised: First of all, the complete lack of recalcitrant lignin and the low cellulose content should make the seaweeds a better raw material for complete biological degradation to methane (Section 1.4.1). Secondly, the complex composition of seaweeds (Section 1.3.3) implies that a partial fermentation to a specific intermediate such as ethanol has to involve microbes with a broad

substrate range. Land plants usually have a much simpler composition in the sense that cellulose generally is a dominant compound.
The brown seaweeds selected for this study were *L. hyperborea* and *A. nodosum*. They are among the most abundant Norwegian seaweeds (Section 1.3.2), and they are also the only two species that are economically exploited in Norway (Section 1.3.8). Thus, efficient harvesting technology is already available.

In the anaerobic digestion of organic matter to methane, it is the hydrolytic and acidogenic steps that are specific for the raw material (Section 1.4.6). Thus, the work on methane production was concentrated on these initial steps, without attempting to optimise the final yield. Due to the complex composition of the seaweeds, mixed substrate utilisation (Section 1.4.6) will be the key factor for an efficient degradation and a high final yield. However, the presence of polyphenols in seaweeds (Section 1.3.7) may inhibit biodegradation, and was therefore a factor that was studied in particular.

In the production of a specific digestion intermediate such as ethanol, specific microbial strains have to be utilised (Section 1.2.3). In this case, the complex composition of seaweeds represents a challenge, since ethanol has to be produced from different substrates. Thus, it will be difficult to find a single organism that can utilise all the different substrates, and a mixture of microorganisms may be necessary. One of the main components of brown seaweeds, alginate, may even have a too low reduction level to be directly converted to ethanol (Section 1.4.2). In this study, only the possibility of ethanol production from the easily extractable storage carbohydrates mannitol and laminaran (Section 2.1.4) was investigated.

Most of the experiments were carried out in batch, which is a simple model system to work with and efficient for screening new process conditions. However, it is a system with large changes in concentrations that never reaches steady state, usually following a pattern of diauxic substrate utilisation. Results should therefore be interpreted with care, and not generalised or considered as directly applicable for continuous processes.

2 Experimental overview

2.1 Algae and media

2.1.1 *Laminaria hyperborea*

L. hyperborea stipes were harvested in early September at the Norwegian coast (59 ° North, 5° East), and arrived at the laboratory within one day after harvesting. The stipes were washed and scrubbed with a brush in 3 % NaCl to remove epiphytes. The peripheral tissue, which tends to accumulate polyphenols, was mechanically removed by a potato peeler (see Figure 2.1). Stipes, peeled stipes and peel were separately milled in a Wiley Laboratory Mill to pass a sieve with 4 mm holes.

Freeze dried samples of stipe from degradation studies (Appendix Paper 3) were applied to NIR spectroscopy to correlate spectra to their content of alginate (Appendix Paper 1).

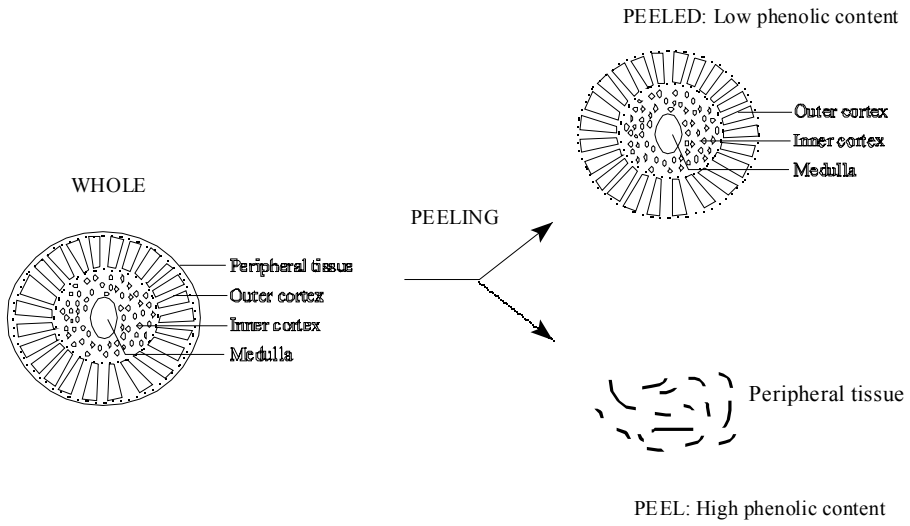

Figure 2.1 A cross-section of *L. hyperborea* stipe before peeling, and the peeled stipe and peripheral tissue after peeling. The content of polyphenols, protein and iodine in the peripheral tissue is high compared to the rest of the stipe (from Moen, 1997).

L. hyperborea fronds used for extraction of laminaran and mannitol (Appendix Papers 5 and 6) were harvested in October and November at the same location as the stipes. Fronds used in studies of hydrolysis and acidogenesis were harvested in March (Appendix Paper 4). The fronds arrived at the laboratory within one day after harvesting, and were immediately milled to pass a sieve of 7 mm holes.

2.1.2 *Ascophyllum nodosum*

A. nodosum was harvested from seawater with a 26–30 ‰ salinity at Munkaunet in the Trondheimsfjord, Norway (63 °north, 10 °east). The algal tissues used for the anaerobic batch digestions were collected early mid April (Appendix Paper 2). Within one hour after harvesting, the seaweeds were milled in a Wiley Laboratory Mill to pass a sieve with 4 mm holes, and stored at 4 °C for one day. In contrast to *L. hyperborea* stipes, the polyphenols in *A. nodosum* cannot be mechanically removed.

2.1.3 Seaweed suspensions for the digestion studies

All the digestion studies (Appendix Papers 2, 3 and 4) were carried out in suspensions of 20 % wet weight algae and 80 % tap water. In some of the experiments extra nutrients were also added (Appendix Papers 2 and 3). The algae suspensions had VS concentrations in the range 13-30 g L^{-1}. To prevent clogging in the tubing, the medium utilised in the continuous experiment (Appendix Paper 4) was treated with an Ultra Turex to reduce the particle size.

2.1.4 Seaweed extracts and media for ethanol production

Contrary to the media utilised in the digestion studies, all the media utilised for ethanol production were autoclaved. Both defined media and extracts from *L. hyperborea* fronds were applied. The seaweed juice used in Appendix Papers 5 and 6 was prepared by extraction of fresh autumn fronds of *L. hyperborea*: 1 kg milled (7 mm sieve) wet weight fronds was extracted in 1 L tap water at pH 2.0 and 65 °C (Percival and McDowell, 1967) for 1 h, yielding extracts with approximately 20 g L^{-1} of both mannitol and laminaran. The extractions

were carried out in 3 L fermentors (Applicon, Schiedam, Netherlands). The seaweed particles were removed by filtering through a nylon cloth with a mesh size of 90 μm, and the final extracts were stored at –20 °C. The pH was adjusted to about 4.5 with 4 g concentrated NH_3 solution (25 %) per kg extract before autoclaving (121°C, 20 min).

2.2 Inocula and microorganisms

2.2.1 Anaerobic mixed inoculum

Anaerobic inocula (Appendix Papers 2, 3 and 4) were originally obtained from a wide range of sources including decomposed seaweeds, marine sediments and cattle manure. The mixed microbial culture was adapted to anaerobic degradation of *L. hyperborea* stipe through several previous batch fermentations. Cultures showing alginate lyase activity and methane formation were stored at –40 °C in 15 % glycerol. The inoculum was either used directly, or a pre-culture was run to consume and remove the glycerol. See the Appendix Papers for further details.

2.2.2 Strains used for ethanol production

The bacterium *Zymobacter palmae* T109 (ATCC 51623) was used in Appendix Papers 5 and 6. In Appendix Paper 6 *Pichia angophorae* (CBS 5830), *Kluyveromyces marxianus* (NCYC 1426) and *Pacchysolen tannophilus* (NCYC 614) were also utilised. All cultures were maintained at –80 °C in 15 % glycerol. Inocula were taken from pre-cultures grown in mannitol medium.

2.3 Reactor systems

2.3.1 10 L working volume

The batch experiments in Appendix Papers 2 and 3 were done in a Fermentor Drive Assembly Model No. M1200-2002 FS-314 equipped with fermentors Model F-14 (New Brunswick Scientific Co. Inc. New Jersey) with 14 L total and 10 L working volume. These fermentors were immersed in a temperature controlled water bath at 35 °C, operated with continuous stirring, and sparged with nitrogen which had passed through a Cu-column at 360 °C to remove traces of oxygen. The pH in the fermentors was maintained at 7.0 by automatic addition of 3M HCl or 3M NaOH. CO_2 and CH_4 concentrations in the outlet gas were automatically recorded by Binos 100.2 M gas analysers.

2.3.2 1 L working volume

The experiments in Appendix Papers 4, 5 and 6 were carried out in 3 L fermentors (Applicon, Schiedam, Netherlands) with 1 L working medium. The pH was automatically controlled by adding NaOH or HCl. CO_2 and CH_4 in outlet gas, stirrer speed and dissolved oxygen (Rosemount, Oxynos 100 gas analyser) were logged automatically. The reactors were sparged with air or nitrogen. Temperature was automatically controlled by a heating jacket.

The continuous experiments in Appendix Papers 4 and 6 were also carried out in the small reactors. Two automatically controlled pumps were applied; one to regulate the medium feed, and one to maintain a constant working volume by pumping out excess liquid. See Appendix Papers for further details.

2.4 New method: NIR determination of alginate content

2.4.1 Background

Alginate may contribute up to 35 % of the *L. hyperborea* dry weight (Section 1.3.3). This alginate may be extracted, precipitated and finally quantified by weighing (Haug, 1964). Such an approach may lead to underestimates, due to low extraction yields and losses. Especially in the case of highly degraded alginates, as observed during biological degradation, losses will be high due to poor precipitation. As an alternative, the total alginate content may be quantified by the so called calcium-acetate method (Haug, 1964; Jensen et al., 1985). This method is based on calcium ion-exchange followed by atomic absorption analysis. However, other charged polymers may interfere with the ion-exchange analysis. This method is therefore unsuitable for analysis of brown seaweeds containing high levels of sulphated polysaccharides (Myklestad, 1968a; Myklestad, 1968b). Both these methods are also time consuming, and a faster and simpler method would be beneficial.

On this background, a study (Appendix Paper 1) was carried out to evaluate the use of near infra-red (NIR) spectroscopy of dry seaweed material as a method for direct alginate quantification. The samples originated from the degradation experiments presented in Appendix Paper 3 and a previous work (Moen et al.,1997). They spanned a wide range of alginate concentrations, determined according to the calcium acetate method. Two types of freeze dried material were utilised: stipe and peeled stipe (see Figure 2.1). NIR reflectance spectra were recorded for each sample and used as a base for the data analysis. See Appendix Paper 1 for further details.

2.4.2 Near infra-red spectroscopy and multivariate analysis

Visible and NIR reflectance measurements (400-2500 nm) were carried out by a scanning spectro-photometer Model 6500 from NIRSystems Inc. (Silver Springs, USA). Every second nm wavelength was recorded, giving a total of 1050 spectral variables. Spectra were obtained at room temperature by averaging 50 succeeding scans. Each sample was measured in three re-packed replicates, and the average value was used in the data analysis.

Spectroscopic measurements of powders often display light scattering effects. These scattering phenomena can arise from variations in water content, surface roughness and particle size (Antti and Sjøstrøm, 1996). In Appendix Paper 1, multiplicative scatter correction (MSC) was used as a transformation method to compensate for these effects. MSC is known to give improved performance when applied to NIR reflectance data prior to calibration (Geladi et al., 1985; Isaksson and Næs, 1988). The method separates multiplicative or scattering variations from additive or chemical information, and gives a better linear fit between spectral data and chemical composition.

The spectral data (X-data) were related to the alginate concentration and material type (Y-data) by using the multivariate calibration method denoted partial least squares (PLS) regression. This is a linear method that reduces the original spectral data to a few components, representing the directions of largest variance.

A PLS2 model that included both alginate content and material type in the Y-data, was first calculated. Then a PLS1 model with only the alginate concentration as Y-data was run. In this case, the samples were sorted according to alginate concentration, and every third sample was removed to be used as a validation set. In this way, the test samples spanned the whole experimental region. The remaining samples were used for calibration of the model. See Appendix Paper 1 for further details.

All the data analyses were performed by the Unscrambler Software Version 5.5 (Camo A/S, Trondheim, Norway).

2.4.3 Main results

The data models showed that NIR spectroscopy can be used for direct quantification of alginate in *L. hyperborea* stipe material. Based on NIR spectra of 52 samples, a 9 component PLS1 model was determined, and validation by a 26 sample test set showed that alginate concentration could be determined with a root mean square error of prediction of 2.1 % (Figure 2.2). Seaweed material with higher and variable polyphenol content would probably

be less suitable for NIR spectroscopy, unless a revised PLS2 model can be calibrated for both alginate and polyphenol content.

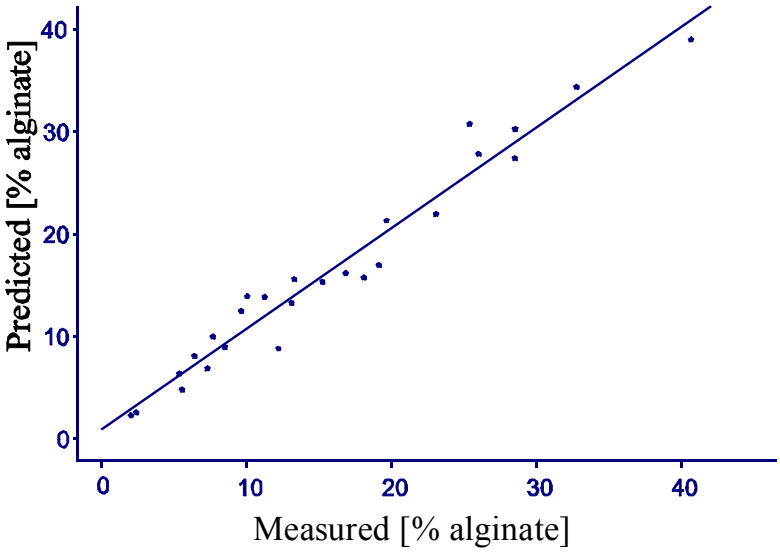

Figure 2.2 PLS1 model based on 52 samples validated by a test set of 26 samples. Predicted versus measured alginate concentrations (from Appendix Paper 1).

2.5 Discussion

The chosen concentrations of algae in the digestions were based on earlier studies at the laboratory (Østgaard et al., 1993; Moen, 1997). They give homogenous suspensions in stirred reactors which enable representative sampling.

Anaerobic digesters can be nutrient limited, and extra nutrients were added in experiments carried out in the 14 L reactors. Especially brown algae harvested in the autumn have a low nitrogen content. The experiments carried out on spring fronds of *L. hyperborea* (Appendix Paper 4), were not added extra nutrients, since pilot studies showed that nutrient supply did not improve the degradation (unpublished results).

The 1:1 ratio of fronds to water applied for the extractions gave a reasonable mixing in the reactor. Higher concentrations of fronds gave inhomogeneous suspensions where only parts of

the reactor where mixed. More concentrated extracts may be achieved by repeated extractions with the same water.

The powder utilised for NIR measurements originated from about 200 ml freeze dried reactor samples (Appendix Paper 3). Since the further digestion experiments were carried out in 1 L working volume (Appendix Paper 4), the required volume for freeze drying was obviously too big. The reduction of reactor volume made it possible to carry out more experiments, but the NIR method could no longer be applied due to large sample demand. However, samples taken from additional fermentations in 10 L working volume reactors, showed that the NIR method worked very well (unpublished results).

3 Methane

3.1 Anaerobic degradation of *Ascophyllum nodosum*

Appendix Paper 2 also contains data on aerobic degradation of this seaweed. This is considered as beyond the scope of this thesis, and will not be discussed here.

3.1.1 Effects of polyphenol fixation on the degradation pattern

The anaerobic batch degradation of *A. nodosum* at 35 °C and pH 7 is described in full detail in Appendix Paper 2. The degradation pattern was carefully recorded, and the possible influence of the reactive polyphenols was investigated. This was assessed by pre-treating one batch of *A. nodosum* with a balanced dosage of formaldehyde to inactivate the polyphenols. See Appendix Paper 2 for further details. The abbreviations ANAEROB and ANAFORM were used for the degradation of *A. nodosum* and for the degradation of formaldehyde pre-treated *A. nodosum*, respectively.

The general characteristics of the biodegradations are summarised in Figure 3.1. The initial CO_2-production (Figure 3.1A) and the conversion of VS (Figure 3.1C) occurred at higher rate in ANAFORM than in ANAEROB. The soluble COD concentration was initially increasing due to the dissolution of organic matter (Figure 3.1D), and COD remained high until methane production was detected (Figure 3.1A). Then HCl was required (Figure 3.2B) due to the consumption of acetate (Figure 3.2A). Figure 3.1E shows that the formaldehyde treated material in ANAFORM resulted in a low polyphenol concentration in the reactor. ANAEROB maintained much higher concentrations of polyphenols until formaldehyde finally was added also to this system after 450 h. Both reactors were reinoculated at 160 h. This was of particular importance for the ANAEROB reactor, since the high polyphenol concentration might have killed part of the microbial community in the original inoculum. ANAEROB was reinoculated a second time after the formaldehyde addition. Methane production was detected shortly after this reinoculation, suggesting that the polyphenols were most toxic for the

methanogenic bacteria. In ANAFORM, methane production was detected after 330 h (Figure 3.1A). Although H_2S production was not quantified, it was readily smelled in the outlet gas.

About 2 g L^{-1} of mannitol was initially dissolved from the algae. It was completely consumed within the first 20 h in ANAFORM. However, mannitol concentration remained high for a long time in ANAEROB, until most of it was consumed quickly between 90 and 116 h (results not included). This resulted in a rise in base consumption (Figure 3.1B), and increased production of acetate (Figure 3.2A), ethanol (Figure 3.2B) and CO_2 (Figure 3.1A). The simultaneous decrease in COD concentration (Figure 3.1D) was caused by biomass growth and possibly H_2 production. Hence, the high polyphenol concentration delayed the utilisation of mannitol.

Shortly after inoculation, ANAFORM produced acetate (Figure 3.2A), butyrate (Figure 3.2C), propionate (Figure 3.2D) and CO_2 (Figure 3.1A). Acetate

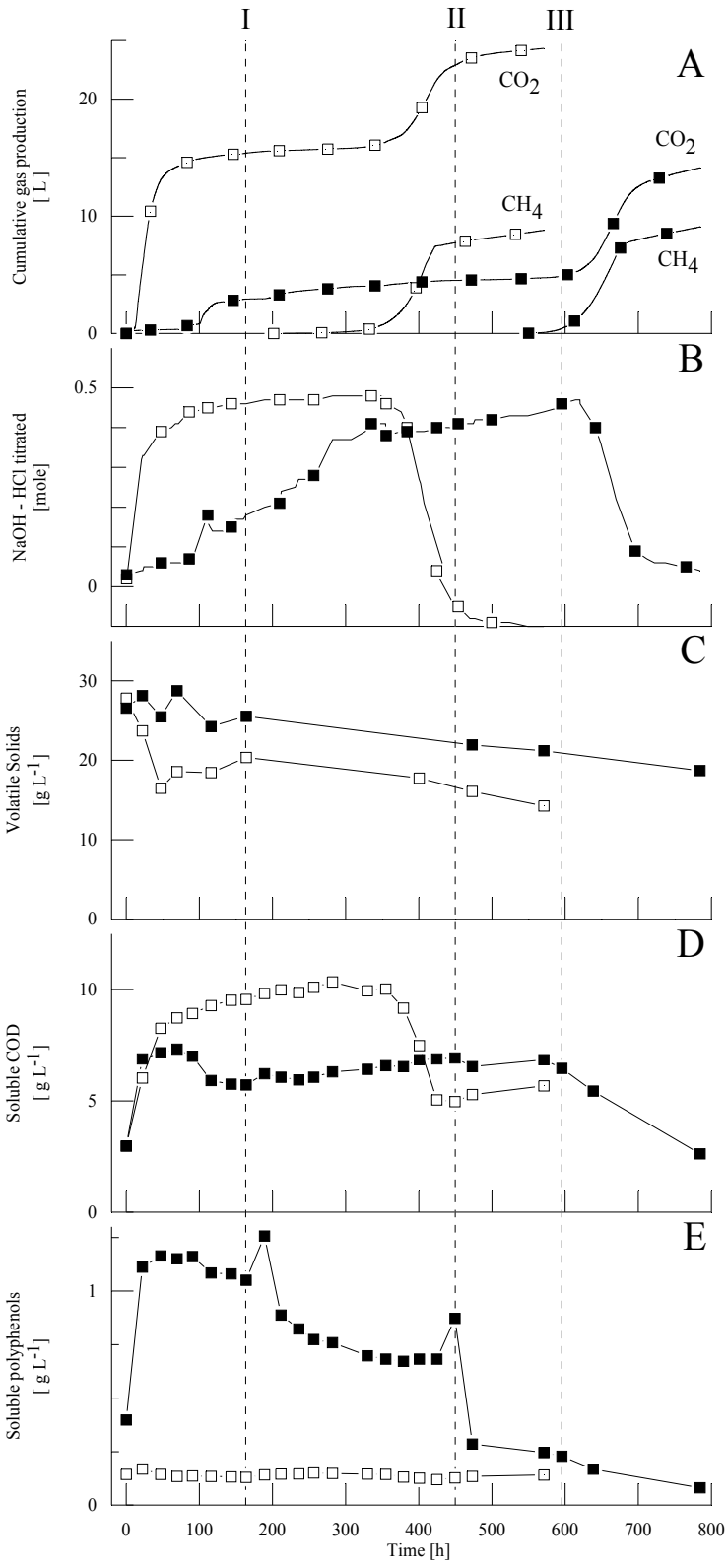

Figure 3.1 Degradation pattern for ANAEROB (■) and ANAFORM (□). (A) Accumulated CO_2 and CH_4; (B) Total net consumption of NaOH-HCl; (C) Volatile Solids; (D) Soluble COD; (E) Soluble polyphenols. The dotted lines indicate: I) Reinoculation; II) Formaldehyde added to ANAEROB; III) Reinoculation of ANAEROB (from Appendix Paper 2).

was the main organic product in both reactors and was later converted to methane (Figure 3.1A). In ANAEROB, both the dissolution of alginate, seen as a lower COD values, and the fermentation of mannitol and uronic acids to VFAs were inhibited. This reactor also temporarily accumulated ethanol, lactate and succinate. These reduced compounds are typically produced when excess electrons are discharged. Excess electrons can also be discharged as H_2, but this pathway may have been inhibited by the polyphenols in ANAEROB.

The initial degradation of alginate in ANAFORM was 0.16 g L^{-1} h^{-1}. In ANAEROB the alginate was lost at a much lower rate. ANAFORM showed a high soluble alginate lyase activity, whereas the activity in ANAEROB was insignificant. The viscosity of the isolated alginate confirmed these differences in degradation activity. Also the total soluble uronates and unsaturated uronates in ANAEROB remained intact until the soluble phenol concentration was reduced by addition of formaldehyde at 450 h. See Appendix Paper 2 for further details.

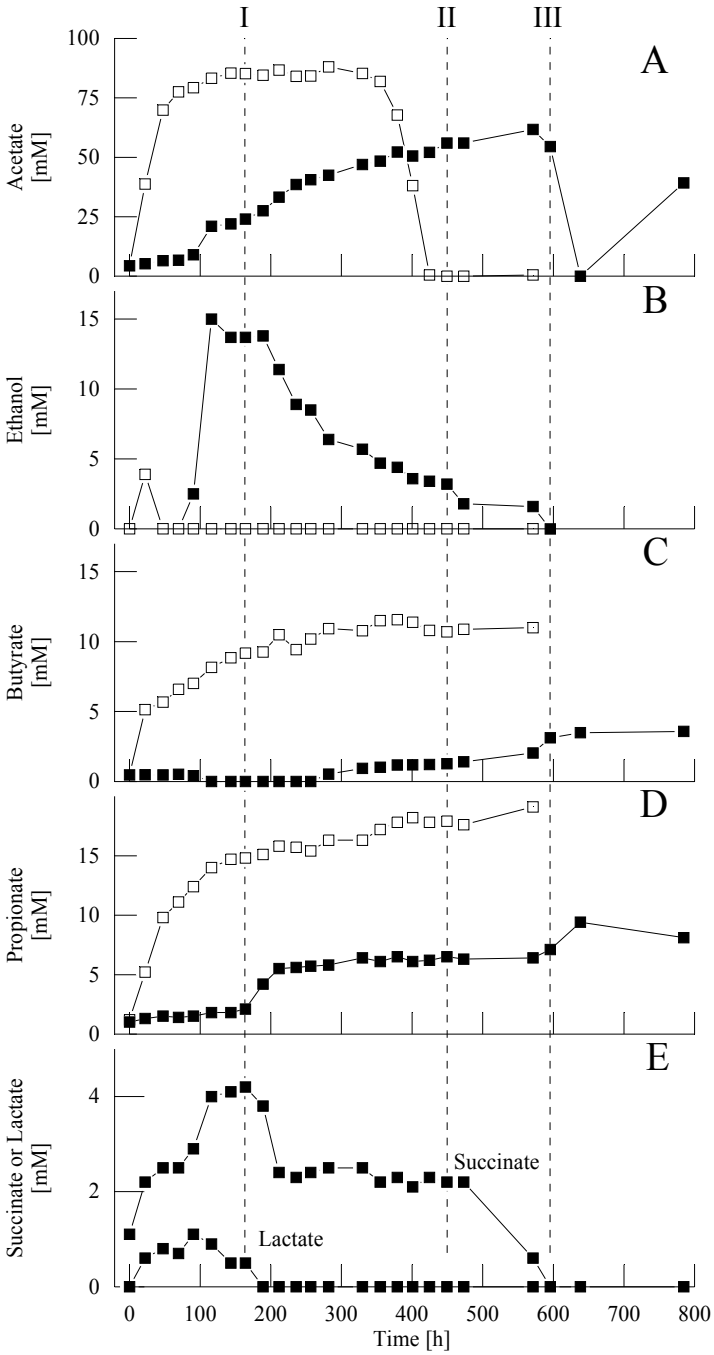

Figure 3.2 Concentration of: A) acetate; B) ethanol; C) butyrate; D) propionate; E) succinate and lactate, in ANAEROB (■) and ANAFORM (□). The dotted lines indicate: I) Reinoculation; II) Formaldehyde added to ANAEROB; III) Reinoculation of ANAEROB (from Appendix Paper 2).

Figure 3.3 shows that the soluble ANAEROB fraction strongly inhibited both the crude enzymes in ANAFORM and the purified *Haliotis tuberculata* M-lyase. The soluble ANAFORM displayed a much lower inhibitory effect, if any, on the M-lyase. Pre-incubation with additional protein (bovine serum albumin, BSA) in the test reduced the inhibitory effect of soluble ANAEROB. An increase in protein binding efficiency at increasing molecular weight of polyphenols has commonly been observed (Scalbert, 1991). Boettcher and Targett (1993) found the relative distribution of polyphenols among molecular size fractions for *A. nodosum* to be: 12 ± 1% < 1 kDa; 4 ± 1% from 1 kDa to 10 kDa; 72 ± 5% from 10 kDa to 100 kDa; 12 ± 5% > 100 kDa. Obviously, the relatively large fraction of high molecular weight polyphenols in this alga will efficiently bind proteins. The inhibitory effect on alginate lyases was probably due to this nonspecific binding by polyphenols to the enzymes since the added BSA clearly reduced the inhibitory effect. BSA will compete with microbial proteins such as enzymes and thereby reduce the inhibitory effect of the polyphenols (see Section 1.3.7).

The guluronate content of the remaining alginates increased rapidly in ANAFORM, but no increase was observed before 784 h in ANAEROB (Appendix Paper 2). As discussed in Section 1.4.2, poly-guluronates might be less accessible to alginate lyases.

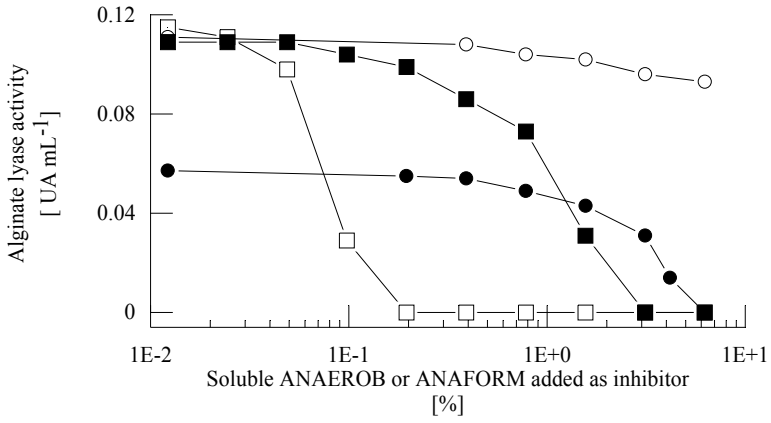

Figure 3.3 Inhibitory effect on alginate lyase activity (*H. tuberculata* M-lyase or crude ANAFORM) by soluble fractions from ANAFORM or ANAEROB after 22 h. M-lyase added soluble ANAFORM (○); M-lyase added soluble ANAEROB (□); M-lyase added soluble ANAEROB and bovine serum albumin (■); soluble ANAFORM added soluble ANAEROB (●) (From Appendix Paper 2).

3.1.2 Main findings

The dominant factor for conversion of organic matter during anaerobic degradation of *A. nodosum* was the inhibitory effect of the polyphenols on alginate lyase activity, mannitol consumption, as well as methane production. The inhibitory effect on alginate lyases was probably due to the nonspecific binding of polyphenols to proteins. The anaerobic degradation

was greatly stimulated when the polyphenols were fixed with low amounts of formaldehyde. Methane was produced from the accumulated acetate, but not until the polyphenols were inactivated. An accumulated content of guluronate in the remaining alginate showed that Ca-crosslinking may limit the enzymatic access to the polymer.

3.2 Anaerobic degradation of *Laminaria hyperborea* stipes

3.2.1 Degradation of tissue containing different levels of polyphenols

L. hyperborea stipe tissues were degraded under anaerobic batch conditions at 35 °C and pH 7 as described Appendix Paper 3. Polyphenols tend to accumulate in the peripheral tissue of *L. hyperborea* (Table 1.3). By peeling off the peripheral tissue three different materials, containing different concentrations of polyphenols, were obtained (see Figure 2.1), denoted PEELED, WHOLE and ENRICHED.

Figure 3.4 presents the basic patterns of the digestions. As expected, Figure 3.4E shows that the initial soluble phenol concentration increased in the sequence PEELED < WHOLE < ENRICHED. The concentrations were similar to or lower than the concentrations observed for formaldehyde treated *A. nodosum* (Figure 3.1). The CO_2-production was lowest in WHOLE within the first 50 h (Figure 3.4A). After this initial period, the CO_2-production rates stabilised at notably lower and similar levels in all digestions. Figure 3.4B shows that the lag phase in methane production increased with the amount of peripheral tissue present, suggesting that methanogenesis was inhibited by the dissolved polyphenols (Figure 3.4E). Such an inhibition was also observed in the degradation of *A. nodosum* in Section 3.1. According to Scalbert (1991), polyphenols are potent inhibitors of methanogenesis.

The degradation rate of VS during the first 21 h increased in the sequence ENRICHED < WHOLE < PEELED (Figure 3.4D), yielding a total conversion of organic matter of 34 %, 40 % and 57 %, respectively. The peripheral tissue was apparently less degradable than the other tissues. All experiments rapidly accumulated acetate, ethanol, formate and succinate

(Appendix Paper 3). The production of acetate halted at 50 h in ENRICHED. In WHOLE and PEELED, acetate was produced throughout the experiment. Notably, the relatively early methane production in these experiments did not lead to a decrease in the acetate concentration. Thus, acetate was probably still produced after the initiation of methane production. In the degradation of *A. nodosum*, methane production led to a quick consumption of acetate (Section 3.1.1). The soluble COD concentrations at the end of the *L. hyperborea* digestions were between 18 and 20 g L^{-1}. These high concentrations were partly caused by the glycerol introduced with the inoculum. The inoculum for the degradation of *A. nodosum*, taken from a pre-culture where the glycerol had been consumed, gave much lower COD concentrations in the reactors (Figure 3.1D).

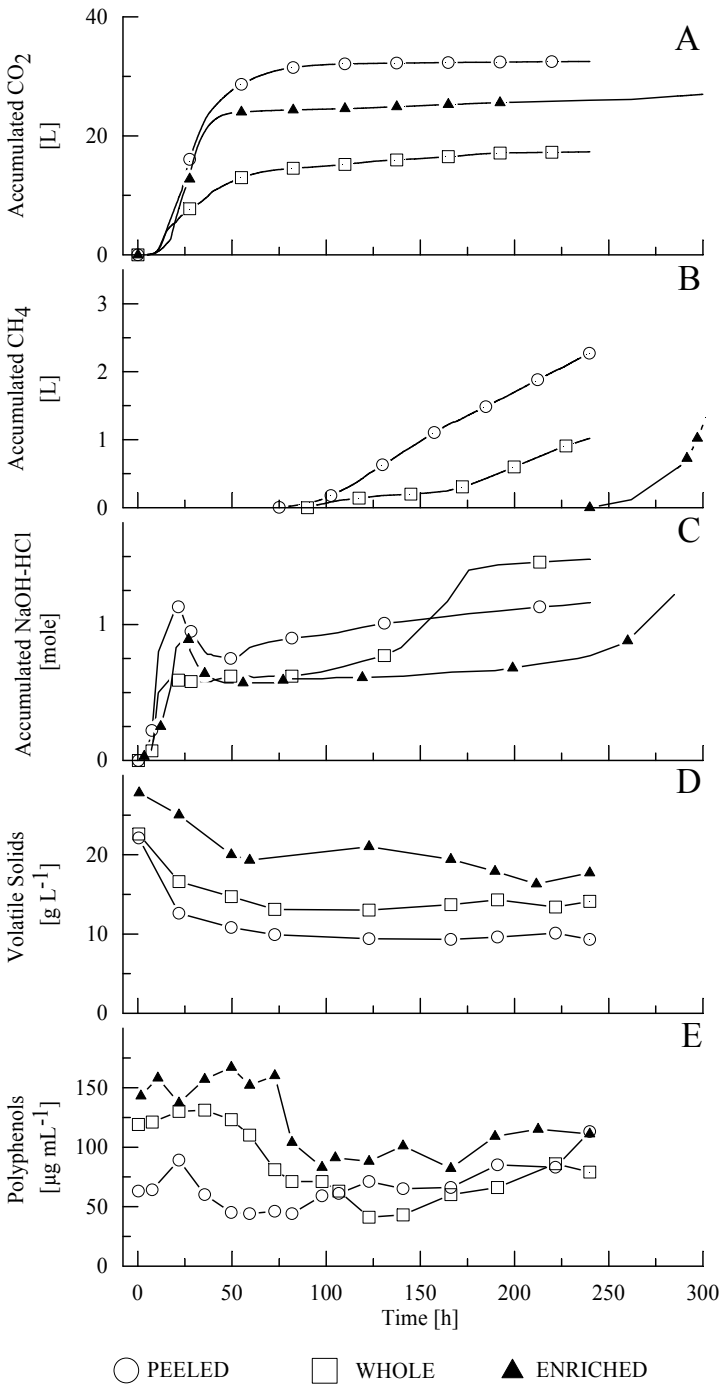

Figure 3.4 Digestion patterns for different *L. hyperborea* materials. (A) Accumulated CO_2 production; (B) Accumulated CH_4; (C) Accumulated molar net consumption NaOH-HCl; (D) Volatile solids; (E) Soluble polyphenol concentration (from Appendix Paper 3).

Figure 3.5 presents the degradation of alginate. During the first 50 h, 0.23 g L^{-1} h^{-1} alginate was converted in PEELED, while 0.12 and 0.18 g L^{-1} h^{-1} was converted in WHOLE and

ENRICHED, respectively (Figure 3.5A). Initially, all fermentations temporarily accumulated soluble uronates (Figure 3.5B) and unsaturated uronates (Figure 3.5C). The insoluble alginate fraction increased with the amount of peripheral tissue added (Figure 3.5A). In spite of the high stability and activity of alginate lyase (Appendix Paper 3), 13-50 % of the alginate seemed to be recalcitrant. Østgaard et al. (1993) also reported that a large fraction of alginates remained insoluble during fermentation of *L. saccharina*. The actual analysis of total alginate is based upon ionic exchange (Haug, 1964), and it could be questioned whether the total values shown in the ENRICHED and WHOLE were too high. Chelation of divalent metal ions by brown algal polyphenols may have occurred (Ragan et al., 1979).

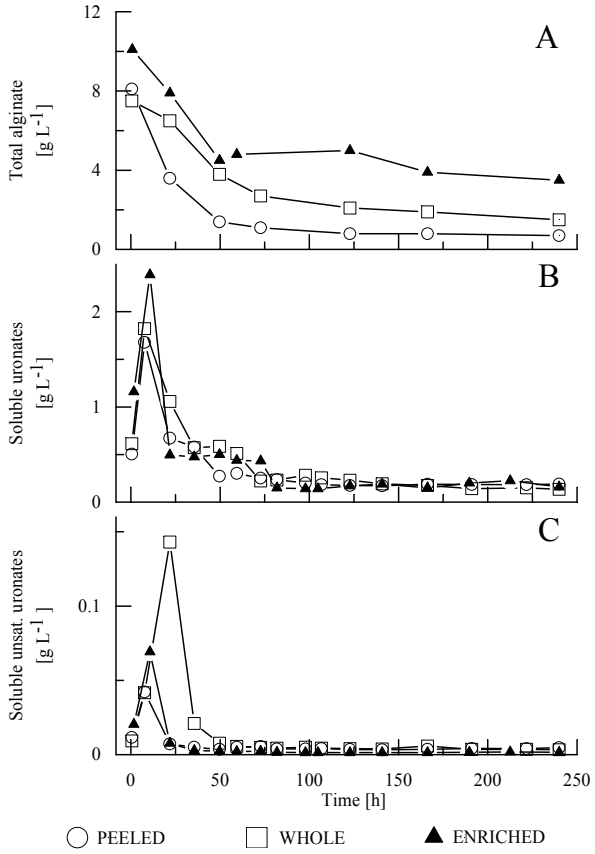

Figure 3.5 Alginate degradation during anaerobic digestion of different *L. hyperborea* materials. (A) Total alginate (determined by ionic exchange); (B) Soluble uronates; (C) Soluble unsaturated uronates (from Appendix Paper 3).

NaOH or HCl were titrated to keep the pH neutral (Figure 3.4C), and during the first 20 h the sodium concentration increased between 65 and 110 mM. The dissolution of alginates was probably improved since the Na/Ca-ratio increased. According to Haug (1964), changes in the equilibrium between non-gelling and gelling ions affect the dissolution of alginates from algal particles.

Figure 3.6 shows the characteristics for the extracted alginates during digestion. Figure 3.6A shows that the initial loss of alginate was 0.12- 0.24 g L^{-1} h^{-1}. After 50 h, no intact alginate could be isolated due to the rapid loss in viscosity (Figure 3.6B). The alginate concentrations in ENRICHED found by the extraction procedure were clearly lower than the values found by the ionic exchange method (Figure 3.5A). The high content of polyphenols may have affected those results. The extracted alginates were analysed by ^{1}H-NMR spectroscopy to determine

the chemical composition of the alginate. The guluronate content of the remaining alginate increased in all samples during the first 22 h (Figure 3.6C). Later, the guluronate content in extracted alginate increased in PEELED and decreased in ENRICHED during fermentation. During the digestion of *A. nodosum*, guluronate also accumulated in the remaining alginate, probably because G-blocks are more protected against enzyme attack. At enhanced polyphenol concentrations, the degradation and extraction of alginate were apparently limited by both calcium binding and associations between polyphenols and alginates. Polyphenols also directly inhibit alginate lyase by non-specific binding (Figure 3.3).

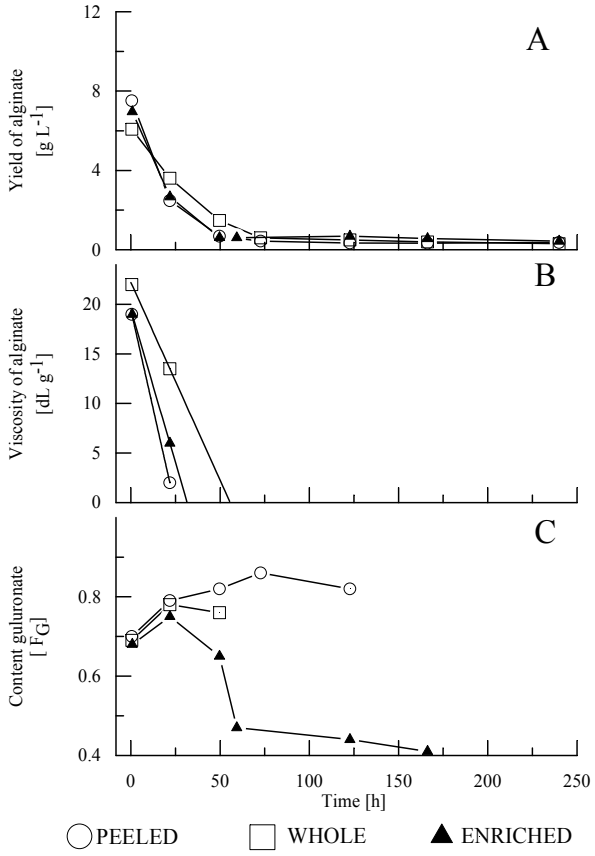

Figure 3.6 Characteristics of extracted alginate during anaerobic digestion of different *L. hyperborea* materials. (A) Yield (extracted alginate); (B) Intrinsic viscosity; (C) Guluronate content in extracted alginate (from Appendix Paper 3).

3.2.2 Main findings

The peripheral tissue was less biodegradable than the other *L. hyperborea* stipe materials. Apparently, the polyphenols dissolved from the peripheral tissue gave a prolonged lag phase for the methane production. After a rapid consumption of the mannitol, alginate lyases were induced and alginate was digested at a rate of 0.12-0.23 g L^{-1} h^{-1}. Later the degradation rate of alginate declined almost to zero, and 13-50 % remained insoluble. Alginate dissolution was partly improved because the Na/Ca-ratio increased notably. Biological degradation significantly changed the composition of the alginate during digestion. Ca-crosslinked guluronate residues or associations between alginate and polyphenols probably limited the further dissolution and degradation.

3.3 Anaerobic degradation of *Laminaria hyperborea* fronds

3.3.1 Hydrolysis and acidogenesis

As noted in Section 1.3.3, the relative constant chemical composition of *L. hyperborea* stipe is contrasted by the high seasonal variations in the composition of fronds (Table 1.3). Thus, fronds will probably show different degradation patterns throughout the year. While the experiments with *A. nodosum* and *L. hyperborea* stipe were carried out in working volumes of 10 L, the following experiments were carried out in smaller reactors with working volumes of 1 L only (Section 2.3).

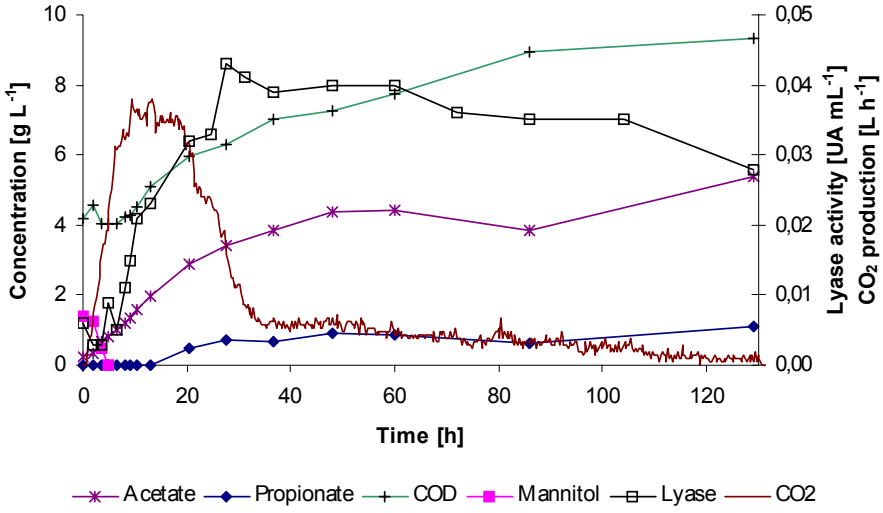

Figure 3.7 Batch fermentation of *L. hyperborea* fronds (from Appendix Paper 4).

Figure 3.7 shows the results of an anaerobic batch fermentation of *L. hyperborea* fronds (see Appendix Paper 4). Within 5 h, all the mannitol (1.4 g L^{-1}) was consumed. In the same period, acetate was produced, while the soluble COD concentration remained stable around 4 g L^{-1}. At this point, alginate lyase activity started to rise concomitant with an increase in COD and an initiation of propionate production. The total increase in COD was 5.3 g L^{-1}, and is probably a measure of the dissolution of alginate. A maximum lyase activity of 0.043 UA mL^{-1} was reached after 27 h, and was only slightly reduced during the rest of the fermentation. From about 20 h, the dissolution of alginate seemed to go slower, seen as a slower increase in COD. A rapid decrease in CO_2 production was also observed at this point. The total CO_2

production for the digestion was 1.1 L. After mannitol was exhausted, the COD and acetate curves had a similar development, suggesting that uronic acids were directly fermented to acetate. As described in Section 1.4.2, the redox balance is maintained during the glycolytic degradation of uronic acids to pyruvate. The further conversion of pyruvate to acetate produces excess electrons, and thereby disturbs the redox balance. However, homoacetogenic bacteria may use these electrons to produce extra acetate from CO_2. The total production of acetate was 0.086 mol, yielding a molar acetate/CO_2 ratio of 1.9. This high ratio suggests that the homoacetogenic pathway played an important role in the digestion of fronds.

The initial COD concentration of 4.2 g L^{-1} accounts for mannitol, acetate and other unidentified dissolved organics. A COD concentration of 1.9 g L^{-1} can be calculated from the initial mannitol and acetate concentrations. Together with the dissolved alginate concentration of 5.3 g L^{-1} COD, this adds up to 7.2 g L^{-1} COD. The end concentrations of 5.4 g L^{-1} acetate and 1.1 g L^{-1} propionate add up to a total COD of 7.4 g L^{-1}. This is 1.9 g L^{-1} less than the measured COD concentration of 9.3 g L^{-1}. The much higher COD concentrations (18-20 g L^{-1}) measured in the degradation of stipes were caused by the glycerol introduced with the inoculum. In the frond degradation experiments, as was the case for the fermentations of *A. nodosum*, the inoculum glycerol was removed by running a pre-culture (see Appendix Paper 4). Traces of formate were temporarily observed within the first 20 h. Laminaran was not detected in the medium. Carbon balance including CO_2, acetate and propionate accounted for 110 % of the carbon in mannitol and dissolved alginate. This imbalance, in addition to the to the production of biomass (not quantified), indicate that other substrates than mannitol and alginate also were utilised. See Appendix Paper 4 for further details.

High stability of alginate lyase was also observed in anaerobic degradation of *L. hyperborea* stipes (Appendix Paper 3). However, the polyphenols in the peripheral tissue of *L. hyperborea* stipe can complex with alginate lyases and reduce both their activity and stability (Moen et al., 1997; Appendix Paper 3). *L. hyperborea* fronds contain very low concentrations of polyphenols (Haug and Larsen, 1958b), and the high lyase stability shown in Figure 3.7 is in good agreement with these earlier findings.

The fronds were harvested in March, and contained low concentrations of mannitol and no detectable laminaran. However, the content of these carbohydrates varies considerably during the year, with the highest concentrations in the autumn. The effects of extra carbohydrates

were investigated by running fermentations of fronds with extra mannitol or glucose added. Addition of extra glucose led to a diauxic pattern where glucose was exhausted before alginate lyase was activated (Appendix Paper 4). Mannitol from the fronds was consumed simultaneously with the glucose. Addition of 9.5 g glucose gave a fermentation pattern where the molar acetate/CO_2 ratio was 1.0. Experiments where extra mannitol was added did not delay the initiation of alginate lyase activity (Appendix Paper 4). However, mannitol addition led to a lower and delayed maximal enzyme activity. Addition of 10 g extra mannitol yielded a molar acetate/CO_2 ratio of 0.8 for the whole fermentation. Thus, fermentation of more reduced carbohydrates gave lower acetate/CO_2 ratios. However, fermentation of glucose and mannitol still produced acetate as the dominant organic compound. Low concentrations of propionate was produced, but no other VFAs were observed. The initial consumption of mannitol or glucose resulted in a decrease in soluble COD concentration. This was caused by biomass growth and possible some H_2 production. Ghosh (1981) reported that batch digestions of kelp, where about one third of the dry weight was mannitol, produced a biogas containing 34-55 % H_2. Thus, during the fermentation of mannitol rich seaweeds, the energy potential for methane production may be reduced due to H_2 formation in the acid-forming step.

3.3.2 Continuous methane production

None of the batch fermentations showed methane activity, and to verify the suitability of methane production from the acidification step, the batch reactor on pure seaweed (Figure 3.7) was run until methane was detected in the outlet gas. It was then operated as a continuous system with a HRT of 20 d, and run for another 3 months. After steady state was reached, no acetate and only low concentrations of propionate (0.4 g L^{-1}) were detected in the reactor. The soluble COD concentration was 2.1 g L^{-1}, which gave a COD removal of 77 %. The recorded VS reduction was between 53 and 56 %. The biogas contained about 50 volume % methane, and the yield was 0.20-0.25 L CH_4/ g VS consumed. The two feeding regimes, once in 24 h or every h, gave similar methane production.

3.3.3 Main findings

The main organic product in the acidogenesis of *L. hyperborea* fronds and fronds added mannitol or glucose was acetate. Low concentrations of propionate were produced, but no other VFAs were observed. The fermentation of pure fronds resulted in a high acetate/CO_2 ratio, suggesting that the homoacetogenic pathway played an important role in the degradation of uronic acids. Addition of extra glucose or mannitol yielded much lower acetate/CO_2 ratios. Excess electrons were used in biomass growth and possible also for the production of H_2, seen as an initial decrease in COD concentration. Growth on glucose and frond alginate showed a diauxic development with glucose as the preferred substrate. Glucose and mannitol were consumed simultaneously. Mannitol did not inhibit the initiation of alginate lyase activity, but resulted in a lower and delayed maximum activity. Thus, the seasonal changes of mannitol and laminaran in *L. hyperborea* fronds will result in different digestion characteristics for these algae throughout the year. *L. hyperborea* fronds were also successfully applied for methane production in a continuous reactor system.

3.4 Discussion and conclusions

Generally, the absence of lignin and the low content of cellulose in brown algae make them a better material for bioconversion than land plants. However, some brown algae have a high content of polyphenols that are potent inhibitors of microorganisms. The effects of high concentrations of polyphenols were clearly seen in the batch fermentation of *A. nodosum*: mannitol utilisation was delayed for 90 h, alginate lyase activity was negligible, and methane was not produced until the polyphenols were fixed with formaldehyde. Fermentation of *L. hyperborea* stipe added extra peripheral tissue containing polyphenols also showed a clear inhibition on methanogenesis. Thus, brown seaweeds with a low content of polyphenols are best suited for methane production. High contents of sulphur may also reduce the methane yield due to competition from sulphur reducing bacteria. Tables 1.2 and 1.3 show that *A. nodosum* has a higher sulphur content than *L. hyperborea*. In conclusion: *L. hyperborea* is a much better substrate for methane production than *A. nodosum*.

The guluronate content increased in the remaining alginate during the fermentations. The reason was probably that the G-blocks in the gel matrix stabilised the tissue and limited the access of the enzymes. The experiments carried out at the lowest polyphenol concentrations showed a remarkably stable alginate lyase activity throughout the fermentations. The dissolution of the gel structure was also probably influenced by the ratio of non-gelling and gelling ions. Titrations with NaOH increased the Na^+ concentration and destabilised the alginate gel by exchange of calcium ions with sodium ions.

L. hyperborea fronds were readily digested, but in contrast to stipe material, their composition will vary considerably during the year. Autumn fronds of *L. hyperborea* contain high levels of the storage carbohydrates mannitol and laminaran. Digestion of fronds harvested in March, when the content of storage carbohydrates is at the lowest, gave a high acetate/CO_2 ratio. Thus, in the digestion of alginate the homoacetogenic pathway seems to have played an important part. Addition of glucose or mannitol to the medium resulted in a higher production of CO_2 and a much lower acetate/CO_2 ratio. The fermentation of mannitol and glucose also gave a simultaneous reduction in soluble COD concentration. This was caused by biomass growth and probably some production of H_2.

In the anaerobic degradation of complex organic material to CH_4 and CO_2, it is the initial steps of hydrolyses and acid formation that are specific for the raw material. Once acetate or H_2 are formed, methane is readily produced. Both the batch experiments and a continuous culture showed that methane was formed from acetate. The continuous experiment resulted in a stable production of biogas with a methane content of about 50 %, and a soluble COD removal of 77 %. The VS reduction was between 53 and 56 %. G-block alginate and algal cell walls (see Section 1.4.5) probably constituted most of the remaining VS.

4 Ethanol

4.1 Ethanol production from mannitol by *Zymobacter palmae*

4.1.1 Glucose and mannitol as substrates

Ethanol fermenting bacteria include the genera *Zymomonas* and *Saccharobacter*, but they have not been reported to ferment mannitol. A novel ethanol producing bacterium isolated from palm sap, *Zymobacter palmae*, has been reported to be facultatively anaerobic and able to ferment hexoses, α-linked di- and tri-saccharides, and sugar alcohols including mannitol (Okamato et al., 1993). The purpose of Appendix Paper 5 was to evaluate *Z. palmae* as a potential organism to carry out the fermentation* of mannitol in seaweed extract to ethanol.

Batch fermentations showed that *Z. palmae* was able to produce ethanol when grown on glucose both under oxygen limiting and anaerobic conditions (Appendix Paper 5). Growth halted in both experiments before glucose was exhausted, but ethanol production continued. From Fermentations 1 and 2 in Table 4.1 it is seen that growth rate and biomass yield were lower under anaerobic conditions, while ethanol yield was a little higher than in the aerated reactor. Under aerobic conditions (DO=35 %), no ethanol was produced and propionate was the dominant organic product (not shown in Table 4.1). Shake flask experiments showed that *Z. palmae* could not utilise laminaran as a substrate.

Initial experiments with mannitol fermentation in shake bottles and reactors demonstrated a clear dependency on air access (Appendix Paper 5). Under anaerobic conditions no growth or mannitol consumption was observed. Increasing oxygen transfer rate (OTR) gave both increased growth rates and biomass yield. However, growth was, as in the glucose experiments, clearly inhibited before the exhaustion of the substrate. The ethanol concentration was generally observed to decrease after substrate depletion.

* In this chapter the term "fermentation" is applied to all experiments, regardless of whether fermentative or respiratory metabolism is involved (see Section 1.2.1).

The inability of mannitol to support strictly anaerobic growth was probably caused by a deregulation of the redox balance (Quain and Boulton, 1987). Anaerobic growth on glucose is balanced because the 2 NADH produced in the bacterial Entner-Doudoroff pathway are consumed when ethanol is produced from pyruvate, thereby regenerating NAD^+. Mannitol probably enters this pathway via fructose produced by a mannitol dehydrogenase, an enzyme that also converts NAD^+ to NADH (Section 1.4.3). The overall result is an excess production and accumulation of NADH under anaerobic conditions, since complete regeneration of NAD^+ presumably does not take place. The reason may be a lack of transhydrogenase, which converts NADH to NADPH, although general textbooks claim that prokaryotes possess this enzyme (Gottschalk, 1986; Ingraham et al., 1983). This would lead to a compartmentation of the redox couples NAD^+/NADH and $NADP^+$/NADPH. NADH is predominantly a catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes (Van Dijken and Scheffers, 1986).

Table 4.1 Operating constants and kinetic parameters (from Appendix Paper 5).

dS/dt, dP/dt and Y_{PS} were calculated from end concentrations or, when substrate was totally consumed, the last sample before substrate exhaustion. μ and q_p were calculated for the initial exponential phase of growth and represent maximum values. For the calculation of q_P, biomass was estimated as the average of two succeeding samples. Y_{XS} was calculated for the growth period.
M=mannitol, G=glucose, A=air, N=nitrogen, OTR=oxygen transfer rate, CER=CO_2 emission rate, dS=substrate consumption, dP=ethanol production

	1	**2**	**3**	**4**	**5**	**6**	**7** (big)	**8** (small)
Fermentation								
Initial substrate concentration [g L^{-1}]	18 G synthetic	16 G synthetic	38 M synthetic	38 M synthetic	16 G 19 M synthetic	17 G 21 M synthetic	17 M extract	17 M extract
Air/N_2 [L L^{-1} h^{-1}]	0.05 N	0.20 A	0.20 A	0.20 A	0.20 A	0.05 N /0.10 A		
OTR [mmol O_2 $L^{-1}h^{-1}$]	0	3.56	4.75	6.19[a]	4.19	0/2.91		
Max. CER [mmol CO_2 $L^{-1}h^{-1}$]	8.68	20.10	4.26	19.91	17.30	8.04		
(h after inoculation)	(14.7)	(13.8)	(10.5)	(6.7)	(10.5)	(19.0)		
Specific growth rate [μ [h^{-1}]	0.36	0.63	0.63	0.71	0.70	0.34/0.05[c]		
Specific ethanol productivity q_P [g g^{-1} h^{-1}]	0.74	0.77	0.73	0.04	1.28	0.74/0.59[c]	0.02	0.03
dS/dt [g h^{-1}]	0.45	0.96	0.45	0.98	1.03/0.82[b]	0.32/0.85[d]	0.61	1.06
dP/dt [g h^{-1}]	0.18	0.29	0.17	0.05	0.39/0.32[b]	0.14/0.28[d]	0.37	0.56
Ethanol yield Y_{PS} [g g^{-1}]	0.39	0.31	0.38	0.05	0.37/0.39[b]	0.44/0.33[d]	0.61	0.53
Biomass yield Y_{XS} [g g^{-1}]	0.06	0.13	0.10	0.08	0.10	0.08/0.02		

a) dissolved oxygen maintained at 25 %. The value represents the maximum OTR, which coincided with the maximum CER.
b) calculated for the periods 0-18 h and 18-32 h. Glucose was exhausted at 18 h.
c) calculated for the initial growth and the renewed growth initiated after aeration.
d) calculated for the periods 0-23 h and 47.5-75 h.

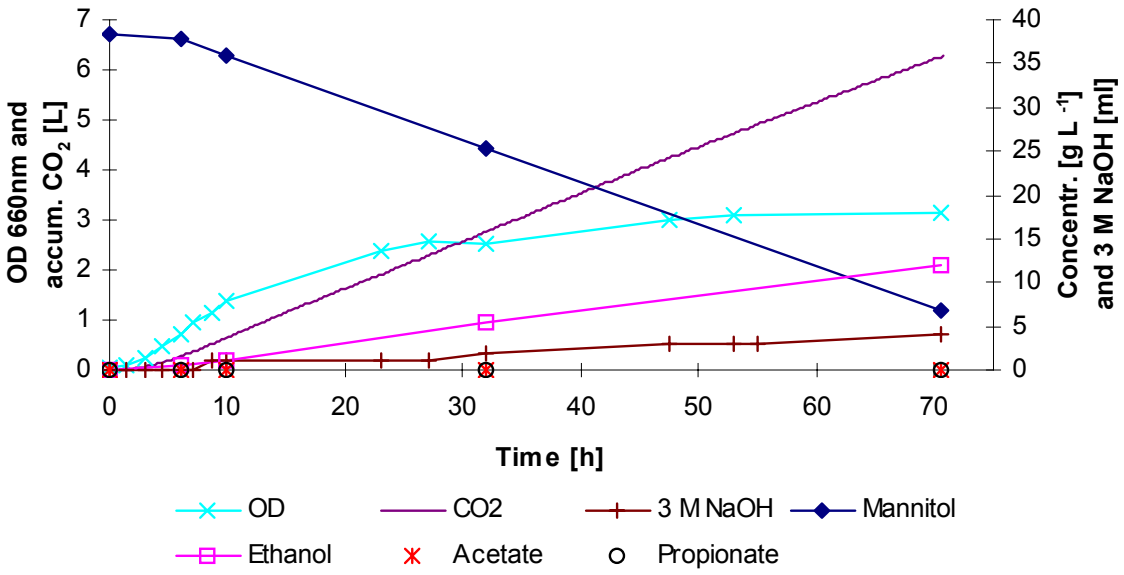

Figure 4.1 Mannitol metabolism of *Z. palmae*: optical density, accumulated CO_2, consumption of 3 M NaOH, mannitol, ethanol, acetate and propionate concentrations in a fermentor aerated with 0.20 L air (L medium min)$^{-1}$ (OTR=4.8 mmol O_2 l^{-1} h^{-1}) (from Appendix Paper 5).

A fermentation carried out at an OTR of 4.8 mmol O_2 L^{-1} h^{-1} and an initial mannitol concentration of 38 g L^{-1} is presented in Figure 4.1 (Fermentation 3 in Table 4.1). Dissolved oxygen quickly dropped below the detection limit, and a maximum CO_2 emission rate (CER) of 4.3 mmol CO_2 L^{-1} h^{-1} was reached at 10.5 h, and maintained throughout the experiment. The growth rate was very low from 20 h and onwards, in spite of the remaining mannitol in the medium. From Figure 4.1 it is readily seen that the ethanol production was not directly growth associated, giving similar ethanol yields both during growth and stationary phases. The total ethanol yield of 0.38 g ethanol (g mannitol)$^{-1}$ was the maximum achieved in mannitol medium, see Table 4.1. Carbon balance at 70 h including biomass, CO_2, ethanol and acetate accounted for 81 % of the carbon consumed. This mismatch in the balance could be due to evaporation of ethanol, which is known to be a problem in ethanol fermentations. Carbon balances earlier in the fermentation period accounted for more of the carbon. When organic acids were analysed, only traces of acetate were identified by HPLC. Thus, with the carbon source present in the medium and under oxygen limiting conditions, there seemed to be almost no production of organic acids. The values of q_P, dS/dt, dP/dt and Y_{PS} were similar to those of the anaerobic fermentation on glucose (Fermentation 1 in Table 4.1). Growth rate

μ was the same as in the aerated glucose fermentation, while Y_{XS} was in the range between the anaerobic and aerated glucose fermentations (Table 4.1).

Another fermentation in mannitol medium where oxygen concentration was maintained at 25 % DO (Fermentation 4 in Table 4.1), resulted in a significant production of acetate and propionate. No ethanol was detected. At 10 h there was a clear reduction of the growth, and the tendency for the period 10-32 h was nearly a complete halt in growth, accumulation of organic acids and a gradual decrease in activity. Thus, a first phase of inhibition of growth was followed by a gradual inhibition of activity as the concentration of organic acids increased. This pattern was contrasted by the oxygen-limited experiment of Figure 4.1, where only traces of organic acids were found after 70 h, and the activity was constant for the whole period. The acid production in the high DO fermentations shows that under aerobic conditions the bacteria followed a different fermentation pattern than under oxygen limiting conditions. The unusual accumulation of organic acids under aerobic conditions may imply that the bacteria possess a limited respiratory metabolism.

Ethanol yield was highly sensitive to the oxygen supply (Table 4.1), since excess aeration led to production of organic acids and thereby reduction of the yield. This means that to optimise ethanol yield one has to balance the need of oxygen for NADH oxidation against the minimisation of organic acid production. Exhaustion of the substrate should also be avoided, since this led to ethanol consumption and production of organic acids. The best procedure for optimisation of ethanol yield seems to be a start up at a low OTR, and then gradually increase the OTR to about 4.8 mmol O_2 L^{-1} h^{-1} in the stationary phase. A further increase in OTR may also be possible as long as the culture is oxygen limited. This would satisfy the growing oxygen demand, but at the same time avoid excessive production of organic acids.

4.1.2 Glucose and mannitol as mixed substrate

A substrate mixture of glucose and mannitol was used to test whether their metabolisation would occur simultaneously or successively. In an aerated fermentation with mixed substrate, glucose was the preferred substrate (Fermentation 5 in Table 4.1). The maximum CER at 10.5 h coincided with growth limitation. The maximum cell density at 27 h of 1.3 g L^{-1} was the

highest achieved in the entire series of experiments, and the total yield of 0.40 g ethanol (g glucose + mannitol)$^{-1}$ was also the highest for an aerated reactor. The q_P was considerably higher than in the other experiments (Table 4.1). This indicates that the mixture of both laminaran and mannitol found in a seaweed extract might have a positive effect on the ethanol production. In such a case, however, laminaran has to be hydrolysed to glucose first. Carbon balance based on biomass, CO_2, ethanol, propionate and acetate accounted for 96 % of the consumed carbon in glucose and mannitol.

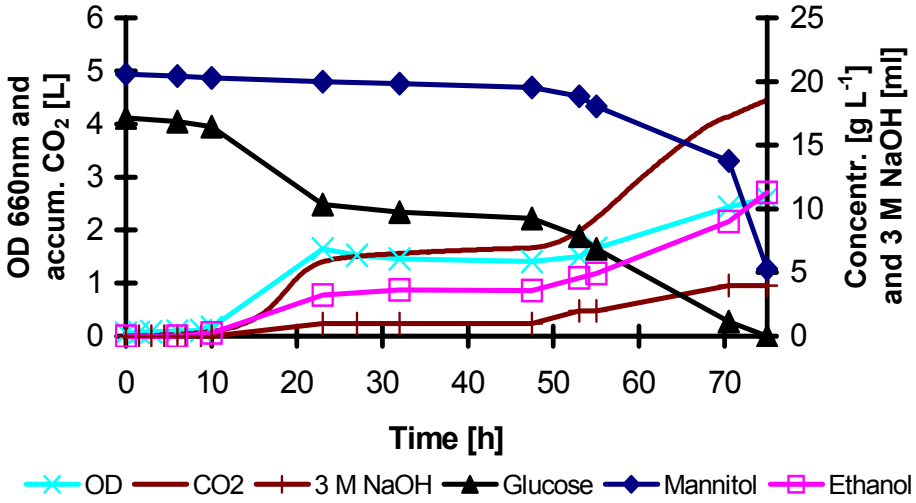

Figure 4.2 Mixed glucose and mannitol metabolism of *Z. palmae* in a fermentor sparged with 0.05 L N_2 (L medium min)$^{-1}$: optical density, glucose, mannitol, and ethanol concentrations (from Appendix Paper 5).

An anaerobic fermentation of a mixed substrate (Fermentation 6 in Table 4.1) is presented in Figure 4.2. Based on the previous experiments, it was expected that the glucose would be consumed and that mannitol would remain unaffected. In the first phase of the experiment both CO_2 development and growth was similar to that of the anaerobic culture on glucose. However, after initial growth and glucose consumption, the growth totally ceased after 23 h, with 10.3 g L^{-1} glucose still remaining in the medium. More unexpected, the consumption of substrate also ceased, and in the following day only 1 g L^{-1} glucose and 0.5 g L^{-1} mannitol was consumed. The reason may be a disturbance of the redox balance, caused by the small

consumption of mannitol. In all the other experiments, termination of growth was not associated with a loss of substrate consumption activity. In an attempt to regain activity, the reactor was aerated after 47.5 h. The cells started to grow again, but now both glucose and mannitol were consumed simultaneously, with a pronounced increase in mannitol consumption when all the glucose was gone. There was also an increasing mismatch in the carbon balance throughout this experiment, accounting for only 66 % at 75 h. Strong mismatch in the carbon balance was observed in the experiments running for the longest time, and could at least partly be caused by ethanol evaporation.

In all experiments, cell dry weight concentration never exceeded 1.3 g L^{-1}, irrespective of initial substrate concentration and oxygen supply. This could be due to depletion of an essential nutrient in the medium. The experiments suggest an anabolic growth factor, since only the growth is halted, not the activity. Halt in the biomass growth could also indicate product inhibition. Okamoto et al. (1994) reported that an ethanol concentration of about 50 g L^{-1} inhibited growth. The halt in growth in our aerobic experiments was observed at different concentrations of acetic and propionic acid. Thus, the accumulation of organic acids was probably not the direct cause of inhibition.

4.1.3 Seaweed extract

Two identical shake flasks with 75 and 150 mL of seaweed extract (Section 2.1.4) were inoculated with the pellet of a pre-culture grown on synthetic mannitol medium. Figure 4.3 clearly shows that the bacteria were able to utilise the mannitol in the extract for ethanol production. The ethanol production and mannitol consumption were faster in the small volume, again demonstrating dependence on oxygen supply. The total ethanol yields for both flasks were actually higher than the theoretical yield of 0.51 g g^{-1} (Fermentations 7 and 8 in Table 4.1), indicating that *Z. palmae* can utilise other carbon sources in the extract. Both dS/dt and dP/dt in the small volume were higher than in all the fermentor experiments (Table 4.1). After the depletion of mannitol, a small production of organic acids was observed. Analyses of the laminaran content showed no consumption, confirming earlier shake flask experiments.

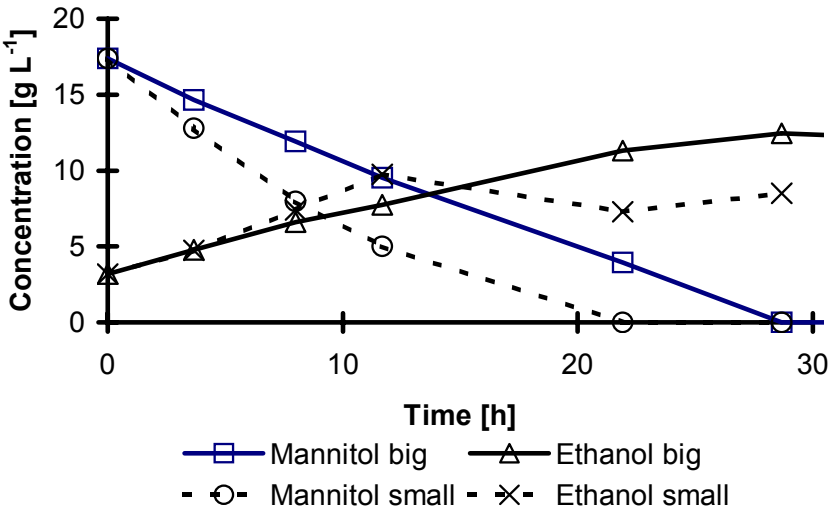

Figure 4.3 Ethanol production from seaweed extract by *Z. palmae*: mannitol and ethanol concentrations in two identical shake flasks with small and large working volumes (from Appendix Paper 5).

4.1.4 Main findings

Z. palmae metabolised glucose under strictly anaerobic conditions. Anaerobic growth on mannitol was not observed, probably due to a deregulation of the redox balance. Excessive aeration led to a change in the fermentation pattern, with pronounced production of acetate and propionate. These acids also accumulated when the substrate was depleted, concomitant with a consumption of ethanol. However, under oxygen-limiting conditions the bacteria readily produced ethanol in synthetic mannitol medium, yielding 0.38 g ethanol/g mannitol.
Z. palmae was also successfully applied for fermentation of mannitol from *L. hyperborea* extracts. Laminaran, however, was not utilised, indicating a lack of β-(1→3)-glucanase.

4.2 Ethanol production from seaweed extract

4.2.1 *Pichia angophorae* in batch culture

Today, the most important microorganisms applied for ethanol production are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*. However, both these ethanol producers have a very narrow substrate range. Yeasts lack transhydrogenase (Van Dijken, 1986), and experiments done with *Saccharomyces* concluded that strict anaerobic growth on mannitol was not possible (Quain and Boulton, 1987). Thus, fermentation of sugar alcohols by yeast requires a supply of oxygen. The bacterium *Zymobacter palmae* has a broad substrate range and can convert mannitol to ethanol (Okamoto, 1993). In Appendix Paper 5, it was shown that *Z. palmae* could produce ethanol from mannitol in seaweed extract, if some supply of oxygen was provided.

A successful utilisation of seaweed extract for ethanol production necessitates that both mannitol and laminaran are efficiently converted to ethanol at high yields. This may be achieved by two different organisms in a two-step process, where each step is optimised for maximum ethanol yield. Ethanol production may also be carried out by a single organism if it can utilise both substrates. In this case, an effective single step process may only be achieved if both substrates can be converted to ethanol with high yields under similar conditions. In Appendix Paper 6, the possibility of ethanol production from *L. hyperborea* extracts was evaluated, focusing on the yeast *Pichia angophorae* and its potential of utilising both mannitol and laminaran as substrates.

Initial experiments with seaweed extract showed that *P. angophorae* was able to utilise both mannitol and laminaran for ethanol production (Appendix Paper 6). Figure 4.4 shows a fermentor experiment carried out in a defined medium with mannitol as the only carbon source (Fermentation Pa synt in Table 4.2). The growing biomass made the oxygen concentration drop and the culture was oxygen limited from 5 h. The initial specific growth rate, before oxygen limitation occurred, was 0.41 h^{-1}. In the period 16-75 h the cell mass increased about 3 times, while CER was very stable, increasing slowly from 3.5 up to 4.5 mmol L^{-1} h^{-1}. The total yield was 0.40 g ethanol (g mannitol)$^{-1}$. The same ethanol yield and a very similar fermentation pattern were observed for *Z. palmae* under similar conditions

(Figure 4.1). However, *P. angophorae* reached a much higher cell mass and had an initial specific ethanol productivity of 0.22 g (g cell h)$^{-1}$, which was more than 3 times lower than productivity of *Z. palmae*.

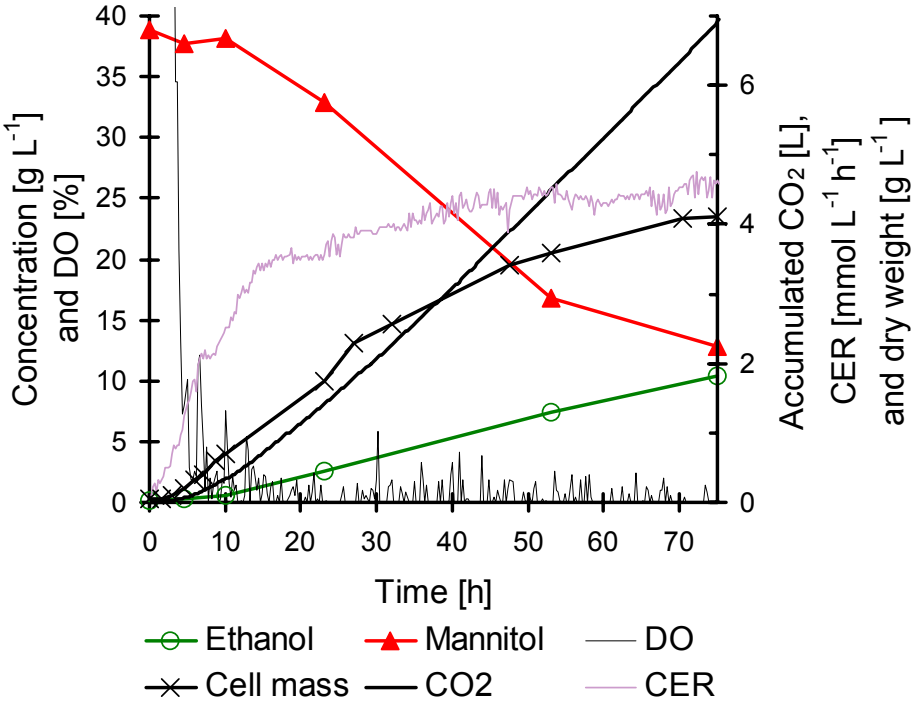

Figure 4.4 Fermentation with *P. angophorae* at pH 4.5 in a defined mannitol medium with OTR = 4.7 mmol L^{-1} h^{-1}. CER= CO_2 emission rate (from Appendix Paper 6).

A series of fermentor experiments were carried out to test the influence of pH and OTR on the relative consumption of mannitol and laminaran by *P. angophorae* in seaweed extract (see Table 4.2). The data of one batch fermentation are plotted in Figure 4.5 (Fermentation Pa sea 3 in Table 4.2). Measurements of optical density or cell dry weight were not possible due to high particle content (about 2 g L^{-1} total solids) in the seaweed extract. Both substrates were consumed simultaneously, but while the consumption rate of mannitol increased with the growing biomass, laminaran consumption rate remained constant for a while, and then fell strongly at the end of the fermentation. The maximum ethanol concentration and maximum CER coincided with the exhaustion of mannitol. Total yield was 0.26 g ethanol (g substrate)$^{-1}$. In the following period, CER was low and laminaran was consumed slowly together with ethanol. Some production of acetate and propionate was also observed in this period. The reason for this late decrease in laminaran consumption rate may be that *P. angophorae* was

unable to hydrolyse polymeric branching points due to a lack of β-(1→6)-glucanase. The main polysaccharide chain in laminaran is hydrolysed by β-(1→3)-glucanase. Thus, an incomplete hydrolysis would be expected if the yeast only has enzymes for hydrolysis of (1→3)-β-glucosidic linkages. The fermentor culture was oxygen limited from 12 h until exhaustion of mannitol. Then the oxygen concentration increased rapidly, interrupted by a short, but typical, drop of oxygen around 24 h. The initiation of ethanol consumption could be due to this higher oxygen levels and a transfer to aerobic metabolism. The fermentation characteristics observed in the other reactors were similar, except for the fermentor aerated with 5.9 mmol O_2 L^{-1} h^{-1} (see Table 4.2). Instead of reaching a CER peak at exhaustion of mannitol, CER was stable from around 15 h to exhaustion of mannitol at 28.2 h. This was similar to the experiment in synthetic mannitol medium (Fermentation Pa synt in Table 4.2), also carried out at low OTR.

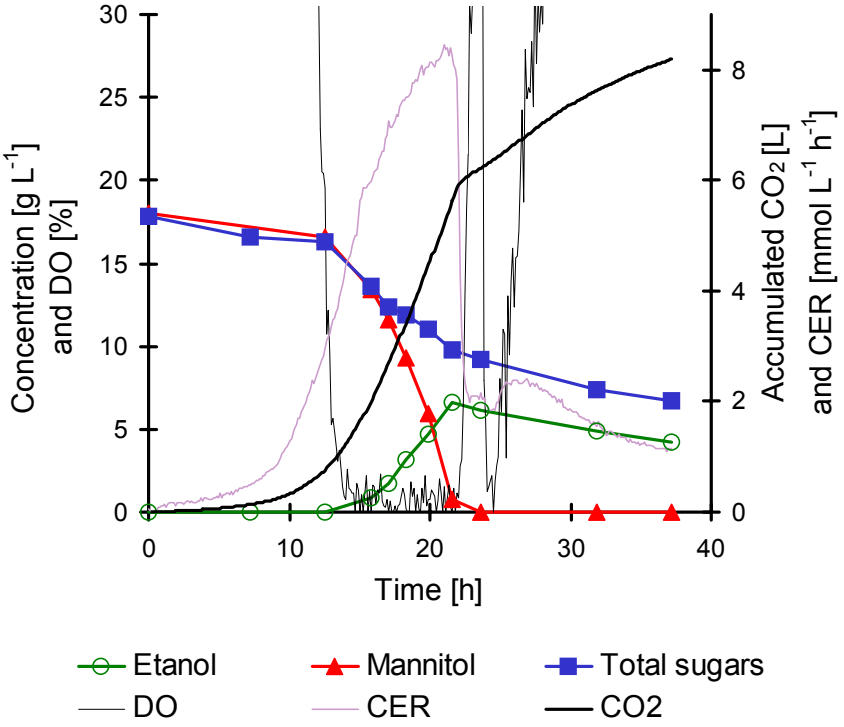

Figure 4.5 Fermentation with *P. angophorae* at pH 4.5 in seaweed extract medium with OTR = 15.4 mmol L^{-1} h^{-1}. CER= CO_2 emission rate (from Appendix Paper 6).

Table 4.2 Operating constants and kinetic parameters

OTR=oxygen transfer rate, CER=CO_2 emission rate, dS=substrate consumption, dP=ethanol production, Pa=*Pichia angophorae*, Zp=*Zymobacter palmae*, sea=seaweed extract, synt=synthetic mannitol medium, M=mannitol, L=laminaran

Fermentation	pH	OTR [mmol O_2 L^{-1} h^{-1}]	Max. CER[a] [mmol CO_2 L^{-1} h^{-1}]	Ethanol yield [g g^{-1}]	dS/dt [g L^{-1} h^{-1}]	Max. dM/dt[c] [g L^{-1} h^{-1}]	Max. dL/dt[c] [g L^{-1} h^{-1}]	dP/dt [g L^{-1} h^{-1}]
Pa synt	4.5	4.7	4.7 (75.0)	0.40	0.35	0.54		0.14
Pa sea	4.5	5.9	9.7 (23.0)[d]	0.43	1.00	1.26	0.84	0.36
Pa sea	4.5	10.1	30.8 (23.1)	0.23	1.10	2.25	0.80	0.26
Pa sea	4.5	15.4	28.0 (21.2)	0.26	1.20	2.62	0.85	0.31
Pa sea	4.5	16.5	26.5 (20.4)	0.25	1.10	2.51	0.89	0.27
Pa sea	4.5	17.5	26.7 (19.7)	0.26	1.10	2.40	0.80	0.29
Pa sea	4.0	15.4	23.3 (23.1)	0.20	0.90	2.03	0.81	0.18
Pa sea	5.0	15.4	28.1 (18.4)	0.27	1.10	2.78	0.90	0.31
Pa sea	5.5	15.4	25.9 (15.5)	0.18	1.10	2.75	0.92	0.19
Zp/Pa sea[b]	6.0	4.9	5.5 (7.6)	0.18	0.94			0.17
			8.7 (40.9)	0.20	0.85			0.14

a) Number in brackets indicate hours after inoculation
b) Two growth periods: 0-11 h for Zp and 24-50.5 h for Pa.
c) Average calculated for the interval of 3 succeeding sample points.
d) In this case max CER did not coincide with the exhaustion of mannitol. Mannitol was depleted after 28.2 h at a CER of 9.2 mmol CO_2 L^{-1} h^{-1}.

Higher OTRs led to faster consumption of mannitol and therefore shorter periods to reach mannitol depletion (see Table 4.2). The total substrate consumption rates (dS/dt), however, were very similar and were not significantly affected by OTR (Appendix Paper 6). The maximum laminaran consumption rate was very stable for all the OTRs ranging from 0.8 to 0.9 g L^{-1} h^{-1}. Laminaran consumption was initiated simultaneously with mannitol consumption, showing that the pre-growth in mannitol medium did not repress the laminarase. The laminaran consumption was not directly affected by the oxygen supply. However, indirectly laminaran consumption decreased with higher OTRs, because of the preference for mannitol as substrate at high OTR. To gain a high ethanol yield it is important to have a low OTR. In the range from 10.1 to 17.5 mmol O_2 L^{-1} h^{-1} the ethanol yield was from 0.23 to 0.26 g ethanol (g substrate)$^{-1}$, while the two fermentations at lowest OTR achieved a yield of 0.40 and 0.43 g g^{-1} (Table 4.2). Thus, higher OTR led to a preference for mannitol, but reduced the ethanol yield. After mannitol was exhausted, a decrease in laminaran consumption rate was observed. The reason was probably that the remaining laminaran contained high levels of branching points. In this period, from mannitol depletion until 37 h, laminaran consumption rate was constant at the lowest OTRs, but increased at the two highest. High OTR led to a

preference for mannitol consumption, and more main chain laminaran may have remained non-hydrolysed until mannitol was depleted.

Consumption rate of mannitol increased at higher pH (Table 4.2). At pH 4.0 the maximum rate was 2.0 g L^{-1} h^{-1}, while it was in the range 2.6 to 2.8 g L^{-1} h^{-1} for the higher pH. The maximum laminaran consumption rate was less affected by pH, spanning the range 0.8 – 0.9 g $L^{-1}h^{-1}$ (Table 4.2). After mannitol was exhausted, the highest pH values showed a positive effect on laminaran consumption. This could be due to more main chain laminaran remaining non-hydrolysed through the period with mannitol present. However, both fermentors also had end concentrations of 5.3 and 5.9 g L^{-1} total sugars, the lowest concentrations achieved in any of the batch experiments. Table 4.2 shows that to achieve maximum ethanol yield, pH should be kept between 4.5 and 5.0. However, the optimisation of ethanol yield requires a lower OTR than used in these pH experiments.

4.2.2 *Pichia angophorae* in continuous culture

Continuous fermentation of seaweed extract was carried out at four different OTRs: 4.1, 5.4, 10.1 and 18.4 mmol O_2 L^{-1} h^{-1}. The system was run at a dilution rate of 0.10 h^{-1}, that is 1/4th of the maximum growth rate in defined medium. The extract fed to the continuous reactor contained 24.1 g L^{-1} mannitol and 20.7 g L^{-1} total sugars, that is about 7 g L^{-1} more carbohydrate than the extract used in the batch experiments. Substrate utilisation was somewhat different in the continuous culture; while mannitol was the preferred substrate in batch cultures, laminaran consumption rate was now greater or similar to the consumption rate of mannitol (Appendix Paper 6). The continuous medium feed resulted in steady state concentrations of total sugars in the range 6.5 to 8.5 g L^{-1} for all OTRs, the lower value probably representing sugars inaccessible to degradation. Thus, the continuous culture experienced laminaran limitation. Mannitol concentrations were for the three lowest OTR in the range of 18-20 g L^{-1}, decreasing to 10 g L^{-1} at 18.4 mmol O_2 h^{-1} L^{-1}. This shows that complete utilisation of mannitol would require a very high OTR at this dilution rate. Ethanol yield, on the other hand, decreased with higher OTR, with a maximum of 0.27 g ethanol (g substrate)$^{-1}$ at 4.1 mmol O_2 h^{-1} L^{-1}. Dissolved oxygen concentration was maintained at zero in the whole OTR range.

The maximum consumption rates for laminaran in the batch experiments were in the range 0.8-0.9 g L^{-1} h^{-1}, which is somewhat lower than the continuous rates. However, the maximum mannitol consumption rates in batch were much higher than in the continuous system, probably due a higher cell concentration at the end of the batch fermentations. This higher cell mass did not result in enhanced laminaran consumption in the batch reactors, since laminaran at this stage probably was enriched in high levels of branching points. The actual cell mass was not quantified, but for different fermentations with a similar CER to OTR ratio, CER should be proportional to the cell mass. In the continuous fermentor at 4.1 and 5.4 mmol O_2 L^{-1} h^{-1}, the average CER was 3.8 and 5.0 mmol CO_2 L^{-1} h^{-1}, respectively. For the batch fermentation at 5.9 mmol O_2 L^{-1} h^{-1}, a maximum CER of 9.7 mmol CO_2 L^{-1} h^{-1} was reached, probably due to a higher cell biomass. Also, more than twice as much substrate was consumed in this batch fermentation. Thus, the lower mannitol consumption rate in continuous systems at low OTR was probably caused by a lower cell mass concentration. It was only at the highest OTR of 18.4 mmol O2 L^{-1} h^{-1} that the continuous system had a CER (26 mmol CO_2 L^{-1} h^{-1}) similar to the batch fermentations, see Table 4.2. The limiting factors for the cell mass concentration were OTR and hydraulic retention time (10 h). Thus, the solution for improving the utilisation of mannitol while maintaining a high ethanol yield would be to increase the retention time, or maintain a higher cell biomass in the reactor by biomass retention.

4.2.3 *Zymobacter palmae* versus *Pichia angophorae*

Figure 4.6 shows the data for a culture of *Z. palmae* grown in seaweed extract. As seen in section 4.1, the activity of *Z. palmae* ceased before mannitol was exhausted, probably because of lack of some growth factor. However, when *P. angophorae* was added after 24 h to this non-active culture, both substrates started to be consumed. This clearly shows that *P. angophorae* is a more suitable organism for ethanol production from seaweed extract. It can utilise both substrates, and is not inhibited before the substrate is consumed.

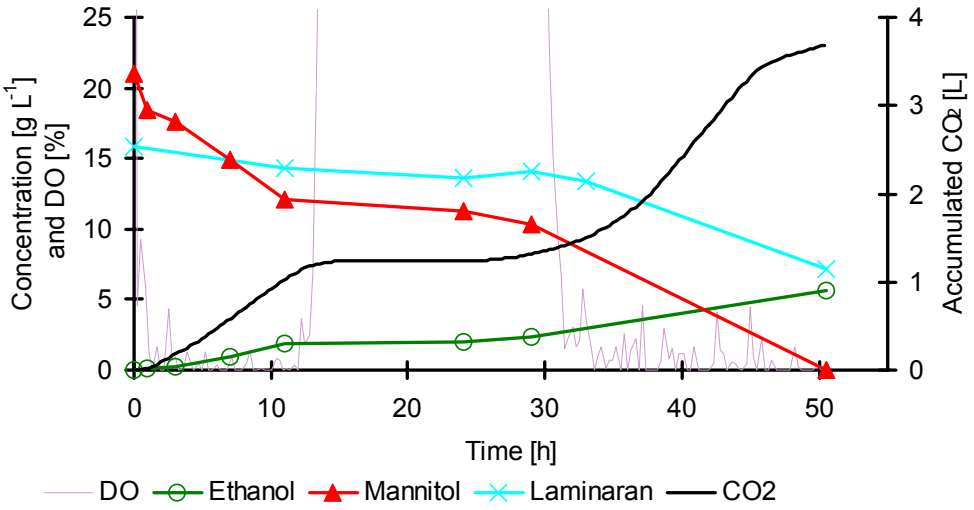

Figure 4.6 Fermentation with *Z. palmae* in seaweed extract with OTR = 4.9 mmol L^{-1} h^{-1}. At 24 h the fermentor was inoculated with *P. angophorae* (10 ml from the culture aerated with 5.9 mmol O_2 L^{-1} h^{-1}, which was running simultaneously with the *Z. palmae* culture) (from Appendix Paper 6).

4.2.4 Main findings

P. angophorae was shown to ferment mannitol and laminaran in seaweed extracts simultaneously to ethanol. In batch experiments, mannitol was the preferred substrate, and higher OTR and pH favoured mannitol consumption. However, optimisation of ethanol yield required a pH between 4.5 and 5.0. Maximum yield was 0.43 g ethanol (g substrate)$^{-1}$, at pH 4.5 and 5.9 mmol O_2 L^{-1} h^{-1}. In continuous culture, the preference of substrate was shifted towards laminaran.

4.3 Discussion and conclusions

Ethanol can be produced from the mannitol and laminaran contained in extracts from *L. hyperborea* fronds. *Z. palmae* was able to produce ethanol from mannitol, while *P. angophorae* could utilise both carbohydrates for ethanol production. Aeration has to be

controlled carefully to prevent production of organic acids. However, utilisation of seaweed carbohydrates for ethanol production is probably only of economic interest when integrated with a balanced and total utilisation of the seaweed material. The largest organic fraction of *L. hyperborea* is alginate, and this seaweed is today exploited on industrial scale for alginate production. In the alginate extraction process, mannitol and laminaran is washed out and disposed into the sea, representing an organic load for the recipient. In such a case, seaweed extract with mannitol and laminaran may be considered to be non-cost material for ethanol production. The excess organic residues in such a process could be fermented to methane.

This work was not concerned with the final ethanol concentration, which certainly would be a critical factor in industrial production of ethanol. Running a distillation on a low ethanol concentration will simply not be economical. In our laboratory, extractions carried out with less water than described above gave up to 30 g L^{-1} of both laminaran and mannitol. This would yield a maximum ethanol concentration of no more than 3 %. Thus, an optimisation of the extraction process is necessary to obtain higher ethanol concentrations. Carrying out several extractions in the same water, or access to dried seaweed material may also be of help.

An obvious practical problem with ethanol production is that the microbial culture may have to be protected against contamination of other microbes.

5 General discussion and conclusions

5.1 Summary of results

Polyphenols proved to be the most important limiting factor in the biodegradation of *A. nodosum* and *L. hyperborea*. High concentrations of polyphenols resulted in an inhibition of the microbial community, and particularly the methanogenic bacteria were affected. Thus, brown seaweeds with a low content of polyphenols are most suitable for methane production. Generally, the guluronate content increased in the remaining alginate during the fermentations. The reason was probably that the G-blocks in the gel matrix stabilised the tissue and limited the access of the enzymes. Digestion of *L. hyperborea* fronds harvested in March, when the content of mannitol and laminaran is at the lowest, gave a high acetate/CO_2 ratio. Thus, in the digestion of alginate the homoacetogenic pathway seems to play an important part. The main organic product of the fermentation was acetate, which was easily converted to methane.

Ethanol was produced from the mannitol and laminaran contained in extracts from *L. hyperborea* fronds. *Z. palmae* was able to produce ethanol from mannitol, while *P. angophorae* could utilise both carbohydrates for ethanol production. Thus, *P. angophorae* is a more suitable organism for ethanol production from seaweed extract. The oxygen supply was a critical factor during ethanol production. Some supply of oxygen is necessary for the fermentation of mannitol, while a too high aeration resulted in reduced ethanol yields and production of organic acids.

5.2 Bioenergy potential of Norwegian seaweed resources

The annual Norwegian production of brown seaweeds is estimated to be $5 \cdot 10^6$ tons fresh weight (Indergaard and Jensen, 1991). Not all of this is easily harvested due to difficult seafloor conditions and low seaweed density. It has been estimated that $1 \cdot 10^6$ tons/year are exploitable (Indergaard and Østgaard, 1992) with the current harvesting technology. From average TS and VS concentrations of 20 and 65 %, respectively, and a biogas yield of 70 %, it

has been estimated a biogas production of 91 kg/ton wet weight seaweed (Indergaard and Jensen, 1991). A gas density of 0.7 kg/m^3 and an energy content of 5 kWh/m^3 yield 650 kWh/ton fresh weight algae. Thus, the exploitable energy potential of methane production from natural crops of brown seaweeds is about 650 GWh/year. In comparison, the energy content of the wood annually used in Norwegian households for heating is about 6.5 TWh (The Norwegian Bioenergy Association, pers. com.).

It has also been estimated that $1 \cdot 10^6$ tons of *L. hyperborea*, harvested in the period June-January (8 months), can give 27 500 m^3 ethanol or 177 GWh (Aasen, 1997).

5.3 Possible applications

Along the Norwegian coast, *L. hyperborea* is the most suitable seaweed for energy production. This kelp is the dominant brown seaweed, it has a low polyphenol content, and equipment for harvesting is already developed by the alginate industry. Due to the complex composition of this alga, methane production is a most obvious process for microbial energy production.

Ethanol production from such a mixture of substrates seems difficult, and alginate, as one of the dominant constituents, may have a too high degree of oxidation to directly serve as a substrate for ethanol production. However, mannitol and laminaran accumulate in *L. hyperborea* in the autumn, and these carbohydrates may serve as substrates for ethanol production. Ethanol production is probably only of economic interest if it is integrated with industrial alginate production, where mannitol and laminaran are treated as waste. Actually, the first extraction step in industrial alginate production is principally the same as the procedure utilised for mannitol and laminaran extraction in this work. Figure 5.1 pictures alternative paths for energy production from *L. hyperborea*: methane production from the whole plant, or an integrated utilisation where ethanol and methane are produced from the alginate industry waste. The alternating accumulation and consumption of mannitol and laminaran in the light and dark seasons make ethanol production interesting only in the summer and autumn. Methane may be produced from the algae throughout the year, but alginate is a more valuable product than methane gas. Thus, the most economic solution for methane gas production seems also to be in combination with alginate production.

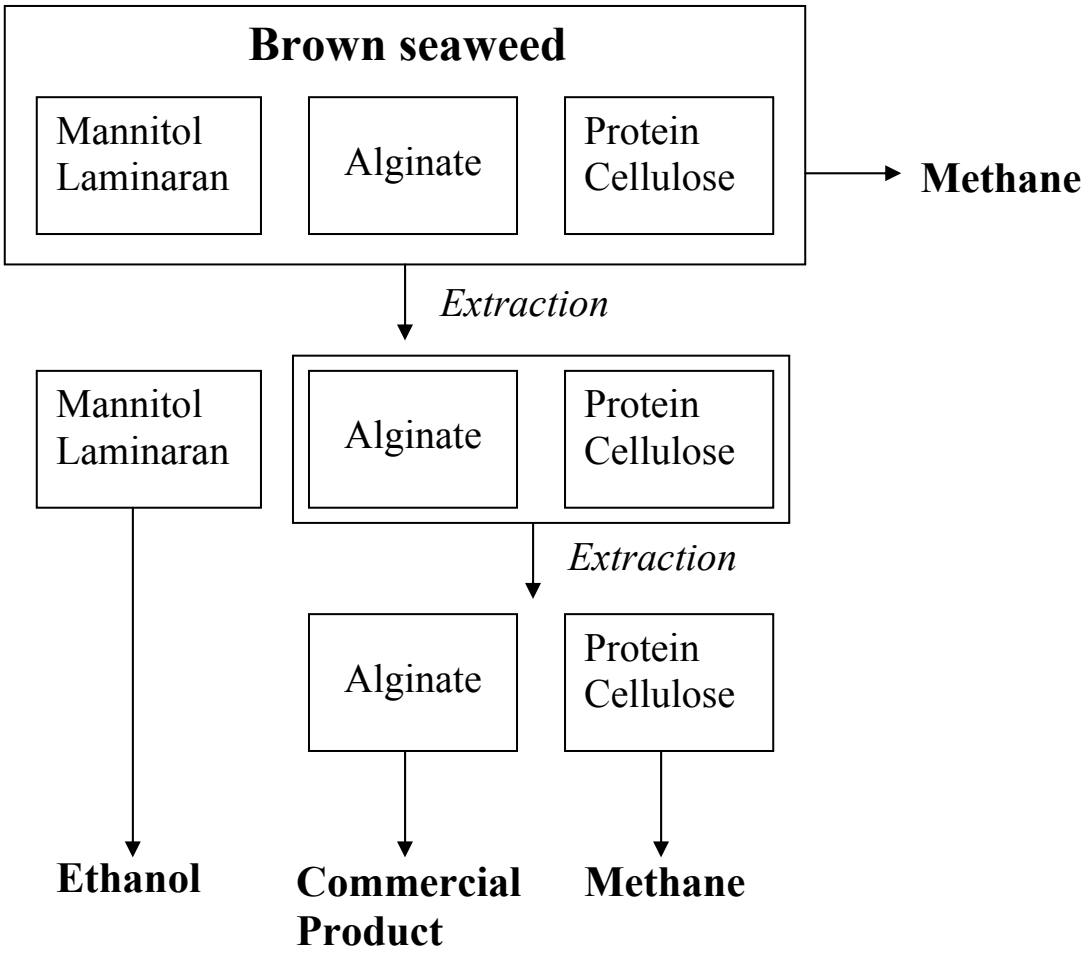

Figure 5.1 Proposed utilisation of different fractions of *L. hyperborea*: Methane production from the whole material, and methane and ethanol production from alginate industry wastes.

5.4 Economic considerations

The dominant cost will be the harvesting price of the raw material. Today, the harvesting costs for *L. hyperborea* utilised in the alginate industry is 120 NOK/ton (FEMS BioPolymer, pers. com.). Fermentation and gas cleaning costs have been estimated to half of the raw material costs (Bird, 1986). Thus the energy cost of biogas produced from *L. hyperborea* would be 0.3 NOK/kWh, assuming the yield given in 5.2.

It has been estimated that for ethanol production based on waste raw material from the alginate industry the price would be 0.7 NOK/L ethanol or 0.1 NOK/kWh (Aasen, 1997). This estimate was based on mannitol and laminaran extracted from $1 \cdot 10^6$ tons of *L. hyperborea*. It also included utilisation of methane, produced from raw material residues, as a local energy source for the process. A comparison of methane and ethanol production was also carried out based on a raw material price of 100 NOK/ton: the production costs were estimated to 0.7 and 0.3 NOK/kWh for ethanol and methane production, respectively. Thus, ethanol production is economical only if the market price of ethanol, on an energy basis, is more than twice as high as the methane price (Aasen, 1997).

5.5 Future research

Future research should be concentrated on continuous or semi-continuous fermentations. Such systems may be operated at steady state for a long period, reducing the number of variables to be considered. To optimise the yield, methane production should be tested out in a two-stage system for independent optimisation of VFA and methane production. Methane producers are slow growing microbes and immobilisation on biofilm carriers or granulation such as UASB (Lettinga, 1993) may reduce the hydraulic retention time. The substrates mannitol and laminaran require different supplies of oxygen to optimise ethanol yield, and a two-staged system may be beneficial also here.

To obtain higher concentrations of mannitol and laminaran in the extract, several extractions may be carried out in the same liquid. Fermentations of such extracts should be optimised for maximum ethanol concentration. In practical applications, it will not be economical to sterilise large liquid volumes. Alternative means to prevent contamination of the ethanol producing culture should be investigated. Fermentations at low pH may be a solution.

The experiments in this work were designed to achieve a homogenous reactor mixture. For practical applications, it may be more economically feasible to work with more concentrated suspensions. Solid phase fermentation of seaweeds should therefore also be investigated.

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1 Svein Jarle Horn, Einar Moen and Kjetill Østgaard (1999) Direct determination of alginate content in brown algae by near infra-red (NIR) spectroscopy. J. appl. Phycol. 11:9-13.

2 Einar Moen, Svein Horn and Kjetill Østgaard (1997) Biological degradation of *Ascophyllum nodosum*. J. appl. Phycol. 9:347-357.

3 Einar Moen, Svein Horn and Kjetill Østgaard (1997) Alginate degradation during anaerobic digestion of *Laminaria hyperborea* stipes. J. appl. Phycol. 9:157-166.

4 Svein Jarle Horn and Kjetill Østgaard (2001) Alginate lyase activity and acidogenesis during fermentation of *Laminaria hyperborea*. J. appl. Phycol. 13:143-152.

5 Svein Jarle Horn, Inga Marie Aasen and Kjetill Østgaard (2000) Production of ethanol from mannitol by *Zymobacter palmae*. J. ind. Microbiol. Biotechnol. 24:51-57.

6 Svein Jarle Horn, Inga Marie Aasen and Kjetill Østgaard (2000) Ethanol production from seaweed extract. J. ind. Microbiol. Biotechnol. J. ind. Microbiol. Biotechnol. 25:249-254.