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An Investigation of the Composition, Microbial Digestion and Potential Applications of an Industrial By-product of Seaweed Extraction

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Commercial preparation of seaweed extract from *Ascophyllum nodosum*, for use as fertilizer and soil improver, produces a residue which requires remediation. This residue is rich in nutrients and offers potential for other added-value products. The residue's composition and microbial flora were studied, a microbial digestion system was developed, and extracts were screened for anti-hyaluronidase and anti-elastase activities.

The residue had a pH of 8.61±0.39, 16% (w/w) TS which comprised 40.6% minerals, 29.5% fibre, 20.9% lipid, 4.9% protein and 0.48% polyphenols. The microbial digestion included an initial 3-day anaerobic phase during which pH decreased from 9.12 to 7.89. At day 3, solubilized material was decanted to delay metabolite inhibition, an inoculum was added, followed by a 10-day aerobic digestion. The rate of digestion (decrease in insoluble material of 28.6±14.2% over 13 days) was influenced by the initial insoluble (R^2=0.773) and soluble (R^2=0.672) matter, the pH at the beginning of the aerobic phase (R^2=0.528) and by accumulation of solubilized digestive products. A compositional analysis of the insoluble material after digestion showed the lipid content of the residue was 96% digested, and protein content increased by 82.4%. Organic acid analysis (HPLC) revealed the presence and consumption of oxalic, formic, lactic and acetic acids during the anaerobic and/or aerobic phases. Microorganisms of *A. nodosum* residue (*Brochothrix thermosphacta*, *Bradyrhizobium japonicum, Pseudomonas sps.* and *Enterobacter amnigenus*) and the inocula employed (*Leclercia adecarboxylata, Serratia sp., Klebsiella pneumoniae, E. coli* and *Aspergillus versicolor*) were identified and characterised.

Soluble material collected from the digestion exhibited stronger anti-hyaluronidase activity than *A. nodosum* plant (IC_{50} values of 0.25 mg/mL and 0.70 mg/ml, respectively). *A. nodosum* and *L. hyperborea* seaweeds were treated and screened for hyaluronidase and elastase inhibitors. *A. nodosum* treated with bromelain showed potential for anti-elastase activity. The data shows that seaweed residue has further potential for commercial exploitation.

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Abbreviations

A	Alcalase
AqE	Aqueous extract
B	Bromelain
BCA	Bicinchononic acid
bSA	Bovine serum albumin
FAME	Fatty acid methyl esters
GC	Gas chromatography
HAase	Hyaluronidase
HLE	Human leucocyte elastase
IC_{50}	Inhibitory concentration (50%)
IM	Insoluble material
MIC	Minimal inhibitory concentration
PCA	Plate count agar
Pp	Pepsin
PPE	Porcine pancreatic elastase
Px	Protamex
Q&C	Quatrano and Caldwell media/agar (Quatrano and Caldwell, 1978)
SD	Standard deviation
SFE	Supercritical fluid extraction
SM	Soluble material
TS	Total solids
TVC	Total viable count

1. INTRODUCTION

1.1 Seaweeds

The term algae describes a large and diverse assembly of eukaryotic organisms that contain chlorophyll and carry out oxygenic photosynthesis. They are morphologically less complex than land plants as they have no true roots or leaves. Most algae are microscopic and unicellular, but, in this group, there are also multicellular organisms, the macroalgae or seaweeds (Hufford, 1978). Seaweeds are marine algae. These primary producers contribute an estimated 40 percent of the planet's photosynthetic productivity, and provide a foundation for aquatic food webs and habitats for many of consumers and associated marine flora and fauna (McLaughlin *et al.*, 2006). Macroalgae are very important ecologically. They dominate the rocky intertidal zone in most oceans and in temperate and polar regions dominate rocky surfaces in the shallow subtidal (down to 50 m) zone. This sea environment is rather stable in temperature, humidity and salinity. They live attached to the bottom by a special structure called the holdfast.

Algae are a large and diverse group of organisms with many different life-cycle strategies, a wide range of forms and they occupy a range of habitats. They are divided into three groups identified by colours (Table 1.1): red seaweed (Rhodophyta), green seaweed (Chlorophyta) and brown seaweed, the Phaeophyta (Barrett and Yonge, 1958). All of them have chlorophyll-like pigments, although this is only obvious in the green seaweed. In the red seaweeds chlorophyll is masked for phycoerythrin and phycocyanin, giving them a colour from red or pink to purplish-brown. The brown to olive green colour is given in the Phaeophyta by fucoxanthin (Morrisey *et al.*, 2001).

Green seaweeds accumulate starch and some fat or oils as energy reserves. The red group accumulates floridean, starch and floridoside, whereas the brown polysaccharides accumulate alcohols and laminaran. The biggest group is the red seaweed with approximately 6500 species of which 275 have been found in Ireland. Two commercially important seaweeds from this group are *Palmaria palmata* and *Chondrus crispus* (Fig 1.1: a and b). Both seaweeds are eaten and can be used as an ingredient in different recipes. The Phaeophyta group has 2200 species described,

Figure 1.1: Red and green seaweeds. *Pamaria palmata* (a), *Chondrus criptus* (b), *Ulva rigida* (c) and *Enteromorpha compresa* (d).

with 147 found in Ireland. The Chlorophyta group has around 1200 species described and 80 are found in Ireland. *Ulva rigida* and *Enteromorpha compresa* (Figure 1:c and d) are common examples of green seaweeds which are associated with macroalgal blooms during the summer (Morrisey *et al.*, 2001).

Table 1.1 Three algal divisions and their significant characteristics (Davis *et al.*, 2003).

Division	Common name	Pigments	Storage product	Cell wall
Chlorophyta	Green algae	Cholophyll a, b; α, β and γ carotenes and several xanthophylls	Starch (amylose and amylopectin) (oil in some)	Cellulose in many (b-1,4-glucopyroside), hydroxyproline glucosides; xylans and mannans; or wall absent; calcified in some
Phaeophyta	Brown algae	Cholophyll a, c; β carotene and fucoxanthin and several other xanthophylls	Laminaran (β-1,3 glucopyranoside, predominantly); mannitol	Cellulose, alginic acid, and sulfated mucopolysaccharides (fucoidan)
Rhodophyta	Red algae	Chlorophyll a (d in some Florideophyceae);R- and C-phycocyanin, allophycocyanin; R- and B-phycoerythrin. α- and β carotene and several xanthophylls	Floridean starch (amylopectin-like)	Cellulose, xylans, several sulfated polysaccharides (galactans) calcification in some; alginate in corallinaceae

1.1.1 Brown Seaweeds

This thesis is concerned with brown seaweeds, especially *Ascophyllum nodosum*, and therefore this group will be described in some detail. Phaeophyta grow in distinct horizontal bands across the seashore. Each species occupies a characteristic level on the shore in response to environmental conditions (Figure 1.2). These conditions include the time that the plants are exposed to air (being covered or uncovered by the tide), the intensity of the wave action, water turbidity, light penetration, types of substratum, competition between different species and the tidal range (Hiscock, 1979; Morrisey *et al.*, 2001). The seaweed species living on the rocky intertidal habitats are thus under the alternate influence of aquatic and aerial climatic regimes and must be adapted to short-term environmental variations over 12 hour tide cycles (Lüning *et al.*, 1990). Seaweed at immersion are exposed to a higher availability of nutrients and to lower light intensities than when emerged. At emersion, seaweeds may be exposed to strong desiccation and to direct solar radiations (Connan *et al.*, 2007).

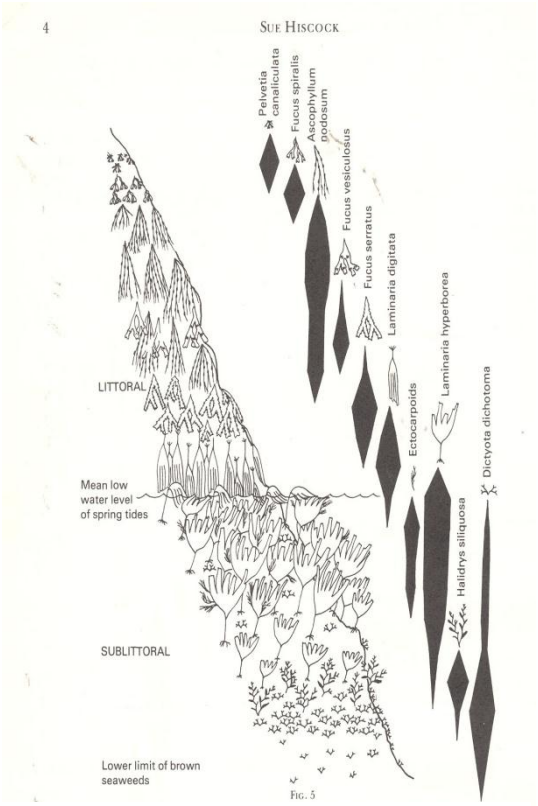
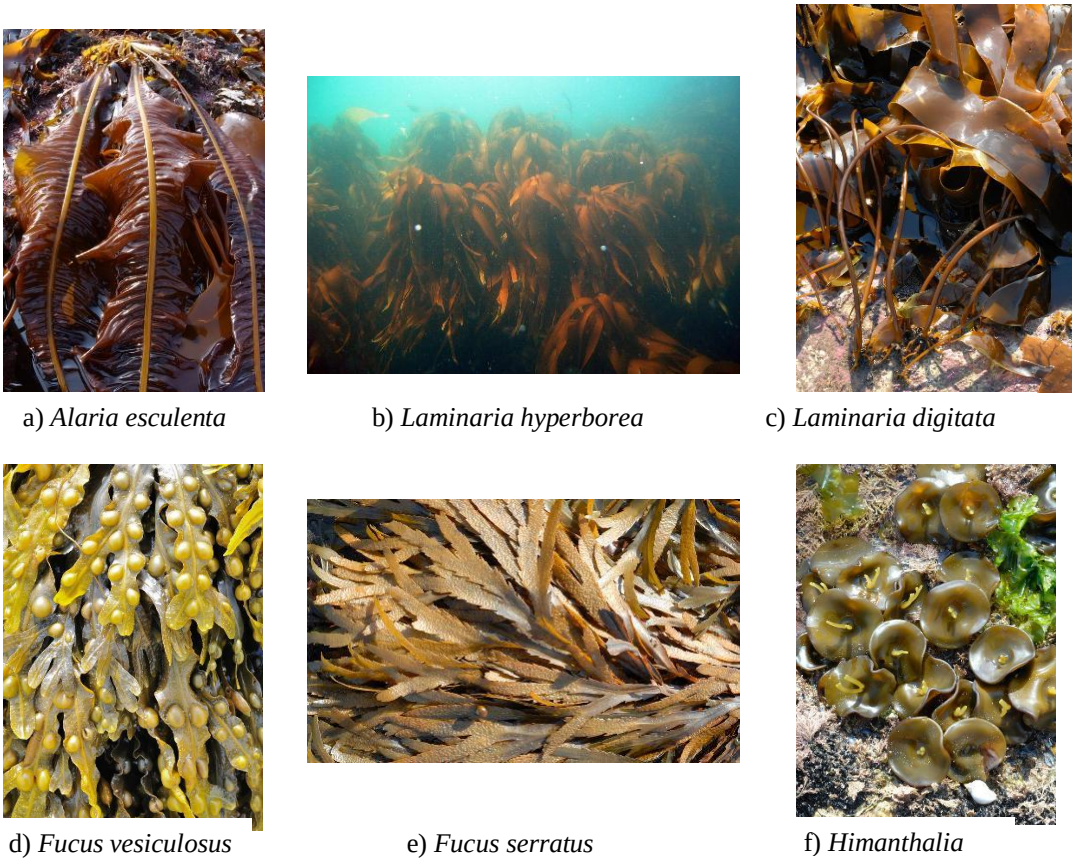


Figure 1.2: Vertical zonation of brown seaweed at a typical sheltered rocky site (Hiscock, 1979).

Phaeophyta species are found on bedrock or on large stable boulders, with few settling on pebble beaches. Other species are intolerant to the air exposure, like the kelps which dominate the lower shore whilst other species as Channelled Wrack (*Pelvetica canaliculata*) can only live in the high intertidal (between tidal) shore and seems to need to be exposed for most of the tidal cycle.

Phaeophyta absorbs medium wavelength, green light (560–490 nm) which enables them to photosynthesise while submerged at depts up to 30-50 m in clear waters but at only 10-15 m in turbid waters, such as estuaries. The majority of Phaeophyta live in the intertidal and sublittoral zones (below low water). Brown seaweeds are best suited to cooler water temperatures, whereas red seaweeds generally penetrate slightly deeper that the deepest browns.

Common Phaeophyta species on the Irish coasts include the kelps (Laminarians) *Alaria esculenta*, *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria saccharina* and *Sacchoriza polyschides*, the wracks (Fucoids) *Fucus vesiculosus*, *Fucus serratus*, *Ascophyllum nodosum* and *Pelvetia canaliculata* and the species *Himnthalia elongata*. Some examples are shown in Figure 1.3. The kelps and wracks provide most of the biomass and most of the commercially utilised species.



a) *Alaria esculenta* b) *Laminaria hyperborea* c) *Laminaria digitata*

d) *Fucus vesiculosus* e) *Fucus serratus* f) *Himanthalia*

Figure 1.3: Different examples of brown seaweeds (Guiry and Guiry, 2009).

1.1.2 *Ascophyllum nodosum*

Ascophyllum nodosum (Linnaeus) Le Jolins is a brown seaweed which belongs to the order Fucales, phylum Phaeophyta. This species is one of the most abundant intertidal seaweeds which are so characteristic of the rocky Irish west coast (Guiry and Hession, 1998). *A. nodosum* is commonly named knotted wrack. It is also known as asco, rockweed, yellow weed, sea whistle or yellow tang in English and feamainn bhuí or feamainn bhuí bhoilgíneach in Irish. *Ascophyllum* comes from the Greek words *askos* which means a wine-skin, *phullon* a leaf and *nodosum* full of swellings.

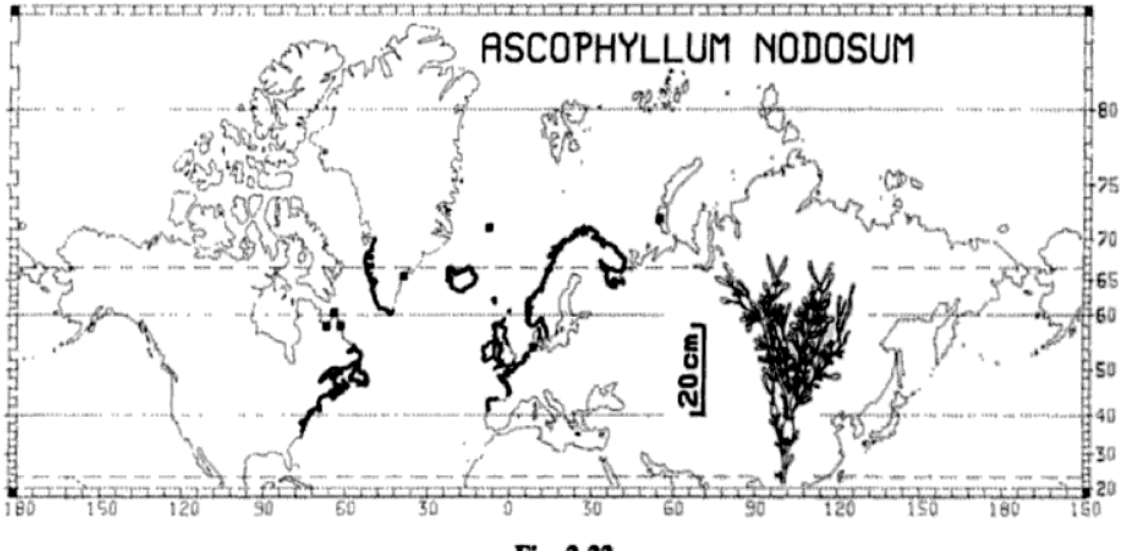


Figure 1.4: *Ascophyllum nodosum* distribution (Lüning *et al.*, 1990)

A. nodosum is a very common wrack of the middle-shore zone in wider Europe and along the Atlantic coast of North America (Figure 1.4). It is found on most of the coast of Ireland. This species requires a substantial substratum for firm anchorage due to the bulk of the adult plant. In some extremely sheltered areas it can be found unattached on the shore, or lying on the sea floor (Hiscock, 1979). It will not grow on mud or small stones. Generally *A. nodosum* is found growing on rocks, large stones, piers and other solid objects of attachment in estuaries and semi-sheltered bays. It tends to be more abundant in sheltered places, where it forms dense and luxuriant beds. Sometimes it is found as a co-dominant species with *Fucus vesiculosus* is these areas.

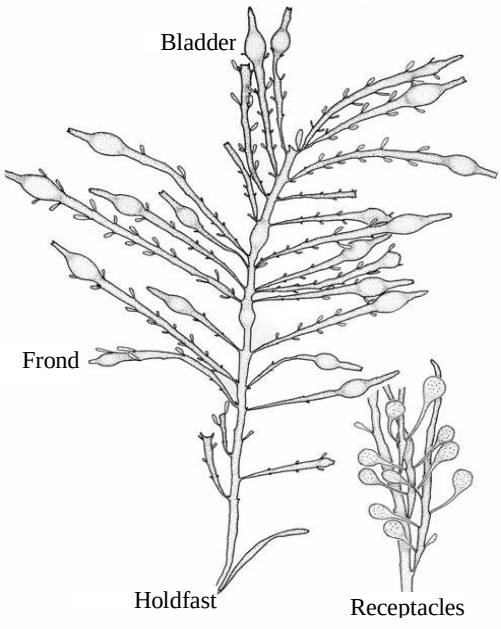


Figure 1.5: *Ascophyllum nodosum* plant. Receptacles structure on the right.

A. nodosum is described as a branched, untidy looking plant. It is an olive green to yellow seaweed which turns to black when dried. Generally, it grows up to 2 metres in length after 3 or 5 years. The structure of the plant is shown in Figure 1.5. The holdfast is found at the bottom of the plant to link to the substratum. The fronds are long, tough, leathery, elastic, irregularly-branched and without midrib. There

are egg-shaped bladders in the fronds which contain gas. They are smooth outside and hollow inside (Figures 1.5 and 1.6). The first bladder is formed after 3 years of growth, and subsequently a new bladder each year. This can give an idea of the age of the plant. When the plant is submerged by the tide, the bladders float the fronds up toward the light, maximising the potential for photosynthesis. *A. nodosum* provides shelter and food for many small fish and other marine organisms. The receptacles are the reproductive structures (fruiting bodies) and have a rough outer surface (Figures 1.5 and 1.6). Receptacles grow out laterally from the sides of the fronds, usually in pairs, from November to May. They are yellow in male plants and green in female. When they fall off at the end of the season their point of attachment is usually colonised by the filamentous red seaweed *Polysiphonia lanosa*, which develops small purple-red tufts of fine branches (Barrett and Yonge, 1958; Morrisey *et al.*, 2001).

The *Ascophyllum* zone supports a diverse epibiota: plants and animals growing on the fronds. This includes species living attached to the seaweed (sessile epibiota) and adjacent rocks, mobile fauna living amongst the seaweed and large predatory species such as fish, birds and others that may feed in the *Ascophyllum* biotope. A variety of fauna such as barnacles, limpets, amphipods, isopods, crabs, winkles and sponges can be found. Ascidians where there are strong tidal currents, and other brown, red and green seaweeds were also associated with *A. nodosum*. Up to 31 species of fish and 26 species of birds use the *A. nodosum* zone as a habitat for feeding, reproduction or sheltering purposes (Kelly *et al.*, 2001).

Figure 1.6 a) *A. nodosum* plant from Fenit Island (Co. Kerry) b) *A. nodosum*'s bladders and receptacles (Guiry and Guiry, 2009).

1.2 Brown Seaweeds in Ireland

Phaeophyta are an important resource on the Irish coast. The Irish coastline is over 3000 km long. The Atlantic seaboard consists of exposed open coast interspersed with many rocky bays and indentations which are ideal for the settlement of seaweeds (Morrisey *et al.*, 2001). Ireland has a long history and culture associated with seaweeds. The Irish word for seaweed, *feamainn* or *feamuinn* has as its stem, *feam* a rod, as in the English sea-rod (*Laminaria hyperborea*), a common kelp in Ireland. Marine plants have been gathered for food since at least the Middle Ages, while the use of seaweed as fertilizer and animal fodder dates back at least several centuries (Kelly *et al.*, 2001). They were also widely used in seaweeds baths (McLaughlin *et al.*, 2006) or iodine preparations (Childs, 2008).

At various times in its long history of poverty and destitution, the west of Ireland has wisely used its seaweed resources, particularly kelps, wrack and a number of food species. The production of kelp ash was a major industry on the west coast of the country in the 18^{th} and 19^{th} centuries. The burnt ash was used for pottery glazing and in the manufacture of glass and soap. Drift and cut seaweeds were widely used to cultivate potatoes in lazy-beds, where drift or cut seaweed and sand were alternately layered in raised beds with large drainage channels in between, and this persists to the present day on some remote islands (Guiry, 2008). The edible seaweeds *Palmaria palmata* (Dulse) and *Chondrus crispus* (Carrageen moss) have also been used in the diet and as health remedies of Irish coastal populations (McLaughlin *et al.*, 2006).

1.3 Uses of Seaweeds

The current uses of seaweeds in Ireland may be summarised by the following main categories:

- Sea-vegetables
- Maërl
- Biotechnology/ Biomedicine
- Biopolymers
- Agriculture/Horticulture
- Cosmetics and Thalassotherapy

1.3.1 Sea Vegetables

Seaweeds are traditionally used in human and animal nutrition (Fleurence, 1999). Edible marine algae have attached special interest as good sources of nutrients including protein, long-chain polyunsaturated fatty acids, dietary fibres, vitamins and minerals (Sánchez-Machado *et al.*, 2004; Shahidi, 2008). As a dietary supplement they have had the longest and perhaps the most significant use (Marsham *et al.*, 2007). Brown seaweeds regarded as sea-vegetables include *Laminaria spp*. (Kombu), *Laminaria saccharina* (Sweet Kombu), *Himanhalia elongata* (Sea Spaghetti) and *Alaria esculenta* (Atlantic Wakame). Few of them have high demand and are supply-limited, so aquaculture development is being researched. This market continues to grow both at home and abroad because more and more people are eating sea-vegetables (Irish_Seaweed_Centre, 2000). The major producer of such seaweeds is China, followed by other Asian countries.

1.3.2 Maërl

Maërl is made up several species of calcareous red algae found in subtidal beds along the west coast of Ireland, especially in Bantry Bay (County Cork) where the commercial harvesting is carried out. Approximately 10,000 tonnes of maërl is harvested *per* year in Ireland (Guiry, 1996-2008; Irish_Seaweed_Centre, 2000). Maërl is used as a soil improver for organic farming and horticulture and also is a good filtration agent for the purification of water destined for human consumption and for water filtration in marine and freshwater aquaria.

1.3.3 Biotechnology/ Biomedicine

The biotechnological/biomedical applications of seaweeds is a very diverse field of research and development. There is research focused on marine algae and their constituents as nutraceuticals and functional foods for their potential health promotion mostly attributed to their ω3 fatty acids, antioxidants and other bioactives (Shahidi, 2008). There are a wide variety of recorded medicinal and pharmaceutical uses such as antiviral activity from sulphated polysaccharides (Witvrouw *et al.*, 1994), carrageenans (Cáceres *et al.*, 2000) or fucoidan (Malhotra *et al.*, 2003; Kusaykin *et al.*, 2008); antibiotic activity (Smit, 2004); coagulation properties (Shanmugam *et al.*, 2002); activities related to cellular growth like mitogenic activity (Kawakubo *et al.*, 1997); anticancer and antitumor properties (Hamann *et al.*, 1996; Smit, 2004; Athukorala *et*

al., 2006); anticoagulant activity, for example, sulphated fucans from the brown seaweeds *Fucus vesiculosus* and *Ascophyllum nosodum* (Trento *et al.*, 2001) or *Lessonia vadosa* (Chandía and Matsuhiro, 2008); anti-inflamatory activity on the immune response (Smit, 2004; Kusaykin *et al.*, 2008) enzyme inhibitors and stimulants (Smit, 2004); anti-diabetic activity (El Gamal, 2010); and antioxidant properties (Athukorala *et al.*, 2006; O'Sullivan *et al.*, 2011).

Laminarans, extracted from brown seaweed, are used for many of the applications mentioned in the previous paragraph but in addition they have shown other advantageous properties in vitro. These include immunomodulation, wound healing, reduction of serum cholesterol levels, transplantation immunology, therapeutic and prophylactic effects, increased resistance to bacterial, viral and parasitic infections (used to prevent infection after surgery), prevention of opportunistic infections in immuno-compromised individuals (HIV sufferers, geriatric patients), development of immunisation strategies, chelation therapy to remove heavy metals and nutraceutical properties (Irish_Seaweed_Centre, 2000).

1.3.4 Biopolymers

"Polysaccharides produced by the marine macroalgae form the basis of an economically important and expanding global industry" (Renn, 1997). Key products are agars, agaroses, alignates and carrageenans (Table 1.2). Alginates are extracted from native brown seaweed. Due to their ability to retain water and their gelling properties are valuable/exploited in an industrial setting (Irish_Seaweed_Centre, 2000; Rayment *et al.*, 2009). Alginates are used in the food industry as thickening and welling agents in, for example, ice-creams and desserts. They are also used in pharmaceutical industry as binding agents for tablets, production textiles, electrodes and in water processing as well as many other applications.

Table 1.2 Seaweed Polysaccharides of biotechnological importance (Renn, 1997).

Polysaccharide	Application
Agars	Foods, microbial culture matrix, dental impression, laxatives and as a raw material for production of agaroses.
Agaroses	Used in matrix for electrophoresis, immunoassays, microbial and cell culture and chromatography and immobilised systems.
Alginates	Used in food, as a tableting agent, dental impression media and matrices for immobilised systems.
Carrageenans	Foods, toothpaste binders, air freshener gels, personal care products and pet foods.

1.3.5 Agriculture/Horticulture

Seaweeds have been used as fertiliser and soil improvers in Europe for centuries (McLaughlin *et al.*, 2006). In the past few decades, interest in using seaweed extracts in horticultural and agricultural crops has increased considerably (Leclerc *et al.*, 2006). The use of seaweeds and by-products for agriculture and horticulture is popular in many countries because the quality and yield of crops are enhanced. In addition this practice improves the condition of the soil by the incorporation of the organic matter (Canales-López, 1999). Seaweeds as fertilizers contribute many minerals, nutrients, trace metals and plant hormones, especially cytokinins and substances mimicking cytokinin activity (Leclerc *et al.*, 2006). The additional benefits of using seaweeds as soil conditioner include anti-fungicide activity (McLaughlin *et al.*, 2006; Jayaraj *et al.*, 2008).

1.3.6 Cosmetics and Thalassotherapy

Thalassotherapy (a spa treatment with seawater and/or seaweed), has been used in Europe for many years. There is an historical precedent that dates back at least 2,500 years. Seaweed baths and treatments are growing in popularity as more people become aware of the health benefits and relaxation derived from their use. This practice provides relief to stress, muscle pain and various other aches that are associated with the general wear and tear on the human body (Guiry, 1996-2008). Seaweed baths release excess fluids from congested cells and dissolves fatty wastes through the skin replacing them with minerals, especially potassium and iodine (Page, 2004).

The main species used in the cosmetics and thalassotherapy sector in Ireland are *Chondrus crispus* (Figure 1b), *Mastocarpus stellatus*, *Fucus* spp. (Figure 3), *Laminaria* spp. (Figure 3), and *Ascophyllum nodosum* (Figure 5; Irish_Seaweed_Centre, 2000).

1.3.6.1 Hyaluronidase and Elastase inhibitors

Ageing of the skin results from two main factors: genetic programming and permanent actinic damage due to environmental stress (Lee and Choi, 1999). Environmental stressors include exposure to ultraviolet (UV) radiation, smoking, wind and chemical exposure. These result in roughness, fine lines, sagging, irregular pigmentation and decreased skin elasticity (Huang and Miller, 2007).

In recent years there have been various technology milestones in anti-aging skincare, including ingredients such as retinoid, alpha hydroxyl acids (AHAs) and beta hydroxyl acids (BHAs). The most current and in-demand skin-care ingredients for both cosmetics and dermatology are peptides (Deloire, 2008). Peptides have long been recognised for their role in wound healing but their efficacy in improving the appearance of photo-damaged skin and ageing skin has only recently been reported. There are a number of studies describing the importance of peptides in skin biology as regulating homeostasis and biological phenomenon of the skin cells: cell proliferation, migration, inflammation or protein synthesis and regulation among others (Zhang and Falla, 2009). Some of these peptides have proved extremely efficient in anti-ageing applications. Kollaren, ChroNOline, KTTKS, pal-KITTS and pal-KT are some recent examples (Farris, 2004; Osborne *et al.*, 2005; Huang and Miller, 2007; Deloire, 2008; Osborne *et al.*, 2008; Chirita *et al.*, 2009; Zhang and Falla, 2009). The mechanism of action has been identified for specific peptides, some of which stimulate the synthesis of different types of collagen from fibroblasts (Kollaren, KTTKS or pal-KTTKS), others inhibit matrix metalloproteases (MMPs) and elastase activities (ECM-Protect) and others regulate extracellular matrix protein synthesis (ChroNOline).

Hyaluronidase plays an important role in skin maintenance. Levels of hylauronic acid (HA) decrease with age leading to wrinkling and altered elasticity (Ghersetich et al., 1994). Hyaluronidase is an enzyme which acts on the polysaccharide hyaluronan (HA), which is one of the main structural components of the skin (Botzki et al., 2004). Hydrolysis of HA by hyaluronidase is a normal event in the turnover/remodelling of skin structure.

Elastase is the enzyme responsible for elastin degradation in the extracellular matrix (ECM). Elastic fibres are a relatively minor component of skin, contributing only 2 to 4% of the total dry weight (Graham Smith and Rolland Finlayson, 1965; Uitto, 2008). They provide elasticity and resilience to the skin. Elastin is highly insoluble and its biosynthesis declines precipitously after the third or fourth decade of life (Uitto, 2008).

With age the elasticity of skin is significantly decreased by elastase activity. Previous studies have shown that elastase activity is increased with age or with UV radiation in mouse skin (Bissett *et al.*, 1987; Labat-Robert *et al.*, 2000). Both hyaluronidase and elastase are also involved in panniculopatia oedemato fibrosclerotica, commonly called "cellulitis". Cosmetic treatment of cellulitis involves of inhibition of these enzymes (Lee *et al.*, 2001).

Previous studies on the literature have shown that a wide variety of naturally-derived compounds have the ability to inhibit various forms of hyaluronidase enzymes from mammalian, insect and reptilian sources (Kim *et al.*, 1995; Fujitani *et al.*, 2001; Mio and Stern, 2002; Lee *et al.*, 2005; Machiah *et al.*, 2006; Sumantran *et al.*, 2007). Some phlorotannins from brown seaweed have also shown to inhibit hyaluronidase (Shibata et al., 2002). While a number of elastase inhibitors have been found in plants or seed extracts (Lee and Choi, 1998; Lee and Choi, 1999; Baylac and Racine, 2004; Kim *et al.*, 2007; Moon *et al.*, 2010) very few have been found in seaweeds. These correspond to a ketosteroid from the red seaweed *Hypnea musciformis* (Bultel-Poncé *et al.*, 2002) and a fucoidan fraction from *A. nodosum* (Senni *et al.*, 2006).

Research active in Shannon Applied Biotechnology Centre, Institute of Technology Tralee, during this project was the study of hyaluronidase and elastase inhibitors. The industrial residue and related samples were screened for enzyme inhibitory activity.

1.4 Irish Seaweed Industry

The seaweed industry in Ireland and Europe grew as seaweeds began to be utilised for their chemical properties. In Ireland, the development of a commercial seaweed industry essentially revolutionised the economy of the north and west coasts (McLaughlin *et al.*, 2006).

Five hundred species of seaweeds have been identified in Ireland. Considering the relative size of the island, this is a high proportion of the marine algae of the whole of the North Atlantic basin. Among these 500 species, 19 are commercially important on a world-wide scale. Of these, 16 are currently being exploited in Ireland.

Research carried out by the Food and Agriculture Organization (FAO) estimated an annual quantity of 7.5-8 million tonnes of wet seaweed (derived from two sources, naturally growing seaweed or cultivated, farmed seaweeds) which is collected and used by the industry (McHugh, 2003).

The Irish industry is broadly based, with the product being supplied to agriculture/horticulture, cosmetics, thalassotheraphy, the biopharma sector (functional foods/nutraceuticals), and for human consumption. The Marine Institute (2006), reported that 32,000 tonnes of wet weed was harvested in 2006. There are 2 companies involve in the agriculture/horticulture sector, 5 in biotechnology, 6 in talassotheraphy, 13 in food and 9 in cosmetics. In total they employ around 300 people full time and 320 part time. The turnover is estimated to be 10 million euro for the horticulture/agriculture sector, and approximately 5 million euro for biopharma, health, food cosmetics and thalassotheraphy (Marine_Institute, 2006).

These companies involved in the seaweed industry are mostly distributed along the west coast of the country. One of the largest seaweeds processing companies is Arramara Teoranta, located in counties Galway and Donegal. It employs 32 people full time and the seaweed is bought from 267 harvesters based mainly in counties Galway, Mayo, Sligo and Donegal. The main seaweed crop used in Ireland today is *A. nodosum* (Irish_Seaweed_Centre, 2000).

1.4.1 *A. nodosum* in the Irish Seaweed Industry

There are currently aproximately 400 people involved in the harvesting and processing of *Ascophyllum nodosum* in Ireland. Seaweed harvesting is an activity that has been of significant economic importance to coastal communities for over fifty years. Kelly *et al.* (2001) said that it contributed in excess of IR£ 1 million per annum directly to coastal communities and it is steadily rising.

Hession *et al* (1998), reported the mean annual harvest of *A. nodosum* on the west coast of Ireland was in the region of 35,000 tonnes (wet weight). The entire resource is harvested by hand. Harvesting is a year round activity, however, it is more common between the months of June to October and occurs mainly at low spring tides (i.e., in two week cycles). *A. nodosum* is harvested, transported by track to one of the processing plants where the seaweed is cleaned (sand, silt, small stones and any other debris are removed). Then it is chopped up, dried and milled (Kelly *et al.*, 2001). High quality meal depends on good clean seaweed raw material.

A. nodosum is harvested on 3 or 5 year cycles. The growing period depends on the region/location and the rate of growth associated with individual beds. It requires a recovery time of only 3-4 years and so the fallow period of 3-5 years employed in Ireland is quite sufficient for sustainability. A report in 1998 (Hession *et al.*) found that

the potential sustainable harvest of *A. nodosum* was 74,845 wet tonnes; and the amount harvested, when this studied was carried out, was of the order of 35,000 tonnes, as previously mentioned. Indeed, there does not seem to be any species in Ireland currently being commercially utilised to its full potential (Irish_Seaweed_Centre, 2000; Marine_Institute, 2006).

1.5 Composition of Brown Seaweed

The importance of the brown seaweeds is related to their composition. The chemical composition of Phaeophyta varies considerably between species, throughout the year (Ragan and Jensen, 1978) and between habitats (Haug and Larsen, 1958). The absence of lignin and low content of cellulose in brown seaweeds should make them a simple material for bioconversion. Compared to terrestrial plants, seaweeds are easily degraded by microorganisms, probably due to their very low content of refractory compounds, such as lignocelluloses and lignin (Hanssen *et al.*, 1987; Moen *et al.*, 1997b). According to Kusaykin *et al*. (2008) "brown seaweeds dominating in Northem seas are a real treasure-trove of physiologically active polysaccharides".

Seaweeds have a complex composition. Complete degradation of the material necessitates the presence of microorganisms with a broad substrate range (Horn, 2000).

The cell walls of brown algae are made of cellulose, alginates, sulphated fucans and protein (Kloareg *et al.*, 1986). Figure 1.7 showns the cell wall structure.

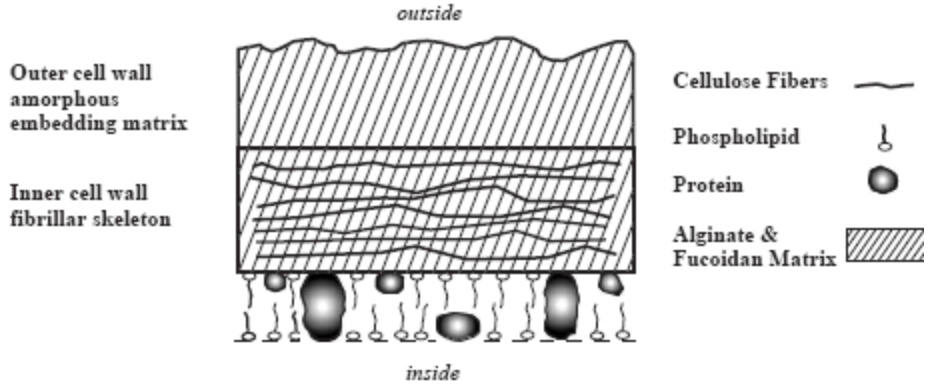


Figure 1.7. Cell wall structure in brow algae (Davis *et al.*, 2003).

1.5.1 Alginate

Alginate is the major structural organic component in the matrix of brown algae and accounts for 10-45% of the thallus dry weight (Kloareg and Quatrano, 1988; Moen *et al.*, 1997c; Horn *et al.*, 1999). It also occurs as capsular polysaccharides in soil bacteria (Smidsrod and Draget, 1997). Brown algal cells are embedded in a three-dimensional continuous network. The bulk of the alginate is located in this intercellular matrix as a gel containing sodium, calcium, magnesium, strontium and barium ions (Smidsrod and Draget, 1997). In the intercellular matrix alginate may account for more than 80% of the organic matter, for example alginate contributes up to 40% of the *Laminaria hyperborea* dry weight (Horn *et al.*, 1999), or up to 30% of *Ascophyllum nodosum* dry weigh (Moen *et al.*, 1997b). Alginate cements cell together, giving both mechanical strength and flexibility to the algal tissue (Smidsrod and Draget, 1997; Rayment *et al.*, 2009).

Alginates are salts of alginic acid, a linear copolymer of β-1,4-D-manuronic acid (M) and α-1,4-L-guluronic acid (G). The residues are organisazed in blocks of polymanuronic acid (M-M), polyguluronic acid (G-G) and heteropolymeric sequences of guluronic and manuronic acid (M-G) (Moen *et al.*, 1997c). See Figure 1.8. The proportion of these two constituents changes depending on the genus of the algae and from which part of the plant the polysaccharide is extracted (Davis *et al.*, 2003; Leal *et al.*, 2008).

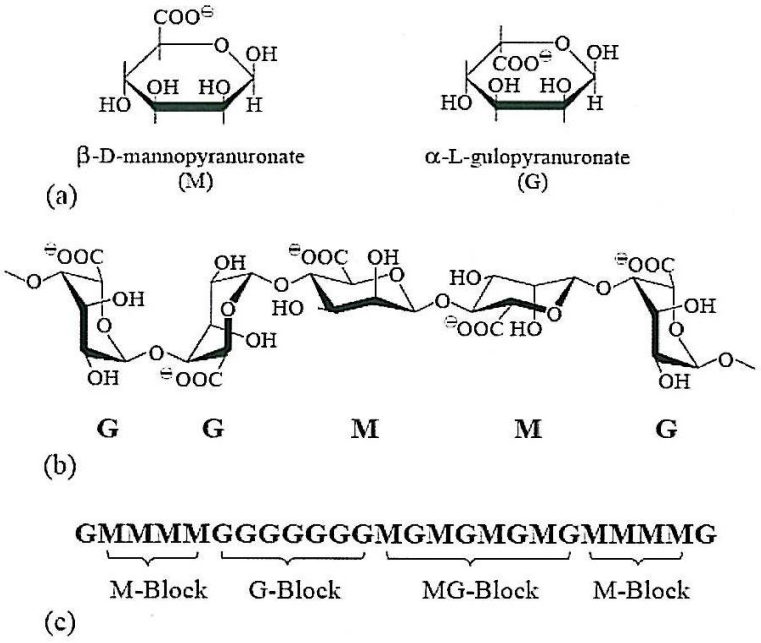


Figure 1.8: Alginate structure: a) structural monomers M and G; b) alginate polymer; c) alginate polymer sequence (Smidsrod and Draget, 1997).

Sequences containing more than one G residue combine with other similar sequences binding calcium ions in between them to form junction zones in calcium alginate gels in the so-called egg-box model (Figure 1.9). Such cooperative ionotorpic gelation only occurs when the length of the G-blocks involved in the dimerization exceeds a certain length (Stokke *et al.*, 1991).

Enzymatic degradation of alginate is catalysed by alginate lyases (EC 4.2.2.3). These enzymes have been found in microorganisms, marine algae, marine molluscs and echinoderms. Alginate lyases may have different specificities against different block structures of the alginate polymer (Moen and Ostgaard, 1997), and they function best around neutral pH (7-8; Moen *et al.*, 1997b).

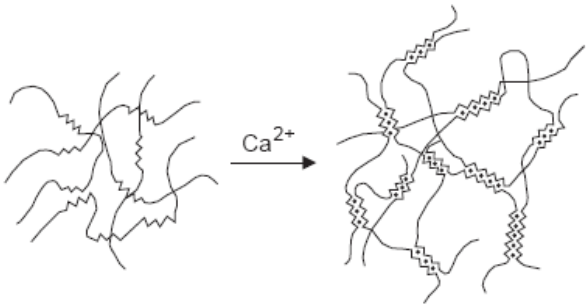


Figure 1.9: Schematic representation of the calcium-induced gelation of alginate in accordance with the "egg-box" structure (Davis *et al.*, 2003).

1.5.2 Cellulose

Cellulose is a linear and unbranched polysaccharide consisting of β-(1→4) bonded D-glucose units (Figure 1.10). They occur as ordered fibres, mostly in the inner cell wall (Figure 1.7; Davis *et al.*, 2003). Hydrogen bonding between polysaccharides chains, which may be composed of up to 3000 monomers, gives the fibres their strength. Cellulose content of *Ascophyllum nodosum* and *Laminaria* were determined to be 7% and 20% respectively (Davis *et al.*, 2003).

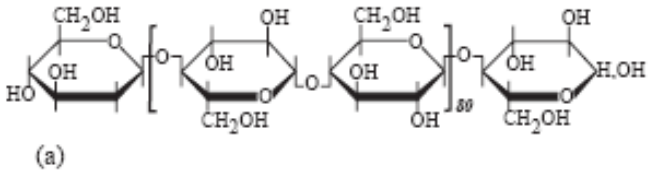


Figure 1.10: Algal cellulose (Davis *et al.*, 2003).

1.5.3 Fucoidans

Algal fucoidan (Figure 1.11) is mainly composed of sulphated fucose. Brown seaweeds produce families of sulfated fucoidans among other polysaccharides. They frequently contain other sugars besides L-fucose, namely, D-xylose, D-galactose and D-glucuronic acid (Rioux *et al.*, 2007). However, additional sugars like D-manose and D-glucose, have also appeared (Duarte *et al.*, 2001). Fucoidan from *Ascophyllum nodosum* has been identify with the following structural characteristics: [→3)-L-α-Fuc*p*-(2SO^-_3)-(1→4)-α-L-Fuc*p*-(2,3SO^-_3)-(1→]n. (Chevolot *et al.*, 2001).

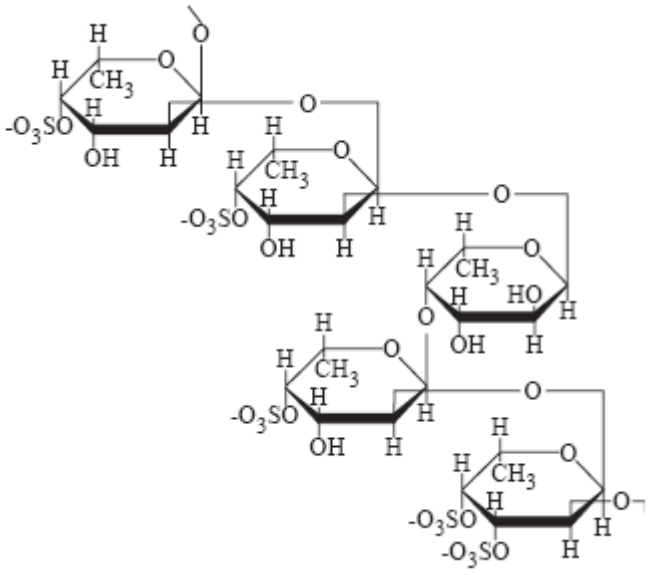


Figure 1.11: The structure of fucoidan, a branched polysaccharide sulfate ester with l-fucose building blocks as the major component with predominantly að1-2Þ-linkages (Davis *et al.*, 2003).

Fucoidans may constitute up to 25-30% of the algal dry weight, depending on the seaweed species and, to a lesser extent on season (Kusaykin *et al.*, 2008). The fucoidans of *Pelvetica canaliculata* represent up to 40% of the cell wall dry weight (Descamps *et al.*, 2006). Fucoidans are found in most brown algae, but are most abundant in species that grow in the intertidal zone. When the seaweeds are exposed to the atmosphere at low tide, many of them exude a slime that provides the seaweed with essential protection against desiccation. This slime is a complex proteoglycan where fucoidan is one of the building units (Horn, 2000).

Until now, enzymes degrading fucoidans have only been found in marine organisms: microbes and invertebrates such as the mollusc *Pecten maximus* (Daniel *et al.*, 1999). *Proteobacteria* and *Bacteriodetes* sps associated with brown seaweeds, as well as sea cucumber and sea urchin are the best producers of fucoidanases (Kusaykin *et al.*, 2008). Bacterial fucoidanolytic activity has been estimated at optimum pH 7.5 and 20-25^oC temperature (Descamps *et al.*, 2006).

1.5.4 Proteins

The protein content of seaweeds varies greatly and demonstrates a dependence on such factors as season and environmental growth conditions (Dawczynski, 2007). Generally, the protein content of brown seaweeds is low, between 3 and 15% of the dry weight, in comparison with green and red seaweeds (10 to 47% of the dry weight). Aspartic and glutamic acids together constitute a large part of the amino acid fraction in most of the seaweeds (Fleurence, 1999).

1.5.5 Storage Carbohydrates

Mannitol and laminaran are storage carbohydrates in brown seaweeds. Both of them vary seasonally, being produced and accumulated during the light season and consumed during the winter (Ragan and Jensen, 1978; Horn and Østgaard, 2001). Mannitol can constitute up to 30% of their dry weight (Davis *et al.*, 2003). Laminaran (Figure 1.12) is composed of (1,3)-β-D.glucan with β(1,6) branching (Rioux *et al.*, 2007). The variations in the degree in branching affect to the solubility of the polysaccharide. For example laminaran containing only β-(1,3)-linked residues is water-insoluble, while branched laminaran tend to be water soluble (Read *et al.*, 1996; Horn, 2000).

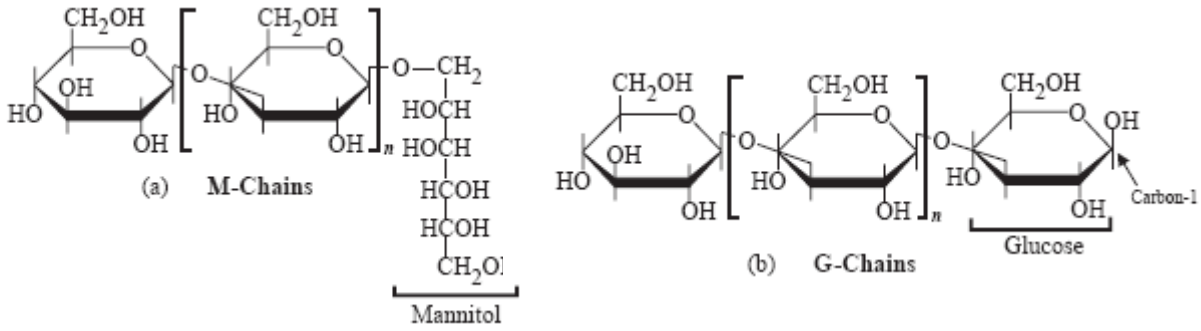


Figure 1.12: The structure of Laminaran. In (a) mannitol is attached to the reducing end (M-chains), whereas in (b) glucose is attached to the reducing end (G-chains). (Davis *et al.*, 2003).

Glucanases are relatively widespread, and many microorganisms can hydrolyse laminaran to its glucose monomer, a good substrate for fermentation. Mannitol, on the other hand, is not readily fermented (Horn *et al.*, 2000).

1.5.6 Polyphenols

Phaeophyta have cell vacuoles called physodes, which contains polyphenols (Haug and Larsen, 1958). Brown seaweed often acumulate large quantities of phenols (Moen *et al.*, 1997c). Polyphenols in marine brown algae, the phlorotannins, are formed by the polymerisation of phloroglucinol (1,3,5 trihydroxybenzene, Figure 1.13) and have a molecular weight ranging from 126.5 Da to 650 kDa (Moen *et al.*, 1997b). Phlorotannins found in the brown alga *Eisenia bicyclis* and *Ecklonia kurome* (from the Japanese coast) are shown in Figure 1.13.

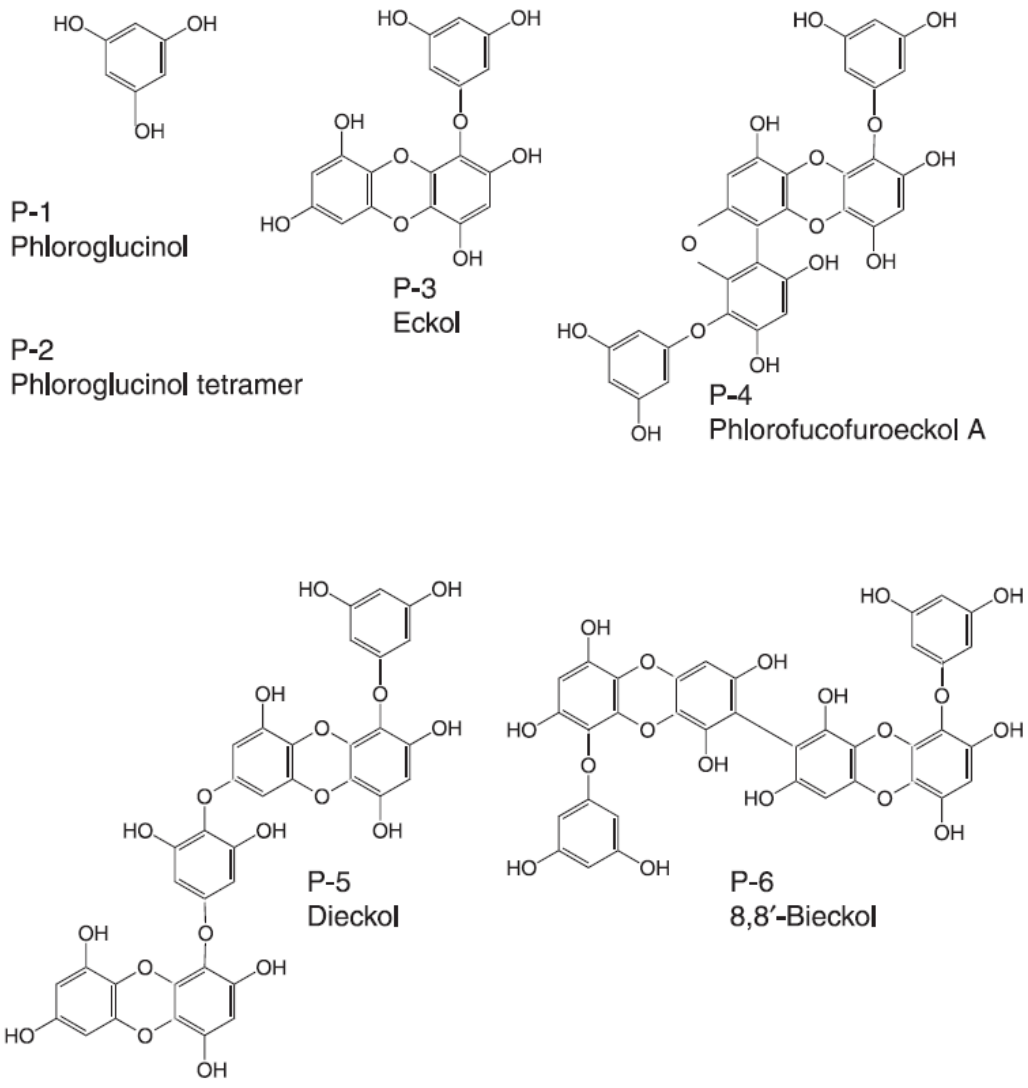


Figure 1.13 Phlorotannins found in the brown alga *Eisenia bicyclis* and *Ecklonia kurome* (Shibata *et al.*, 2002).

The dry matter of *Aschophyllum nodosum* contains up to 14% polyphenols, this content varies significantly with season, locality, salinity, light and nutrient concentration (Haug and Larsen, 1958; Ragan and Jensen, 1978; Connan *et al.*, 2007). Haug and Larsen (1958) reported that these reducing compounds were responsible for the formation of brown-colored substances in alkaline seaweed extracts.

Phlorotannins play an essential role in the protection of brown algae against grazers; they also take part in the thallus anchoring to the substrate and in the elaboration of the cell wall (Connan *et al.*, 2007). Reactive compounds such as polyphenols may have toxic effects on the microbes. The presence of phenolic compounds in the algae may lead to discolouring of extracted alginates, and is also responsible for oxidative reductive depolymerisation (ORD) leading to a loss in viscosity (Moen *et al.*, 1997c). Formaldehyde leads to a structural change in the physodes with the result that extracts of material treated with formaldehyde show no reducing power (Haug and Larsen, 1958).

1.6 *Ascophyllum nodosum* composition

The composition of this brown seaweed varies with different factors such as location, salinity and seasons. For *A. nodosum*, the summer season (mid June to August) is characterized by a high content of dry matter, but during these months there are no individual chemical constituents at maximum. The first part of winter season is characterized by significant accumulation of mannitol, laminaran and alginate. Tocopherols, vitamin B12 and ether-soluble materials are at high levels, whereas photosynthetic pigments, protein, niacin, iodine and calcium are at low levels. As the winter season progresses, levels of storage reserves (mannitol, laminaran) decrease, and some inorganic constituents reach their maximum values (Ragan and Jensen, 1978).

In the following table the chemical composition of *A. nodosum* is described together with notes on changes through the year.

Table 1.3 *Ascophyllum nodosum* chemical composition

Component	Percentage	Notes	Ref.
Water	67-82	Decreased with salinity. Lowered during the spring	1
Ash	18-24	Increased from autumn to spring.	1,2,5
Proteins	4.8-9.8[(1)], 1.2[(2)] and 5-12[(7)]	Increased from autumn to spring.	1,2,7
Lipids	1.9-4.8[(1,7)] and 1.2[(2)]	Increased from early spring to late autumn.	1,2,7
Sugars (general)	42-64[(7)] and 69.6[(2)]		2, 7
Alginic acid	24-29	Maximum Sep-Nov; minimum May-Jun	1,7,5
Laminaran	1.2-6.6[(1)] and 10[(7)]	Increased from spring to late autumn.	1,7
Mannitol	6.8-10.4[(1)] and 4.2[(7)]	Increased from early spring to early autumn	1,7
Fucoidan	4-10		3
Fibre (cellulose)	3.5-4.6	Quite constant during the year.	1
Polyphenols	0,5-14	Lowered during the spring (April to May). Increased greatly with salinity	3,4,5,6
Iodine	0.06-0.09	Max. Feb-May; min. Aug-Nov	1,5
K	2-3		1
Na	3-4		1,7
Ca	1-3	Max. in May; min. Aug-Sep	1,5,7
Mg	0.5-0.9	Max. in Jul; min. in Mar	1,5,7
S	2.5-3.5		1
P	0.1-0.15		1

Water content is given as a percentage of fresh weight and all other components as a percentage of the dry weight.

References: 1.(Jensen, 1960), 2.(Rioux *et al.*, 2007), 3.(Horn, 2000), 4.(Haug and Larsen, 1958), 5.(Ragan and Jensen, 1978), 6.(Ragan and Jensen, 1977) and 7.(Morrisey *et al.*, 2001).

1.7 Products from *Ascophyllum nodosum*

Ascphyllum nodosum is the most important species of seaweed collected in Ireland and has been used for the past 40 years. The weed is mainly used as the raw material for seaweed meal (section 1.4.1), which is mainly exported for the extraction of alginates. However, horticulturists, market gardeners organic farmers and fruit growers are increasingly using both dried seaweed meal and liquid extracts in soil conditioning and crop spray applications (Morrisey *et al.*, 2001). This seaweed is not normally used directly as a foodstuff but may appear as a constituent of health food tablets.

This brown seaweed is also used as a stock feed additive. It may comprise up to 5% of the diet for poultry, sheep, cattle, pigs and horses. The trace elements and vitamin components of *A. nodosum* meal are the active ingredients for growth of cattle, milk production, colour in eggs and improving wool colour in sheep (Doty *et al.*, 1987). In a study carried out by Gardiner *et al*. (2008) results showed that *A. nodosum* extract in

pigs may provide a dietary means to improve gut health and potentially reduce pathogen carriage by intestinal coliform reduction.

A. nodosum liquid extracts are commercially available (Figure 1.14). Some reports have indicated enhanced plant yield, quality and health in different crops following application, although the mechanisms of action have not been fully determined. For example, it was reported a yield increase (14-25%) and heavier clusters on grape (Norrie *et al.*, 2002), and similar results were also obtained on "Italia" table grapes (Colapietra and Alexander, 2006). An application of this product to fungal infected carrot, showed the plants significantly reduced disease development by up to 57% for *Alternaria* and 53.3% for *Botrytis* fungal pathogens (Jayaraj *et al.*, 2008). Hanssen *et al*. (1987), showed a significantly better results in lettuce plants when using *A. nodosum* liquid extract from an anaerobic digestion (16-24 days) than *Laminaria hyperborea* o *L. saccharina*. Fan *et al*., (2011) showed that *A. nodosum* extract enhanced the nutritional quality of spinach (by stimulation of flavonoid synthesis), which protected the nematode *C. elegans* against oxidative and thermal stress.

Figure 1.14 Products from Acadian Seaplants Limited (Canada) a) Stimplex: all-natural Plant Growth Regulator (PGR), b) Acadian: pure extract made from Ascophyllum nodosum.

It may be important to clarify that, as was previously stated, the composition of this species changes with the location. So, extracts obtained from the same species in different places may be similar but not identical in composition.

The majority of the research in this project has been carried out on an *A. nodosum* residue, which remains following an extraction process of this liquid extract product. Several applications of seaweed wastes have been previously reported: biogas production (Carpentier *et al.*, 1988; Kerner *et al.*, 1991), bioethanol conversion (Ge *et al.*, 2011) and seaweed composting (Tang *et al.*, 2007; Tang *et al.*, 2011) among others. However, the objective of this research was to specifically investigate the potential for microbial digestion and characterization of the residue, derived from the seaweed extraction described in Section 1.8.

1.8 Origins of the seaweed residue evaluated in this project

The seaweed fertalizer extraction process is carried out by a local company in Tralee, County Kerry. It is a biotechnology company which develops and commercialises products derived from marine raw materials. Its products include bio-stimulants for improved plant growth, disease suppressants for plant protection, active compounds for skin care applications and powders and liquid extracts for animal health.

For the seaweed extraction process a dry meal of *Ascophyllum nodosum* is used as a raw material. This meal is produced by Arramara Teoranta, as explained in section 1.4.1.

In Tralee, dried *A. nodosum* meal is processed by an alkaline digestion, followed by a neutralisation and clarification of the resulting digest (Figure 1.15). Seven hundred and fifty kilograms of dried meal (Figure 1.16a) is mixed with 5100 litres of tap water and 50kg of potassium carbonate. All the ingredients are heated at 85-95^{o}C at 3-5 cyclical bar range, for 24 hours. This process occurs at the same time in three autoclaves which are heated by steam.

Following this process, the digest is filtered through decanters which separate the liquid portion from the cake. The liquid is then treated to remove sand and is supplemented by mineral addition. The final stage is evaporation to reach a concentration of 10% total solids (Figure 1.16b). This product is dispensed into containers of 5, 25 and 1000 litres. The waste left after filtration (1500kg) is the *Ascophyllum nodosum* residue on which this research project is based (Figure 1.16c).

The seaweed residue is currently removed for land-spreading. This land-spread solution is finite, because it requires local authority permission and constitutes a production cost that reduces competitiveness. For each batch of seaweed extract produced, 1500 kg of seaweed residue requires remediation. The residue is still rich in nutrients and offers the potential for other added value products.

The harsh conditions associated with the initial commercial seaweed extraction suggest that seeking a more complete digestion of the original material would be either hazardous or economically un-viable. However, if a cost-effective, more complete digestion were possible by alternate means, then a supplementary product or extract might arise.

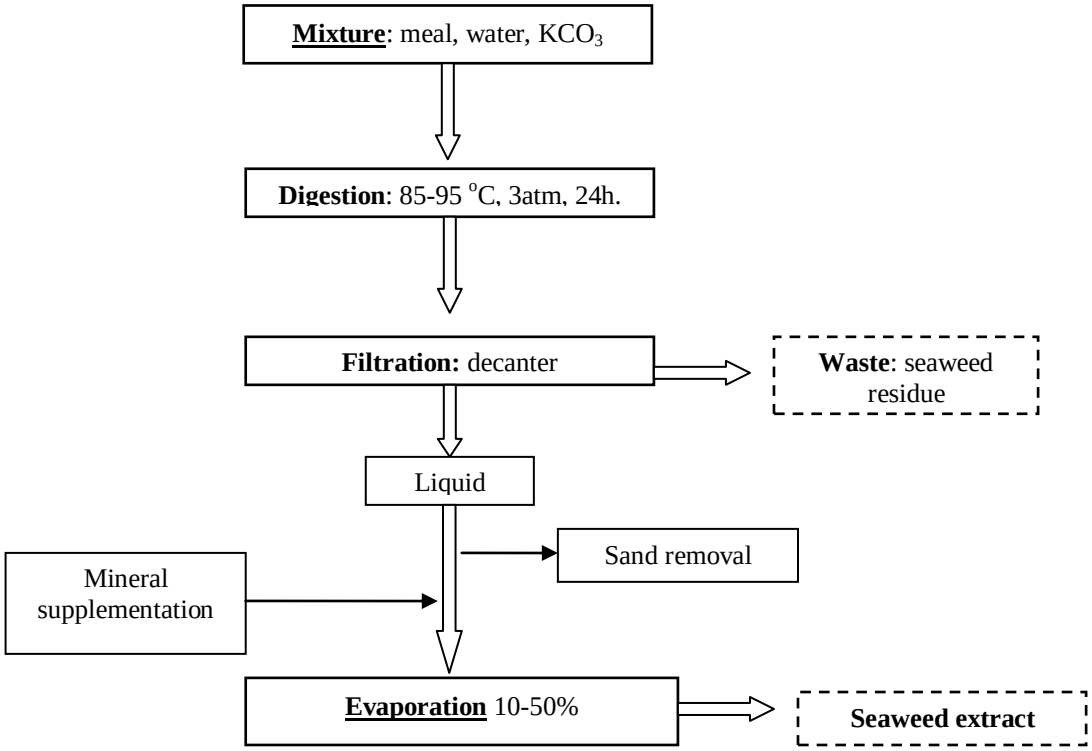


Figure 1.15 *A. nodosum* liquid fertilizer extraction process.

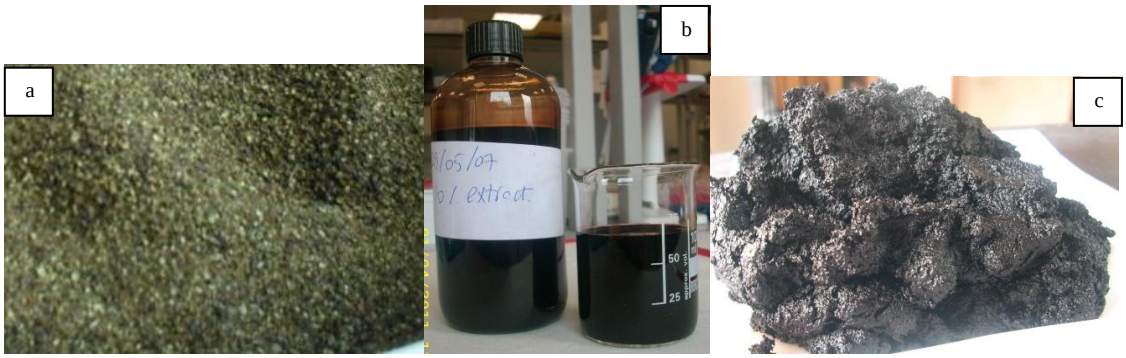


Figure 1.16 *A. nodosum* meal (a), liquid fertilizer (b) and residue (c)

1.9 Microbial digestions of brown seaweeds

The development of a digestion system for *Ascophyllum nodosum* residue from the liquid fertilizer extraction industry is a novel proposal. Different types of microbial digestions have been carried out in brown seaweeds before with different objectives. Hanssen *et al* (1987), investigated the potential of *Laminaria hyperborea*, *Laminaria saccharina* and *Ascophyllum nodosum* as sources of biogas by anaerobic digestion

(batch and semi-continuous cultures at 35^{o}C). It was found that the gas production of the two Laminaria species was almost double than the obtained for *A. nodosum*. Retentions times of less than 8 days gave a partially digested material which was tested as an additive to peat briquettes; while retention times of 16-24 days resulted in extensive degradation which yielded a water soluble extract after filtration and evaporation. This material was tested as a fertilizer on lettuce, finding that *A. nodosum* liquid extract showed significantly better results than the *Laminaria sp*., or than other fertilizers.

Kerner *et al.* (1991), worked out an anaerobic digestion system for two waste sludges produced during the industrial extraction of alginate from two algal species: L*aminaria hyperborea* and *Ascophyllum nodosum*. The process compromised batch and semi-continuous cultures at 35^{o}C and stirring intermittently. The pH was maintained around neutral value by adding 4M NaOH. The volatile solids were reduced by 20-40 % (batch) and 40-50 % (semi-continuous), and the advantage of generating an energy source (methane) as a by-product was found. Moen *et al.* (1997b), carried out aerobic and anaerobic digestions of *A. nodosum*. Batch reactors were operated at 35^{o}C and pH 7, and feed with algal material, nutrients and inocula adapted to seaweed degradation. An inhibitory effect of the polyphenols was found on both alginate lyases activity, probably due to the non-specific binding by polyphenols to the enzymes, and methane production. Aerobically, less influence was found by phenols, as the polyphenol concentration was reduced. The anaerobic degradation was greatly stimulated when the polyphenol concentration was fixed by adding a low concentration of formaldehyde (0.1%).

Laminaria hyperborea stipes were aerobically degraded at different levels of polyphenols (Moen *et al.*, 1997c), by manipulating the amounts of peripheral tissue. It was found that the degradation of organic matter was clearly depressed by increasing the amount of peripheral tissue, as also the alginate lyase activity, presumably due to the release of reactive polyphenols. When carrying out an anaerobic degradation of the same seaweed (Moen *et al.*, 1997a), it was shown the alginate lyases were less affected by the polyphenols. This relation between the phenol content in *L. hyperborea* under aerobic and anaerobic incubation seemed to work in opposite direction of what happened in *A. nodosum*.

In some cases, only isolated species of microorganism have been investigated, e.g. Quatrano and Caldwell (1978) characterized a marine bacterium isolated from the tissue of the brown seaweed *Fucus distichus*. This strain utilizes all the major polysaccharides of brown and red algae as a carbon sources, by the release of extracellular hydrolases into the medium. This study was extended to the isolation of marine bacteria capable of producing different specific lyases for alginate degradation (Doubet and Quatrano, 1982). Horn *et al.* (2000), fermented *Laminaria hyperboera* extract for ethanol production. The yeast *Pichia angophorae* was shown to ferment mannitol and laminaran in seaweed extract simultaneously to ethanol, requiring a pH 4.5-5 and 30^{o}C temperature. Descamps *et al*. (2006), reported a Flavobacteria strain which secretes fucoidan –hydrolase activity.

In other cases, enzymatic degradation of different components of brown seaweed has been studied. For example, Daniel *et al*. (1999), showed *A. nodosum* fucoidan degradation by an enzymatic activity from the digestive glands of the mollusc *Pecten maximux*. In this case, pH 5.5 and 30^{o}C temperature was required.

A digestion of complex organic matter is carried out by a range of microorganisms living in an ecological community. A complete process for anaerobic digestion of brown algae requires a mixed microbial population (Moen *et al.*, 1997a). Polymeric and particulate matter is not directly accessible for microorganisms, and the first step in the anaerobic break down is hydrolysis. Protein, polysaccharides and lipids are depolymerised to mono- and oligomers by extracellular enzymes. There is no energy gain in this depolymerisation, and the same microorganisms, accompanied by other non-hydrolytic fermentative, utilize the products of this hydrolysis. Notably, the presence of readily available substrates may inhibit the production of hydrolytic enzymes (Horn and Ostgaard, 2001). Incomplete digestion could be related to suboptimal adaption of the microflora. Inhibition of digestion may occur due to high concentration of substances such as polyphenols, heavy metals, sulfides salts and volatile acids (Moen *et al.*, 1997a).

Considering all this published information, a microbial digestion of *Ascophyllum nodosum* residue will be developed, as also, an increase of the knowledge in this area will be contributed. As stated by Montgomery (2004), the biological world does not produce materials that it cannot decompose, however the residue contains added potassium carbonate and materials that are largely resistant to chemical and physical digestion, and therefore represents a challenge when pollution is to be avoided.

1.10 Objectives of Research

This research represents the first undertaken analysis and the first attempt at microbial digestion of the by-product of seaweed extraction, generated by a local company. The ultimate aim of the research is to determine an alternate fate of the by-product – other than land-spread. This project was divided into the following main objectives.

The first objective of this project was to study the composition of *Ascophyllum nodosum* residue. Although the composition of the seaweed itself has been broadly determined and the composition of the commercial extract established, the composition of the residue was completely unknown. It was felt that an essential early component of the project was to determine a proximal analysis of the residue following the extraction procedure. Further, as seaweed is a natural product whose composition is subject to seasonal variation, it is important to determine what, if any variations occur in the residue components. Consequently the composition of the residue was monitored on a monthly basis for a full calendar year (Jan-Dec 2007).

The subsequent aim of this research, following discussions with the industrial partner, was to develop methodology for the microbial digestion of the residue. There are several possible outcomes to digestion: (1) the development of a novel product or products which could be exploited commercially, (2) the development of a novel protocol or protocols, or (3) resolubilisation of the residue with subsequent recycling back into the extraction process. The factors affecting the microbial digestion of *A. nodosum* residue were also studied. The compositional change in the residue during the digestion process was monitored. In addition, knowledge gleaned from the development of this process could be useful in solving similar challenges for other raw materials and by-products.

The third objective of this research was to study and characterize the microorganisms present in *A. nodosum* residue and involved in its digestion process and to determine their requirements to improve the digestion. Basic properties of microbial population isolated from the seaweed residue and from the inocula employed in the digestion process were studied. This was followed by identification of the microorganisms involved, with the utilization of phenotypic pattern techniques. These studies informed the development of the subsequent research. The industrial partner was informed of the progress of the research and was most helpful in pointing to, from an industrial perspective, feasible end-points to each element of the project.

The last objective was to screen the digest of the seaweed residue and the brown seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea* for inhibitors of hyaluronidase and elastase activities. These two enzymes are involved in the turnover of the main components of the skin matrix. To-date seaweed-derived peptides have not been evaluated for this application, although seaweed-derived extracts are common and acceptable, marketable ingredients in cosmetic products. Methodology to generate protein and peptide fractions from the seaweeds and a commercial extract was also developed.

2. MATERIAL AND METHODS

In this research project the composition of *A. nodosum* residue was studied and methodology for the microbial digestion was developed. Some studies were carried out in the microbial populations of the residue and of the different inocula used. Also digestion samples and *A. nodosum* and *Laminaria hyperborea* seaweeds were screened for anti-hyaluronidase and anti-elastase activities. The methodology and materials employed for all these studies are explained in the following sections.

2.1 Samples: collection and storage

Residue samples were collected after the extraction process. They were carried to the Institute of Technology Tralee and placed directly into the fridge (4ºC) until further analysis. A small portion of each sample was kept in the freezer (-80ºC) for later analysis.

For the compositional analysis, residue samples were collected every month over a full calendar year (Jan-Dec 2007). For the analysis of *Ascophyllum nodosum* meal, extract and residue from the same extraction process, the samples were collected on 28th June 2007. Meal was kept at room temperature in dark conditions, and the residue and the extract were stored at 4^{o}C.

For the microbial digestion, only freshly obtained residue samples were used, with duration of storage typically 1-7 days.

Fresh seaweed samples of *A. nodosum* and *L. hyperborea* were collected on 19th October 2009 at Fenit Island (Co. Kerry, Ireland; Lat: 52.17 Long: 9.52), and stored at -80^{o}C.

2.2 Compositional Analysis

Several chemical tests were used to analyse the composition of the *A. nodosum* residue. Monthly analysis was carried out during a full calendar year. The analysis involved study of total solids, moisture, ash, pH, total nitrogen (protein), lipids, fibre and polyphenols. The changes of the different measures during the year were noted. Metal analysis and fatty acids analysis were also studied in selected samples. The methods utilized are listed in the following sections.

2.2.1 Total Solids and Moisture

The total solids content of a sample is calculated from the amount of material left as a residue upon drying at 105ºC to a constant weight. All the results in this research are reported on a dry weight basis.

A) Materials

- Crucible
- Balance (Mettler Toledo, Switzerland)
- Convection Oven (Lab-Line Instruments, Inc., USA)

B) Method

An amount of sample, between 3 and 10 g, was removed from different points of the original sample (to ensure that it was representative), weighed and placed in a pre-weighed crucible. The sample was accurately weighed on an analytical balance and its weight was recorded. The crucible with the sample was placed in a convection oven at 105 +/- 2 ^{o}C and dried to a constant weight (± 0.1% change in moisture content upon 1 hour of reheating). The crucible with the sample was removed from the oven and placed in a desiccator. It was allowed to cool to room temperature and it was weighed. For each month, between 3 and 6 replicates were tested, depending of the amount of sample that was received.

C) Calculation

Total solids and moisture percentages were calculated as follows. Results are given by percentage of fresh sample.

$$\% \text{ Total Solids} = \left[\frac{(\text{Weight of dried sample plus crucible} - \text{weight of crucible})}{\text{Weight of wet sample}} \right] \times 100$$

$$\% \text{ Moisture} = 100 - \left[\frac{(\text{Weight of dried sample plus crucible} - \text{weight of crucible})}{\text{Weight of fresh sample}} \right] \times 100$$

2.2.2 Ash

The ash content of a sample is calculated from the amount of material left as a residue upon combustion at 550ºC.

A) Materials:

- Crucible
- Balance (Mettler Toledo, Switzerland)
- Muffle Furnace (Carbolite, UK).

B) Method:

Previously dried samples (section 2.2.1) were placed into a furnace at 550ºC (Aguilera-Morales *et al.*, 2005) for 6 h. They were cooled to room temperature in a desiccator and weighed. Between 3 and 6 replicates were tested for each monthly sample.

C) Calculation:

Total percentage of ash on a dry weight basis was calculated as follows:

$$\% \text{ Ash} = \left[\frac{(\text{Weight of ash sample plus crucible} - \text{weight of crucible})}{\text{Weight of dry sample}} \right] \times 100$$

2.2.3 Kjeldahl Method for Total Nitrogen Determination

This method involves the determination of the total nitrogen content of samples and the subsequent conversion of this quantity into total protein, based upon the assumption that the nitrogen originates mainly from protein (Roca *et al.*, 2003). The method is divided in three steps:

1) Digestion: mineralization of the nitrogen from the proteins with sulphuric acid. Potassium Sulphate is used to raise the boiling point of the mixture, and Selenium is added as a catalyst. This step proceeds according to the reaction:

$$\text{N from proteins} + H_2SO_4 \Longrightarrow (NH_4)_2SO_4 + H_2O + CO_2 + \text{other products}$$

2) Distillation: the NH_4 from the digestion is transformed to ammonia when the digest is made alkaline with sodium hydroxide. The ammonia is volatile and is readily removed by steam distillation and trapped in a receiving flask of boric acid.

$$(NH_4)_2SO_4 + 2NaOH \Longrightarrow 2\,NH_3 + Na_2SO_4 + 2H_2O$$

$$NH_3 + H_3BO_3 \Longrightarrow NH_4^+:H_2BO_3 + H_3BO_3$$

3) Titration: the complex $NH^{4+}:H_2BO_3$ is soluble and makes the boric acid alkaline. Using a pH indicator (e.g. universal indicator), titration with hydrochloric acid (HCl) quantifies the ammonia present.

$$NH_4^+:H_2BO_3 + HCl \Longrightarrow NH_4Cl + H_3BO_3$$

The total volume of HCl used is proportional to the amount of nitrogen present in the sample. One mole of HCl is equivalent to 14 grams of nitrogen. The total protein can be determined by multiplying the total nitrogen content by 6.25 or an equivalent value reflecting the proportion of nitrogen (approximately 16%) in proteins (Sánchez-Machado *et al.*, 2004; Aguilera-Morales *et al.*, 2005; Dawczynski, 2007).

A) Materials:

- H_2SO_4 (Concentrated; 96%, Riedel- deHaën, 107208)
- Kjeltabs (Thompson & Capper LTD.; AA08): each tablet contains 3.5 g Potassium Sulphate and 3.5 mg Selenium.
- NaOH 10M (AnalaR, 102525P)
- Boric Acid 1% (Merck, 100165)
- pH indicator (Hopking & Williams, 886030)
- HCl 0.1 M
- Kjeldahl heating block (Tecator, Sweden)
- Kjeldahl distillation apparatus (2100 Kjeltec Distillation Unit, Foss Tecator, Sweden).

B) Method:

Digestion: residue sample was dried in an oven at 105 ± 2ºC until a constant dry weight was achieved (Section 2.2.1). A known weight (between 2 and 3 g) of dry sample was placed in the digestion tube. Two Kjeltabs and 25 ml of H_2SO_4 were added. The tubes were placed in the heating block and heated for 2 hours at 400 ºC. The tubes were allowed to cool after this step.

Distillation and Titration: a digestion tube was placed in the Kjeldahl distillation apparatus, which automatically added 90 ml of 10M NaOH. This volume was selected

empirically to ensure alkalinisation of the digest. The apparatus proceeded with steam distillation (for 5 minutes) after addition of the sodium hydroxide. The ammonia vapour was collected in a collection flask with 100 ml of Boric acid (1% w/w). After the distillation, a few drops of pH indicator were added. The distillate was then titrated with 0.1mol/L HCl until a yellow end-point became evident. Each monthly sample was studied in triplicate.

C) Calculation:

The amount of protein present in the sample was calculated as follows. Final results are given in percentage of dry sample.

$$\% \text{ Protein} = \left[\left(\text{X ml of HCl} \times \frac{\text{0.1 mol HCl}}{\text{1000 ml HCl}} \times \frac{\text{14 g Nitrogen}}{\text{1 mol HCl}} \times \frac{\text{100 g Protein}}{\text{16 g Nitrogen}} \right) / \text{g. dry sample} \right] \times 100$$

2.2.4 Soxhlet Extraction

This method was described by Soxhlet in 1879 and it is commonly used for the extraction of lipids from solid samples. The oil and fat from the solid material are extracted by repeated washing (percolation) with an organic solvent in special glassware called “soxhlet apparatus”.

A) Materials:

- Petroleum Ether (40-60 ^{o}C): Methylated spirits (1:1); (Romil Ltd., H601).
- Cellulose Thimbles (Lennox, single thickness, 18 x 55 mm).
- Soxhlet Apparatus: flask, extraction chamber and condenser.
- Electro mantle (Electrothermal, England)
- Vacuum rotatory evaporator (Bibby Sterilin Ltd, England)

B) Method:

Before finalising the experimental procedure, a pilot study was carried out with the seaweed residue to optimise the process for lipid extraction. Different mixtures of solvents were tried as well different times of extraction. The following criteria were determined: (1) use of the residue in its wet form, (2) use a mixture of Petroleum ether

and methylated spirits in the proportion 1:1 and (3) run the extraction of each sample for 5 hours.

A known amount of the wet residue sample, between 5 and 8 g, was weighed into a cellulose thimble and placed into the extraction chamber. Solvent mixture (150 ml) was added to a weighed flask. Once ready, the soxhlet apparatus (Figure 2.1) was attached to the heating mantle. The heat was regulated to allow approximately 1 cycle of solvent extraction *per* 40 seconds. The condenser was cooled with cold tap water. Each monthly sample was run for 5 hours in triplicate.

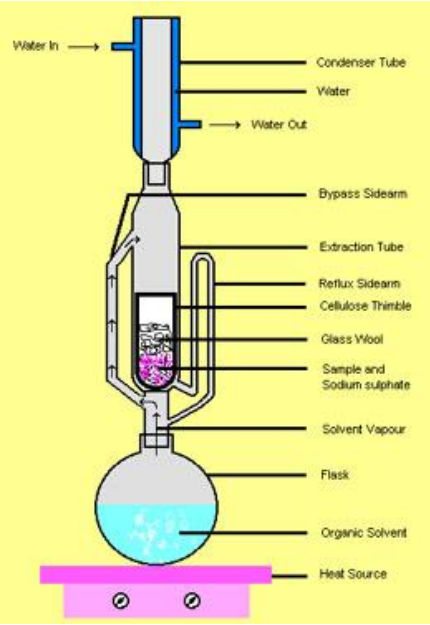


Fig. 2.1: Soxhlet Extraction Apparatus.

After the extraction, solvent and lipid extract were left in the flask. The solvent was evaporated by vacuum rotatory evaporation with a temperature controlled bath (40^{o}C) (Punín Crespo, 2005, 2006). Then the flask was allowed to cool in a dessicator and weighed. Each sample was studied in triplicate. The amount of lipid present in the commercial extract was measured using the glass separating funnel technique (Section 2.2.5).

C) Calculation:

$$\% \text{ Lipid} = \left[\frac{(\text{Weight of lipid sample plus flask} - \text{weight of flask})}{\text{Weight of dry residue sample*}} \right] \times 100$$

*Dry sample was calculated using % total solids for each month.

2.2.5 Glass separating funnel technique for lipid extraction

The lipid content determination in the extract sample was carried out using a different method of the soxhlet extraction, due to the sample being liquid. This is a simple technique where the sample and an immiscible solvent are mixed in a glass separating funnel so that the lipid contained in the sample transfers to the solvent.

A) Materials:

- Petroleum Ether (40-60 ^{o}C; Romil Ltd, H601)
- Glass funnel
- Vacuum rotary evaporator (Bibby Sterilin Ltd, England)
- Round flask

B) Method:

One millilitre of the extract sample was place in the glass funnel and diluted with deionised water. Petroleum ether (10 ml) was added and mixed with the sample. The mixture was allowed to settle until two layers were clearly distinguishable: petroleum ether with lipids at the top and the extract underneath. Very carefully both layers were separated, using a pre-weighed flask for the petroleum ether and the lipids. This process was repeated several times to ensure no lipid was left in the extract. The solvent was evaporated by vacuum rotary evaporation with a temperature controlled bath (40^{o}C) (Punín Crespo, 2005, 2006). The flask was allowed to cool in a dessicator and weighed.

C) Calculation:

Calculation was done as follows in the next formula. Final result is given as percentage (w/w) of dried sample.

$$\% \text{ Lipid} = \left[\frac{\text{(Weight of lipid sample plus flask – weight of flask)}}{\text{Weight of extract dry sample*}} \right] \times 100$$

*Dry sample of the extract was calculated using % total solids.

2.2.6 Fibre

Seaweeds are rich in fibre. Brown algae fibres are mainly cellulose and insoluble alginates. The amorphous, slimy fraction of brown algae fibres consists mainly of water-soluble alginates and/or fucoidan. The typical algae carbohydrates are not digestible by the human gastrointestinal tract and, therefore, they are dietary fibres (Dawczynski, 2007).

A) Method:

Fibre content was determined as the difference between total solids the percentage of protein, lipid and ash (Roca et al., 2003). This calculation assumes that carbohydrates that are not part of the fibre were either not present or present in very low concentration in the *A. nodosum* residue due to the high temperature used in the extraction process. It was felt that any soluble carbohydrate would have been removed. Other methods for determination of fibre content require the use of enzymes to remove/breakdown carbohydrates and protein present in the samples. These enzymes have pH optima in or around neutral pH. As the extraction process uses very high concentrations of potassium carbonate which remains in the residue, the pH of the residue is in the range 8.06 – 9.51. Furthermore there is an overwhelming buffering effect which made reduction of the pH extremely difficult (Section 3.3.2). Consequently determination of fibre content by difference was the most practical method available.

B) Calculation:

The calculation was done based on the next formula:

$$\text{Fibre \%} = 100 - (\text{Ash \%} + \text{Protein \%} + \text{Lipid \%})$$

2.2.7 Measuring the pH

The pH-meter was calibrated following the standard procedure with pH 7 and 9 standards. The pH was measured in a solution that contained 5 g of residue sample and 45 ml of deionised water. This was stirred for 1 minute. Every monthly sample was measured in triplicate. The pH was directly measured in the commercial extract, without addition of water.

2.2.8 Polyphenols assay

Water soluble polyphenols were measured and compared with Gallic acid by means of a standard curve. Gallic acid is also soluble in water.

A) Materials:

- Gallic Acid (Sigma, 67384-100G)
- Folin Ciocalteu's phenol reagent (Merck, 9001.0500)

- Sodium Carbonate 7% (Riedel-deHaën; 13418)
- Spectrophotometer (Shimadzu, UVmini-1240, China)

B) Method:

Fifty ml of deionised water were added to approximately 5 g of residue sample at room temperature. For *A. nodosum* meal, milled sample was used. The samples were kept overnight in dark conditions. Next morning, the polyphenol content was measured following a method described by Slinkard and Singleton (1977). This method was slightly modified because of the nature of the residue. A test volume of 0.125 ml was taken from the liquid part of the overnight sample. Deionised water (0.5 ml) and Folin Ciocalteu's phenol reagent (0.125 ml) were added and the sample was held at room temperature for 6 minutes. Sodium carbonate (7%; 0.125 ml) and water (2 ml) were added in this order. It was held for 90 minutes before the absorbance was read at 760 nm. A standard curve for gallic acid (GA) was obtained. The polyphenol concentration of each sample was determined using the equation of the standard curve. Each residue sample was tested in duplicate.

C) Calculation:

The calculation was done in two steps. Firstly, µg GA equivalent/ml of sample were calculated from the absorbance reading following the equation of the GA standard curve (Figure 2.2), and second step to yield the final result, expressed as mg GA equivalent/ g dry residue sample.

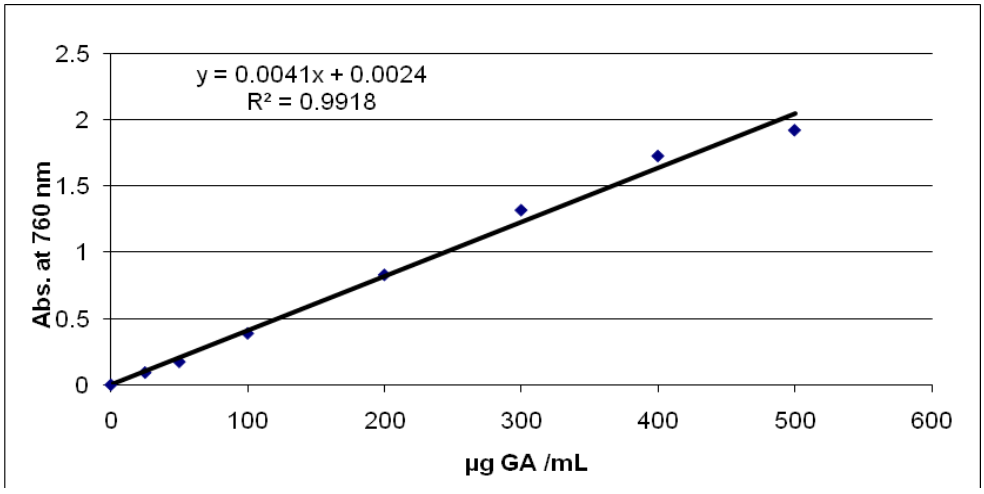


Figure 2.2 Standard curve for gallic acid.

Step 1:

µg GA equivalent/ml of sample = (Absorbance at 760nm – 0.0024) / 0.0041)

Step 2:

$$\text{mg GA equivalent/g dry residue sample} = \left(\frac{\text{µg GA equivalent}}{\text{1 ml}} \times \frac{\text{50 ml}}{\text{g of dry sample*}} \right) / 1000$$

*Dry sample was calculated using the % of TS for each month.

2.2.9 Metal Analysis

The metal analysis was carried out by two different students taking part of their 3rd and 4th year final projects respectively. For this analysis, three different methods were utilised: atomic emission spectrometry, atomic absorption spectrometry and colorimetric techniques. Depending on what type of mineral is being determined either atomic absorption or atomic emission can be used. Minerals such as sodium and potassium are determined using atomic emission and most of the other minerals are determined using atomic absorption (James, 1999).

2.2.9.1 Atomic emission and absorption spectroscopy

In the atomic emission the sample is subjected to a high energy thermal environment to produce atoms in an excited state which produces a collection of emission lines. The emission spectrum can be used for both quantitative and qualitative analysis.

In atomic absorption, if light of the correct wavelength hits a free ground state atom, the atom will absorb the light as it enters its excited state. The light must be of a specific wavelength and the amount of light which will be absorbed as it passes through a cloud of atoms is measured. The specific selection of wavelength allows determination of an element in the presence of others. The atom cloud is produced by aspirating the sample in solution into a flame aligned in a light beam.

Potassium and Sodium were studied by Atomic Emission Spectroscopy, while Iron, Calcium, Copper, Magnesium, Manganese, Zinc and Cadmium were studied by Atomic Absorption Spectroscopy.

A) Materials:

- Atomic emission and atomic absorption spectrophotometer (Perkin Elmer, AA-100, USA)
- Hot plate (Stuart, England)
- Glass wool
- Concentrated HCl
- Mineral standard solutions and light bulbs (S&J Juniper & C^{o}, England):
    - Potassium (Merck, Germany)
    - Sodium (Merck, Germany)
    - Iron (Merck ,Germany)
    - Calcium (Spectrosal, England)
    - Copper (Merck, Germany)
    - Magnesium (Reagecon, Ireland)
    - Manganese (IVA Analytical, Ireland)
    - Zinc (Merck, Germany)
    - Cadmium (Reagecon, Ireland)

B) Method:

The mineral composition was analysed on the ash sample originally from the *A. nodosum* residue (section 2.2.2). Concentrated HCL (2.5 mL) was added to the weighed ash sample (in a crucible). It was heated to boiling for 5 minutes (more HCl was added when necessary, to maintain the original volume). The sample was transferred to a beaker with the help of distilled water bringing the final volume up to 40 ml. The sample was boiled for 10 minutes and allowed to cool down. The sample was then filtered through glass wool into a volumetric flask and made up to a volume of 100 ml by adding more deionised water. This solution was used to measure the mineral compositions by atomic absorption or emission in the spectrophotometer.

Standard curves were established for each mineral (Table 2.1). Depending on which mineral was being determined the atomic emission or the atomic absorption spectrophotometer was set up according to the manufacturers requirements (different lamps, wavelengths, etc., following the standard operating procedure provided by the Institute). The standards and the sample were measured. In a few cases, the ash solution was diluted to get a more accurate result within the range of the standard curve.

Table 2.1 Studied minerals' standard curves.

	Standard curve	
Mineral	**Equation**	**R^2**
Potassium	y = 0.1771x +0.032	0.9999
Sodium	y = 0.1169x+ 0.0937	0.9995
Iron	y = 0.0227x + 0.0046	0.9996
Calcium	y = 0.0485 + 0.0107	0.9995
Copper	y = 0.0533x - 0.0003	0. 9988
Magnesium	y = 0.3607x - 0.0017	0.9973
Manganese	y = 0.0611x + 0.004	0.9995
Zinc	y = 0.1555x + 0.0072	0.996
Cadmium	y = 0.1049x + 0.0029	0.9984
Phosphorus	y = 0.6045x + 0.0508	0.9795

C) Calculation:

The concentration of each mineral in the ash solution was calculated from the equation of the standard curve (Table 2.1). This equation is $Y=MX_1+C$, where "Y" is the absorbance reading recorded and "X_1" is calculated as the concentration of a mineral in the analysed sample. For these minerals where the ash solution was diluted, "X_1" is multiplied by the dilution factor obtaining "X_2". The calculation continues with the next formula:

$$\text{mg of "mineral"/g fresh residue} = \left(\frac{x_{1 \text{ or } } x_2 \text{ µg of mineral*}}{1 \text{ ml}} \text{ x } 100 \text{ ml } \text{ x} \frac{1 \text{ mg}}{1000 \text{ µg}} \right) / \text{ g fresh residue}$$

** Take the value of "X_2" if a dilution of this mineral was made and "X_1" if not.*

A final calculation to get the concentration of each mineral in the ash sample was done as follows (James, 1999):

$$\% \text{ mineral in ash} = \frac{M}{W \text{ x } V}$$

Where M is the concentration of the dilute ash solution (from standard curve); W is the weight of ash used originally; and V, the volume of ash solution diluted to 100 ml.

2.2.9.2 Colorimetric Techniques

Colorimetric methods can be used to determine the concentration of a wide variety of minerals. Colorimetric techniques are based on the change in colour of a reagent when it reacts with a specific mineral in solution, the intensity of the colour is a measure of its concentration. The concentration of the mineral is determined by measuring the absorbance of the solution at a specific wavelength using a spectrophotometer (Thomas and Chamberlin, 1980).

Colorimetric techniques were used to measure the concentration of phosphorus in the ash sample.

A) Materials:

- Spectrometer (Thermo, UVA 131104, England)
- Phosphorus standard solution
- Ammonium molybdate solution: 100 ml of 10% ammonium molybdate added to 150 ml of sulphuric acid previously dissolved in 150 ml of distilled water.
- Stannous chloride solution: 2.5 g of stannous chloride in 100 ml of glycerol.

B) Method:

A standard range of 0.2-0.6 ppm was prepared in duplicate from the phosphorus standard solution. The appropriate amount of reagent was then added (1 ml of ammonium molybdate solution and 2 drops of stannous chloride solution) to the standards, blank and previously prepared ash sample solution (section 2.2.9.1) which had all been made up to 100 ml in volumetric flasks.

The blank was used to zero the spectrometer at 508 nm wavelength. The absorbance readings for the standards and the sample were recorded.

C) Calculation:

A standard curve was utilized to determine the phosphorus present in the sample (Table 2.1). The same two formulas presented in the previous section (2.2.9.1) were used, obtaining the results as mg of phosphorous per g of fresh residue and as percentage of phosphorus in the ash sample.

2.2.10 Fatty Acids Analysis by Gas Chromatography

Fatty acids analysis was carried out by gas chromatography (GC) after a preliminary saponification followed by methyl esterification of the lipid sample. The conversion of the fatty acids to the methyl ester form allows for subsequent separation by GC.

A) Materials:

- 0.5 M NaOH in methanol
- Boron Trifluoride in methanol, 20%. (Merck; Art. 801663)
- Saturated NaCl
- n-hexane
- Fatty acid methyl esters (F.A.M.E.) Mix C18-C20. Cat. Number:18916-1AMP. Supelco^{TM} Bellefonte USA.
- F.A.M.E. Mix GLC-20. Cat. Number: 1892-1AMP. Supelco^{TM} Bellefonte USA.
- F.A.M.E. Mix C4-C24, Cat. Number: 18919-1AMP. Supelco^{TM} Bellefonte USA.
- GC-17A Gas Chromatograph (Shimadzu, Japan)
- GC Software: Class-VP 7.2.1 SP1
- GC Column: JW Scientific DB1301, 30m x 0.320mm ID, 0.25µm film.

B) Method:

Lipid extraction was done by Soxhlet technique (see Section 2.2.4). The sample was then removed from the flask by adding 8 ml of NaOH in methanol and heating on steam bath until the lipid was dissolved. This took between 10 and 20 minutes during which time the flask was covered to prevent evaporation. Four millilitres of the dissolved solution were taken and 5 ml of boron trifluoride in methanol was added. This mixture was boiled for 2 minutes on a hot plate. After cooling to room temperature, 4 ml of saturated NaCl solution was added. Finally 2 ml of n-hexane were added to the bottom of the sample. The Fatty Acids Methyl Esters (F.A.M.E.) were extracted from the top of the solution, for the GC analysis. The analysis was carried out on two residue samples: 2nd May 2008 and 12th May 2008.

Different concentrations of the standard mixtures were prepared in n-hexane. GC analyses of the standard mixtures and the methyl-esterified samples from the residue were run under the following conditions:

- Oven: 70 ºC (3 min) to 150 ºC (at 8 ºC /min), to175 ºC (at 2 ºC /min), to 185 ºC (at 1 ºC /min), to 260 ºC (at 2 ºC / min).
- Carrier: helium, 32 cm/sec.
- Detector: Flame Ionisation Detection (FID).
- Injector:1 µl, 70 ºC, split 100:1.

C) Interpretation of the chromatographs:

FAME peaks from the three standard mixtures were identified and associated with a retention time following the next criteria:

- Length of the carbon chain: the greater the number of carbon atoms, the longer the retention time.
- Number of double bonds between the carbon atoms: more double bonds results in a shorter retention time. For example between C-14:0 and C-14:1, they will both of them appear close together on the chromatogram because both have 14 carbon atoms, but C-14:1 will appear first because the double bond.
- Closely eluting FAME have different concentrations in the standard mixture, which is also useful to differentiate them.

The FAME peaks from the *A. nodosum* residue were identified after GC by comparison with the retention times of these ones from the standard mixtures.

2.2.11 Statistical Analysis

For all the results obtained in the monthly compositional analysis (sections 2.2.1 to 2.2.8), the average and the standard deviation of each month was determined. The general composition of the residue was determined on samples collected monthly over a 12 month period, and all samples were included in calculating an overall average and a standard deviation.

The results of the analysis of the *A. nodosum* meal, extract and residue from the same extraction process were also analyzed by calculating the average and the standard deviation.

2.3 Microbial digestion

The digestion system for *A. nodosum* residue was designed on a trial and error basis initially, and optimised by evidence-directed strategy. The techniques used in each part of a digestion, the different inocula employed, culture conditions and the parameters measured are described in the following sections.

2.3.1 Initiating the digestion

To prepare for each digestion trial, conical flasks of appropriate volume were filled with deionised water and closed with cotton and aluminium foil at the top. They were sterilized in an autoclave (pbi international, Milano) at 121^{o}C and 1 bar for 15 minutes after which they were allowed to cool down to room temperature.

A. nodosum residue was weighed in an analytical balance (Mettler Toledo, Switzerland), at room temperature and was placed aseptically into a sterilized flask. Different residue: water ratios were used for the digestion trials, varying from 1.2:1 (minimally agitatable consistency) to 1:8 (highest volume of water added the industrial partner considered feasible for industrial scale). For each trial, several identical flasks were started at day 0 and incubated under the same conditions. Each flask was stopped at different time (day) to analyse the digestion progress.

2.3.2 Incubation type

Three types of incubations were used for the microbial digestion of *A. nodosum* residue: aerobic, anaerobic and mixed anaerobic/aerobic. These are explained in the following sections.

2.3.2.1 Aerobic: Shaker incubation and forced aeration

For aerobic incubation, flasks were closed with cotton wool. Two different types of incubations were used to ensure sufficient aeration of the digest.

I) Shaker incubation

Flasks were placed in the shaking incubator (New Brunswick Scientific, USA) at 30^{o}C and shaken at 200 revolutions per minute (rpm) to improve the oxygen exchange in the surface of the digestion sample. Initial trials were shaken at 75 rpm, which was increased until 200 rpm during the development of the digestion process.

II) Forced aeration

In this alternative, air was pumped into the digestion through silicone tubing attached to an air-pump (Second Nature Wishper 200, Wilinger Bros, UK) at a rate of 1.3 litre air/ min. An air-stone was attached to the end of the silicone tube in the digestion flask so ensure small bubble formation and to aid diffusion of air throughout the flask. Flasks were incubated at 30^{o}C. Air-pump was used for digests greater than one litre.

2.3.2.2 Anaerobic

For anaerobic incubation, the head-space in each digestion flask was reduced by using a smaller flask size and increasing the digestion volume. Before starting the digestion, nitrogen gas was pumped into each flask (2-3 bar) for 30 seconds at room temperature, to displace oxygen with nitrogen. Flasks were sealed with an airlock (Bubbler airlock, Young's Ubrew, UK) and incubated at 30^{o}C and 100 rpm.

2.3.2.3 Mixed anaerobic/aerobic

For combined anaerobic/aerobic digestions both anaerobic and aerobic (shaker incubation) techniques described above were applied in sequence. As small flasks were used during the anaerobic incubation, digestion samples were transferred (under sterile conditions) into a bigger flask (1L) to improve the oxygen exchange, when incubated aerobically.

2.3.3 Inocula

Digest mixtures were inoculated with different types of inocula: A, B and C.

2.3.3.1 Inoculum A

An abundant sample of the fresh seaweed *A. nodosum* was left accidentaly in a partially closed plastic bag at room temperature for 4 months. When it was inspected after this period, the seaweed was partially liquefied. The inoculum sample was collected by adding water to the digested matter and collecting the resulting liquid. The liquid was filtered through a strainer, collected in sterile universals bottles and kept frozen at -80 ^{o}C (glycerol was not used to preserve this inoculum). This inoculum was used in trial INOC-A. Inoculum A samples were thawed at room temperature. Each flask of INOC-

A was inoculated at day 0 with 6 ml of this inoculum (10% v/v), under sterile conditions.

2.3.3.2 Inoculum B

Inoculum B was a mixed microbial culture provided by a local company, but originally obtained from fresh *A. nodosum* plant, which was left with deionised water in a flask (closed with cotton wool) at room temperature for 2 weeks. After this time, a sample was taken from the flask, and grown up in Quatrano and Caldwell (Q&C) media (Quatrano and Caldwell, 1978) at 30^{o}C and 200 rpm in a shaker incubator. This media contained per litre of distilled water: NaCl, 25g; $MgSO_4 \cdot 7H_2O$, 5.0g; KCl, 1.0g; $CaCl_2$, 0.2g; K_2HPO_4, 0.1g; $FeSO_4 \cdot 7H_2O$, 0.02g; peptone 2% and beef extract 2%. From this, inoculum B samples were kept in sterile universal bottles at -80^{o}C, with 15% of sterilized glycerol (Moen *et al.*, 1997a; Moen *et al.*, 1997b; Moen *et al.*, 1997c; Horn and Ostgaard, 2001). Inoculum B samples were defrosted at room temperature and grown up in Q&C media for 24 hours at 30^{o}C and 200 rpm before use, to degrade and remove the glycerol. After this, the digestion flasks were inoculated with 10% (original digestion volume) of the inoculum B, under sterile conditions.

2.3.3.3 Inoculum C

A little amount of the residue sample was placed in a beaker, covered with foil paper and kept under dark conditions at room temperature for polyphenol determination. After some weeks, a white fungus was found growing on the surface of the residue in one of the samples which had not been used (Figure 2.3). A sample from the surface was taken, under sterile conditions, and grown in Q&C media at 200 rpm and 30^{o}C. This sample was kept in 15% sterile glycerol at -80^{o}C. Inoculum C samples were defrosted at room temperature and grown up in Q&C media for 24 hours at 30^{o}C and 200 rpm before use. After this, the digestion flasks were inoculated with 10% (original digestion volume) of this inoculum, under sterile conditions at day 0.

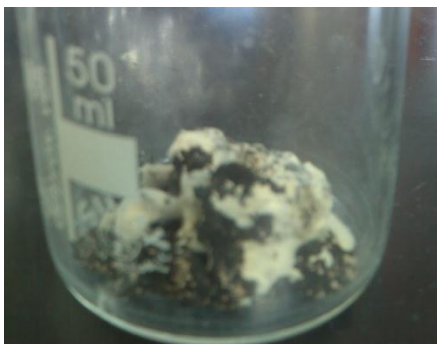


Figure 2.3 Fungus growing in *A. nodosum* residue.

2.3.4 Collection of the solubilised material

In some of the trials solubilised material was decanted during digestion to avoid metabolite inhibition of the digestive process. The required volume of the digestion sample was centrifuged (Table 2.2) under aseptic conditions, such that a pellet of insoluble material (IM) was separated from the supernatant containing soluble material (SM). The IM was returned to the digestion flask with the rest of the sample for further digestion while 200 ml of soluble material were removed and replaced with an equal volume of sterile deionised water.

2.3.5 Measured Parameters

Digestion progress was determined by measurement of insoluble matter (IM), soluble matter (SM) and pH before, during and after each trial. TS of each residue sample were also measured prior to the digestion process.

2.3.5.1 Residue and Inoculum Total Solids

The total solids of the residue sample used in each digestion and the inoculum were measured and calculated as done in Section 2.2.1.

2.3.5.2 pH

The pH-meter was calibrated following the standard procedure with pH 7 and 9 standards. The pH was measured at the end of each digestion flask. In trials 9-12 the pH was measured continuously (recording the pH every 1-2 hours) during the 3 first days under anaerobic incubation. To fit the pH-meter inside of a digestion flask, a 1 litre flask with 100 g of residue and 800 ml of sterile deionised water was used.

2.3.5.3 Separation of soluble and insoluble matter

Insoluble matter (IM) and soluble matter (SM) were measured when digestion was finished on each flask. The sample was separated into IM and SM by using two different methods: filtration and centrifugation. Day 0 was considered a control.

I) Filtration

Filtration method was used in trial INIT-1 as a simple method to separate IM and SM. The filter papers were very prone to blockage and the method was discontinued.

A) Material:

- Funnel
- Filter paper (Whatman No. 1)
- Beakers
- Crucibles
- Balance (Mettler Toledo, Switzerland)
- Convection Oven (Lab-Line Instruments, USA)

B) Method:

The digestion sample was passed through a weighed filter paper in a funnel. The digestion flask was rinsed with 100 ml of deionised water, which was also filtered. After the filtration, the IM was left in the filter paper and the DS (the liquid that passed the filter paper) were collected in a weighed beaker. IM and the filter paper were dried in a crucible in a convection oven at 105^{o}C to a constant weight. The SM was first dried in a steam bath until 5 ml of sample was left, and later in a convection oven at 105^{o}C until a constant weight was recorded.

C) Calculations:

First of all, the total solids present in each flask were calculated using the TS% of the residue sample (Section 2.2.2),

$$\text{Total solids (g)} = \frac{\text{Fresh residue (g) x TS\%}}{100}$$

The IM and SM percentages were calculated as follows,

$$\text{IM \%} = \left[\frac{\text{Dried sample plus crucible plush filter g} - (\text{Crucible g} + \text{Filter g})}{\text{Total solids g}} \right] \text{x } 100$$

$$\text{DS \%} = \left[\frac{\text{Dried sample plus beaker g} - \text{beaker g}}{\text{Total solids g}} \right] \text{x } 100$$

II) Centrifugation

Centrifugation method was used in trials 2 onwards. Some optimisation occurred through this time as to the most suitable centrifuge bottles, rotor speed and rotor/centrifuge.

A) Materials:

- Centrifuge bottles: 50 ml (Corning, Mexico); 250 ml (Beckman, USA)
- Centrifuges: see Table 2.2
- Beakers
- Crucibles
- Balance (Mettler Toledo, Switzerland)
- Convection Oven (Lab-Line Instruments, USA)

B) Method:

Digestion samples were placed into the centrifuge bottles. The digestion flask was rinsed with 100 ml of deionised water, which was also added to the centrifuge bottles. Samples were centrifuged at different speeds depending on the centrifuge and rotor used (Table 2.2), for 15-20 minutes. After this, pellet (IM) and supernatant (SM) were separated. The pellet was placed in a weighed crucible and dried in a convection oven at 105^{o}C until a constant weight was recorded. Deionised water was used to rinse the US left in the centrifuge bottles. The supernatant was placed in a beaker, from which three aliquots of 20 ml each were taken, placed into three weighed little beakers and dried in a convection oven at 105^{o}C until a constant weight was recorded. The total volume of the supernatant was also recorded.

Table 2.2 Centrifuge models and characteristics used for IM and SM separation

Model	Speed (xg)	Time (min)	Used for
Falcon 6/300, UK	3000	20	Initial trials and following changes
Beckman, J2-HS, USA	15300	15	Batch digestion model

C) Calculations:

IM and SM were calculated as shown below. The total solids by weight of each flask were calculated as described in Section (2.3.5.3.I). In the trials where an inoculum was added, the TS contribution of the inoculum was subtracted at the end.

$$\text{IM \%} = \left[\frac{\text{Dried sample plus crucible g} - \text{Crucible g}}{\text{Total solids g}} \right] \text{x} \quad 100$$

$$\text{SM \%} = \left[\frac{[(\text{Dried sample plus baker1 g} - \text{baker1 g}) + (\text{Dried sample plus baker2 g} - \text{baker2 g}) + (\text{Dried sample plus baker3 g} - \text{baker3 g}) / 3 \text{ x } 20] \text{ x Volume ml}}{\text{Total solids g}} \right] \text{x} \quad 100$$

2.3.5.4 Compositional analysis of digestion products

Ash, protein, lipid and fibre contents were measured in the IM remaining at the end of some trials. The methodology employed for these analyses were those described in Sections 2.2.2-6. For the lipid analysis the dried IM was reduced to powder with a mortar prior to the soxhlet extraction.

Polyphenol content was also studied in the SM obtained during digestion, following the methodology of Section 2.2.8.

2.3.5.5 Organic acid analysis

Organic acid analysis was carried out by reverse-phase HPLC. Both polar and non-polar organic acids were studied. SM samples collected at different days of digestion were analysed. The methodology employed followed that used by Lues *et al*. (1998) with slight modifications.

A) Materials:

- KH_2PO_4 (4871, Merck)
- H_3PO_4 (P6560, Sigma)
- Methanol (HPLC grade, Sigma-Aldrich)
- Filters (Filtropur S 0.2 µm. 83.1826.001, SARSTEDT)
- Syringes (10 Ml, Becton Dickson SA)
- Column ($C_{18,}$ Spherisorb, 4.6 x 250 mm, 5µm particle)

- HPLC system (Shimadzu Corporation, Japan): SPD-10A UV-detector, SCL-10A system controller, LC-10AD liquid chromatograph, FCU-10AL mixer.
- Software: CLASS VP Chromatography, data system version 4.3 (Shimadzu Scientific Instruments, Japan)
- Standards: formic, lactic and acetic were purchased from Fluka Analytical; oxalic acid from Merck and citric acid from Sigma.

B) Method:

Digestion samples were kept frozen after their collection and were defrosted and filtered through a 0.20 µm sterile filter prior to the organic acid analysis. Both standard organic acids and digestion samples were run through the HPLC system under the following conditions. A C_{18} column (spherisorb, 4.6 x 250 mm, 5µm particle) was used. The mobile phase was 0.01 M KH_2PO_4/H_3PO_4, pH 2.25 and a buffer/methanol gradient was created as described in Table 2.3. UV detection was carried out at 210 nm. Standard organic acids were run individually at different concentrations. Peak retention times and areas were recorded and a standard curve (area *vs*. concentration) for each organic acid was obtained. The peaks from the digestion samples were identified by comparison with the retention times of those from the standards. The concentration of each organic acid present in the digestion samples was calculated from peak area and the standard curve.

Table 2.3 Buffer/Methanol gradient protocol for organic acids HPLC

Time (min)	Eluent
0-15	100% buffer
15-25	100% buffer – 60% methanol
25-40	60% methanol
40-55	60% methanol – 100% buffer
55-70	100% buffer

2.4 Microbial studies

Microbial studies were carried out in parallel to studies of the analytical composition of *A. nodosum* residue and the microbial digestion. The residue was characterised microbiologically, and the different inocula (A, B and C) were studied. These studies included total viable count, colony isolation and characterization, and further

identification in some cases. The microorganisms growing during the 3-day anaerobic phase were also studied and identified by phenotypic pattern. The methodologies employed are explained in the following sections.

2.4.1 Total Viable Count

Only live cells in a sample are counted when using the total viable count method. A viable cell is defined as the one that is able to divide and form offspring. The usual way to carry out a viable count is to determine the number of cells present in a sample, which are capable of forming colonies on a suitable agar medium. It is assumed that each viable cell can yield one new colony (Madigan *et al.*, 1997).

A) Materials:

- Analytical balance (Mettler Toledo, Switzerland)
- Masticator (Stomacher, England)
- Sterile bags for masticator (Seward, England)
- Sterile universal bottles
- Sterile tips and pipettes
- Sea-water (filtered)
- Marine agar (DifcoTM, France). Composition (per liter): peptone 5.0 g; yeast extract 1.0 g; ferric citrate 0.1 g; sodium chloride 19.45 g; magnesium chloride 8.8 g; sodium sulfate 3.24 g; calcium chloride 1.8 g; potassium chloride 0.55 g; sodium bicarbonate 0.16 g; potassium bromide 0.08 g; agar 15.0 g; strontium chloride 34.0 mg; boric acid 22.0 mg; sodium silicate 4.0 mg; sodium fluoride 2.4 mg; ammonium nitrate 1.6 mg; disodium phosphate 8.0 mg. Final pH 7.6 ± 0.2.
- Q&C agar (composition per litre of distilled water: NaCl, 25g; $MgSO_4 \cdot 7H_2O$, 5.0g; KCl, 1.0g; $CaCl_2$, 0.2g; K_2HPO_4, 0.1g; $FeSO_4 \cdot 7H_2O$, 0.02g; peptone 2% and beef extract 2%; 3% agar (Difco, USA); Quatrano and Caldwell, 1978).
- Plate count agar (Fluka Analytical; Composition (g/L): agar 9.0, dextrose 1.0, tryptone 5.0, yeast extract 2.5)
- Sterile petry dishes
- Glass spreader

- Bunsen
- Incubator
- Hermetic Jar
- Anaerocult (Merck, Germany)
- Colony counter (Stuart Scientific, UK)

B) Method:

The total viable count (TVC) method was used to count the bacterial numbers in the residue, in the inocula and during the 3 day-anaerobic phase. Although the methodology employed was the same, there was some variation in the use of solutions to dilute the sample and agars. First of all, the methodology employed for the residue TVC is described and variations explained thereafter.

The residue sample was taken from the fridge and left at ambient temperature for 15 minutes. A known amount of residue (25 g) was weighed on an analytical balance and placed, under aseptic conditions, into a sterile bottle which contained 225 ml of sterile sea-water (previously filtered). This first dilution (10^{-1}) was transferred to a sterile plastic bag and mixed in a masticator for 5 minutes at ambient temperature. After this time, 1 ml of the mixture was taken and transferred, under aseptic conditions, to a sterile universal containing 9 ml of sterile sea-water to obtain a 10^{-2} dilution. This procedure was repeated 3 more times, until 10^{-5} dilution was reached.

Marine agar was prepared according to the instructions of the manufacturer, sterilized in an autoclave (1 atm. for 15 minutes) and poured into sterile Petri dishes (approximately 25 ml of agar per plate). Each dilution (0.1 ml) was spread in a separate agar plate under aseptic conditions and with the help of a glass spreader. Four agar plates were prepared for each dilution. Duplicate plates were incubated under aerobic conditions and the remaining two plates were incubated anaerobically.

In each trial 2 controls were added to ensure the plates were free from contamination. One of the controls consisted of only marine agar while in the other, 0.1 ml of sterile sea-water was added to the marine agar and spread on the surface in the same manner as the samples. Both controls were incubated also under aerobic and anaerobic conditions.

In both cases, agar plates were incubated in an inverted position to prevent the water condensation on the culture. For anaerobic incubation, agar plates were placed into a hermetic jar with a gas generator envelope (Anaerocult). This operates by generating H_2 and CO_2, when water is added to the envelope in accordance with the manufacturer's instructions. The samples from the January batch were incubated at 30^{o}C for 24 h under aerobic conditions and 48 h under anaerobic conditions.

After incubation, colonies were counted with the help of a colony counter. While the usual practice is to count colonies only on the plates that have between 30 and 300 colonies (Madigan et al., 1997), this was quite difficult to adhere to in this study. It was found that the number of bacteria on the plates consistently fell outside this range.

Different solutions and/or type of agar were used to do the TVC analysis in inocula A and B and during the 3-day anaerobic phase. For the inoculum A TVC, dilutions (up to 10^{-8}) were prepared in sea-water, spread on marine agar plates and incubated at 30^{o}C for 24 h (aerobic) and 48 h (anaerobic). Inoculum B was grown in Q&C media at 30^{o}C and 200 rpm for 24 h prior to the TVC. Ringers solution was used to prepared the dilutions (up to 10^{-8}), which were spread on Q&C agar plates and incubated only aerobically for 24 h at 30^{o}C.

When studying the anaerobic phase of the microbial digestion, the digest (10 ml) was diluted in ringer's solution (90 ml), from which serial dilutions were made. Plate count agar was employed and plates were incubated under aerobic (24 h) and anaerobic (48 h) conditions.

2.4.2 Monitoring microbial flora

The microbial flora was monitored on January and February residue samples. Residue samples were diluted and spread on agar plates. The plates were incubated for a week at room temperature (around 20^{o}C) and were studied every day, with daily observation on the colonies and their different characteristics. This experiment was also carried out in inoculum A, where the plates were incubated at 30^{o}C for one week. The changes and the waves of bacterial populations were observed.

2.4.3 Colony isolation and study

The different types of colonies observed on the TVC plates were selected, described and isolated. Colonies were observed with the help of a magnifier to obtain a better

detail. Each isolate was examined to determine its morphology, motility and Gram reaction among other characteristics.

2.4.3.1 Colony isolation

Different types of colonies were selected from the agar plates used in the TVC procedure. These colonies were described (shape, size and colour) and isolated. Each colony was picked up from the original plate with a sterile loop and spread on a new plate (marine agar was used for residue bacteria / Q&C agar for inoculum B/ plate count agar for the 3-day anaerobic phase) using the streak-plate technique (Harley and Prescott, 1990). This technique consists of spreading the inoculating loop with bacteria by 8-10 strokes over a small area at one edge of the plate (imagining the plate was divided in 4 areas). After this, the loop was flamed, killing any remaining bacteria, and 4 or 5 streaks were done into the second imaginary area, by crossing the streaks in area 1. The loop was again flamed and the streaking procedure was repeated twice more: from area 2 to area 3 and from area 3 to 4 (Figure 2.4). All the plates were incubated under aerobic and/or anaerobic conditions for 24 and 48 hours respectively. By using streak-plate technique it was assumed that one colony comes from one cell (Harley and Prescott, 1990). The isolation technique was used as many times as was needed until colonies were isolated and propagated as pure cultures. After this, several tests were carried out on each type of colony.

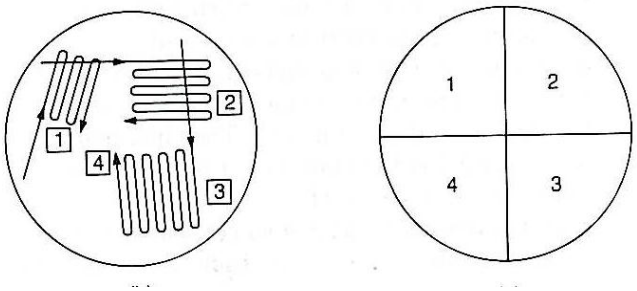


Figure 2.4 Streak-plate technique (Harley and Prescott, 1990).

2.4.3.2 Observation of morphology and motility by phase contrast microscopy

The shape, cell arrangement and the motility of each isolate were observed *in vivo* by phase contrast microscopy. The phase contrast microscope allows improved contrast differences between cells and the surrounding medium, making it possible to see cells without staining. Cells differ in refractive index from their surroundings and hence

bend some of the light rays that pass through them. This effect is amplified by a special ring in the objective lens of a phase contrast microscope, leading to the formation of a dark image on a light background (Harley and Prescott, 1990; Madigan *et al.*, 1997).

A) Materials:

- Sterile yellow tips
- Slides
- Coverslips
- Phase contrast microscope (Nikon, Japan)

B) Method:

Each colony was grown for 18-20 h in agar (marine agar for residue colonies) and/or 4 hours in media (Q&C media for inoculum B, nutrient media for bacteria isolated from the anaerobic phase). For the agar colonies, a loopful of the colony was picked up and spread on a water drop on the micro-slide. For the media colonies a drop of the sample (15 µl approximately) was picked up with a sterile pipette and transferred to a clean micro-slide. The coverslip was slipped slowly over the drop. The sample was then observed on the phase contrast microscope.

2.4.3.3 Gram stain

The Gram stain is a differential stain, which involves the application of more than one dye to a cell so that cells of one physiological type can be distinguished from other types. The stain was discovered in 1884 and divides all bacteria into two groups: gram positive and gram negative. Gram stain involves the application of:

(a) a basic dye which is held loosely by all cell
(b) a mordant, iodine, which forms a dye-iodine complex holding the stain more firmly in the cell
(c) a decolourising agent, ethyl alcohol, which removes the bound dye from gram negative cells but fails to free it from gram positive cells
(d) a counter stain which stains the decolourised gram negative cells a different colour form the original dye.

The basic dye penetrates the bacterial cell and reacts with acidic components of the protoplasm. When iodine is added, a complex is formed. This complex remains in gram positive bacteria when a decolourizer is applied, the cell wall apparently interfering with extraction of their higher cell wall lipid content which is soluble in the decolourizer, and these are counterstained to give a contrasting red colour (Atlas, 1989; Harley and Prescott, 1990).

A) Materials:

- Sterile loop or sterile yellow tips
- Deionised water
- Bunsen
- Crystal violet
- Iodine
- Absolute alcohol
- Safranin (0.5%)
- Microscope (Nikon, Japan)
- Inmersion oil

B) Method:

First of all, the smear was prepared, air dried and heat-fixed. The slide was covered with crystal violet allowing this to act for 30 seconds. After this time, crystal violet was poured off, and washed by holding the slide at an angle downwards and pouring on iodine solution. The slide was finally covered with fresh iodine, allowing this to act for 30 seconds. After that, the iodine was washed off with absolute alcohol until colour ceased to come out, and also with water. Once the slide was cleaned, Safranin was applied for 1-2 minutes, washed with water and dried between blotting paper. The slide was then observed under the microscope to determine if the microorganism was gram positive or negative. The shape of the microorganism(s) present was also recorded (Dart, 1996).

2.4.3.4 Catalase activity

Some bacteria contain flavoproteins that reduce O_2, resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2^-). These are very toxic oxidant agents,

which destroy cellular constituents. Many bacteria possess enzymes that afford protection against toxic O_2 products (Harley and Prescott, 1990). Catalase enzyme catalyses the breakdown of hydrogen peroxide to oxygen and water (Harley and Prescott, 1990; Dart, 1996).

A) Materials:

- Sterile loop
- Slides
- 10% H_2O_2
- Microscope

B) Method:

A few drops of 10% H_2O_2 were placed on a slide and a loopful of microorganisms was rubbed in. The test was considered positive when the evolution of gas was observed either with the naked eye or with the microscope if the result was uncertain (Dart, 1996). *Staphylococcus epidermidis* was used as a positive control.

2.4.3.5 Oxidase activity

The oxidase test is used to determine if a bacteria produces certain cytochrome c oxidases. In this test, the tetramethil-p-pheneylenediamine is oxidised by cytochrome oxidase to a purple compound. If the bacterial strain is oxidase positive, it can utilize oxygen for energy production with an electron transfer chain, but not if it is oxidase negative.

A) Materials:

- Sterile loop
- 1% tetramethyl-p-phenylenediamine in water (w/v; Sigma-Aldrich)
- Filter paper (Wathman n^o 1)

B) Method:

The reagent was prepared fresh prior to the experiment. A loopful of cells from a young culture (16 to 18 h) was rubbed into the paper and few drops of the reagent were added. If a deep purple colour was formed in 5-10 seconds the reaction was considered

positive. Any purple colour formed after 30 seconds was considered negative. *Pseudomonas fluorescence* was used as a positive control.

2.4.3.6 MacConkey Agar

A selective medium is a medium that allows the growth of certain type of microorganisms. MacConkey agar is a selective media for Gram negatives. It contains bile salts and crystal violet, which inhibit the growth of Gram positive bacteria. It is also a differential media because it can be use to distinguish between bacteria that ferment lactose and the ones that do not. Lactose is the only carbohydrate present in the media, which also contains a pH indicator, neutral red (red at pH < 6.8). The bacteria that ferment lactose will form red colonies due to the acid pH by the acid production, while the ones that they do not ferment lactose will appear transparent (Atlas, 1989).

A) Materials:

- Sterile loops
- MacConkey agar (Oxoid, comosition (g/L): peptone 20.0, lactose 10.0, bile salts 5.0, sodium chloride 5.0, neutral red, 0.075, agar 12.0)
- Incubator

B) Method:

MacConkey agar plates were prepared according to the manufacturer's instructions. Each bacterial strain was spread on the plates in duplicates. Plates were incubated at 30^{o}C for 24 h. Results were read and recorded after this time.

2.4.3.7 Endospore Stain

The bacterial endospore is resistant to many agents including bacterial stains. In simple stains bacterial endospores are rarely stained and appear as empty holes in the stained cells. A special procedure is necessary to make spore cells take up stains, like the application of heat during staining. When stained, the spore is reluctant to release the stain and this feature is used to differentiate the spore form the vegetative cell by decolourizing the vegetative cell and staining it with a different colour to the spore. Endospores are produced by four genera of bacteria: *Bacillus*, *Clostridium*, *Sporosarcina* and *Sporolactobacillus*.

A) Materials:

- Sterile loop or sterile yellow tips
- Deionised water
- Bunsen
- Malachita green
- Neutral red
- Microscope (Nikon, Japan)
- Inmersion oil

B) Method:

The smear was prepared, air dried and heat-fixed. The slide was covered with malachite green, heated (bunsen) to steam and maintained steaming for 3 minutes. More malachite green was added where required, to prevent the sample drying out. The slide was washed with water. Neutral red was added and left for 30 seconds. The slide was then washed with water and dried with blotting paper. The slide was then observed under the microscope to determine if endospores were present.

2.4.3.8 Acid-fast stain (Ziehl-Nielsen)

Some bacteria such as those from the gender *Mycobacterium*, do not stain easily with normal procedures due to the great amount of lipid (long chain fatty acids) present in their cellular-wall. Applying heat while staining, the colorant passes through the cell-wall. However, once stained they are very resistant to destaining.

A) Materials:

- Sterile loop or sterile yellow tips
- Deionised water
- Bunsen
- Carbol Fuschin
- Metilene blue
- Acid-alcohol solution: 95% etanol (97 ml), concentrated HCl (3 ml)
- Microscope (Nikon, Japan)
- Inmersion oil

B) Method:

The smear was prepared, air dried and heat-fixed. The slide was covered with carbol fuchsin, heated with a bunsen to steam and maintained steaming for 5 minutes (not boiling). More carbol fuchsin was added as needed, to prevent the sample drying out. The slide was washed with water. Acid-alcohol solution was added and kept for 15 seconds. The slide was washed with water. Methylene blue was added and left for 1 minute. The slide was again washed with water and dried between blotting paper. The slide was then observed under the microscope. Bacteria positive to this test will appear red, while the negatives will be blue.

2.4.4 Inoculum B growth curve

Microbial growth of the inoculum B was studied by determination of turbidity of the growth medium. Two different temperatures (30^{o}C and 35^{o}C) were tested and statistically analyzed.

A) Materials:

- Sterile pipettes
- 250 ml conical flasks
- Spectrophotometer (Shimadzu, UVmini-1240, China)
- Coverslips
- Shaker incubator (New Brunswick Scientific, USA)

B) Method:

A 24 h culture of inoculum B was used to inoculate Q&C media (10% v/v) under aseptic conditions. This new culture was incubated aerobically at 30^{o}C and 35^{o}C, and shaken at 200 rpm for 24 hours. Every 2 h, starting at time 0, an absorbance reading (660 nm), was taken from the growing media. Sterile media was used to zero the spectrophotometer. If the absorbance was too high for accuracy, then appropriate dilutions of the media (in deionised water) were carried out. The OD of these dilutions was then measured. Three trials were carried out at 30^{o}C with each trial studied in duplicate. At 35^{o}C, two trials were performed, also in duplicate. For each trial four flasks were studied: two from 0 to 12 hours and the other two from 12 to 24 hours. (It was necessary to inoculate a second set of flasks 12 hours after the first set to allow for

daytime monitoring of the OD). In some instances, the trials were allowed to continue to grow and absorbance was measured longer than the standard 24 h period.

C) Statistic analysis:

Statistical analysis was carried out on the results obtained at 30 and 35^{o}C, with the aim of testing if there was a significant difference between the microbial growth at both temperatures. First of all, the data collected was divided in three sections: 0 to 8 hours, 8 to 18 hours and 18 to 24 hours. The normality of the data in each section was checked by Kolmogorov-Smirnov Test. Once the normality was proved, the independent sample T-test was applied, determining the significance level (p-value).

2.4.5 Microorganisms identification: inocula B and C

Microorganisms from inocula B and C were identified. For inoculum B a preliminary identification with API 20 E gallery was carried out, and further genetic characterization was studied by the Institue of Biotechnology and Food Science (University of Technology, Bratislava, Slovakia).

2.4.5.1 Inoculum B: API 20 E gallery

The API test kit (bioMerieux, France) consists of enzymatic and carbon compound assimilation test elements (Truu *et al.*, 1999). API 20 E is a standardized identification system for Enterobacteriaceae and other non-fastidious, gram negative rods, which uses 21 miniaturized biochemical tests and a database. API 20 E consisted of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension which reconstitutes the media. During the incubation, the metabolism of the bacteria produces colour changes which are either spontaneous or revealed by the addition of reagents.

Although API 20 E is designed for gram negative bacteria, it was also used for the gram positive bacteria in this study, with the aim of testing the isolated colonies through the biochemical tests.

A) Materials:

- Sterile pipettes
- API 20 E (Biomerieux, France)
- NaCl 0.85 % medium presented on 5ml bottles (Biomerieux, France)
- TDA (Biomerieux, France)
- JAMES (Biomerieux, France)
- VP 1 and VP 2 (Biomerieux, France)
- NIT 1 and NIT 2 (Biomerieux, France)
- Mineral oil (Biomerieux, France)

B) Method:

Each isolate was incubated on a Q&C agar plate for 20 hours. After this, one representative colony was picked up with a sterile loop and placed into 5ml of 0.85% NaCl. The solution was mixed until a homogeneous bacterial suspension was achieved and immediately distributed into the tubes of the strip according to the manufacturer's instructions. The strips were incubated at 37^{o}C for 22 h. Each type of colony was tested in duplicate.

2.4.5.2 Inoculum C

As explained in section 3.3.3.3 Inoculum C was a fungus found growing on a residue sample. An isolation of this fungus was carried out by taking a loop from the surface of the residue under aseptic conditions and spreading on an agar plate. Different types of agar were used to incubate the fungus: Q&C agar, yeast agar (Biokar Diagnosis; tryptone 6 g/l, yeast extract 3 g/l and bacteriological agar 10 g/l) and potato dextrose agar (Merck; potato infusion 4 g/l, D-glucose 20 g/l and agar-agar 15 g/l) were used in an attempt to find which one was the most appropriate for this fungus. Potato dextrose agar seemed to best support growth. Agar plates were incubated at 30^{o}C, until spore production was found. The identification of the fungus was carried out by CABI Europe-UK (www.cabi.org; Bakeham Lane, Egham, Surrey, TW20 9TY, UK).

2.4.6 Anaerobic phase bacterial identification by phenotypic pattern

Microorganisms isolated from the 3-day anaerobic phase were identified by the Biolog Omnilog GEN III microbial identification system (Biolog, Inc., Hayward, CA, USA).

This is a bacterial identification method that establishes identification based on the exchange of electrons generated during respiration, leading subsequently to tetrazolium-based colour changes. This system tests the ability of microorganisms to oxidize a panel of different carbon sources (Truu *et al.*, 1999). A unique biochemical pattern or "fingerprint" is then produced when the results are surveyed. The fingerprint data are analyzed, compared to a database, and identification is generated (Morgan *et al.*, 2009).

The Biolog GEN III MicroPlate analyzes a microorganism in 94 phenotypic tests: 71 carbon sources utilization assays (Figure 2.5, columns 1-9) and 23 chemical sensitivity assays (Figure 2.5, columns 10-12). There is a negative control (well A-1, Figure 22.5) with no carbon source, and a positive control (well A-10, Figure 2.5) used as a reference for the chemical sensitivity assays in columns 10-12.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 PH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 B-Methyl-D-Glucoside	B5 D-Salacin	B6 N-Acetyl-D-Glucosamine	B7 N-Acteul-B-D-Mannosamine	B8 N-Actetyl-D-Galactosamine	B9 N-Acetyl Neuraminic acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Frustose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocicline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCL	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucid Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butiric Acid	H3 α-Hydroxy-Butyric Acid	H4 B-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2.5 Layout of assays in GEN III microplate.

A) <u>Materials:</u>

- Gen III MicroPlates (1030, Biolog, USA)
- Plate Count Agar (Fluka Analytical; Composition (g/L): agar 9.0, dextrose 1.0, tryptone 5.0, yeast extract 2.5)
- Sterile plastic loops (Starstedt)

- Inoculating Fluid IF-A (72401, Biolog, USA)
- Sterile inoculators swabs (3323, Biolog, USA)
- 8-chanel electronic pipette (3711, Biolog, USA)
- Sterile pipette tips (3001, Biolog, USA)
- Turbidimeter (Biolog, USA)
- 65% turbidity standard (3440, Biolog, USA)
- Omnilog (Biolog, USA)
- Omnilog Sofware: Omnilog-PMDC 1.30.01, Omnilog-OL-PM-FM/Kin 1.20.02 (File management/ kinetic plot version)
- Omnilog ID version ML 5.2
- Database: BIOLOG GENIII version 2.5.1.15
- Algorithm version MLID5.dll 2.3.1.395

B) Method:

Before starting, the microplates and the inoculating fluid were pre-warmed to room temperature. Each isolated microorganism was grown in plate count agar at 30^{o}C for 22-24 h. The turbidimeter was calibrated with a standard (65% T), and blanked (100% T) with a clean tube containing uninoculated IF-A. Isolated colonies were picked up from the agar plate with a swab (under aseptic conditions) and put into the IF-A by pressing the cells against the tube wall. A final turbidity range of 98-90% was prepared for each strain. The cell suspension was transferred into the microplate (100 µl/well) with the help of an electronic multichannel pipette. The microplate was then placed into the Omnilog incubator/reader, and it was incubated at 33^{o}C for 24 h. The Omnilog took a reading and recorded a picture of each microplate every 15 minutes. Results obtained from the Omnilog were analysed with Biolog's microbial identification system software (version ML 5.2) and compared to the database BIOLOG GENIII (version 2.5.1.15) at Technopath (Ballina, Co. Tipperary).

2.5 Screening for anti-hyaluronidase and anti-elastase activites

Digested seaweed residue, seaweed meal and the two seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea* were screened for inhibitors for hyaluronidase and elastase activities, which are involved in the turnover of the main components of the skin matrix. To-date seaweed derived peptides have not been evaluated for this application. Methodology to generate protein and peptide fractions from the seaweeds and a commercial extract was also developed. The materials and the methodology used are described in the following sections.

2.5.1 Samples collection and preparation

Samples used in this study included *A. nodosum* residue's digestate at different stages of digestion, two brown fresh seaweeds, and *A. nodosum*'s meal and extract.

2.5.1.1 *A. nodosum* residue digestion samples

Digestion samples were collected at different times from the soluble material separated by centrifugation (Section 2.3.5.3). Samples were freeze-dried (Edwards Modulyo) and screened for hyaluronidase activity.

2.5.1.2 Seaweed samples

Fresh seaweed samples of *A. nodosum* and *L. hyperborea* were collected on 19th October 2009 at Fenit Island (Co. Kerry, Ireland; Lat: 52.17 Long: 9.52). Dehydrated *A. nodosum* meal and the aqueous extract (AqE) from *A. nodosum* were provided by a local company. Fresh seaweeds were cleaned (epiphytes were removed) and chopped on arrival to the lab at the Institute of Technology Tralee. For the following experiments samples were use in fresh and dried format. Seaweed plants were kept frozen at -85^{o}C and were defrosted (at 4^{o}C) the night previous to the experiment; or were dried at 105±2^{o}C to a constant weigh, and milled with a mortar. The AqE was also dried at 105±2^{o}C and milled with a mortar. Dehydrated *A. nodosum* was also milled. All these samples were treated through a series of experiments described in Section 2.5.2.

2.5.2 Obtaining peptides

Samples from section 2.5.1.2 were treated to obtain proteins and generate peptides (Fig 2.6). Firstly, lipid content was removed by supercritical fluid extraction (SFE).

Secondly, carbohydrates were digested to liberate proteins from the cell-wall, and finally, proteins were hydrolysed with four different proteases.

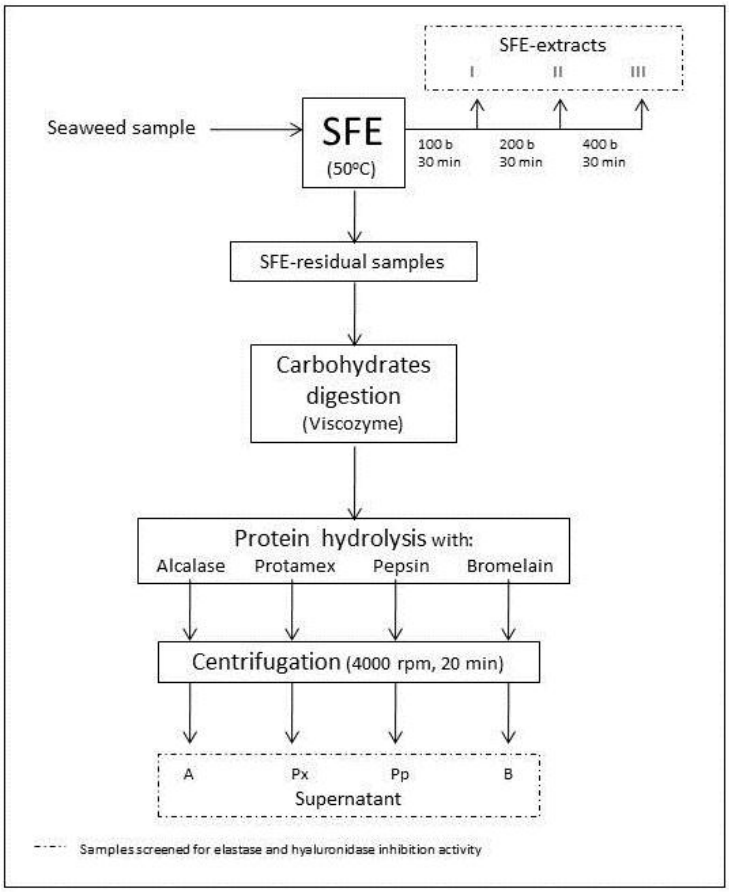


Figure 2.6 Methodology employed to obtain proteins and liberate peptides.
b- bar, A – alcalase, Px – protamex, Pp – pepsine, B – bromelain.

2.5.2.1 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative extraction method whereby supercritical fluids, instead of organic solvents, are used as an extraction medium (King *et al.*, 2001). It is a useful alternative to conventional solvent extraction because of its ability to shorten extraction time and to reduce the amount of organic solvent required (Punín Crespo, 2005, 2006). Supercritical CO_2 is non-toxic, non-flammable, inexpensive and easily separated from the extracts. Its low critical temperature (31.1^{o}C) and critical pressure (7.4 MPa) allow extraction of the thermolabile compounds with minimal degradation (Cheung, 1999). In its supercritical state, CO_2 has both gas-like and liquid-like qualities, and it is this dual characteristic of supercritical fluids that provides the ideal conditions for extracting compounds with a high degree of recovery

in a short period of time. SFE was performed in the Shannon Applied Biotechnology Laboratory (Limerick Institute of Technology).

A) Materials:

- Spe-ed-SFE (4.1) supercritical fluid extractor (Applied Separations Inc., USA)
- Sped-ed™ SFE polypropylene frits 1"/14 mm (Applied Separations, 7956)
- Sped-ed™ polypropylene wool (Applied Separations, 7952)
- Spe-ed™ Matrix, wet support (Pelican Scientific Ltd., UK)

B) Method:

Sample (10g) was packed into the SFE-vessel, as shown in Figure 2.7. For the liquid AqE (fresh), 10 mL of sample were mixed with wet support (6 g), to provide a solid aspect. The vessel was inverted and placed into the SFE system under the following conditions: 50°C of temperature, 100 bar for the first 30 min, 200 bar for the following 30 min and 400 bar for the last 30 min. The temperature of the collection valves was 60°C. Supercritical CO_2 was used for the extraction process. Samples (SFE-extracts) were collected every 30 min in eppendorf tubes and their weight was recorded. The SFE-residual sample (material remaining in the vessel after the extraction) was collected at the end of the extraction process in a plastic bag and its weight was also recorded. SFE-extracts and SFE-residual samples were frozen (-85°C) upon extraction.

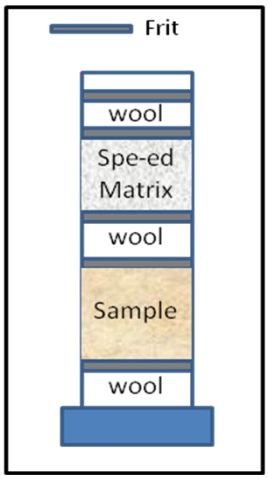


Figure 2.7 SFE vessel, packed with sample

After the SFE, three different types of samples were considered: (1) SFE-extracts (100, 200 and 400 bar), which were directly tested on elastase and hyaluronidase activity assays, (2) SFE-residual samples and (3) non-SFE samples (fresh seaweed samples that were never extracted with SFE). Both SFE-residual samples and non-SFE samples were continued through carbohydrates digestion and protein hydrolysis.

2.5.2.2 Carbohydrates digestion

SFE-residual samples and non-SFE samples were further treated to digest the carbohydrates present freeing the proteins, especially those from the cellular wall. An enzymatic mixture containing carbohydrases (arabanase, cellulase, beta-glucanase, hemicellulase and xylanase) was used for this purpose.

A) Materials:

- Viscozyme enzymatic mixture (Novozymes, Denmark)
- 100 mM Ammonium acetate buffer (pH 4.5)
- Shaker incubator (New Brunswick Scientific, USA)
- Water bath
- Electric mill (Yellow line)

B) Method:

SFE-residual samples and non-SFE sampes were defrosted, milled with an electric mill, homogenized and treated with Viscozyme. For this, sample (5g) was added to 20 mL of 100 mM Ammonium acetate buffer (pH 4.5), and 1 mL of Viscozyme was added. Samples were incubated overnight at 37^{o}C in a shaker incubator. After incubation, samples were placed in a water-bath at 80^{o}C for 20 minutes to deactivate the enzymes. Samples were allowed to cool, and were frozen at -85^{o}C.

2.5.2.3 Protein hydrolysis

Once the carbohydrates were digested and proteins assumed to be freed, a protein hydrolysis of the samples (still containing Viscozyme) was carried out. For this propose, four different types of proteases were used: alcalase (a), protamex (px), pepsin (pp) and bromelain (b).

A) Materials:

- 200mM Na_2HPO_4-NaH_2PO_4 buffer
- Alcalase (Novozymes, PLN05320)
- Protamex (Novozymes, PW2A1046)
- Pepsin (Sigma, P7125)
- Bromelain (Sigma, B4882)
- Water bath
- Eppendorf centrifuge (EBA 12, Hettich zentrifugen, Germany)

B) Method:

Prior to this step, samples were defrosted. Each sample (700 µL) was mixed with 200mM Na_2HPO_4-NaH_2PO_4 buffer (700 µL) of adequate pH according to each protease (Table 2.4). For pepsin water with HCl (pH 2) was used instead of the buffer.

Samples were pre-incubated at the appropriate temperature (Table 2.4) in a water bath for 20 min. The protease was added (E:S ratio 5%) and samples were incubated at the appropriate temperature (Table 2.4). Sub-samples (aliquots) were collected at times 0, 30 and 60 minutes for each sample and enzyme. Enzymes were deactivated at 80^{o}C or 65^{o}C for 20 min (Table 2.4). Samples were allowed to cool down and centrifuged at 1520 xg for 20 minutes. Supernatant was frozen (-85^{o}C), freeze dried (Edwards Modulyo) and screened for Elastase and Hyaluronidase inhibitors.

Due to the possible interference of viscozyme proteins/peptides on hyaluronidase and elastase activity, a control without sample, called viscozyme-control, was run through sections 2.5.2.2 and 2.5.2.3, and tested in elastase and hyaluronidase assays.

Table 2.4 Protein hydrolysis

Enzyme	pH	Temp.	E:S (%)	Enzyme (mL or g)	Substrate (g)	Deactivation Temp. X min.
Alcalase	7.5	50	5	*	**	80 x 20
Protamex	6.5	50	5	*	**	80 x 20
Pepsin	2	37	5	*	**	80 x 20
Bromelain	5	50	5	*	**	65 x 20

2.5.3 Protein content evaluation

Protein content was evaluated on the initial seaweed samples and in the supernatant collected after protein hydrolysis. Kjeldahl total nitrogen determination and BCA methodologies were employed. For Kjeldahl method see section 2.2.3.

2.5.3.1 BCA Protein Assay

The BCA protein assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (biuret reaction) with the sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) using a reagent containing BCA. A purple-coloured product is formed by the chelation of two molecules of BCA with one cuprous ion. This reaction product presents a maximum absorbance at 562 nm (Roca *et al.*, 2003).

A) Materials:

- Pierce BCA Protein Assay Kit (Thermo Scientific, USA). It contained reagent A (sodium carbonate, sodium bicarbonate, bicinchoninc acid and sodium tartrate in 0.1M sodium hydroxide) and B (4% culpric sulphate).
- Microplate reader (Thermo Scientific, USA)
- Bovine Serum Albumin (bSA; Sigma, A2153)

B) Method:

This method was carried out according to the instructions of the Pierce BCA Protein Assay Kit (microplate procedure). Sample (25 µL) was added into the microplate well with 200 µL of the mix working reagent (reagents A and B in a proportion 50:1). The plate was covered and incubated at 37°C for 30 min. After this time, the plate was allowed to cool at RT and the absorbance was measured at 562 nm. A standard curve of bSA (Figure 2.8) was used to calculate the protein concentration of the sample. Each sample was studied in duplicate and a blank was also run to determine the background absorbance.

C) Calculation:

The blank absorbance value was subtracted from the sample value. The equation of the bSA standard curve (Figure 2.8) was used to calculate the protein concentration of the sample. Where dilutions were made, the dilution factor was applied in the calculation. The average and the standard deviation were calculated.

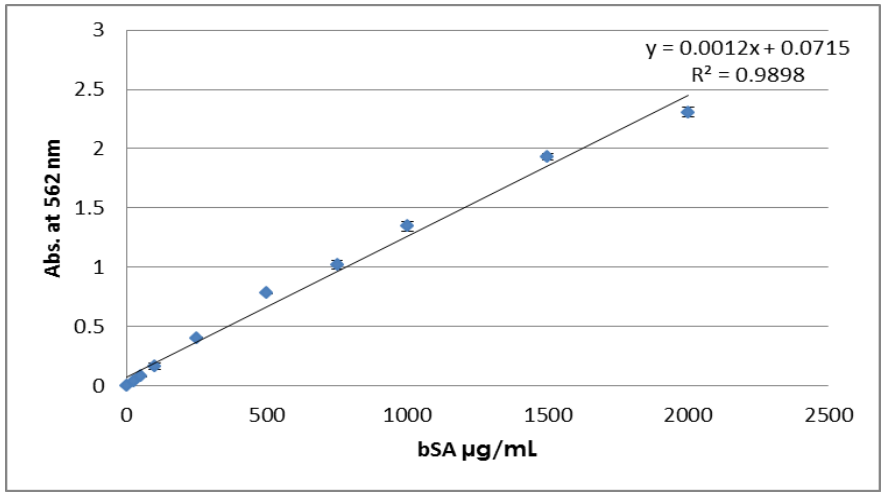


Figure 2.8 bSA standard curve obtained with BCA protein assay (SD, n=2).

2.5.4 Hyaluronidase activity assay

Hyaluronidase (HAase) activity was spectrophotometrically determined by measuring the amount of N-acetylglucosamine (product) formed from hyaluronic acid (substrate). The HAase reaction was carried out following the steps of Kim *et al.* (1995), Lee *et al.* (2005) and Lee *et al.* (2007) and the product of the reaction was measured according to the Morgan-Elson method (Reissig *et al*., 1955), with slight modifications.

A) Materials:

- Bovine testes Hyaluronidase Type I-S (Sigma, H3506)
- Hyaluronic acid from human umbilical cord (Sigma, H1504)
- 0.1M Acetate buffer (pH 3.5)
- 12.5 mM Calcium chloride
- 0.4 N NaOH
- 0.4 M potassium tetraborate
- p-dimethylaminobenzaldehyde (Fluka, 39080)
- Glacial acetic acid 100% (Merck, 1.0063.2511)
- 10N HCl
- Water bath
- Spectrophotometer (Shimadzu, UVmini-1240, China)

B) Method:

Bovine hyaluronidase (50µL; 7900 units/mL) dissolved in 0.1M acetate buffer (pH 3.5) was mixed with 100 µL of a designated concentration of sample, previously dissolved in acetate buffer, and then pre-incubated in a water bath at 37^{o}C for 20 minutes. The positive control was treated with 100 µL of buffer instead of sample. Calcium chloride (100 µL, 12.5 mMol/L; HAase activator) was added to the reaction mixture and then incubated for a further 20 minutes. For the HAase reaction, 250 µL of hyaluronic acid (1.2 mg/mL) dissolved in acetate buffer was added, and incubated in a water bath at 37^{o}C for 40 minutes. For termination of the HAase reaction, 100 µL of 0.4 N NaOH was added. A blank for each sample was run and was now treated with 50 µL of HAase, instead of at the beginning of the test.

The Morgan-Elson colour reaction was started by addition of 100 µL of 0.4 M potassium tetraborate and the subsequent heating in boiling water for 3 min. After cooling to room temperature, 3 mL of DMAB (p-dimethylaminobenzaldehyde) reagent

were added and then incubated in a water bath at 37°C for 20 minutes. For DMAB reagent preparation, 10g of DMAB were dissolved in 100 mL of analytical reagent glacial acetic acid (100%) which contained 12.5% v/v 10 N HCl. This reagent was stored at 2°C, and shortly before use it was diluted with 9 volumes of glacial acetic acid. After 20 minutes incubation, optical density of the reaction mixture was measured at 585 nm. Background absorbance values were subtracted from each sample.

2.5.5 Elastase activity assay

Elastase assay was performed with the EnzChek Elastase Assay Kit (Invitrogen, UK). This kit contains DQTM elastin (soluble bovine neck ligament elastin), which has been labelled with BODIPY FL dye such that the conjugate's fluorescence can be quenched. The non-fluorescence substrate can be digested by elastase or other proteases to yield highly fluorescent fragments. The resulting increase in fluorescence can be monitored with a fluorescence microplate reader.

A) Materials:

- EnzChek Elastase Assay Kit (Invitrogen, UK), which contains DQ-elastin (substrate), porcine pancreatic elastase (enzyme), N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (inhibitor) and 1M tris-HCl pH 8.0 with 2mM sodium azide (reaction buffer).
- Microplate reader (Thermo Scientific, USA)

B) Method:

Samples were dissolved and diluted in the reaction buffer provided by the kit (1M tris-HCl). Sample (50 µl; 0.20 mg/mL) was added to each well of the microplate, followed by 100 µL of Elastase (0.2 U/mL). The plate was pre-incubated at room temperature and dark conditions for 15 minutes. DQ-elastin (50µL; 100 µg/mL) was added and the plate was incubated in the microplate reader (Thermo Scientific, USA) at 25°C for 2 hours. Fluorescence was measured at 495nm (excitation) and 515 nm (emission) every 10 minutes during this time. An inhibitor control (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) at a concentration of 0.20mg/mL (real concentration in assay 0.05 mg/mL), a positive control and a blank for each sample were also tested in each batch. Background fluorescence (blank) was subtracted from each sample.

2.5.6 Statistics

Inhibitory effects of sample on elastase and hyaluronidase activity was expressed as follows:

$$\text{Inhibition (\%)} = (1- B/A) \times 100,$$

where A is the enzyme activity without the sample (positive control) and B is the activity in the presence of the sample (Lee *et al.*, 2001). Samples and their blanks were run in duplicate or triplicate, depending of sample availability. An average value and standard deviation were calculated.

The IC_{50} value is defined as the necessary concentration of a sample to inhibit an enzyme by 50%. The IC_{50} was based on a graph where the percentage of inhibition is represented in relation to the concentration of the sample.

3. RESULTS

3.1 Compositional Analysis of *A. nodosum* residue

The composition of *A. nodosum* residue was studied monthly over a full calendar year. Results obtained in terms of total solids (TS), minerals, protein, lipid and fibre contents are presented in the following sections. Mineral analysis and fatty acid characterisation were also carried out.

3.1.1 Total Solids and Moisture

The results obtained for total solids (TS) and moisture are shown in Table 3.1 and Figure 3.1. There appeared to be little variation during the year for both TS and moisture measures. August presented the highest moisture value (86.96% ± 1.58) followed by May, October and June, while the lowest value was obtained in January (81.68% ± 0.54) and February (81.63% ± 0.27). The average value for TS and moisture was 16.00% ± 1.94 and 84.00% ± 1.94 respectively.

Table 3.1 *A. nodosum* residue total solids and moisture

Residue sample		TS (%)			Moisture (%)		
		Individual	Average	SD	Individual	Average	SD
Jan	1	17.88	18.32	0.54	82.12	81.68	0.54
	2	17.57			82.43		
	3	18.13			81.87		
	4	18.88			81.12		
	5	18.64			81.36		
	6	18.83			81.17		
Feb	1	18.12	18.37	0.27	81.88	81.63	0.27
	2	18.16			81.84		
	3	18.65			81.35		
	4	18.57			81.43		
Mar	1	15.75	16.11	0.43	84.25	83.89	0.43
	2	15.74			84.26		
	3	16.38			83.62		
	4	16.56			83.44		
Apr	1	17.04	16.87	0.22	82.96	83.13	0.22
	2	16.62			83.38		
	3	16.94			83.06		
May	1	14.14	14.06	0.17	85.86	85.94	0.17
	2	14.31			85.69		
	3	13.86			86.14		
	4	13.92			86.08		
	5	14.13			85.87		
	6	13.99			86.01		
Jun	1	14.51	14.63	0.17	85.49	85.37	0.17
	2	14.69			85.31		
	3	14.58			85.42		
	4	14.92			85.08		
	5	14.45			85.55		
	6	14.63			85.37		
Jul	1	16.29	16.55	0.21	83.71	83.45	0.21
	2	16.45			83.55		
	3	16.66			83.34		
	4	16.50			83.50		
	5	16.83			83.17		
Aug	1	9.93	13.04	1.58	90.07	86.96	1.58
	2	13.46			86.54		
	3	13.10			86.90		
	4	13.97			86.03		
	5	14.30			85.70		
	6	13.49			86.51		
Sep	1	17.89	18.35	0.26	82.11	81.65	0.26
	2	18.59			81.41		
	3	18.44			81.56		
	4	18.34			81.66		
	5	18.56			81.44		
	6	18.27			81.73		
Oct	1	14.46	14.35	0.17	85.54	85.65	0.17
	2	14.35			85.65		
	3	14.39			85.61		
	4	14.34			85.66		
	5	14.03			85.97		
	6	14.54			85.46		
Nov	1	16.21	16.22	0.18	83.79	83.78	0.18
	2	16.40			83.60		
	3	16.04			83.96		
Dec	1	17.26	17.24	0.09	82.74	82.76	0.09
	2	17.32			82.68		
	3	17.14			82.86		

Results given as percentage (w/w) of fresh residue sample

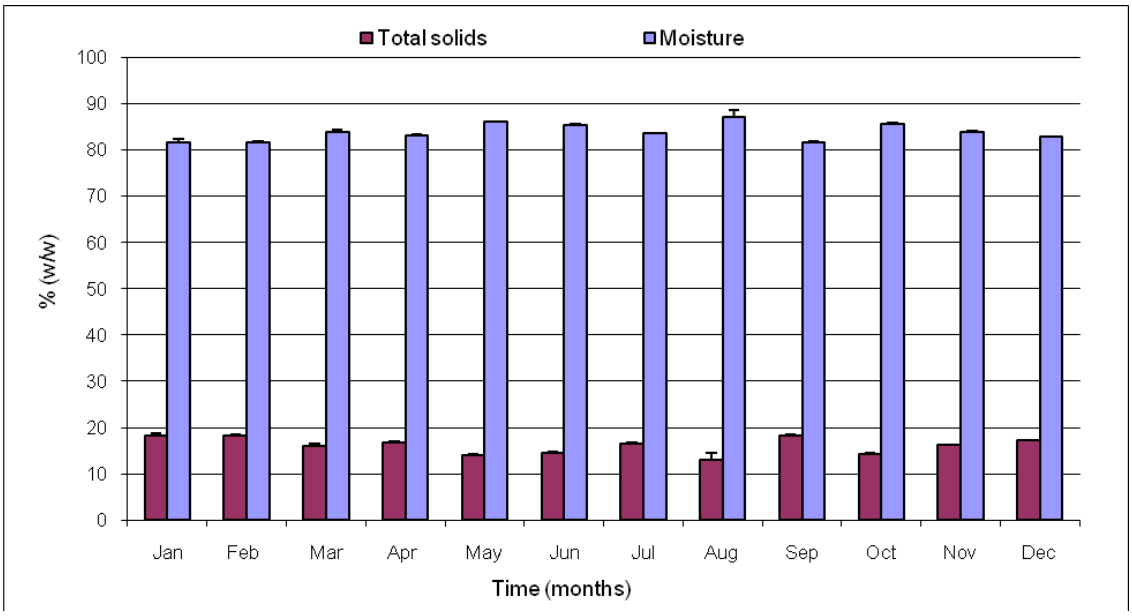


Figure 3.1 *A. nodosum* residue total solids and moisture (average and SD of each month). Results are given as percentage (w/w) of fresh sample.

3.1.2. Ash

The results for ash content are given in percentage (w/w) of dry sample in the following Table (3.2) and Figure (3.2). The highest content of ash was found in February with a value of 46.74% ± 1.96 and a minimum in March with 37.09% ± 0.64. There is a small variation in the ash percentage during the year. Some of the ash samples contained sand that could have led to overestimation of the measurement. The mean value for the ash content is 40.76% ± 2.75.

Table 3.2. Ash content in *A. nodosum* residue

Residue	Sample	Ash (%)	Average	SD	Residue	Sample	Ash (%)	Average	SD
Jan	1	39.73	39.15	1.13	Jul	1	36.73	37.70	0.61
	2	40.84				2	37.99		
	3	39.76				3	38.04		
	4	38.11				4	38.25		
	5	38.37				5	37.49		
	6	38.11			Aug	1	53.91*	40.15	0.48
Feb	1	44.83	46.74	1.96		2	39.77		
	2	46.89				3	40.93		
	3	45.85				4	39.94		
	4	49.40				5	39.84		
Mar	1	37.38	37.09	0.64		6	40.29		
	2	37.78			Sep	1	38.98	38.78	0.29
	3	36.85				2	38.40		
	4	36.32				3	38.41		
Apr	1	43.42	42.72	0.99		4	39.04		
	2	42.02				5	38.94		
	3	N.D				6	38.91		
May	1	42.48	42.49	1.02	Oct	1	41.44	41.25	0.65
	2	40.60				2	41.82		
	3	43.63				3	41.43		
	4	42.58				4	41.85		
	5	42.63				5	40.17		
	6	43.04				6	40.79		
Jun	1	43.62	43.53	0.30	Nov	1	40.14	40.33	0.22
	2	43.36				2	40.29		
	3	43.96				3	40.57		
	4	43.15			Dec	1	39.08	39.17	0.23
	5	43.52				2	39.43		
	6	72.88*				3	39.00		

Results given as percentaje (w/w) of dry sample.

N.D: Not determined; * values not included for the mean.

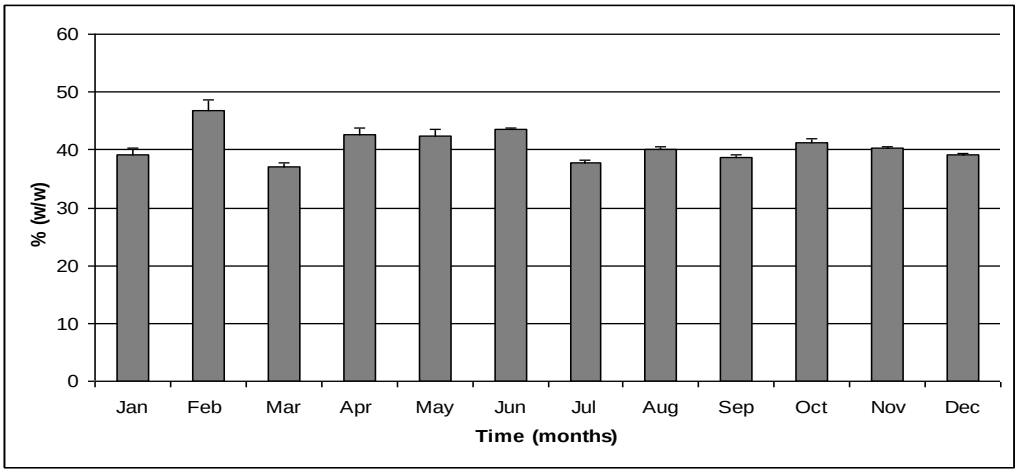


Figure 3.2. Ash content in *A. nodosum* residue (average and SD for each month). Results are given as percentage (w/w) of dry sample.

3.1.3. Protein content

Protein content results obtained by Kjeldahl method are shown in Table 3.3 and Figure 3.3. *A. nodosum* residue had an average protein content of 4.93% ± 1.27 during the year of study. A variation between the maximum in March (8.22% ± 0.11) and the minimum in October (3.66% ± 0.08) was found.

Table 3.3. Protein content in *A. nodosum* residue.

Sample		Protein (%)	Average	SD	Sample		Protein (%)	Average	SD
	1	3.59				1	5.08		
Jan	2	4.01	4.00	0.41	Jul	2	4.99	5.06	0.06
	3	4.41				3	5.12		
	1	5.97				1	4.37		
Feb	2	5.79	6.01	0.23	Aug	2	4.78	4.64	0.23
	3	6.26				3	4.77		
	1	8.10				1	3.83		
Mar	2	8.28	8.22	0.11	Sep	2	3.97	3.81	0.17
	3	8.28				3	3.62		
	1	5.34				1	3.58		
Apr	2	6.14	5.80	0.41	Oct	2	3.71	3.66	0.08
	3	5.92				3	3.70		
	1	3.96				1	4.30		
May	2	4.27	4.22	0.23	Nov	2	4.47	4.28	0.20
	3	4.42				3	4.08		
	1	4.45				1	5.02		
Jun	2	4.40	4.46	0.08	Dec	2	4.77	4.99	0.21
	3	4.55				3	5.19		

Results given as percentage (w/w) of dry sample.

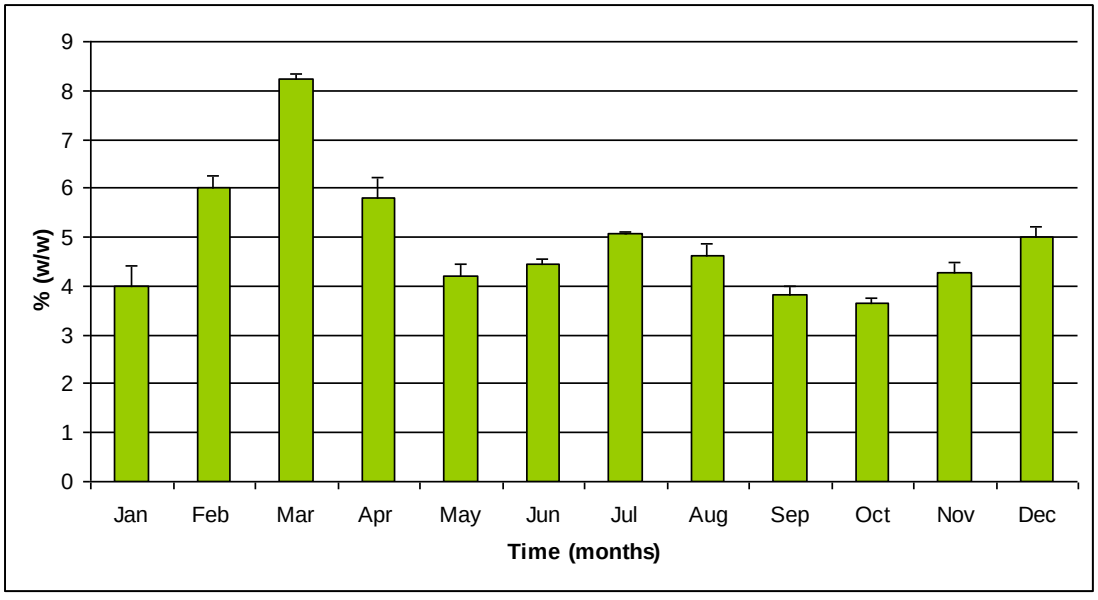


Figure 3.3. Protein content in *A. nodosum* residue (average and SD for each month). Results are given as percentage (w/w) of dry sample.

3.1.4. Lipid content

The results for the lipid content obtained by Soxhlet method are shown in Table 3.4 and Figure 3.4. There was a variation in the lipid content during the year of study. In this case the maximum value was taken in August (38.43% ± 0.06) and the minimum in March (18.21% ± 6.65), followed by January and February, the winter months. The average value for the lipid content was 24.80% ± 7.13.

Table 3.4. Lipid content in *A. nodosum* residue.

Sample		Lipid (%)	Average	SD	Sample		Lipid (%)	Average	SD
	1	17.74				1	22.11		
Jan	2	19.13	19.40	1.80	Jul	2	24.54	23.18	1.24
	3	21.32				3	22.88		
	1	22.86				1	38.47		
Feb	2	19.89	19.41	3.71	Aug	2	53.08*	38.43	0.06
	3	15.49				3	38.38		
	1	12.60				1	20.11		
Mar	2	25.57	18.21	6.65	Sep	2	23.16	20.96	1.92
	3	16.47				3	19.60		
	1	23.61				1	25.69		
Apr	2	21.80	22.25	1.20	Oct	2	30.51	27.00	3.07
	3	21.32				3	24.80		
	1	25.92				1	23.60		
May	2	34.56	30.53	4.35	Nov	2	20.17	19.25	4.87
	3	31.10				3	13.98		
	1	31.15				1	29.63		
Jun	2	35.25	29.58	6.60	Dec	2	19.49	24.49	5.07
	3	22.34				3	24.37		

Results given as percentage (w/w) of dry sample.* Results not included for the mean.

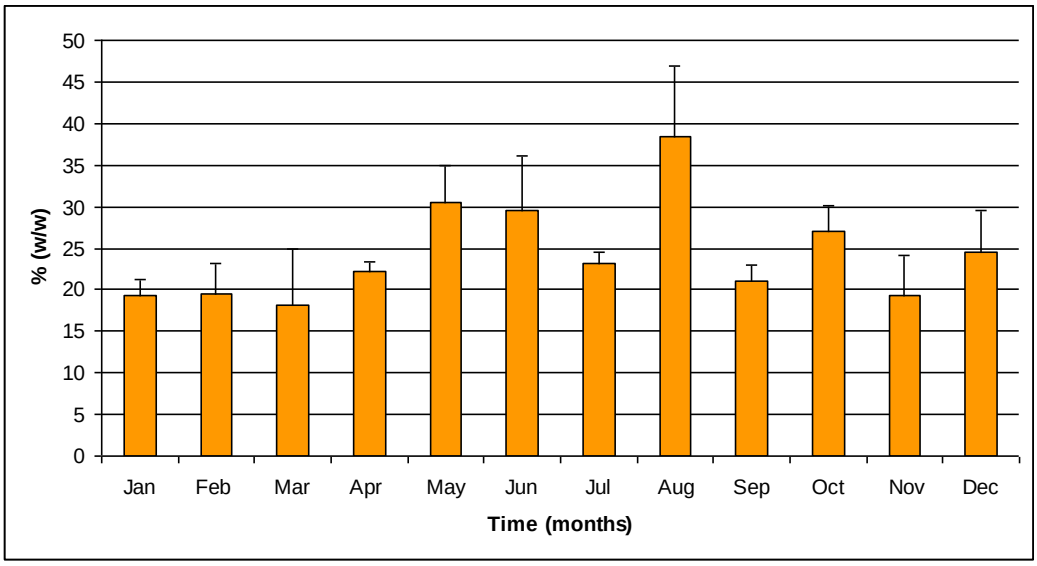


Figure 3.4. Lipid content in *A. nodosum* residue (average and SD for each month). Results given as percentage (w/w) of dry sample.

3.1.5. Fibre content

The results for the fibre content calculation are shown in Table 3.5 and Figure 3.5. Some variation was found (SD = 7.62). August had the lowest value (11.90%), while the highest values are found in January (37.44%), March (36.48%), July (34.06%) and November (36.13%).

Table 3.5 Fibre content in *A. nodosum* residue

Sample	Fibre (%)	Average	SD
Jan	37.44	29.52	7.62
Feb	27.84		
Mar	36.48		
Apr	29.24		
May	22.76		
Jun	22.43		
Jul	34.06		
Aug	11.90		
Sep	36.46		
Oct	28.09		
Nov	36.13		
Dec	31.35		

Results given as percentage (w/w) of dry sample.

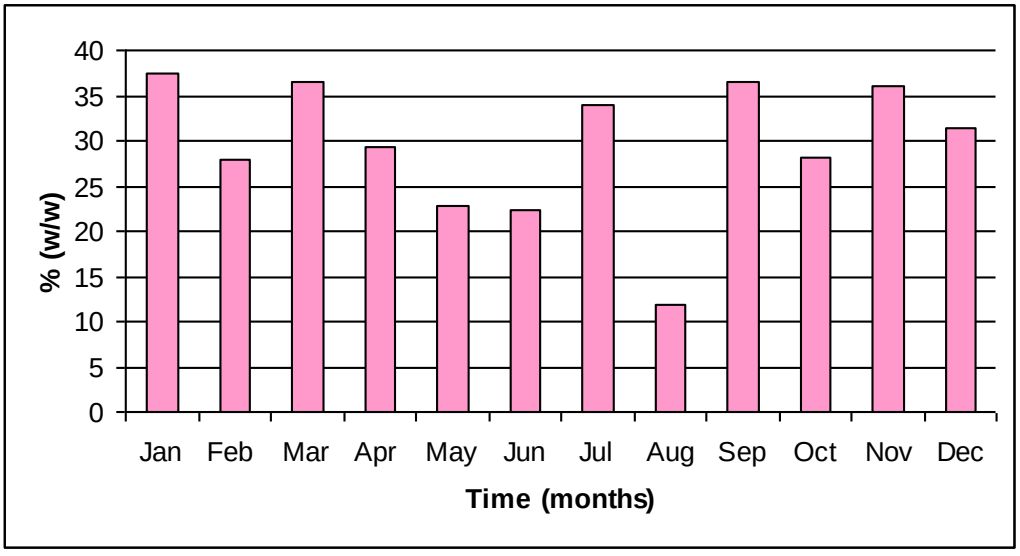


Figure 3.5. Fibre content in *A. nodosum* residue. Results given as percentage (w/w) of dry sample.

3.1.6. General Composition of *Ascophyllum nodosum* residue

Table 3.6 shows the averages of the total solids, moisture, ash, protein, lipid and fibre contents for all the replicates studied in the 12 month analysis. Figure 3.6 displays the general composition of the seaweed residue, which was mainly ash and fibre followed by lipids and proteins. The highest variation was found in the lipid (28.6%), fibre (25.8%) and protein (24.5%) contents, while the ash content only varied by 6.6%.

Table 3.6 General composition of the *A. nodosum* residue and *A nodosum* seaweed

	Residue		*A. nodosum*	
	% (w/w)	SD	% (w/w)	Ref.
Total Solids	16,00	1,94	18-33	
Moisture	84,00	1,94	67-82	1
Ash	40,66	2,68	18-24	1,2
Protein	4,93	1,25	4.8-9.8	1,2,3
Lipid	24,80	7,93	1.2-4.8	1,2,3
Other (fibre)	29,52	7,62	42-62	3

Results given as percentage (w/w) of fresh sample for total solids and moisture, and percentage (w/w) of dry sample for ash, protein, lipid and other (fibre). References: 1. (Jensen, 1960), 2. (Rioux et al., 2007) and 3. (Morrisey et al., 2001).

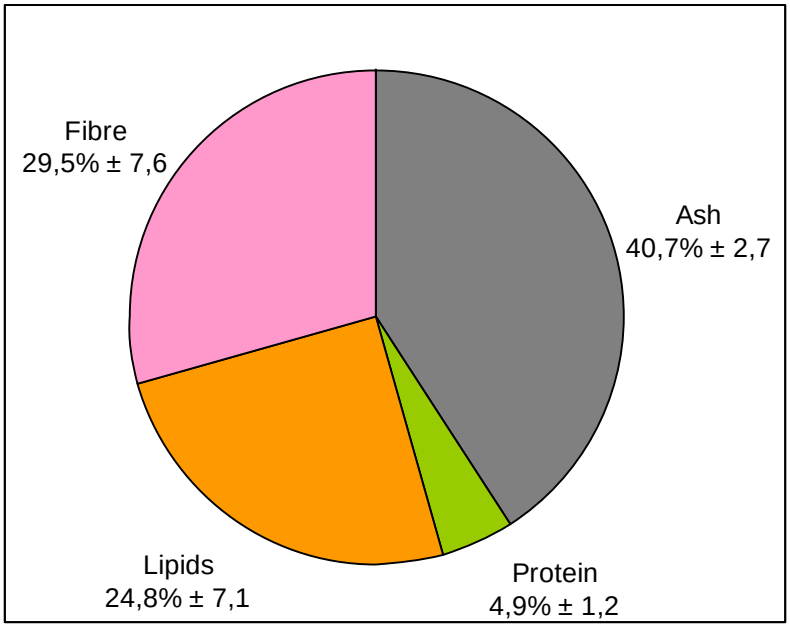


Figure 3.6 General composition of *A. nodosum* residue (average and SD of the 12 months studied). Results given as percentage (w/w) of dry sample.

3.1.7. pH

The pH was also studied monthly over a full year. Results for the pH are shown in Table 3.7 and Figure 3.7. Little variation was observed during the year, finding the highest value in May (9.51 ± 0.02) and the lowest in August (8.06 ± 0.01). The average value for the pH was 8.61 ± 0.39.

Table 3.7 pH of *A. nodosum* residue

Sample		pH	mean	SD	Sample		pH	mean	SD
	1	8.53				1	8.47		
Jan	2	8.55	8.55	0.02	Jul	2	8.43	8.45	0.02
	3	8.57				3	8.45		
	1	8.65				1	8.08		
Feb	2	8.57	8.61	0.04	Aug	2	8.06	8.06	0.01
	3	8.60				3	8.05		
	1	8.41				1	9.17		
Mar	2	8.48	8.40	0.08	Sep	2	9.09	9.10	0.06
	3	8.32				3	9.05		
	1	8.32				1	8.47		
Apr	2	8.34	8.28	0.09	Oct	2	8.57	8.53	0.05
	3	8.18				3	8.54		
	1	9.49				1	8.60		
May	2	9.54	9.51	0.02	Nov	2	8.63	8.63	0.03
	3	9.51				3	8.67		
	1	8.49							
Jun	2	8.57	8.54	0.05	Dec			N.D.	
	3	8.57							

N.D. not determined

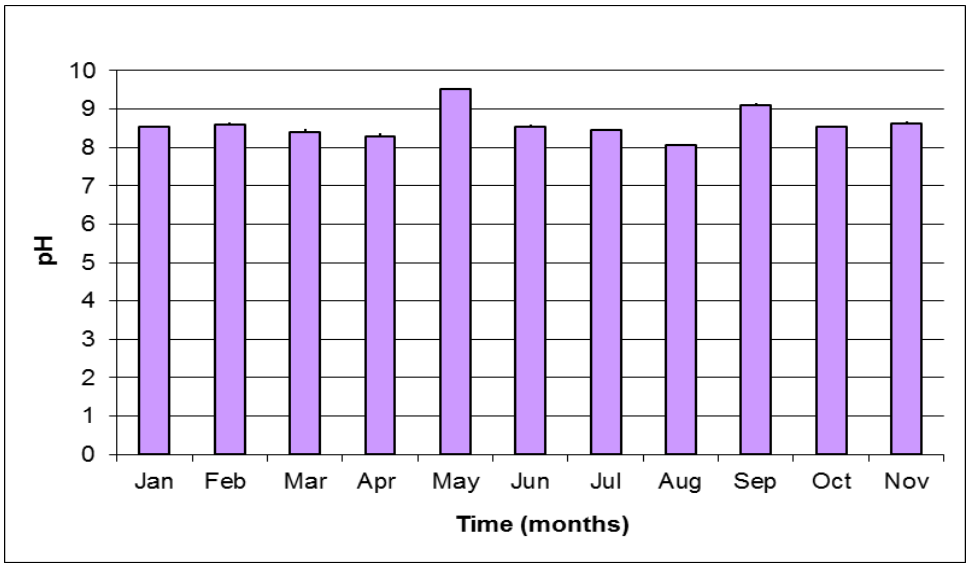


Figure 3.7 *A. nodosum* residue pH (average for each month).

3.1.8. Polyphenols

The polyphenol determinations are shown in Table 3.8 and Figure 3.8. Some variation was found during the year. Maximum values were found in May, June and October (8.31 ± 0.18; 7.56 ± 0.79 and 7.30 ± 0.31 mg GA equivalent/ g dry sample, respectively). Minimum values were found during the winter months, January (2.18 ± 0.49) and December (2.94 ± 1.81).

Table 3.8 Soluble polyphenol concentration in *A. nodosum* residue

Sample		Polyphenols	Average	SD	Sample		Polyphenols	Average	SD
Jan	1	2.53	2.18	0.49	Jul	1	2.34	3.50	1.65
	2	1.84				2	4.67		
Feb	1	4.21	4.37	0.22	Aug	1	3.76	3.63	0.19
	2	4.53				2	3.49		
Mar	1	5.74	5.80	0.09	Sep	1	3.78	4.23	0.64
	2	5.87				2	4.68		
Apr	1	4.00	4.50	0.71	Oct	1	7.09	7.30	0.31
	2	5.00				2	7.52		
May	1	8.19	8.31	0.18	Nov	1	3.75	4.07	0.45
	2	8.43				2	4.39		
Jun	1	7.00	7.56	0.79	Dec	1	1.66	2.94	1.81
	2	8.11				2	4.22		

Results given as mg of Gallic Acid equivalent per g of dry sample.

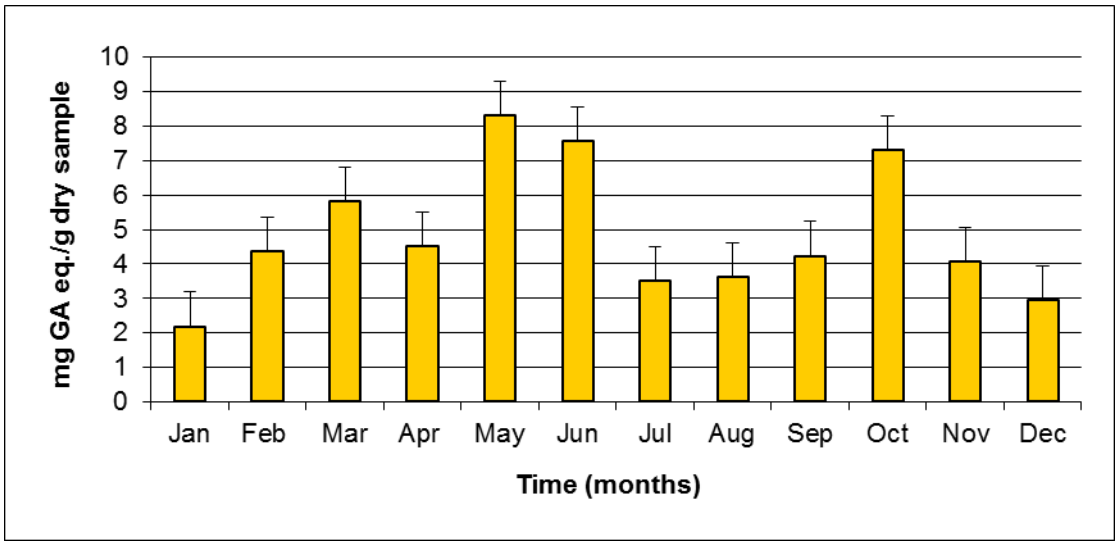


Figure 3.8 Soluble polyphenol concentration in *A. nodosum* residue.

3.1.9. Metal analysis

Metal analysis results are shown in Table 3.9. Of the minerals analysed (K^+, Na^+, etc.) Potassium was the most abundant (49.16% of the total), probably due to the addition of potassium carbonate in the extraction process. Sodium and Calcium were next with a concentration of 17.88% and 16.77% of the total, followed by Magnesium (13.95% of the total) and Iron (1.62% of the total). The rest of the minerals analysed were present in trace amounts. There remains a discrepancy and therefore there may be additional unknown minerals to determine.

Table 3.9 Mineral composition of *A. nodosum* residue

Mineral	% of mineral content analysed	mg/g of wet residue
K^+	49.16	11.813
Na^+	17.88	4.2966
Ca^{2+}	16.77	4.029
Mg^{2+}	13.95	3.349
Fe^{2+}	1.62	0.387
P^+	0.43	0.105
Mn^{2+}	0.13	0.031
Zn^{2+}	0.05	0.012
Cu^{2+}	0.02	$5.55*^{10-3}$
Cd^{2+}	$5.7*^{10-5}$	$1.975*^{10-5}$

3.1.10. Fatty Acid Analysis

Fatty acids were analysed by GC. The FAME standards were identified at different concentrations of the mixture and the retention times were averaged (Table 3.10).

Two residue samples were analysed by GC (Figures 3.9 and 3.10). The FAME found and their retentions times are shown in Table 3.11. There were two unidentified FAME: first one at 5.99 minutes, which is between C-6:0 and C-8:0, and a second one at 39.8 minutes between C-18:0 and C-20:5. FAME peaks found at 30.8 minutes in both samples are not positively identified; they might be C-17:0 or C-17:1. It also happens at 34.77 and 34.82 minutes, where they might be C-18:3 or C-18:2.

Table 3.10 F.A.M.E. standards and their retention times

	Retention Time (min)				
FAME	**10 mg/ml**	**5 mg/ml**	**2,5 mg/ml**	**Average**	**SD**
C-4:0	1,63	1,60	N.D.	1,62	0,02
C-6:0	3,52	3,52	3,52	3,52	0,00
C-8:0	7,42	7,45	7,50	7,46	0,04
C-11:0	12,96	12,96	13,21	13,04	0,14
C-13:0	17,09	17,16	17,58	17,28	0,27
C-14:1	19,57	19,60	20,19	19,79	0,35
C-14:0	19,86	19,87	20,49	20,07	0,36
C-15:1	22,77	22,80	23,54	23,04	0,44
C-15:0	23,09	23,10	23,86	23,35	0,44
C-16:1	26,01	26,03	26,94	26,33	0,53
C-16:0	26,74	26,75	27,71	27,07	0,56
C-17:1	30,18	30,20	31,38	30,59	0,69
C-17:0	31,06	31,06	32,30	31,47	0,72
C-18:3n3	33,94	33,95	35,33	34,41	0,80
C-18:3n6	34,65	34,68	36,10	35,14	0,83
C-18:2	34,83	34,85	36,27	35,32	0,83
C-18:0	36,14	36,15	37,60	36,63	0,84
C-20:5n3	43,07	43,08	44,61	43,59	0,89
C-20:4	43,54	43,54	45,06	44,05	0,88
C-20:0	46,01	46,03	47,62	46,55	0,92
C-21:0	50,49	50,50	52,18	51,06	0,97
C-22:0	54,71	54,73	56,49	55,31	1,02
C-23:0	58,70	58,69	60,62	59,34	1,11
C-24:0	62,53	62,53	64,53	63,20	1,15

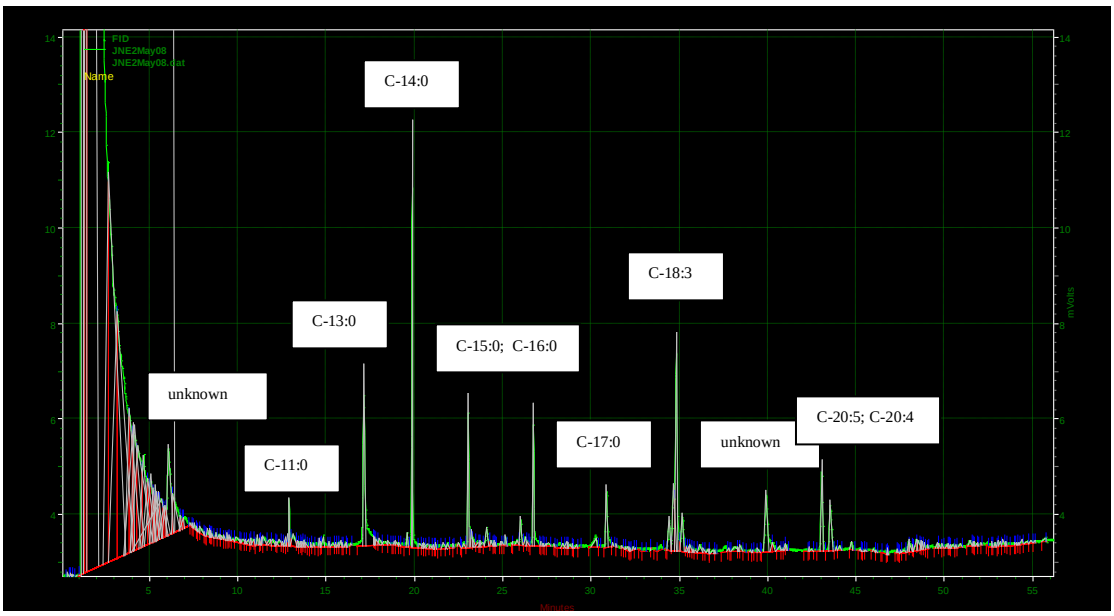


Figure 3.9 FAME analysis by GC from *A. nosodum* residue (2nd May 2008). Time x-axis and mVolt y-axis.

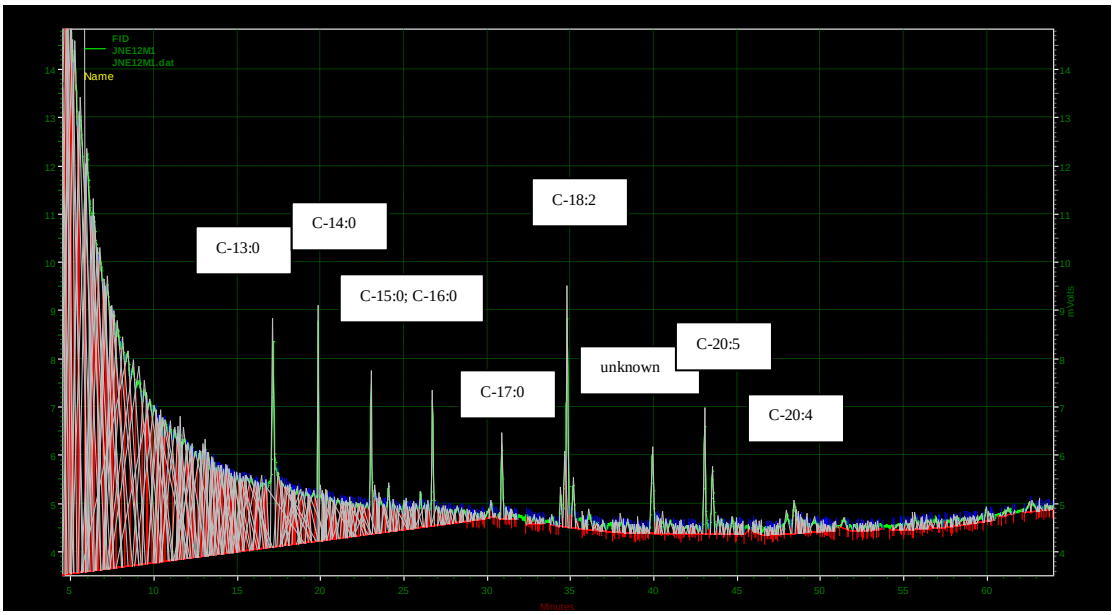


Figure 3.10 FAME analysis by GC from *A. nodosum* residue (12th May 2008). Time x-axis and mVolt y-axis.

Table 3.11 FAME from *A. nodosum* residue

Sample Date			
2nd May 08		**12th May 08**	
FAME	**Retention time (min)**	**FAME**	**Retention time (min)**
Unknown	5.99	C-13:0	17.41
C-11:0	12.98	C-14:0	19.85
C-13:0	17.14	C-15:0	23.02
C-14:0	19.9	C-16:0	26.72
C-15:0	23.04	C-17:0/1	30.82
C-16:0	26.69	C-18:3/2	34.82
C-17:0/1	30.85	Unknown	39.89
C-18:3/2	34.77	C-20:5	43.03
Unknown	39.83	C-20:4	43.48
C-20:5	43.1		
C-20:4	43.49		

3.2. Compositional analysis of *Ascophyllum nodosum* meal, extract and residue from the same extraction process

A compositional analysis was carried out in the meal used, the extract obtained and residue left from the same extraction process. The aim of this study was to find out how the different components of *A. nodosum* meal were distributed during the industrial process. Table 3.12 shows the results obtained for TS, moisture, ash, protein, lipid contents, fibre, polyphenols and pH of *A. nodosum* meal, extract and residue. Only the average values and the standard deviation are shown. For the complete data set see appendix I. The general composition of the three samples is also shown in Figure 3.11.

Table 3.12 *A. nosodum* meal, extract and residue composition

	Meal	Extract	Residue
TS (% w/w)	86.82 ± 0.19	9.68 ± 0.08	18.08 ± 0.23
Moisture (% w/w)	13.18 ± 0.19	90.32 ± 0.08	81.92 ± 0.23
Ash (% w/w dried matter)	23.23 ± 0.18	46.10 ± 2.79	38.89 ± 0.47
Protein (% w/w dried matter)	6.46 ± 0.13	4.59 ± 0.24	5.70 ± 0.73
Lipid (% w/w dried matter)	1.79 ± 0.21	3.53	18.10 ± 2.60
Fibre (% w/w dried matter)	68.52	45.78	37.32
Polyphenols (mg GA eq/g dry sample)	2.26 ± 0.32	39.07	4.02 ± 0.15
pH	5.25	8.78	9.06

The main component in the meal was fibre (68.52%), followed by minerals (23% ± 0.18), proteins (6.46% ± 0.13) and lipids (1.79% ± 0.21). In the extract the principle components were fibre and ash, being present in roughly equal proportions: 45.78% and 46.10%. A similar comparison was noted in the residue, where the fibre presented a value of 37.32% and the ash 38.89% ± 0.47. The residue had greater lipid content than the extract (18.10% *vs*. 3.53%).

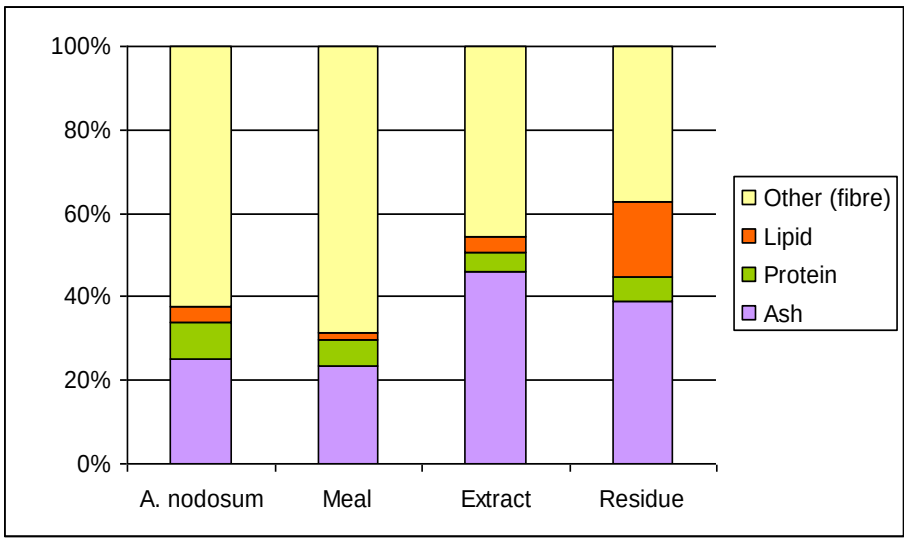


Figure 3.11 General composition of dried *A. nodosum* seaweed, meal, fertilizer extract and residue. *A. nodosum* composition is represented as an average of the intervals reported by the literature (see Table 3.6).

3.3 Development of a microbial digestion: initial trials and modifications

The main objective of this research project was the development of a microbial digestion system for *A. nodosum* residue. Due to the novelty of this proposal, the digestion process was developed on a trial and error basis initially, and optimised by evidence-directed strategy. Different parameters such us addition of an inoculum, aeration type, pH, residue/water content and decantation of soluble matter were studied to determine the optimum conditions. In the following sections the development and optimisation of a basic digestion model is described.

3.3.1 Initial digestion trials

Initial digestion trials 1 and 2 (INIT-1 and INIT-2) were the first experiments carried out in April and May 2007. The conditions employed are described in Table 3.13. Trial INIT-1 started with 55 g of residue and 45 ml of water, which yielded a very thick digestate from which separation of insoluble matter (IM) and soluble matter (SM) was not possible by filtration. Centrifugation was employed as a separation method, from day 6 onwards, allowing a more effective separation of IM and SM, and was used in all subsequent digestions. In trial INIT-2, a smaller amount of residue (35 g) and a greater volume of water (150 ml) were employed. No inoculum was added in initial trials.

The IM decreased from 72.94% to 64.74% in INIT-1 (11.24% decrease, Table 3.14), while it only decreased from 46.18% to 44.99% in INIT-2 (2.58% decrease, Table 3.15). The SM slightly increased in INIT-1 (9.16%, Figure 3.12), while it decreased in initial-2 (-18.50%, Figure 3.13). This suggests that in INIT-2, the natural flora of *A. nodosum* residue (inoculum was not added) used the SM for their growth. The increase (in mid-digestion) of IM in INIT-2 may be due to biomass production.

Table 3.13 Digestion conditions for initial trials

Trial	Residue	Water	Days	Temp.	Incubation	Measured Parameters	IM/SM Separation Techniques
INIT-1	55 g	45 ml	16	30^{o}C	Aerobic (75 rpm)	IM, SM	Filtration and Centrifugation
INIT-2	35 g	150 ml	18	30^{o}C	Aerobic (75-100 rpm)	IM, SM	Centrifugation

Table 3.14 Trial INIT-1

Day	IM %	SM %
0	72.94	26.10
1	73.40	22.96
2	78.86	19.31
3	75.89	20.95
6	N.D	27.20
7	66.56	29.14
8	65.98	N.D
9	66.58	36.46
10	63.74	29.36
14	65.22	29.52
15	68.36	29.95
16	64.74	28.49
Change (%)	-11.24	9.16

Results given as percentage (w/w) of dry sample.

Table 3.15 Trial INIT-2

Day	IM %	SM %
0	46.18	52.44
1	44.53	51.67
2	44.46	50.64
3	46.52	48.99
4	52.07	47.06
5	47.34	46.55
6	45.81	46.53
7	44.87	46.49
8	45.78	46.51
18	44.99	42.74
Change (%)	-2.58	-18.50

Results given as percentage (w/w) of dry sample

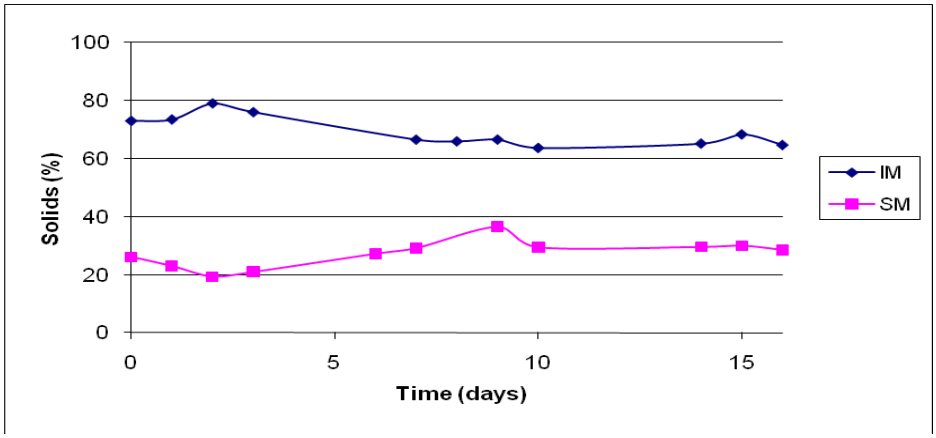


Figure 3.12 Trial INIT-1: IM and SM profiles (w/w of dry sample).

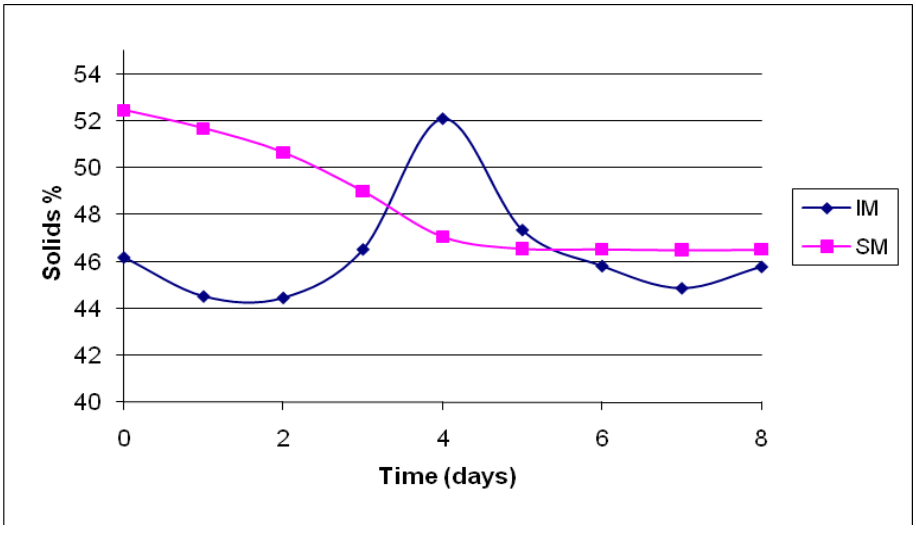


Figure 3.13 Trial INIT-2: IM and SM profiles (w/w of dry sample).

3.3.2 Addition of inocula

In an attempt to speed up *A. nodosum* residue digestion the addition of an inoculum was considered. Three different inocula (A, B and C) were evaluated separately, in terms of IM decrease over digestion.

3.3.2.1 Inoculum A

Inoculum A was derived from decomposing seaweed as explained in Section 2.3.3.1. In the inoculum A trial (INOC-A) *A. nodosum* residue (26 g), acetate buffer (100 ml) and inoculum A (6 ml) were incubated aerobically (100 rpm) at 30^{o}C over 12 days. Acetate buffer was used instead of water with the aim of decreasing the residue's initial pH (3.3.3.1).

Results from INOC-A (Table 3.16) suggested that the microorganisms in this inoculum preferentially metabolised soluble matter, as indicated by the decrease of the SM from 63.68% to 46.80% (26.51 decrease, Table 3.16). The soluble content of this particular residue sample appeared high relative to other samples (INIT-1, Section 3.3.1). It would appear that the microorganisms used the abundance of available nutrients to create biomass (IM increased by 7.20%, Table 3.16).

Table 3.16 Trial INOC-A

Day	IM %	SM %
0	43,31	63,68
1	49,88	54,73
2	44,38	54,11
5	42,29	57,88
6	46,34	55,83
7	45,61	56,43
12	46,43	46,80
Change (%)	+7.20	-26.51

Results given as percentage (w/w) of dry sample.

3.3.2.2 Inoculum B

Inoculum B was derived from decomposing *A. nodosum* seaweed (Section 2.3.3.2). Two experiments were run to evaluate inoculum B. The first experiment included two digestion trials (INOCB-1 and INOCB-2) where inoculum B was employed. The second experiment compared two trials: one with the addition of inoculum B (INOCB-3) and the other without inoculum B (NON-INOCB).

The digestion conditions employed for inoculum B trials 1 and 2 (INOCB-1 and INOCB-2) are given in Table 3.17. Both trials were run under the same conditions, with the only difference being the duration (6 *vs*. 12 days, Table 3.17), to check if longer incubation time would lead to an increase in the digestion yield.

Two different residue samples were employed for INOCB-1 and INOCB-2. In INOCB-1 the IM decreased from 73.99% to 51.55% after 6 days (30.33% decrease) and the SM increased from 31.56% to 36.42% (15.40% increase, Table 3.18). In trial INOCB-2 the IM decreased from 57.84% to 45.56% after 12 days of aerobic incubation (21.23% decrease), while the solubilised material slowly decreased from 39.05% to 36.41% (6.76% decrease, Table 3.19). The residue sample employed for INOCB-2 presented a lower initial value of IM (57.84%) compared to INOCB-1 (73.99%) and higher SM (39.05% compared to 31.56%; Figure 3.14). Also, the residue sample used in INOCB-1 presented a pH value of 8.0, while in INOCB-2 the pH was higher (9.0).

Inoculum B seemed to provide a beneficial outcome, as showed in INOCB-1 (30.33% IM decrease). This result was not repeated in INOCB-2 (21.23% IM decrease); however this could be explained by the low initial amount of IM, high SM and by the high pH of this particular residue sample.

Table 3.17 Digestion conditions for INOCB 1 and 2

Trial	Residue	Water	Inoculum	Days	Incubation	Measured Parameters
INOCB-1	24 g	100 ml	Inoc. B (10 ml)	6	Aerobic, 150 rpm	IM, SM
INOCB-2	24g	100 ml	Inoc. B (10 ml)	12	Aerobic, 150-200 rpm	IM, SM, pH

Table 3.18 Trial INOCB-1

Day	IM %	SM %
0	73,99	31,56
3	66,59	32,57
6	51,55	36,42
Change (%)	-30.33	15.40

IM and SM results are given as percentage (w/w) of dry sample.

Table 3.19 Trial INOCB-2

Day	pH	IM %	SM %
0	9	57,84	39,05
2		56,67	36,20
6		49,96	38,03
9		47,39	36,14
12	9,5	45,56	36,41
Change (%)		-21.23	-6.76

IM and SM results are given as percentage (w/w) of dry sample.

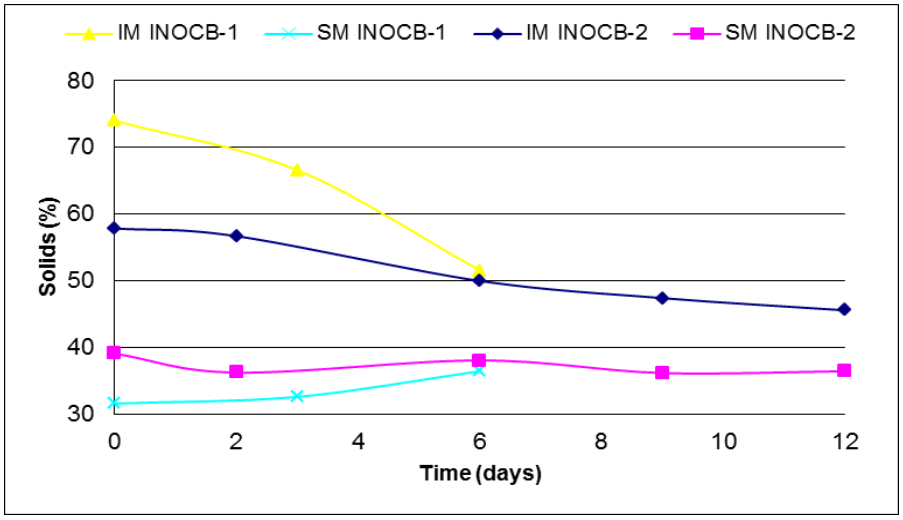


Figure 3.14 IM and SM profiles of Inoculum B trials 1 and 2.
Results given as % w/w of dry sample.

In the second experiment a trial inoculated with inoculum B (INOCB-3) was compared to another where inoculum B was not employed (NON-INOCB). Both trials consisted of residue (50 g) and water (400 ml) incubated under a mixed incubation type (Sections 2.3.2.3 and 3.3.3.2): 3 day anaerobic and 5 day aerobic (shaking incubation). After the anaerobic phase, SM (200 ml) was decanted and replaced with water, a matter studied further in Section 3.3.4. INOCB-3 was inoculated with inoculum B just before the aerobic phase. No inoculum was employed for NON-INOCB. IM, SM and pH were measured during digestion.

Results of INOCB-3 and NON-INOCB are presented in Table 3.20. INOCB-3 trial showed a greater IM decrease (from 47.24% to 33.48) compared to NON-INOCB (from 47.24% to 41.43%). The SM increased up to 28.16% during the aerobic phase of INOCB-3, while it showed a smaller increase in NON-INOCB (up to 21.93%, Table 3.20). The use of inoculum B improved the digestion rate of *A. nodosum* residue (29.13% IM decrease, INOCB-3) in comparison with the no addition of inoculum

(12.30% IM decrease, NON-INOCB). Inoculum B was utilised for the subsequent digestions. The microorganisms in inoculum B seemed to have the greater capacity to digest the solid matter of the residue.

Table 3.20 Trials INOCB-3 and NON-INOCB

	INOCB-3			NON-INOCB		
Day	**pH**	**IM%**	**SM %**	**pH**	**IM%**	**SM %**
0	9,08	47,24	49,7	9,08	47,24	49,7
3	7,24	46,33	48,58	7,24	46,33	48,58
6	8,12	37,34	29,04	8,34	40,64	23,61
8	8,34	33,48	28,16	8,59	41,43	21,93
Change (%)		-29.13	-43.34		-12.30	-55.88

IM and SM results are given as percentage (w/w) of dry sample.

3.3.2.3 Inoculum C

Inoculum C was derived from microorganisms found growing in *A. nodosum* residue (Section 2.3.3.3). In inoculum C trials 1, 2 and 3 (INOCC-1, INOCC-2 and INOCC-3) some variation in the water content was investigated: 50 or 100 ml of water per 7.5-8 g of residue TS (Table 3.20). These trials were inoculated at day 0 with Inoculum C and incubated aerobically at 30^{o}C. Measures of IM and SM were only taken at the last day of digestion. For this reason, the initial and final collected data were compared on the basis of dried weight.

In trials INOCC-1 and INOCC-2, it was found that an initial volume of 100 ml (INOCC-2) resulted in a more favourable decrease in the IM than an initial volume of 50 ml (INOCC-1 Table 3.21) further indicating the importance of adequate dilution of the digested material. In INOCC-3, which is an approximate scale-up of INOCC-2, similar results were found in spite of the longer period (67 days). Inoculum C was considered for further development of the microbial digestion, although the time employed in the digestion (a minimum of 28 days) was considered excessive for the industrial partner.

Table 3.21 Trials Inoculum C

	Initial			Final				
Trial	**Water (ml)**	**pH**	**Residue TS (g)**	**Day**	**pH**	**IM**	**SM**	**IM % Removal**
INOCC-1	50		7,515 g	28		3,629 g / 48.29 %	2,935 g / 39.04 %	51,71
INOCC-2	100		7,45 g	28		2,815 g / 37.79 %	3,76 g / 50.52 %	62,21
INOCC-3	500	8,36	44,297 g	67	9,26	16,558 g / 37.38 %	18,73 g / 42.29 %	62,62

IM and SM results are given as grams (dry sample) and percentage (w/w) of dry sample.

3.3.3 Effect of gaseous conditions on pH

One of the early issues considered in the development of the digestion process was the need to reduce the initial alkaline pH (8.61±0.39) to facilitate bacterial growth. A neutral pH was considered to better suit the microorganisms and their enzymes. Different chemicals (HCl and acetate buffer) and natural sources (moss peat and horse manure) were evaluated to decrease the pH, as well as the effect of different gaseous conditions (aerobic, anaerobic and mixed anaerobic/aerobic) on pH. Forced aeration was also evaluated.

3.3.3.1 Aerobic incubation and attempts to decrease pH

The first digestion trials in *A. nodosum* residue were carried out under aerobic incubation. For the aerobic trial pH-AEROB, 25 g of residue and 100 ml of water were mixed and incubated at 30^{o}C during 7 days. An initial pH of 8.26 increased to 9.04 after 24 hours and 9.15 after 7 days (Figure 3.15, pH-AEROB).

Attempts were made to reduce the pH by addition of HCl (pH-HCl) or acetate buffer (pH-ACETBUF). In trial pH-HCl, 25 g of residue and 100 ml of water were employed. 1M HCl was added (8 ml at time zero, 4 ml after 18 hours and 4 ml at 24 h) while pH was monitored regularly. This trial was incubated aerobically (100 rpm) at 30^{o}C over 14 days. The initial pH 4.00 gradually increased reaching a value of 7.75 at day 6 and 8.86 at day 14 (Figure 3.15, pH-HCl).

In trial pH-ACETBUF, acetate buffer (0.2 M, prepared from sodium acetate and acetic acid) was employed to decrease the residue's pH. A preliminary study using a range of different pH values (4-5.5) for the acetate buffer showed that a pH of 4.8 (buffer) resulted in an initial pH value of 6.16 when mixed with the residue, which increased to pH 8.6 after 11 days of aerobic incubation. So for pH-ACETBUF, acetate buffer pH 4.8 (100 ml) and inoculum A (Section 3.3.2.1) were added to the residue (25 g), instead of water. Incubation was at 30^{o}C for 12 days. The pH results of pH-ACETBUF showed an initial pH of 7.13, which increased to 8.73 after 24 hours and a gradual increase to 9.12 at day 12 (Figure 3.15, pH-ACETBUF).

The addition of HCl and acetate buffer were unsuccessful attempts to decrease the alkaline pH of the residue, as the pH returned to alkaline values after few days (1 to 6). The addition of larger volumes of these chemicals was not considered viable for an industrial process.

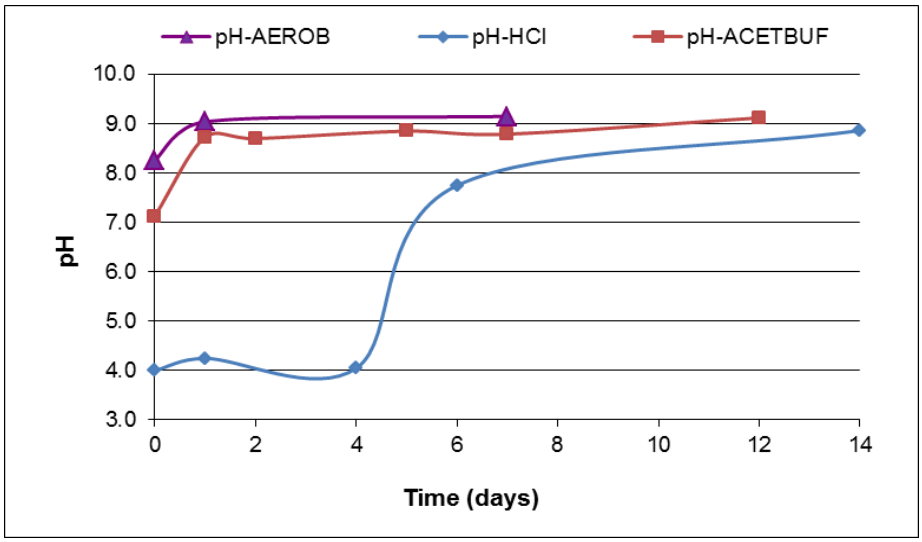


Figure 3.15 pH profiles for pH-AEROB, pH-HCl and pH-ACETBUF.

Natural sources like moss peat and horse manure were considered at this point of the research to change the pH of the residue. Different amounts of moss peat and horse manure were evaluated over several trials. Moss peat trials (pH-MOSSP) consisted on *A. nodosum* residue (25 g) mixed with different amounts of moss peat (5 to 20 g) and 100 ml of water, incubated aerobically (force aeration) at 30°C over 7 days. Also a control where no moss peat was added (only residue) was studied. The moss peat employed presented a pH value of 3.3. Results of pH-MOSSP are shown in Figure 3.16. From this experiment it was determined that up to 20 g of moss peat had to be added per 25 g of residue to obtain an initial pH of 6.25 and 7.38 after 7 days. This was not considered feasible for a large scale application.

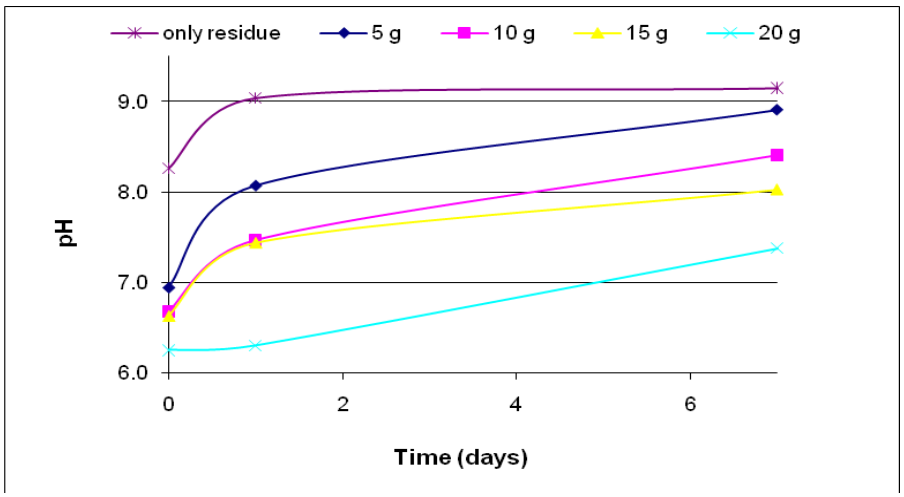


Figure 3.16 Trial pH-MOSSP pH profiles. Different amounts of moss peat (5 to 20 g) were added to *A. nodosum* residue (25 g), incubated aerobically at 30°C during 7 days.

Horse manure trials (pH-HORMA) employed residue (25 g) mixed with different amounts of horse manure (1, 2.5 and 5 g) and water (100 ml), incubated for a week at 30^{o}C under aerobic conditions (forced aeration). The horse manure employed had a pH value of 7.8 and mixed with the residue gave an initial basic pH of 8.6±0.04, which increased to 8.84±0.04 after 24 h and it kept at this value until day 7 (8.82±0.03). No differences were found between the different amounts of horse manure added (1, 2.5 and 5 g). An initial pH of 8.6 was not considered useful for the digestion of *A. nodosum* residue. It was concluded that a significant buffering effect existed in the residue, which made the reduction of the initial pH by the methods outlined (HCl, acetate buffer, moss peat and horse manure), very difficult or impractical.

3.3.3.2 Anaerobic incubation

Anaerobic digestion trials 1, 2 and 3 (ANAEROB-1, ANAEROB-2 and ANAEROB-3) employed a new initial proportion of residue/water: 50 g of *A. nodosum* residue in 400 ml of deionised water. This proportion of water was used from ANAEROB trials onwards. Digestion samples were incubated under anaerobic conditions (Section 2.3.2.2), at 30^{o}C for 8 days. The digestion sample was now more diluted to facilitate microbial attack; and also the volume was increased to reduce the air-space in the digestion flask, for the anaerobic incubation.

In ANAEROB-1 an initial pH value of 9.1 decreased to 7.27 after 8 days. ANAEROB-2 started with a pH of 9.08, which decreased to 7.18 after 48 hours (Figure 3.17). In ANAEROB-3, an initial pH of 9.08 was reduced to 7.24 after 3 days (Figure 3.17) and slightly increased to7.38 at day 8. In ANAEROB-3 soluble material (200 ml) was decanted and replace with water at day 3, further investigated in Section 3.3.4.

It was observed that the pH decreased under anaerobic conditions. This suggested the use of anaerobic incubation to decrease the pH. As a result of this observation, an initial 3-day period under anaerobic conditions was employed, leading to a mixed anaerobic/aerobic incubation procedure.

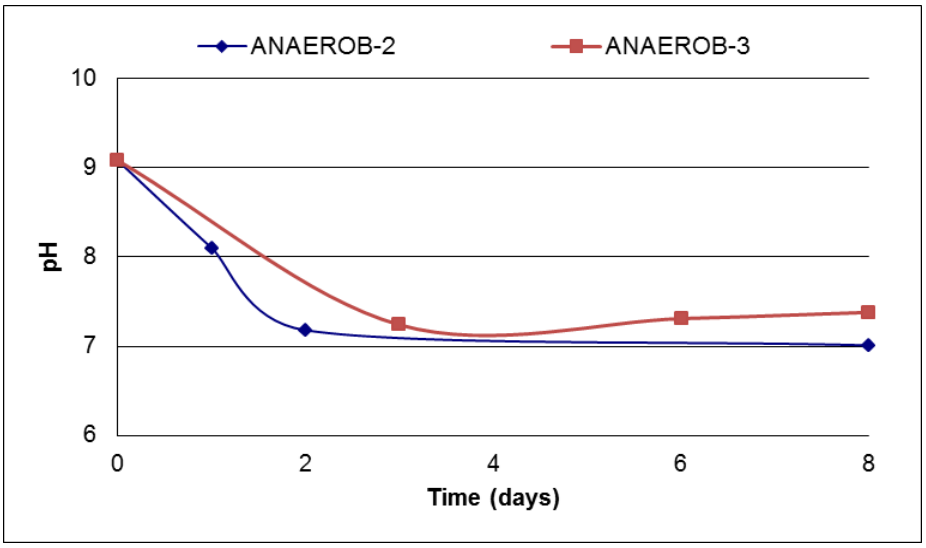


Figure 3.17 pH profile for ANAEROB-2 and 3.

3.3.3.3 Mixed anaerobic/aerobic

Mixed anaerobic/aerobic trials (MIXED) combined a 3-day anaerobic phase, followed by an aerobic phase (Section 2.3.2.3), both at 30^{o}C. A residue/water proportion of 50 g/400 ml was employed. Reduction of the initial pH by anaerobic means before an aerobic digestion was evaluated.

Trial MIXED-1 consisted of a 3-day anaerobic phase, followed by a 5-day aerobic phase. The duration of the aerobic phase was of 10 days (instead of 5) in trial MIXED-2. Both trials were run in duplicates. The pH results for MIXED-1 and MIXED-2 are shown in Figure 3.18. Initial pH values of 9.08 (MIXED-1) and 8.84 (MIXED-2) decreased to 7.2 during the 3-day anaerobic phase, as predicted. During the aerobic phase, the pH increased to 8.4 at day 8 (MIXED-1) and 8.6 at day 13 (MIXED-2).

The mixed anaerobic/aerobic incubation type showed to successfully decrease the initial alkaline pH of the residue during the anaerobic phase, providing a neutral pH at the beginning of the aerobic phase. This incubation type was further employed for the digestion of *A. nodosum* residue. Further study of pH during digestion was carried out in Sections 3.4.1.1 and 3.4.3.1. Also, a possible relation between organic acid production/digestion and pH decrease/increase was investigated by organic acid analysis (Section 3.5.3).

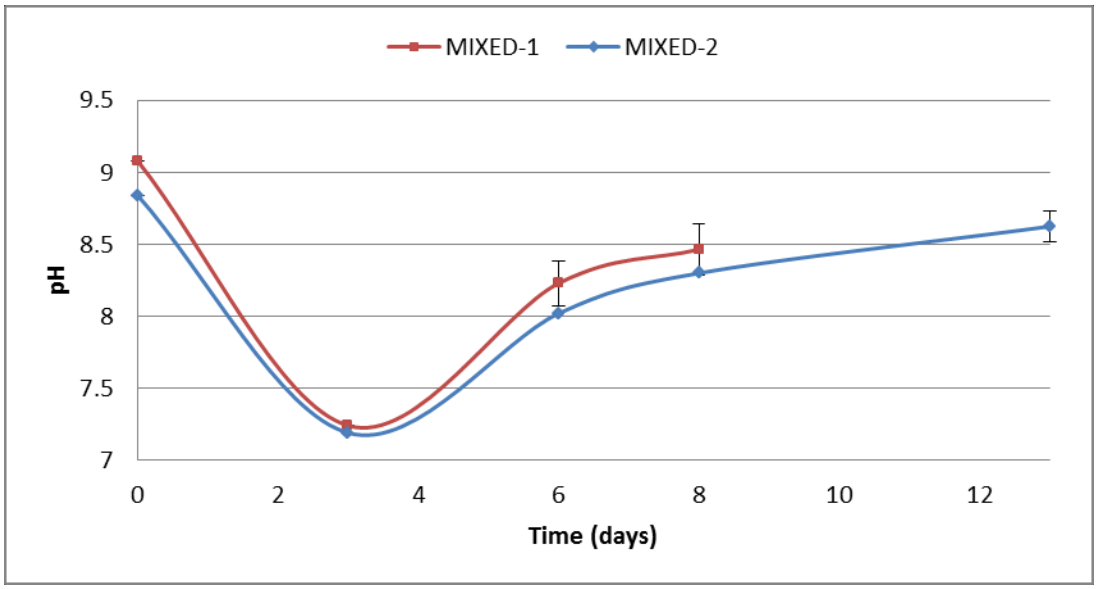


Figure 3.18 pH profiles for trials MIXED 1 and 2 (SD, n=2).

3.3.3.4 Forced aeration

Forced aeration was evaluated as a possible incubation type for aerobic conditions. Air was pumped into the digestion with an air-pump as explained in Section 2.3.2.1. Two experiments were carried out to compare shaking incubation *versus* forced aeration in *A. nosodum* residue digestion.

The first experiment consisted of two trials, FORCED-1 and SHAKING-1, which shared the same residue sample. In both trials, residue (24 g) was mixed with water (100 ml) and inoculum B (10 ml) and it was incubated aerobically for 6 days using forced aeration (FORCED-1) and shaking incubation (150 rpm, SHAKING-1). IM and SM were measured during digestion. Results are presented in Table 3.22. The IM of FORCED-1 decreased from 73.99% to 68.33% over 6 days, while SHAKING-1 exhibited a higher IM decrease to 51.55% (Table 3.22). SHAKING-1 showed a better digestion rate (30.33% of IM decrease) than FORCED-1 (7.65% IM decrease). The SM decreased in FORCED-1, while it increased in SHAKING-1 (Table 3.22).

Table 3.22 Trials FORCED-1 and SHAKING-1

	FORCED-1		SHAKING-1	
Day	**IM %**	**SM %**	**IM %**	**SM %**
0	73,99	31,56	73,99	31,56
3	69,05	31,76	66,59	32,57
6	68,33	20,78	51,55	36,42
Change (%)	-7.65	-34.16	-30.33	15.40

IM and SM results are given as percentage (w/w) of dry sample.

The second experiment consisted of two trials: FORCED-2 and SHAKING-2, which shared the same residue sample. This time, residue (50 g) was mixed with water (400 ml) and a mixed anaerobic (3 days) / aerobic (10 day) incubation type was employed. SM (200 ml) was decanted after the anaerobic phase and replaced with water, a matter studied in the following Section (3.3.4). Inoculum B was added at the beginning of the aerobic phase. Forced aeration (FORCED-2) and shaking incubation (SHAKING-2) were employed during the aerobic phase. Results of IM, SM and pH during digestion are presented in Table 3.23. SHAKING-2 exhibited a further decrease of the IM (from 66.35% to 41.92%) than FORCED-2 (from 66.35% to 63.77%), the former presenting a better digestion rate (36.82% IM decrease *vs.* 3.89%) over the same period of time. The SM decreased from 31.87% to 21.81% in FORCED-2 and it increased from 31.87% to 35.91% in SHAKING-2 (Table 3.23). Similar results in terms of IM and SM were exhibited by trials FORCED-1 and SHAKING-1 (Table 3.22).

These results could indicate that bacterial populations of *A. nodosum* residue and added Inoculum B fed in preference of the IM when incubated with shaking incubation as IM decreased by 33.57 ± 4.59% (average SHAKING 1 and 2) and SM increased by 24.04 ± 1.92%. In contrast, these bacterial populations seemed to feed preferentially on SM when incubated with forced aeration as SM decreased by 32.86 ± 1.83% (average FORCED 1 and 2) and IM slightly decreased 5.76± 2.65.

Table 3.23 Trials FORCED-2 and SHAKING-2

	FORCED-2			SHAKING-2		
Day	**pH**	**IM%**	**SM %**	**pH**	**IM%**	**SM %**
0	8.84	66.35	31.87	8.84	66.35	31.87
3	7.19	62.4	32.8	7.19	62.4	32.8
6	7.6	63.59	32.07	8.02	64.85	32.67
9	8.24	70.88	22.82	8.3	63.03	27.69
13	8.87	63.77	21.81	8.7	41.92	35.91
Change (%)		-3.89	-31.57		-36.82	12.68

IM and SM results are given as percentage (w/w) of dry sample.

When forced aeration was employed, a different smell was noted in the digestion flasks (FORCED 1 and 2) when compared with the flasks in the shaker incubator (SHAKING 1 and 2). The nature of the odour was not investigated but it seemed that different processes were active (different metabolic products and/or different microbial sps dominating) when aeration conditions were changed.

Forced aeration led to no significant improvement in yield of digested materials (5.76 ± 2.65%), compared to shaking incubation (33.57 ± 4.59%). For this reason forced aeration was not considered for further development in the microbial digestion of *A. nodosum* residue.

3.3.4 SM decantation

SM decantation was evaluated to prevent metabolite inhibition of the digestive process. SM was removed by centrifugation and replaced with water as explained in Section 2.3.4. An experiment was carried out with two trials: DECANT and NONDECANT. Both trials shared the same residue sample and consisted of residue (50 g) and water (400 ml), incubated under a mixed incubation type: 3-day anaerobic followed by a 10 day aerobic (shaking incubation). SM (200 ml) was decanted and replaced with water after the anaerobic phase in trial DECANT, while SM was not decanted in trial NONDECANT. Inoculum B (40 ml) was added to both trials at the beginning of the aerobic phase. The following parameters were measured over digestion: IM, SM and pH.

Results for both trials are shown in Table 3.24. The IM in DECANT was digested from 66.35% to 41.92%, while in NONDECANT the IM only decreased to 57.43%. The SM slightly increased from 31.87% to 32.80% during the anaerobic phase of both trials. During the aerobic phase the SM increased by 33.84% in NONDECANT (from 32.8% to 43.9%) and by 82.28% in DECANT (from 19.70% to 35.91%; Table 3.23).

DECANT presented a 36.82% reduction in IM, compared to 13.44% in NONDECANT. These results are also supported by the SM increase during the aerobic phase. This shows the importance of decanting SM to avoid metabolite inhibition. SM decantation was further employed for *A. nosodum* residue digestion process.

Table 3.24 Trials DECANT and NONDECANT

	DECANT			NONDECANT		
Day	**pH**	**IM%**	**SM %**	**pH**	**IM%**	**SM %**
0	8.84	66.35	31.87	8.84	66.35	31.87
3	7.19	62.4	32.8	7.19	62.4	32.8
3*	7.68	67.56	19.7			
6	8.02	64.85	32.67			
9	8.3	63.03	27.69			
13	8.7	41.92	35.91	8.55	57.43	43.9
Change (%)		-36.82			-13.44	

3* measure taken after SM decantation and addition on inoculum B

IM and SM results are given as percentage (w/w) of dry sample.

3.3.5 Developing a digestion model

Following this developmental work, there remained some concern regarding the variability and reproducibility of the digestion; however information was emerging that seemed to suggest a digestion model. Arising from Sections 3.3.1 to 3.3.4, a digestion system was constructed where anaerobic incubation reduced the pH; solubilised material was diluted after a further 3 days (to help to avoid metabolite inhibition) and addition of inoculum B was important. This combination appeared to digest a significant proportion of the solid component of *A. nodosum* residue. As inoculum C trials required additional time, inoculum C was referred for further development. Standardising the inoculum, the timing of anaerobic / aerobic digestions and the initial undigested solid content, led to an improvement in reproducibility, as shown in the following sections.

3.4 Batch digestion system and modifications

This section describes the batch digestion system that emerged as a result of the previous digestion trials (Section 3.3). The batch digestion model consisted of an initial proportion of residue/water of 50g/400ml, which was incubated under anaerobic conditions for the first three days to reduce the pH of the residue. At day 3, solubilised material (200 ml) was decanted and replaced with water to prevent metabolite inhibition. Inoculum B (10% v/v) was added at this stage and the digestion was incubated aerobically (shaken at 200 rpm) for 10 days (Figure 3.19).

The reproducibility of the batch digestion system was evaluated over different residue samples. Some modifications such as regular reduction of pH, regular SM decantation, reduction in the volume of water and combined inocula B and C were also studied. A

final study of the factors affecting the batch digestion system of *A. nodosum* residue was also undertaken.

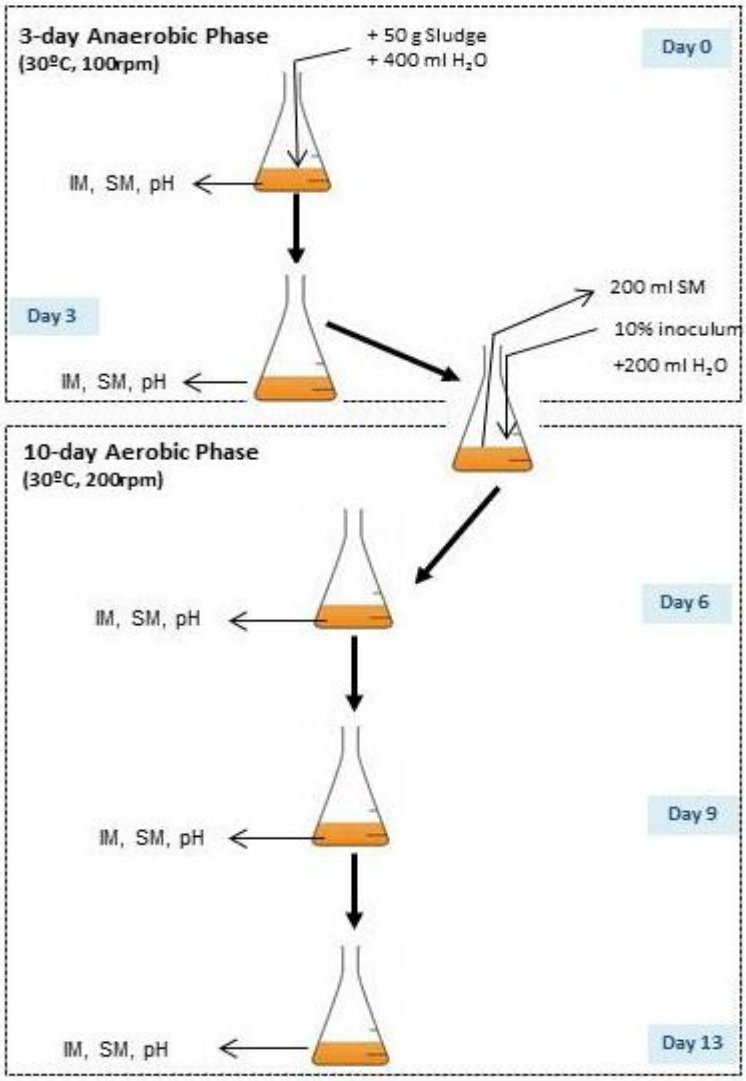


Figure 3.19 Batch digestion model.

3.4.1 Reproducibility evaluation of the batch digestion system

The conditions employed in the batch digestion system were shown to suit *A. nodosum* residue digestion during the developmental work (Section 3.3). A study of the reproducibility of the batch model was carried out in this section. The batch model described in Figure 3.19 was studied over several replicates (trials BATCH 1 to 6) employing different residue samples. IM, SM and pH were measured and studied during the digestion. Each residue sample presented different initial conditions of IM,

SM and pH, which made the digestion process challenging. Results of pH, IM and SM are presented separately in the following sections.

3.4.1.1 pH

Results regarding pH values of digestion trials BATCH 1 to 6 are shown in Table 3.25. The pH showed a decrease during the anaerobic incubation and an increase during the aerobic phase (Figure 3.20). In BATCH 1 to 4 the pH was studied continuously over the anaerobic phase. To fit the pH-meter inside a digestion flask, a 1 litre flask with 100 g of residue and 800 ml of water was employed. By comparing the three first days of these trials (Figure 3.21), it was apparent that pH decreased at a similar rate in each case. The rate of decrease seemed to be dependent on initial pH. In BATCH-3 an initial pH of 8.61 decreased to 7.22 at day 3, while in BATCH-2 a higher initial value of 9.09 dropped to 7.59. In BATCH-4 the pH dropped from 9.51 to 7.67 (Figure 3.21). The pH decreased an average of 1.24 ± 0.24 log value during the anaerobic phase of trials BATCH 1 to 6 (Table 3.25). When each digestion was continued under aerobic conditions, the pH gradually increased an average of 0.98±0.32 log value (Table 3.25), but only rarely returning to the original pH.

Table 3.25 pH values for trials BATCH 1 to 6

	pH							
Day	**BATCH-1**	**BATCH-2**	**BATCH-3**	**BATCH-4**	**BATCH-5**	**BATCH-6**	**Average**	**SD**
0	8.84	9.09	8.61	9.51	8.46	9.26	8.96	0.40
3	7.19	7.84	7.63	8.23	7.42	8.00	7.72	0.38
6	8.02	8.35	7.88	8.47	N.D	8.45	8.23	0.27
9	8.3	8.42	7.68	8.65	8.62	8.55	8.37	0.36
13	8.7	8.67	8.84	9	8.28	8.68	8.70	0.24
pH change								
Anaerobic phase	-1.65	-1.25	-0.98	-1.28	-1.04	-1.26	-1.24	0.24
Aerobic phase	1.51	0.83	1.21	0.77	0.86	0.68	0.98	0.32

N.D. not determined

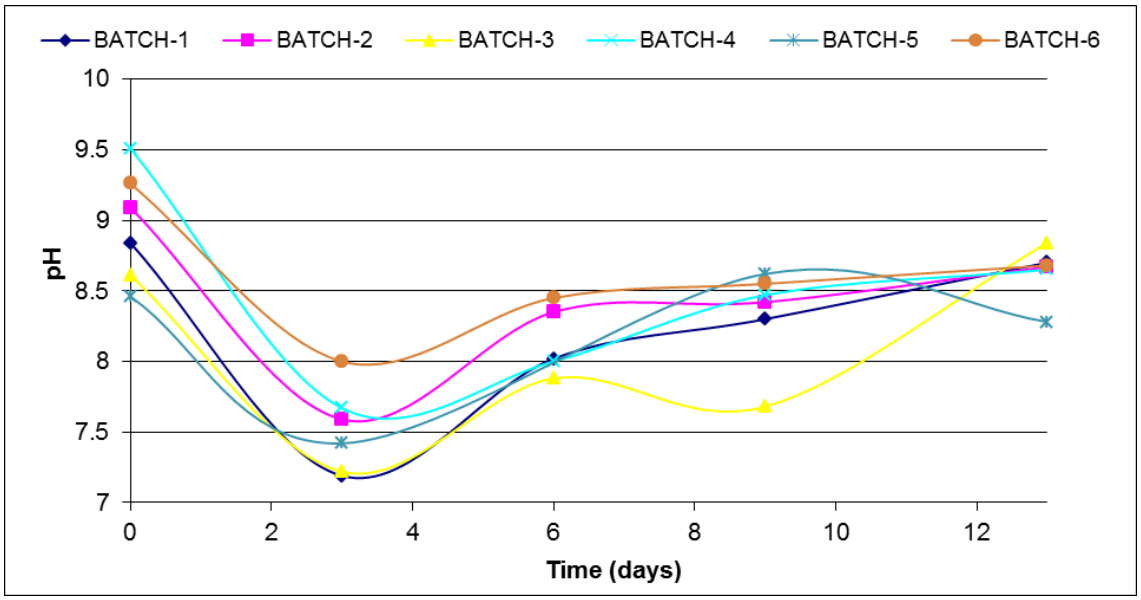


Figure 3.20 Batch digestion model pH profiles (BATCH 1 to 6).

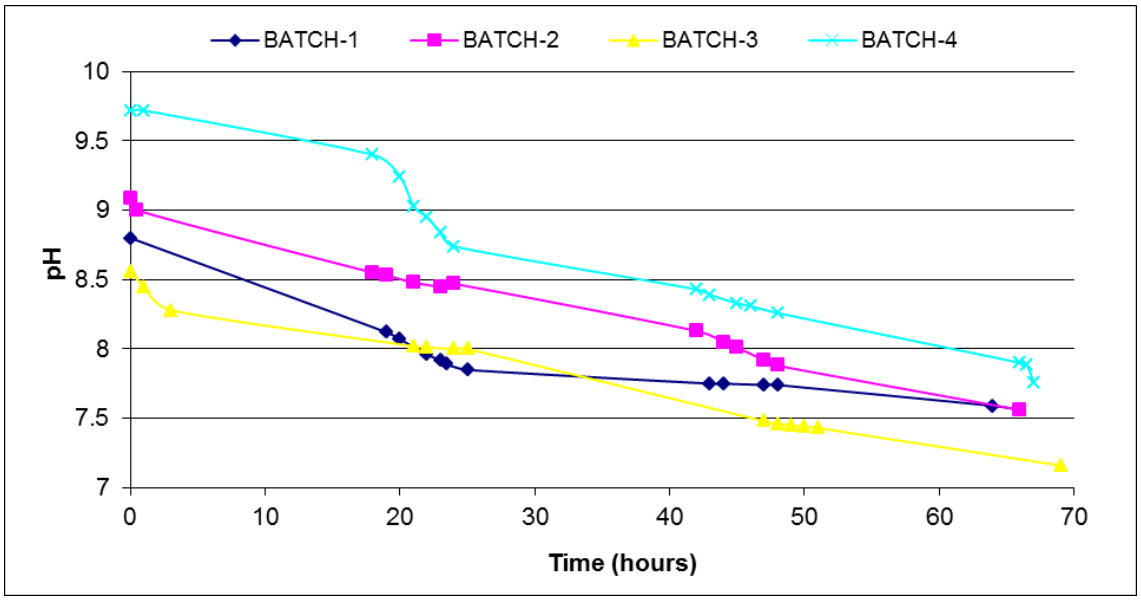


Figure 3.21 pH profiles during the 3-day anaerobic phase (BATCH 1 to 4).

3.4.1.2 IM and SM

The proportion of IM decreased in trials BATCH 1 to 6. It is important to note that the initial amount of IM was quite different in each digestion trial (72.43% ± 10.58, Table 3.26). It varied from 61.62% (BATCH-5) to 84.7% (BATCH-3; Figure 3.22). There was a definite inverse relationship between the initial IM and the initial SM (27.32% ± 8.21, Table 3.27). The initial SM ranged from 18.27% for BATCH-3 and 39.67% for BATCH-6.

The IM decreased at different rates in each BATCH trial (Table 3.36). During the anaerobic phase, the IM slightly decreased an average of 6.44 ± 11.16%. The digestion rate was higher during the aerobic phase with an average value of 29.48 ± 7.56% (Table 3.26). The overall digestion rate varied from 50.15% (BATCH-2, IM decrease) to 17.30% (BATCH-5), with an average value of 33.88 ± 11.52% (Table 3.26). Although the residue samples had different initial values of IM, the final values post-digestion were similar for all trials: 47.05 ± 4.57% at day 13 (Table 3.26, Figure 3.22). There would appear to be a metabolic or system-related obstacle at this level of undigested solids which prevents the digestion continuing further.

The IM increased at very different rates over digestion (Table 3.27). The IM showed a higher increase during the anaerobic phase (24.61 ± 26.34%), than during the aerobic (9.88 ± 11.56%, Table 3.27). There was some variation between the different trials. For example the SM increased by 80.73% in BATCH-2, while in decreased in BATCH-6. This seemed to be related to the IM digestion, as BATCH-2 (with 80.73% SM increase) exhibited the highest IM decrease (50.15%), and on the contrary BATCH-6 (where SM increased) presented a much lower IM decrease (24.36%). At the same time this might be influenced by the initial amounts of IM and SM and the pH. BATCH-2 had an initial amount of IM higher than BATCH-6 (83.53 *vs.* 61.73), while the amount of SM was lower in BATCH-2 (19.56%) than in BATCH-6 (39.67%). In addition, the pH was slightly higher in BATCH-6 compared to BATCH-2 in both day 0 (9.26 *vs.* 9.09) and day 3 (8.00 *vs.* 7.84). Further study of the factors that affect the microbial digestion of *A. nodosum* residue is carried out in Section 3.4.3.

Table 3.26 IM values for trials BATCH 1 to 6

	IM (%)							
Day	**BATCH-1**	**BATCH-2**	**BATCH-3**	**BATCH-4**	**BATCH-5**	**BATCH-6**	**Average**	**SD**
0	66.35	83.53	84.73	76.6	61.62	61.73	72.43	10.58
3	62.4	71.94	70.34	68.42	70.54	58.25	66.98	5.44
6	64.86	69.95	68.44	66.61		51.91	64.35	7.21
9	63.03	66.42	61.05	63.56	59.67	49.82	60.59	5.76
13	41.92	41.64	52.66	48.42	50.96	46.69	47.05	4.57
IM (%) change								
Anaerobic phase	-5.95	-13.88	-16.98	-10.68	14.48	-5.64	-6.44	11.16
Aerobic phase	-32.82	-42.12	-25.14	-29.23	-27.76	-19.85	-29.48	7.56
Total	-36.82	-50.15	-37.85	-36.79	-17.30	-24.36	-33.88	11.52

IM results are given as percentage (w/w) of dry sample.

Table 3.27 SM values for trials BATCH 1 to 6

	SM (%)							
Day	**BATCH-1**	**BATCH-2**	**BATCH-3**	**BATCH-4**	**BATCH-5**	**BATCH-6**	**Average**	**SD**
0	31.87	19.56	18.27	24.01	30.55	39.67	27.32	8.21
3	32.8	30.52	28.32	31.27	30.46	41.16	32.42	4.52
6	32.67	26.5	31.84	31.77		26.86	29.93	2.99
9	27.69	32.25	34.42	31.03	30.52	31.29	31.20	2.20
13	35.91	35.35	32.24	35.85	36.08	35.83	35.21	1.48
SM (%) change								
Anaerobic phase	2.92	56.03	55.01	30.24	-0.29	3.76	24.61	26.34
Aerobic phase	9.48	15.83	13.84	14.65	18.45	-12.95	9.88	11.56
Total	12.68	80.73	76.46	49.31	18.10	-9.68	37.93	36.72

SM results are given as percentage (w/w) of dry sample.

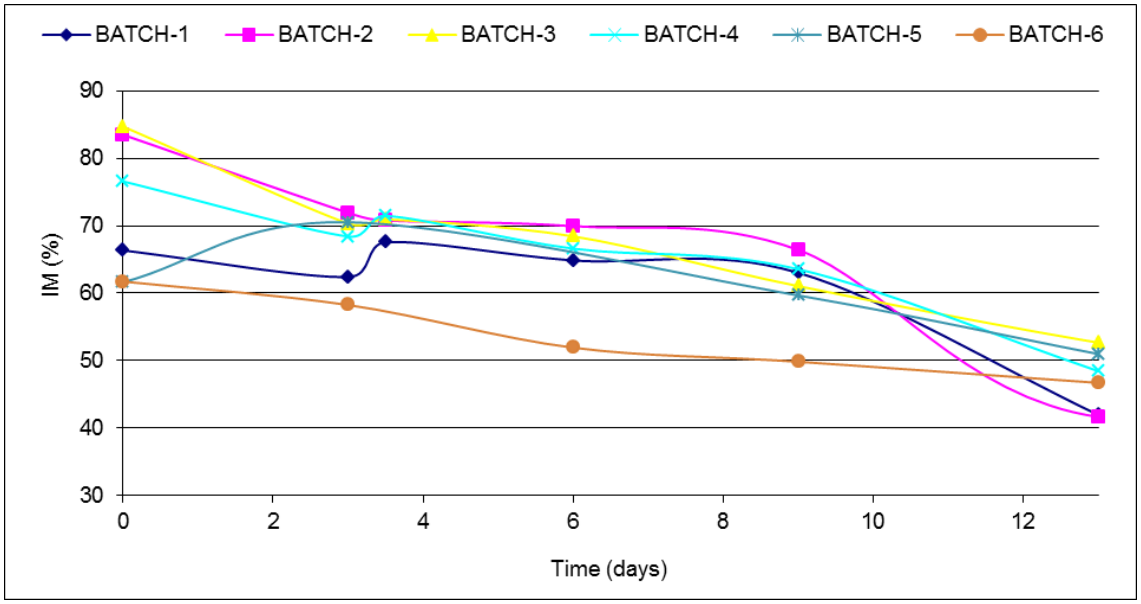


Figure 3.22 IM (% w/w of dry sample) profiles of trials BATCH 1 to 6.

The batch digestion model was continued after day 13 on two occasions. Trials BATCH 2 and 3 were run for 20 and 21 days respectively. In BATCH-3 solubilised material was decanted twice: at days 3 and 13 (200 ml each time), the lost volume being replaced with water. In BATCH-2, which was allowed to run for 20 days, the IM increased from day 13 (41.64%) to day 20 (49.12%), as also did the SM (35.35% to 37.32%). The opposite was the case in BATCH-3, which ran for 21 days. The IM decreased from 52.66% at day 13 to 48.06% at day 16 and to 44.15% at day 21.

This reflected the need for decanting of solubilised material to avoid metabolic inhibition, but also the apparent system related obstacle preventing further decrease in IM, even after extended incubation.

The pH, IM and SM were studied in the batch digestion model over trials BATCH 1 to 6. In terms of reproducibility, the pH was shown to behave in a similar pattern over the 6 trials studied (Figure 3. 20). However, variation was found in the IM decrease and the SM increase over digestion (Tables 3.26 and 3.27). Different initial values of IM and SM seem to affect the digestion rate. Further research at this stage of the project was directed towards making the digestion process of *A. nodosum* residue reproducible and feasible for the Irish seaweed industry.

3.4.2 Modifications to the batch digestion system

Some modifications were studied in the batch digestion system. This included further pH decrease, further SM decantation, reduction in the volume of water, combined inocula B and C and residue wash prior to the digestion process. Some of these modifications were requested by the industrial partner, who pointed at different elements of the project from an industrial and a large scale application perspective. In the following sections these modifications are studied.

3.4.2.1 Regular SM decantation *vs.* regular pH decrease

The value of repeated decanting SM and lowering of pH during the digestion were evaluated. For this an experiment with three digestion trials (BATCH-DECANT, BATCH-pH and BATCH-CONTROL) was carried out. The three trials share the same residue sample and followed the batch model explained in Figure 3.19, with some modifications. In trial BATCH-DECANT solubilised material (200 ml) was decanted every 3 days (days 3, 6 and 9) and replaced with water. BATCH-pH consisted of alternating three day blocks of anaerobic and aerobic incubations (anaerobic incubation days 0-3 and 6-9; aerobic incubation days 3-6 and 9-12), while BATCH-CONTROL followed the batch digestion model (Figure 3.19) without any modifications.

The results obtained for these trials are shown in Table 3.28. Trial BATCH-DECANT presented a similar but slightly faster reduction of the IM (42.48% in 12 days), when compared with BATCH-CONTROL (36.79% in 13 days; Table 3.28 and Figure 3.23). The performance of BATCH-DECANT suggests the importance of metabolite inhibition when digested material reaches a certain concentration.

In contrast to repeated decanting SM, BATCH-pH was slower and less efficient: 30.87% IM decrease (Table 3.28). The pH of BATCH-pH decreased during the anaerobic periods (day 0 to 3 and day 6 to 9), and increased again during the aerobic

phases (day 3 to 6 and day 9 to 12; Figure 3.24). It seemed that the microorganisms, which originally were located in the residue, were maintained in the digestion sample and were able to decrease the pH with anaerobic conditions each time, despite of the addition of the new inoculum B and intermittent aerobic incubation. In BATCH-DECANT, pH increased after day 3 ending with a value of 8.4 at day 12, which is lower when compared with BATCH-CONTROL (9.00 at day 13). It would appear, however, that dilution or removal of digested solutes is more effective in promoting further digestion than taking steps to maintain a near-neutral pH.

Table 3.28 Trials BATCH-DECANT, BATCH-pH and BATCH-CONTROL

	BATCH-DECANT			BATCH-pH			BATCH-CONTROL		
Day	**pH**	**IM %**	**SM %**	**pH**	**IM %**	**SM %**	**pH**	**IM %**	**SM %**
0	9,51	76,60	24,01	9,51	76,60	24,01	9.51	76.60	24.01
3	7,67	68,42	31,27	7,67	68,42	31,27	8.23	68.42	31.27
6	8,43	62,77	32,76	8,62	67,5	32,05	8.47	66.61	31.77
9	8,29	56,77	19,15	7,79	62,28	32,17	8.65	63.56	31.03
12	8,40	44,06	13,6	8,55	52,95	35,42			
13							9.00	48.42	35.85
Change (%)		-42.48	-43.36		-30.87	47.52		-36.79	49.31

IM and SM results are given as percentage (w/w) of dry sample.

Change is calculated from day 0 to 12/13.

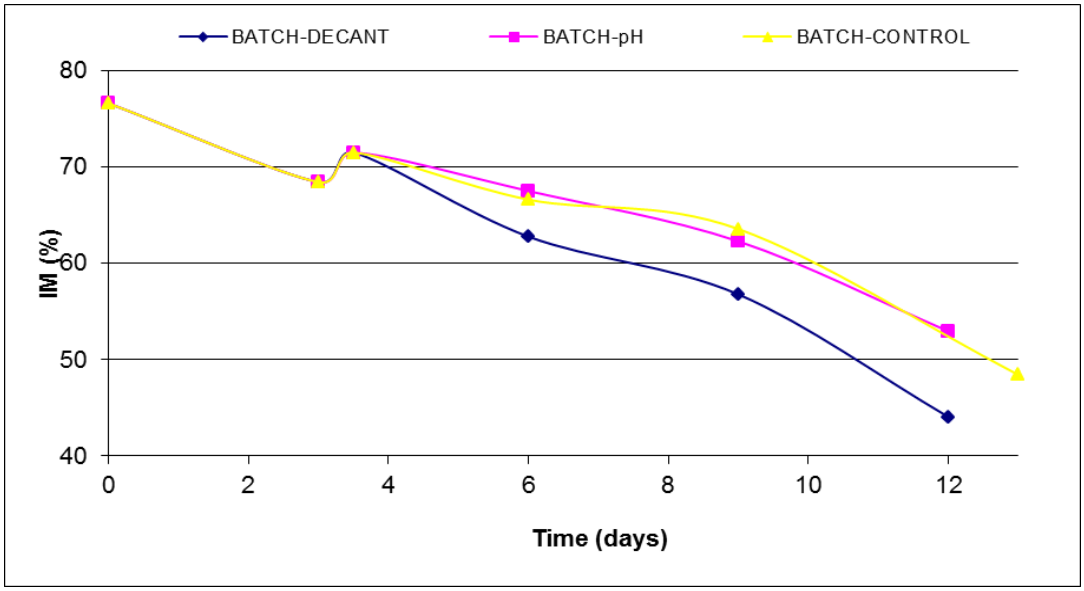


Figure 3.23 IM profiles (% w/w of dry sample) of trials BATCH-DECANT, BATCH-pH and BATCH-CONTROL.

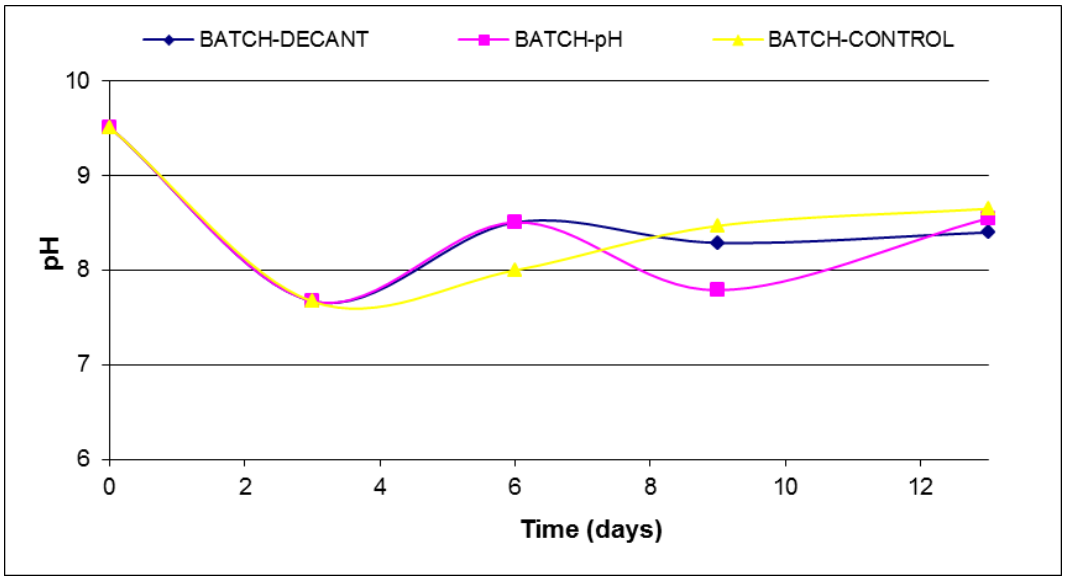


Figure 3.24 pH profiles of trials BATCH-DECANT, BATCH-pH and BATCH-CONTROL.

3.4.2.2 Reduction in the volume of water

Four hundred millilitres of water per 50 g of residue was considered to be a high volume for digestion by the industrial partner. In terms of scaling up this process, space, energy needed and incubation time were considered at this point of the research. A reduction of the volume of water was studied. Trial BATCH-WATER consisted of 50 g of residue mixed with 100 ml of water (instead of 400 ml) following the batch digestion model described at in Figure 3.19. This time, 50 ml of SM (instead of 200 ml) were decanted at day 3 and replaced with water. A reduction in the volume of water was evaluated by comparing the IM removal in trial BATCH-WATER with previous trials (BATCH 2 and 3), which exhibited similar initial values of IM and SM.

Results for trial BATCH-WATER are shown in Table 3.29. This particular sample of residue presented a high initial IM (81.44%) and a low SM (19.52%). The IM decreased to 63.13% at day 14. Trials BATCH 2 and 3 (Section 3.4.1) presented similar value of initial IM, 83.53% and 84.73% respectively (Table 3.26), compared to BATCH-WATER. The IM removal was lower in BATCH-WATER (22.48%) in comparison with BATCH-2 (50.15%) or BATCH-3 (37.85%; Table 3.26, Figure 3.25). It is likely that the reduction in the volume of water concentrated the nutrients created during digestion, and metabolite inhibition took place earlier in the digestion. The pH in BATCH-WATER behaved very similarly to the other BATCH trials: it decreased

during the anaerobic phase (from 8.5 to 7.5) and it increased during the aerobic phase (up to 9.0 at day 14, Table 3.29).

Table 3.29 Trial BATCH-WATER

Day	pH	IM %	SM %
0	8.5	81.44	19.52
3	7.5	68.48	31.23
6	8.8	62.74	26.04
9	9.0	57.15	27.79
14	9.0	63.13	31.20
Change (%)		-22.48	59.84

IM and SM results are given as percentage (w/w) of dry sample.

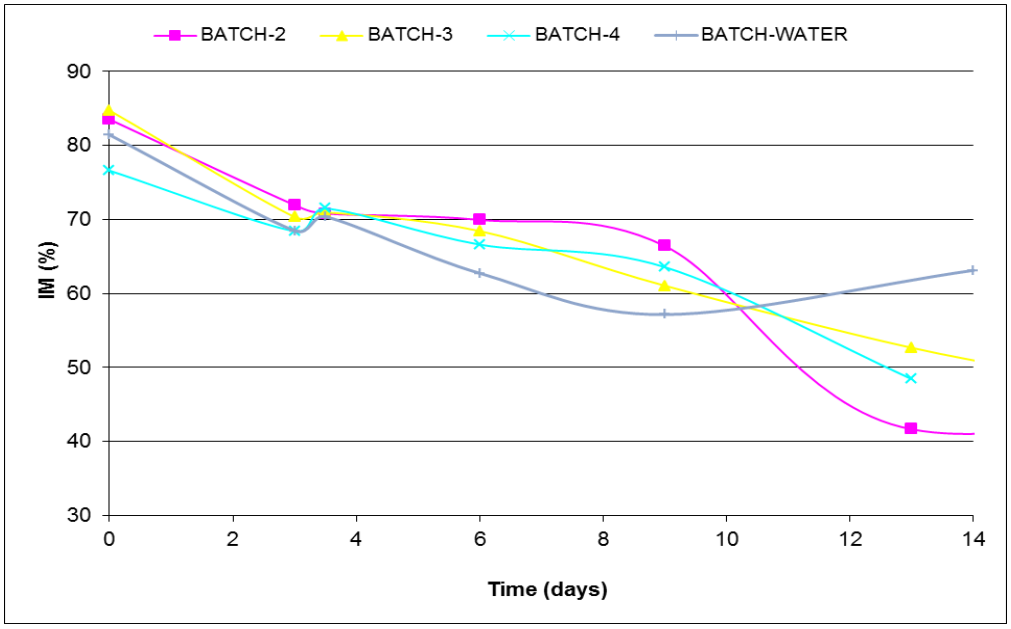


Figure 3.25 IM profiles (% w/w of dry sample) of trials BATCH 2 to 4 and BATCH-WATER.

A second experiment regarding the reduction in the volume of water consisted of two trials: WATER 1 and 2. In both trials, a reduced volume of water (100ml) and a shorter incubation time of 6/7 days (3 anaerobic and 3/4 aerobic) were studied. In WATER 1 and 2 SM was decanted and replaced with water as follows: 50 ml were decanted at day 3 and 20 ml at days 4, 5 and 6. Different residue samples were employed for trials WATER 1 and 2. Inoculum B was added at day 3.

The results obtained are presented in Table 3.30. Both trials exhibited different initial IM: 87.66% (WATER-1) and 64.34% (WATER-2), which lead to a different yield of digestion: 30.82% and 21.17% IM decrease respectively (Table 3.30). In comparison with BATCH-WATER (Table 3.29), an improvement in the IM decrease was found in

trial WATER-1 (30.82% vs. 22.48%) over a shorter period of time (6 days *vs*. 13). These results support once more, the need for diluting the solubilised material during digestion. The pH decreased during the 3-anaerobic days in trial WATER-2 from 9.5 to 7.7. Unusually, the pH only slightly decreased in trial WATER-1 (from 8.3 to 8.0, Table 3.30).

Table 3.30 Trials WATER 1 and 2

	WATER-1			WATER-2		
Day	**pH**	**IM %**	**SM %**	**pH**	**IM %**	**SM %**
0	8.3	87.66	13.16	9.5	64.34	36.70
3	8.0	N.D	N.D	7.7	N.D	N.D
6	8.8	60.64	21.36			
7				8.5	50.72	16.75
Change (%)		-30.82	62.31		-21.17	-54.36

IM and SM results are given as percentage (w/w) of dry sample.

Results exhibited by trials BATCH-WATER and WATER 1 and 2, showed the need for dilution of the residue in order to digest it. A reduction in the volume of water did not enhance the removal of the IM (BATCH-WATER), although it was improved when SM was decanted daily (WATER-1). A residue to water ratio of 50 g / 100 ml resulted in a thick mixture from which decanting a small volume of SM (20 ml) was challenging. This direction of the research was discontinued. However, future work could involve the study of a digestion system where SM is decanted and replaced with water in a continuous mode.

3.4.2.3 Combined inocula B+C

Inoculum C was included in the batch digestion model because it clearly demonstrated its ability to digest the residue, although over a long period of time (minimum of 28 days; Section 3.3.2.3). Two trials (BATCH-B+C 1 and 2) were run following the batch digestion model described in Figure 3.19, with both inocula B and C (5% v/v each) added at the beginning of the aerobic phase. Each inoculum was grown separately for 24 h in Q&C media prior to the digestion.

Both trials BATCH-B+C 1 and 2 exhibited a low initial IM (56%) and a high initial SM (41%, Table 3.31). These were the lowest IM and the highest SM found in *A. nodosum* residue (compared to BATCH 1 to 6 trials, shown in Tables 3.26 and 3.27). In trial BATCH-B+C-1, the IM decreased to 52.21%, while in BATCH-B+C-2 it decreased

further (45.96%, Figure 3. 26). The initial value of the SM was quite high (41%) and only slightly increased over the 13 days in BATCH-B+C-1 (to 43.57%) and in BATCH-B+C-2 (to 48.56%).

Although both inocula have been shown to digest *A. nodosum* residue in the previous sections, in this case the digestion yield was quite low (6.77% IM decrease for BATCH-B+C-1 and 17.01% for BATCH-B+C-2), probably due to the low initial IM. This low rate of digestion could also be influenced by the high initial pH in BATCH-B+C-1, where an initial pH of 9.38 decreased to 8.47 at day 3 (the highest pH value found at day 3 in comparison with trials BATCH 1 to 6, Table 3.25). The initial alkaline pH decreased as expected during the 3-day anaerobic phase, and increased again during the 10-day aerobic incubation for both trials (Table 3.31).

Table 3.31 Trials BATCH-B+C 1 and 2

	BATCH-B+C-1			BATCH-B+C-2		
Day	**pH**	**IM %**	**SM %**	**pH**	**IM %**	**SM %**
0	9.38	56.00	41.53	8.90	56.75	41.95
3	8.47	56.57	46.51	7.81	55.16	46.57
6	8.44	57.50	46.14	8.38	62.38	46.75
9	8.45	54.33	42.44	8.46	57.36	44.91
13	8.85	52.21	43.57	8.38	45.96	48.56
Change (%)		-6.77	4.91		-19.01	15.76

IM and SM results are given as percentage (w/w) of dry sample.

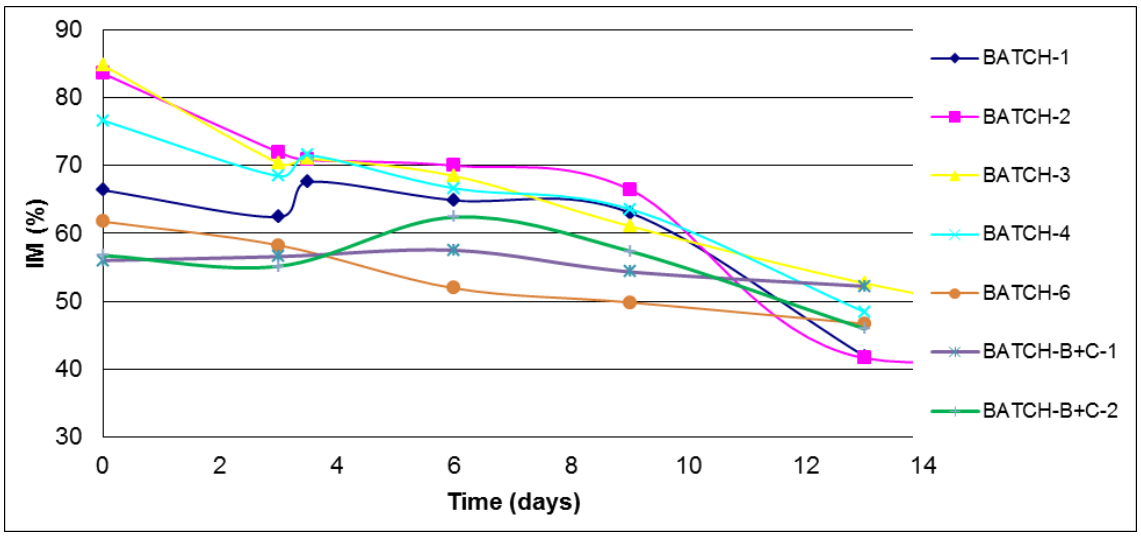


Figure 3.26 IM profiles (% w/w of dry sample) of trials BATCH 1, 2, 3, 4, 6 and BATCH-B+C 1 and 2.

The combination of inocula B+C, in trials BATCH-B+B 1 and 2, did not show an improvement in the digestion of *A. nodosum* residue. However the initial amounts of IM and SM of the residue samples employed were very low and high respectively, compared to the other BATCH trials. It was considered that the initial amounts of IM and SM could have affected the digestion process. Further research concerning a reduction in the initial amount of SM was studied in the following section.

3.4.2.4 Reduction of the initial SM

A. nodosum residue microbial digestion seemed to be affected by the initial amount of IM/SM. An experiment was carried out, where the initial amount of SM was decreased by washing the residue sample prior to the digestion process. The aim was to evaluate if the digestion yield would improve by decreasing the initial SM in samples with high initial SM. Two digestion trials (BATCH-WASH 1 and 2) were carried out as follows. First of all, two residue samples with high initial amount of SM and low IM were selected (from the previous samples). Residue (50 g) was mixed with 400 ml of water. The total volume of SM was decanted by centrifugation and replaced with water.

Results for trials BATCH-WASH 1 and 2 are shown in Table 3.32. The SM decreased from 41.95% to 6.40% in BATCH-WASH-1 and from 35.42% to 5.73% for BATCH-WASH-2 subsequently (Table 3.32). The SM increased during the digestion process, reaching a final value of 28.15% in BATCH-WASH-1 (339% increase) and 30.47% in BATCH-WASH-2 (432% increase). Although the SM showed a big increase over digestion the IM did not show the corresponding decrease (Table 3.32). In BATCH-WASH-1 the IM decreased from 54.29% to 44.25% (18.49% decrease) and in BATCH-WASH-2 from 55.36% to 53.46% (3.43% decrease). The reason why the SM increase was not reflected in the IM decrease over the digestion process was unknown. However, it was hypothesized that bacterial biomass contributed to the amount of IM during digestion.

Table 3.32 Trials BATCH-WASH 1 and 2

	BATCH-WASH-1			BATCH-WASH-2		
Day	**pH**	**IM %**	**SM %**	**pH**	**IM %**	**SM %**
0 (prior to wash)	8.90	56.75	41.95	9.01	61.26	36.42
0 (after wash)	9.58	54.29	6.40	9.63	55.36	5.73
3	8.92	51.41	11.85	8.82	53.76	9.56
6	8.38	59.61	25.09			
9	8.51	55.85	26.45			
13	8.31	44.25	28.15	8.43	53.46	30.47
Change (%)		-18.49	339.84		-3.43	431.76

IM and SM results are given as percentage (w/w) of dry sample.

Change (%) was calculated from day 0 after wash to day 13.

3.4.3 Factors affecting the batch digestion model

The batch digestion system of *A. nodosum* residue was developed by evidence-directed strategy in Section 3.3. Its reproducibility was studied in Section 3.4.1, and some modifications were applied in Section 3.4.2. Some of the modifications studied resulted in an improvement of the IM digestion, such as regular SM decantation (BATCH-DECANT, Section 3.4.2.1), while other modifications did not show an improvement when compared to the BATCH digestion system (BATCH 1 to 6). These modifications included: regular lowering of the pH (BATCH-pH), reduction in the volume of water (BATCH-WATER, WATER 1 and 2), the use of a combined inoculum (BATCH-B+C 1 and 2) and lowering the initial amount of SM (BATCH-WASH 1 and 2). Variations in the initial values of IM, SM and pH probably affected the digestion process.

Some of the batch digestion trials showed a good digestion yield (>36% IM decrease, BATCH 1-4), while others exhibited lower decrease of IM (<25%; BATCH 5-6, BATCH-B+C and BATCH-WASH; Table 3.33). Each residue sample presented different characteristics in terms of IM, SM and pH, giving each digestion trial different initial values. The initial IM varied from 54.3% (BATCH-WASH-1) to 84.7% (BATCH-3), while accordingly, the SM varied from 41.9% (trial BATCH-B+C-2) to18.2% (BATCH-3, Table 3.33). The initial pH also varied from 8.46 (BATCH-5) to 9.51 (BATCH-4), giving some variation in the pH values at the beginning of the aerobic phase (pH 8.82 to 7.19, Table 3.33).

Table 3.33 Recapitulation of trials BATCH 1 to 6, BATCH-B+C and BATCH-WASH

	Initial			Final				
Trial	pH	IM (%)	SM (%)	pH	IM (%)	SM (%)	IM decrease (%)	SM increase (%)
BATCH-1	8.84	66.35	31.87	8.70	41.92	35.91	36.82	12.68
BATCH-2	9.09	83.53	19.56	8.67	41.64	35.35	50.15	80.73
BATCH-3	8.61	84.73	18.27	8.84	52.66	32.24	37.85	76.46
BATCH-4	9.51	76.60	24.01	9.00	48.42	35.85	36.79	49.31
BATCH-5	8.46	61.62	30.55	8.28	50.96	36.085	17.29	18.12
BATCH-6	9.26	61.73	39.67	8.68	46.69	35.83	24.36	-9.68
BATCH-B+C-1	9.38	56.00	41.53	8.85	52.21	43.57	6.77	4.91
BATCH-B+C-2	8.9	56.75	41.95	8.38	45.96	48.56	19.01	15.76
BATCH-WASH-1	9.58	54.29	6.4	8.31	44.25	28.15	18.49	339.84*
BATCH-WASH-2	9.63	55.36	5.73	8.43	53.46	30.47	3.43	431.76*
Average	9.13	65.70	25.95	8.61	47.82	36.20	25.10	31.04
SD	0.41	11.75	13.49	0.25	4.41	5.98	14.95	33.69

*Not included in the average and SD calculation.

The microbial digestion of *A. nosodum* residue was shown to be affected by the initial IM, SM and pH. To further study these factors, some correlations were drawn within the batch trials represented in Table 3.33. Trials BATCH-DECANT, BATCH-pH and WATER were not considered comparable to the batch digestion system due to the modifications employed. These correlations are studied over the following sections.

3.4.3.1 pH

Two correlations were drawn regarding the pH. In the first correlation the initial pH and the IM decrease over digestion were studied (Figure 3.27), and no relationship was found (R^2=0.11). The second correlation evaluated the pH at the beginning of the aerobic phase and the IM digestion (Figure 3. 28). some degree of correlation (R^2=0.52) was shown. It appeared that the lower the pH at day 3, the higher the digestion of *A. nodosum* residue.

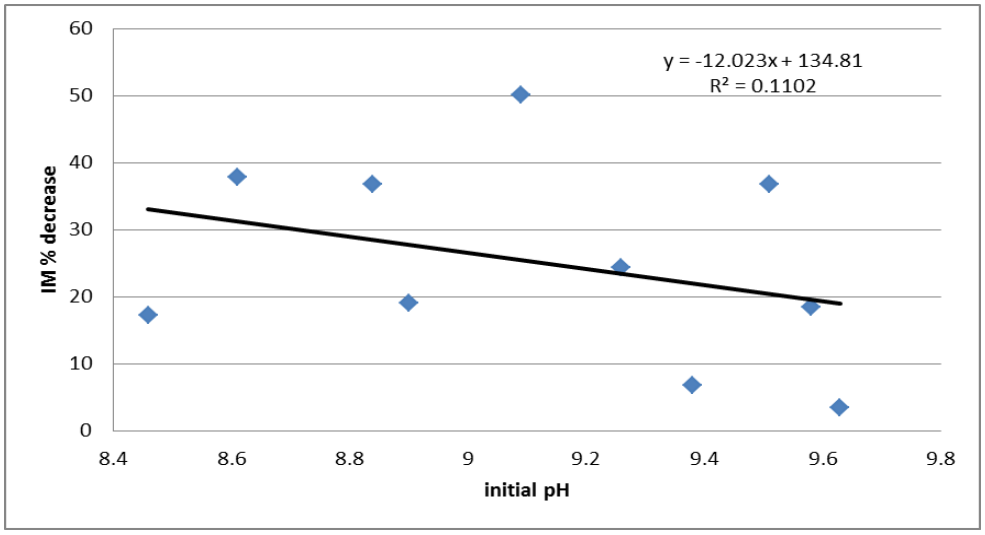


Figure 3.27 Correlation line between the initial pH and the IM decrease.

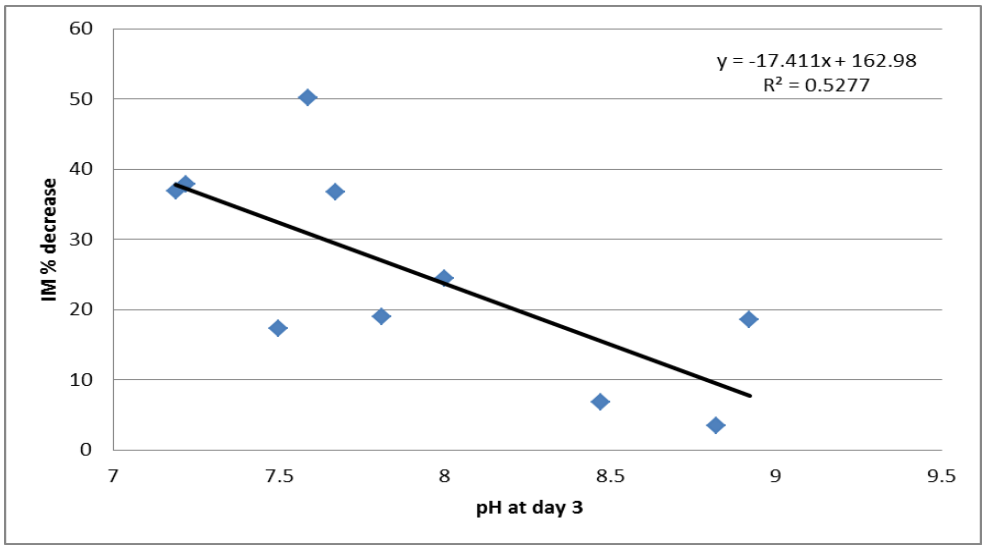


Figure 3.28 Correlation line between the pH at day 3 (beginning of aerobic phase) and the IM decrease.

3.4.3.2 Initial IM and SM

Extremes of initial IM and SM were observed to cause digestions to accelerate or fail when digesting the IM of *A. nodosum* residue (Table 3.33). The correlation between the initial amounts of IM and SM and the residue digestion was studied (Figures 3.29 and 3. 30). In both cases some degree of correlation was found. Figure 3.29 shows the correlation (R^2=0.77) between the initial amount of IM and the residue digestion. It was shown that the higher the initial IM the better the residue digestion, while conversely, with low initial IM the efficiency of digestion was reduced.

An inverse correlation (R^2=0.67) was found between the initial amount of SM and the IM decrease (Figure 3.30). In this case the lower the initial SM the better the digestion of the residue was found, and vice versa.

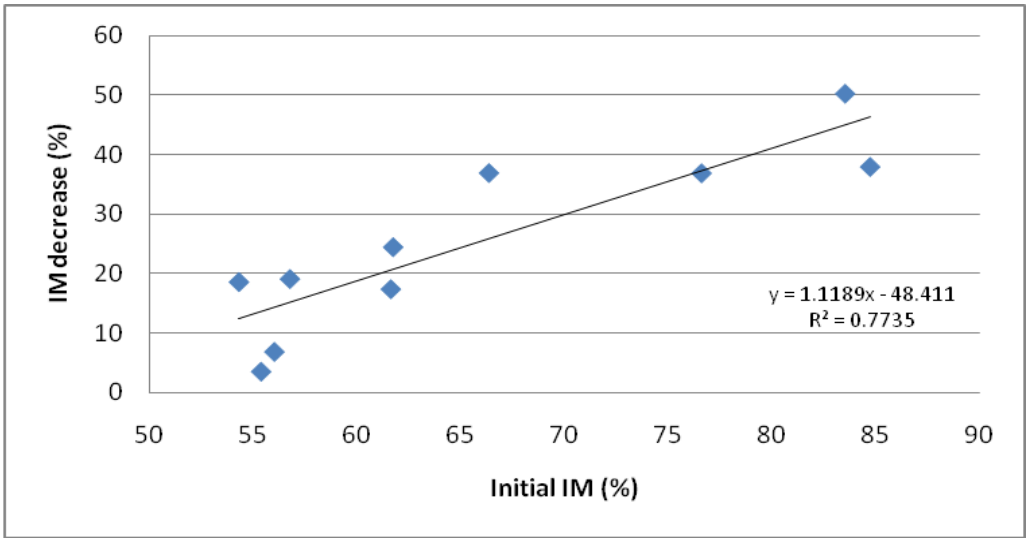


Figure 3.29 Correlation line between the initial IM and the IM decrease.

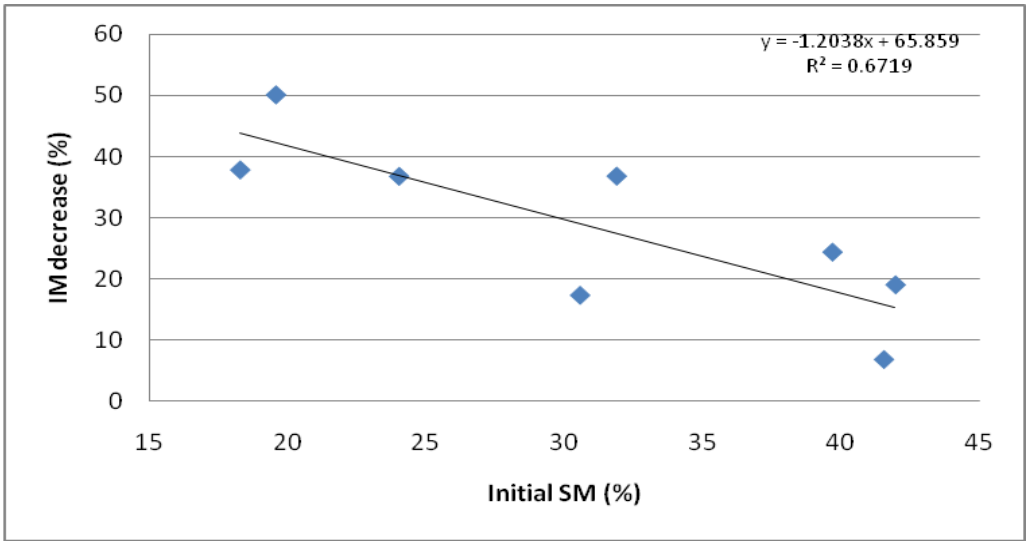


Figure 3.30 Correlation line between the initial SM and the IM decrease.

Factors such as the pH at day 3, the initial amounts of IM and SM have been shown to correlate with the digestion of *A. nodosum* residue. These and other factors will be discussed further in Section 4.3.

3.5 Compositional analyses during and after digestion

The composition of the IM was studied after digestion (day 13) in terms of ash, protein lipid and fibre contents. The polyphenol content and the organic acids were analysed during the digestion process. The aim of this study was to determine the compositional changes before, during and after digestion. The analysis of the organic acids was carried out to investigate any possible relationship between pH changes found during the anaerobic and aerobic phases and organic acid levels (Sections 3.3 and 3.4.1.1).

3.5.1 Composition of the IM at day 13 and comparison to the initial composition

A compositional analysis was carried out on the dried IM samples from the last flask (day 13) of trials BATCH-4, BATCH-DECANT and BATCH-pH. The results obtained for ash, protein, lipid and fibre analysis are shown in Table 3.34. Although the trials employed (BATCH-DECANT and BATCH-pH) were modifications of the batch digestion system and did not follow exactly the same procedure as BATCH-4, the results suggest that the microorganisms acted in a similar manner in the three trials. Fibre and minerals were the main components found with mean values of 46.03±1.80% and 33.51±3.07% respectively, followed by the protein (18.41±0.87%) and the lipid (2.05±1.39%).

Table 3.34 Last day IM composition of trials BATCH-4, BATCH-DECANT and BATCH-pH

Trial	Ash	Protein	Lipid	Fibre
BATCH-4	33,57	17,60	3,34	45,49
BATCH-DECANT	30,41	19,33	2,21	48,04
BATCH-pH	36,55	18,31	0,58	44,56
Average:	33,51	18,41	2,05	46,03
SD:	3,07	0,87	1,39	1,80

Results are shown as percentage (w/w) of dried matter

The composition of the IM after digestion (Table 3.34) was compared to the initial composition of the residue (Table 3.6) in Table 3.35 and Figure 3.31. The most significant changes were found in the lipid and the protein content. The lipid content decreased by 95.99%, which may indicate the utilisation of the lipid present in *A. nodosum* residue over the digestion. The protein content increased by 82.36, probably due to enzyme production and/or microbial growth during both anaerobic and aerobic phases. The ash and fibre contents decreased by 60.04% and 24.26% respectively. Ash minerals were possibly transferred into the SM and lost by decantation. The fibre

remained high during the digestion, suggesting that the bacteria present were not able to digest this material and/or new fibre content was added to by the microbial biomass.

Table 3.35 Compositional changes after the batch model digestion

	Day 0 (%)	Day 13 (%)	Change %
Lipid	24.80	2.05	- 95.99
Protein	4.90	18.41	+ 82.36
Ash	40.70	33.51	- 60.04
Fibre	29.50	46.03	- 24.26

Day 0 shows percentage comopsition fo the residue (dry weight)

Day 3 shows percentage composition of the digest (IM, dry weight)

Percentage change values refer to change in absolute weight for each of the components.

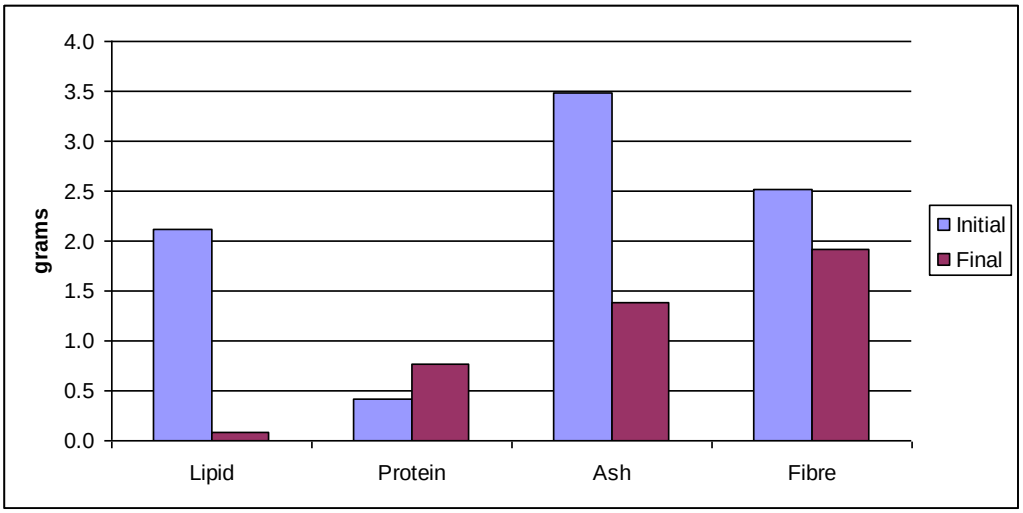


Figure 3.31 Compositional changes during the batch model digestion.

3.5.2 Polyphenol content

The polyphenol content was studied in the SM during the batch digestion model (BATCH-5). An initial concentration of 0.128 g/L soluble polyphenols was found (Figure 3.32). This concentration increased during the 3-day anaerobic phase (0.188 g/L at day 3) and decreased during the aerobic phase (0.09 g/L at day 10), increasing again during the last 3 days (0.146 g/L at day 13; Figure 3.32). This suggests that a portion of polyphenols were liberated during the anaerobic period and metabolised under aerobic conditions, being further released in the last few days of digestion. This profile may be instrumental in determining the extent to which digestions preceded.

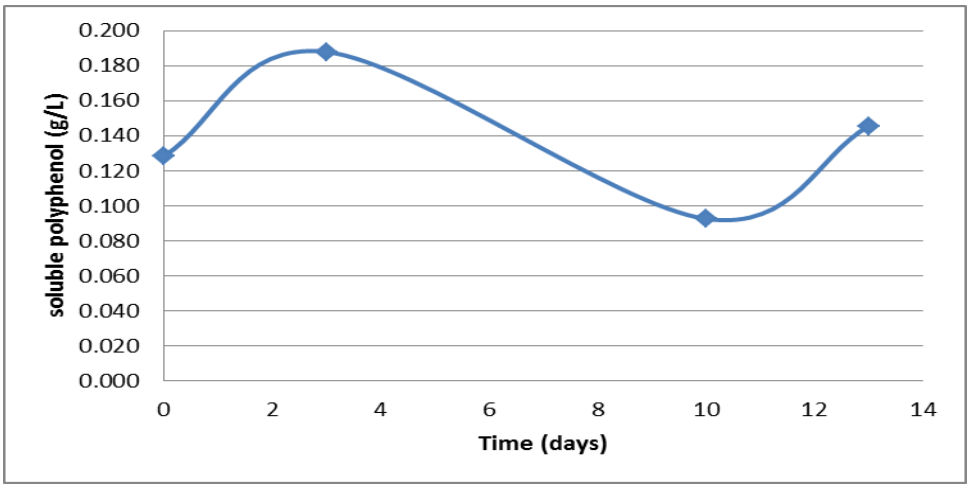


Figure 3.32 Polyphenol concentration and SD during the 13 day digestion process (n = 2).

3.5.3 Organic acid analysis

Organic acid analysis was carried out with the aim of relating organic acid levels to pH changes during both anaerobic and aerobic phases of digestion. Organic acids were identified and quantified by reverse phase HPLC during the digestion process. For this, SM samples of trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2 were studied for comparison, with several standards. For each standard three different concentrations were analysed. The average of the retention time and the equation of the standard curve (concentration *vs*. area) are presented in Table 3.36.

Formic, lactic, acetic and probably oxalic acid were identified in the digestion samples (SM). However other peaks at 3.4 minutes (unknown 1), 7.6 minutes (unknown 2) and 10.9 minutes (unknown 3) were detected in the three trials as possible organic acids, but they were not identified (Figure 3.33). Citric acid was not found in any of the samples.

These three trials were run as the batch digestion model, with some differences between them. In trial BATCH-6 the digest was inoculated with inoculum B at the beginning of the aerobic phase (Section 3.4.1), while in BATCH-B+C a combination of incoula B and C was used (Section 3.4.2.3). In BATCH-WASH the residue was washed prior to the digestion process, to reduce the initial amount of SM, and also Inocula B and C were used (Section 3.4.2.4). When studying each identified organic acid through the different trials some differences were found in terms of initial concentrations and changes over the 13-day digestion (Figure 3.34). The most abundant acid at day 0 was formic acid (10-16 mM), followed by acetic acid (4-8 mM), lactic (≤2 mM) and oxalic

(<2 mM; Figure 3.34). In BATCH-WASH all the organic acids were reduced to less than 1 mM, when the SM was decanted and replaced with water (Figure 3.34). This suggests that the organic acids present in *A. nodosum* residue are soluble and free, as they left with the residue wash.

The oxalic acid was clearly metabolised during the anaerobic phase of BATCH-6 as it decreased from 1.5 to 0.5 mM (Figure 3.35) and it remained constant during the aerobic phase (approximately 0.6 mM). In BATCH-WASH oxalic acid increased slightly over the 13 days from 0.13 to 0.33 mM (Figure 3.35). Formic acid (Figure 3.36) was completely digested in BATCH-6 from day 0 to 9. It was accumulated during the 3 first days of BATCH-B+C, metabolised at days 3-6 and slightly accumulated until day 13. Lactic acid was accumulated over days 0-3 (BATCH-6 and BATCH-WASH) and 0-6 (BATCH-B+C), and was totally consumed after that (Figure 3.37). Acetic acid started at different concentrations in each trial (0.5-8.0 mM) having a different profile during digestion process, but finishing with very similar concentration in the three cases (2.7-3.3 mM; Figure 3.38).

Table 3.36 Organic acid standards analysed by reverse phase HPLC

		Standard curve	
Organic Acid	**Retention time (min)**	**Equation**	**R^2**
Oxalic	4.50 ± 0.02	y = 1E+09x	0.9734
Formic	6.06 ± 0.08	y = 5E+07x	0.9999
Lactic	8.35 ± 0.11	y = 5E+07x	0.9959
Acetic	9.18 ± 0.09	y = 4E+07x	0.9772
Citric	3.14 ± 0.28	y = 2E+08x	0. 9855

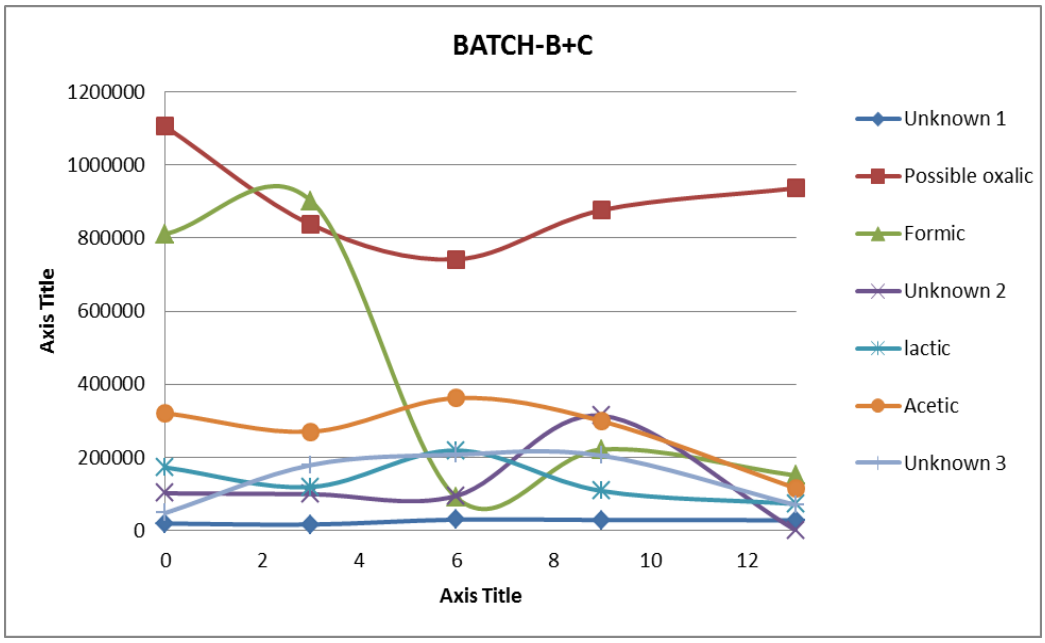


Figure 3.33 Organic acid profile (by reverse phase HPLC) during the 13 day digestion process in trial BATCH-B+C 1.

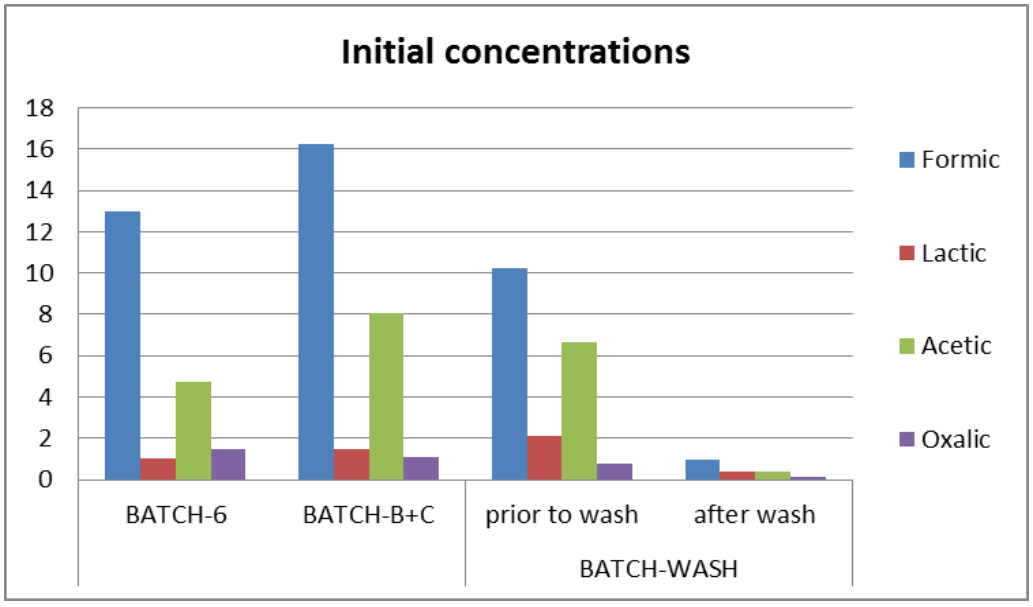


Figure 3.34 Initial concentrations of organic acids in the SM of trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2.

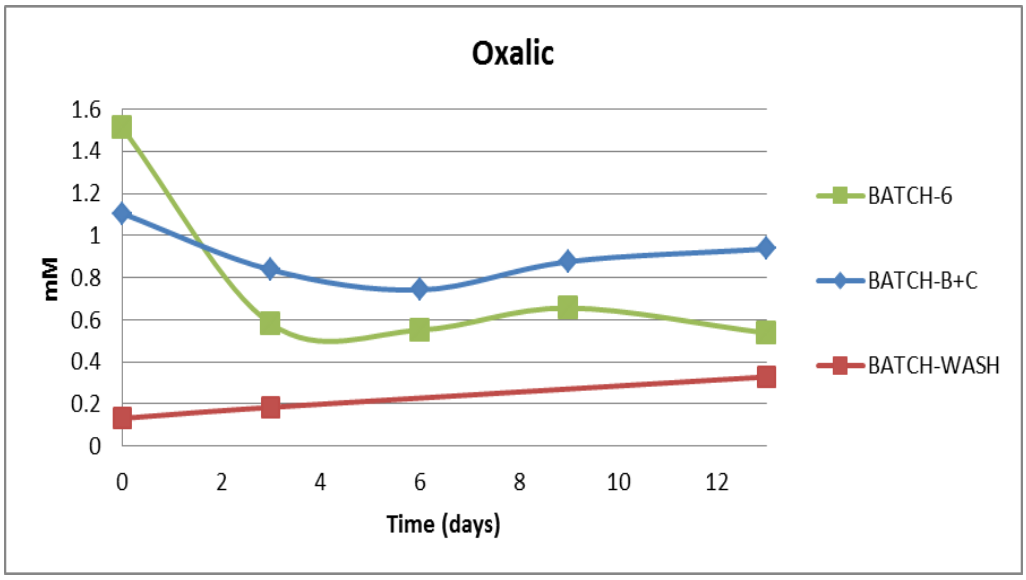


Figure 3.35 Oxalic acid profiles (by reverse phase HPLC) during the 13 day digestion process in trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2.

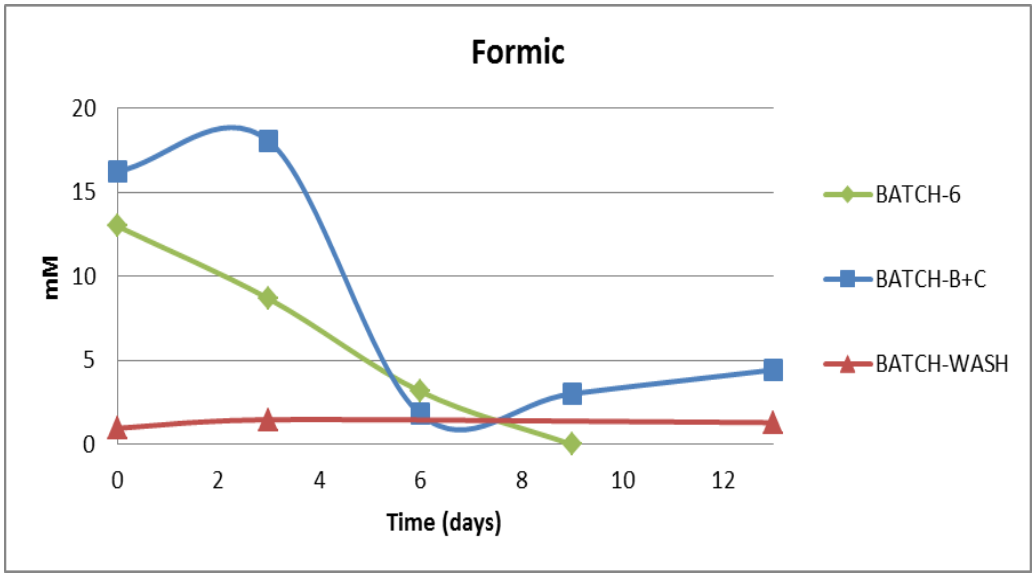


Figure 3.36 Formic acid profiles (by reverse phase HPLC) during the 13 day digestion process in trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2.

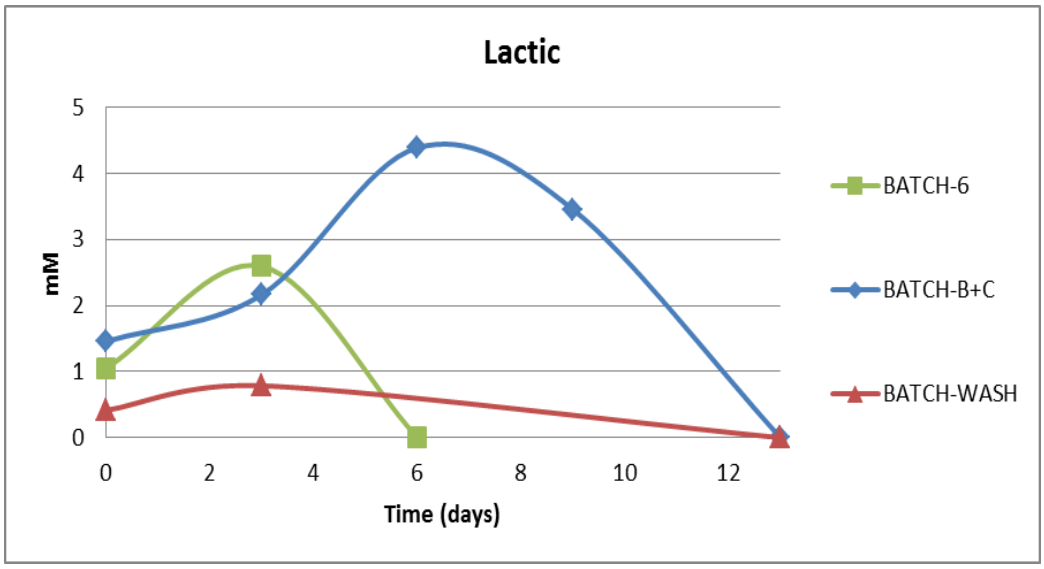


Figure 3.37 Lactic acid profiles (by reverse phase HPLC) during the 13 day digestion process in trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2.

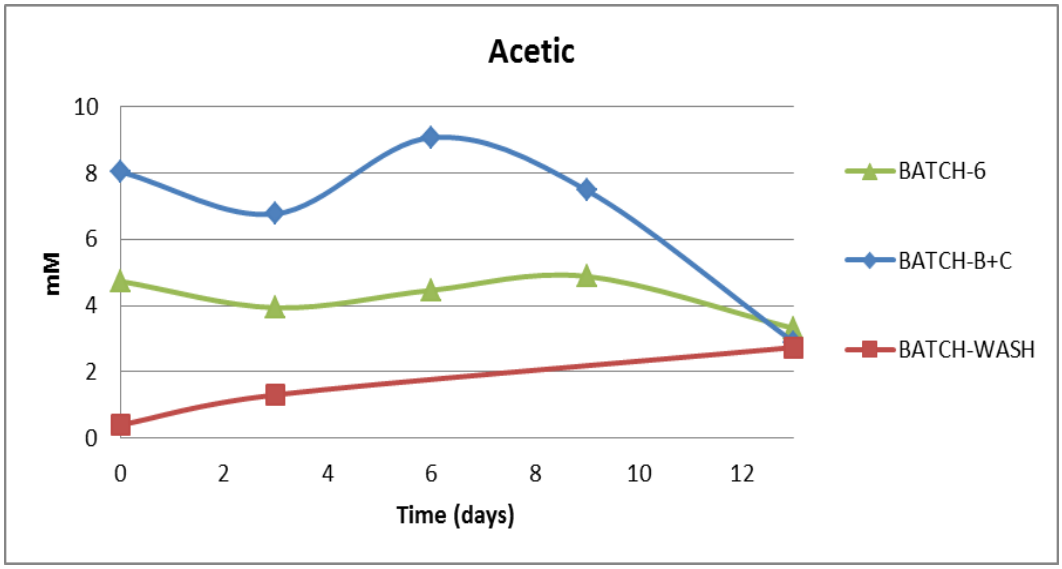


Figure 3.38 Acetic acid profiles (by reverse phase HPLC) during the 13 day digestion process in trials BATCH-6, BATCH-B+C-1 and BATCH-WASH-2.

As shown in the previous figures (3.33 to 3.38) individual organic acids behave differently in each digestion trial. No consistent relationship between the concentration of organic acids and the pH decrease (anaerobic phase) or increase (aerobic phase) during digestion was found.

3.6 Microbiology studies

The following sections describe the microbiological studies carried out in this research. Microbial characterization of the residue was carried out, with the aim of understanding what types and species of microorganisms are able to grow on the residue. The conditions of the industrial extraction procedure result in a residue with distinct properties. The microbial populations capable of growth under these conditions were investigated. The microorganisms growing during the 3-day anaerobic phase of the batch digestion system, which may be related to the reduction of pH, were also studied. The different inocula used in the digestion trials were examined to determine the numbers and variety of microbial flora involved.

3.6.1 Microbial studies of the residue

Initially, a total viable count was carried out. Colonies were also isolated and a preliminary characterization was performed.

3.6.1.1 Total Viable Count

The TVC was carried out on samples from the January (2007) residue (Table 3.37). While counting, it was found that the colony numbers were higher than 300 in dilution 10^{-3} and lower than 30 in 10^{-4} for both aerobic and anaerobic plates. This is shown by the results expressed as CFU (colony forming units)/ml. The calculated value obtained from the 10^{-3} dilution ($5.03*10^6$) was approximately 5-6 times higher than that obtained from the 10^{-4} dilution ($9.17*10^5$) for the aerobic count, and also for the anaerobic count (Table 3.37). This showed that an unusual collapse in populations seemed to occur between these two dilutions. The TVC was carried out in triplicate and on each occasion this phenomenon was observed. These findings could be related to aggregates of bacteria or to an unusual co-dependent relationship between the microorganisms present in the residue sample.

Table 3.37 January residue TVC

	Aerobic (24 h)		Anaerobic (48 h)	
	CFU/ml	**SD**	**CFU/ml**	**SD**
10^{-3}	$5,03*10^6$	$9,23*10^5$	$2,78*10^6$	$3,64*10^5$
10^{-4}	$9,17*10^5$	$9,32*10^5$	$6,83*10^5$	$8,18*10^5$

CFU: colony forming units

3.6.1.2 Monitoring microbial flora

As explained in Section 2.4.2, the plates prepared for TVC from January and February residue samples were incubated at room temperature (approximately 20^{o}C) and monitored for a week. For both January and February samples, it was noted that there was significantly more than a 10-fold difference in the numbers of colonies on the 10^{-3} and 10^{-4} dilution plates. This can be clearly seen in photographs taken of 10^{-3} and 10^{-4} dilution plate for samples of residue taken in both January (Figure 3.39, a-b) and February (Figure 3.39, c-d; aerobic incubation), and also plates from January residue incubated anaerobically (Figure 3.39, e-f).

During this time, a succession of different colonies was also observed. For example, two or three morphologically different white colonies were observed on the first days (up to 48 hrs); while orange, bright, and small yellow colonies were observed on the following days (48 to 120 hours; Figure 3.40); some of these types of colonies disappeared completely by day 6 or 7. The same phenomenon was observed under aerobic and anaerobic conditions. The growth and also the change in colony type were found to be slower when the plates were incubated anaerobically. The succession of colonies made it difficult to obtain a valid colony count, and could indicate interdependent relationships between the different microorganisms which take part in the digestion of *A. nodosum* residue.

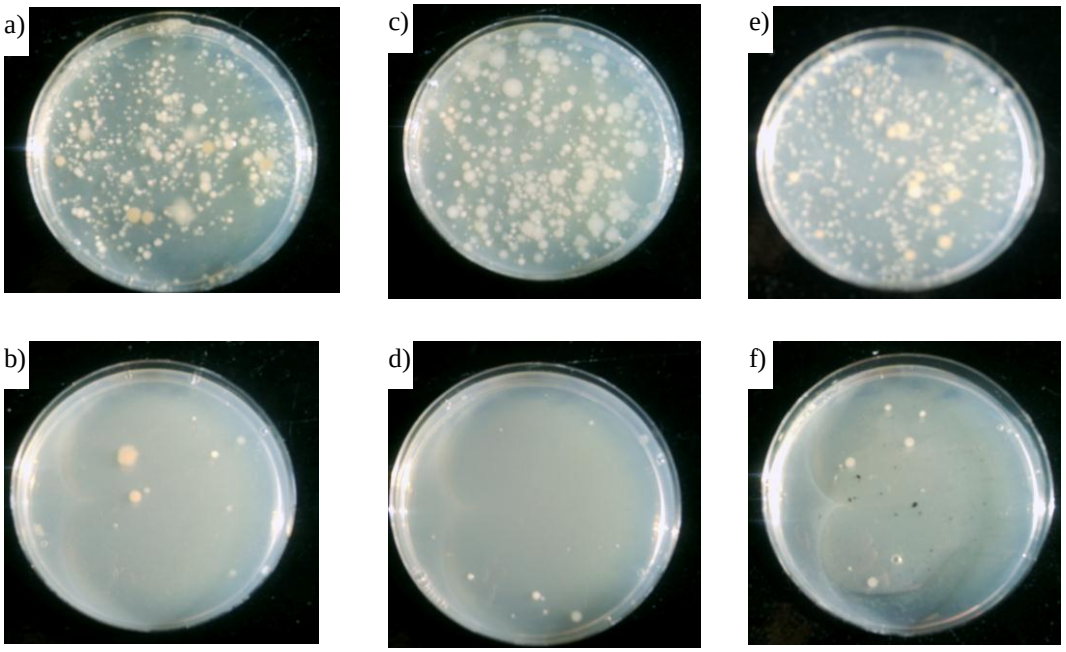


Figure 3.39 Residue dilutions plates after 72 h incubation at room temperature. a (10^{-3}) and b (10^{-4}) dilutions of Jan residue incubated aerobically; c-(10^{-3}) and d (10^{-4}) dilutions of Feb residue incubated aerobically; e (10^{-3}) and f (10^{-4}) dilutions of Januray residue incubated under anaerobic conditions.

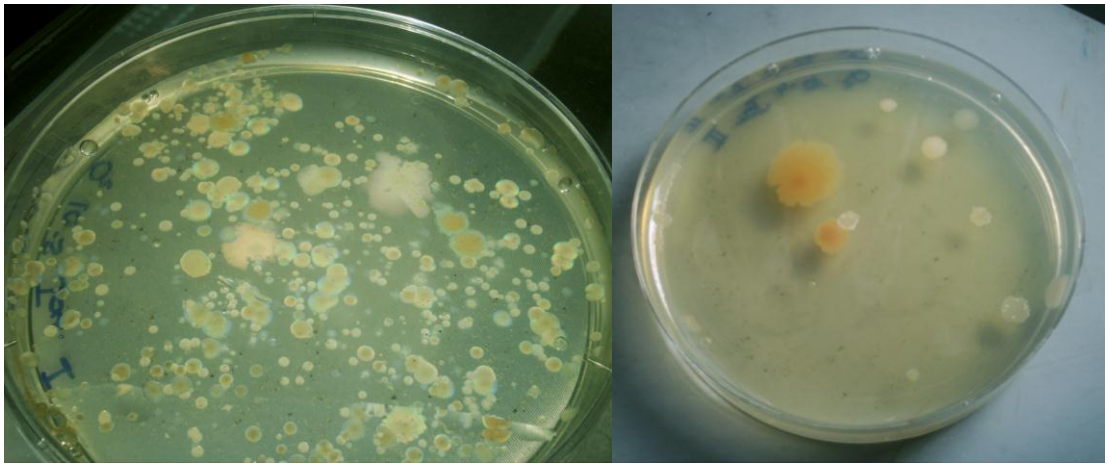


Figure 3.40 Different types of colonies observed during the week monitoring of the residue dilutions culture.

3.6.1.3 Preliminary colony studies

From a January residue sample, 9 different colonies were selected from the 10^{-3} and 10^{-4} dilution plates. The appearance of these colonies on agar was described and the colonies were sub-cultured in order to obtain pure cultures. Several tests were carried out on these colonies after 24 h incubation at 30^{o}C. The results are shown in Table 3.38.

This preliminary study showed a diversity of microorganisms: six isolates were able to grow in both aerobic and anaerobic conditions. All were gram negative; a variety of bacterial shapes were found and both catalase positive and catalase negative organisms were obtained.

This was a preliminary study, and therefore of limited benefit in identifying these organisms. It did however; show that there was variation in the flora isolated. Further study of selected sample microorganisms was carried out and described in Section 3.6.5, where the microorganisms growing during the 3-day anaerobic phase were counted, isolated, characterised and identified.

Table 3.38. *A. nodosum* residue colonies: description and basic characteristics

Colony	Description	Aerobes	Facultative anaerobes	Anaerobes	Gram Stain	Bacterial Shape	Catalase
1	White, irregular, raised and undulate margin	+			-	vibrios	+
2	White, circular, flat and entire margin		+		-	vibrios	+
3	White, circular, convex and entire margin	+			-	bacillus	+
4	Yellow, circular, flat and entire margin		+		-	coccus	-
5.1	Yellow-orange, irregular, umbonate and entire margin		+		-	rods	+
5.2	Yellow-white, irregular, umbonate and entire margin			+	-	rods	-
6	Cream-coloured, big circular, flat and entire margin		+		-	rods	+
7	White little plunctiform, convex and entire margin		+		-	bacillus	+
8	White very little plunctiform, flat and entire margin		+		-	bacillus	-

3.6.2 Inoculum A: Total Viable Count

A TVC was carried out in triplicate on inoculum A, which was used in trial INOC-A. Colonies were counted after 24 hours of incubation for the aerobic plates, and after 96 h for the anaerobic plates. The results, expressed as CFU/ml and presented in Table 3.39, show that inoculum A had a relatively low number of microorganisms.

Table 3.39 TVC of inoculum A

	CFU/ml	SD
Aerobic (24h)	$7.90*10^6$	$1.27*10^6$
Anaerobic (96h)	$9.90*10^6$	$2.75*10^6$

CFU: colony forming units

The plates for the TVC were incubated at 30^{o}C and monitored for a week. A succession of different microorganisms was observed; starting with 1 or 2 different types of colonies visible during the first days and continuing with more different types becoming visible during the following days. The odour of the plates also changed during this time. This is a mixed culture and co-metabolism could have easily happened.

This succession of microbial populations (i.e. a different population appeared to replace a previous population as the culture proceeded) made colony counting somewhat difficult because of the continuous change of the bacterial numbers and species. Accordingly, the results obtained in the TVC (Table 3.39) show only the counts obtained at a particular point in time (after 24 or 96 hrs.) and do not reflect the changing microflora.

3.6.3 Inoculum B

Inoculum B was used in digestion trials 5-9 and also in the batch digestion model. It seemed quite effective at digesting the solid part of *A. nodosum* residue when the conditions were appropriate. A TVC was carried out on this inoculum. Colonies arising from this procedure were then isolated, examined and identified. A growth curve was carried out at both 30^{o}C and 35^{o}C.

3.6.3.1 TVC

Table 3.40 shows the results for inoculum B, where plates were incubated aerobically for 24 hours. A high initial number of bacteria (2.05*10^{9} CFU/ml) was found. After 24 hours incubation, there seemed to be a single type of colony when viewed with the eye (see Figure 3.41).

Table 3.40 TVC of inoculum B

	CFU/ml	SD
Aerobic (24h)	2.05*10^{9}	5.25*10^{8}

CFU colony forming units

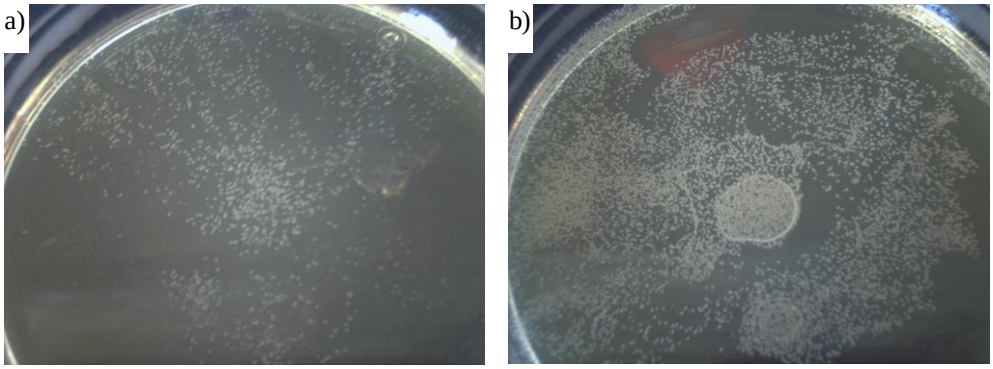


Figure 3.41 Petri dishes showing inoculum B colonies after 24 h of aerobic incubation. a) 10^{-6} dilution and b) 10^{-4} dilution.

3.6.3.2 Growth curve

Several previous digestions of brown seaweeds used an incubation temperature of 35°C (Hanssen *et al.*, 1987; Kerner *et al.*, 1991; Moen *et al.*, 1997b). Because of that, it was decided to study the effect, if any, of this higher temperature. A growth curve for inoculum B was carried out at two different temperatures: 30°C and 35°C, with the aim of studying which temperature was best suited to encourage the growth of the microorganisms in this inoculum.

Figures 3.45 and 3.46 show the replicate values and the average optical densities (O.D.) from the growth curves at 30°C and 35°C, respectively. There is greater variation between the replicates at 30°C, when compared with those at 35°C. The average O.D. values of the incubations at 30°C and 35°C are shown together in Figure 3.44. As might be expected the 35°C incubation resulted in a slight steeper growth curve for inoculum B.

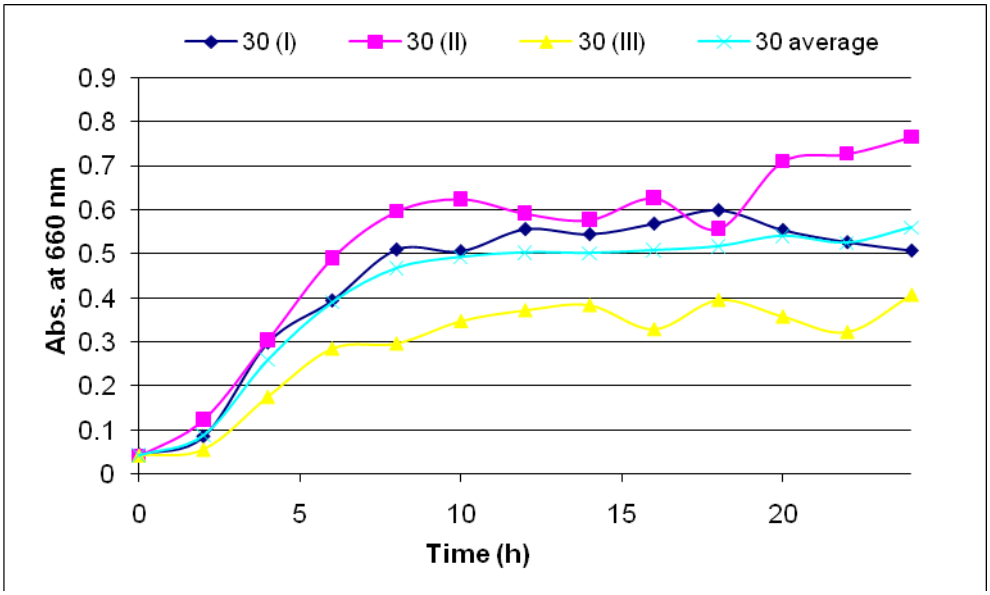


Figure 3.42 Inoculum B growth curve at 30°C.
This shows the growth of inoculum B in each of the three different trials, together with the average of the three.

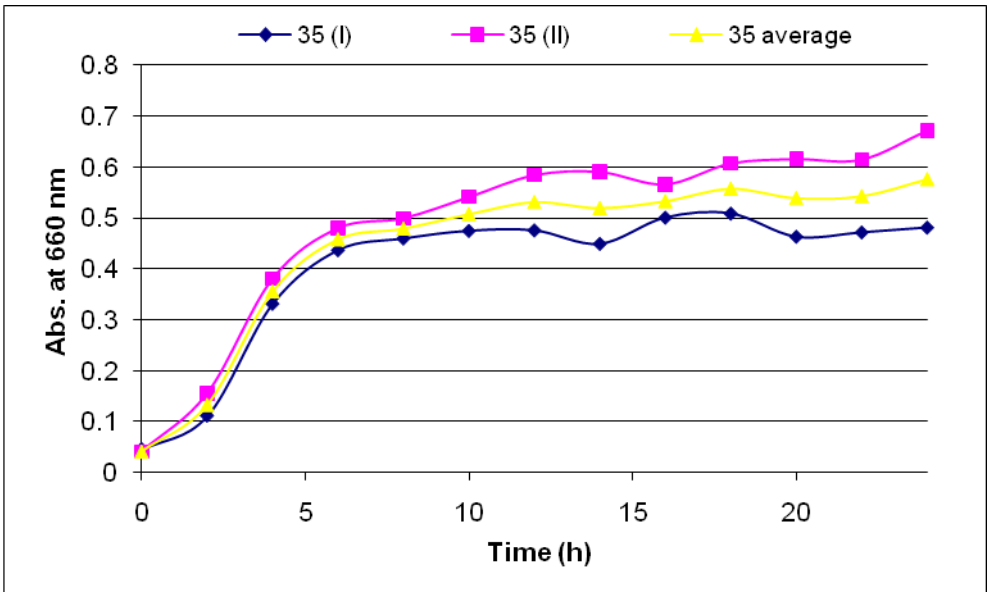


Figure 3.43 Inoculum B growth curve at 35°C.
This shows the growth of inoculum B in each of the two different trials, together with the average of the two

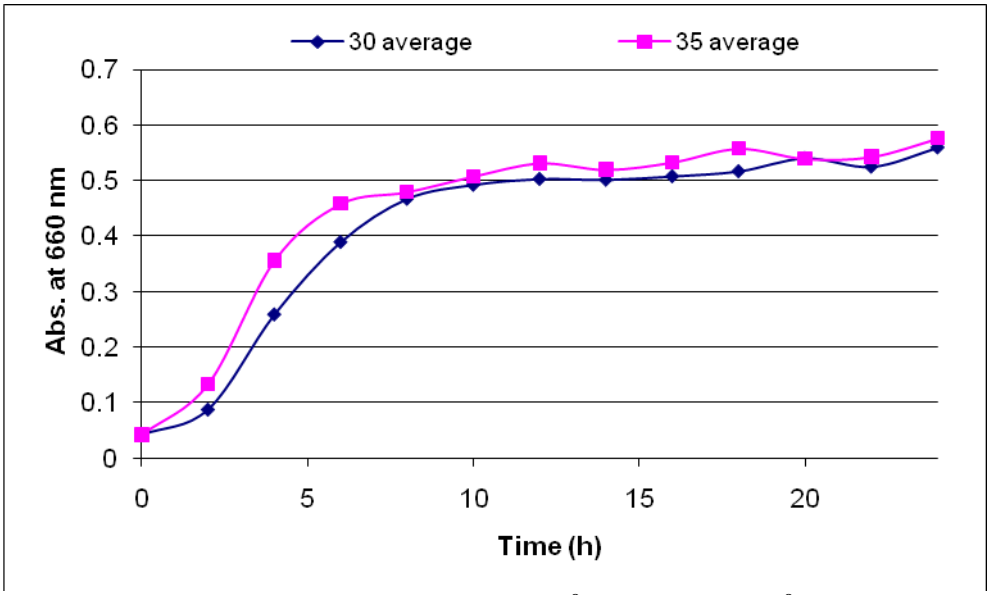


Figure 3.44 Inoculum B growth curve: average of 30°C *vs.* average of 35°C.

Statistical analysis was carried out to determine if there were significant differences between bacterial growth at 30°C and 35°C. The data was divided into three intervals: 0 to 8, 8 to 16 and 16 to 24 hours. The normality of the data was proved with Kolmogorov-Smirnov test: all data possessed a normal distribution. The T-test (independent samples) gave rise to the following results (Table 3.41): no significant differences were found in any of the intervals between the bacterial growth at 30°C and 35°C. It can be concluded that while inoculum B incubated at 35°C seemed to grow slightly faster than incubated at 30°C, the difference is not significant.

Table 3.41 T-test independent samples (30°C and 35°C)

Interval	T	Sig. (2-tailed)
0 to 8 h.	0.838	0.406
8 to 16 h.	0.810	0.422
16 to 24 h.	0.637	0.527

Some of the flasks were incubated for longer than 24 hours. It was found that the O.D. values were still increasing after 50 hours of incubation, which means bacterial growth was still occurring. In some cases, as in Figure 3.45a, the growth curve seemed to have a second and third log phase, suggesting that more than one microorganism was present in inoculum B. This theory was supported by phase contrast cell observation and Gram stain analysis, where different shapes of microorganisms and gram positive/negative

microorganisms were observed (Figure 3.46). The proportion of Gram positive and negative microorganisms changed during the incubation period. This would also suggest a succession of microorganisms, as was evident in some of the previous studies (*A. nodosum* residue and Inoculum A; Sections 3.6.1.3 and 3.6.2 respectively).

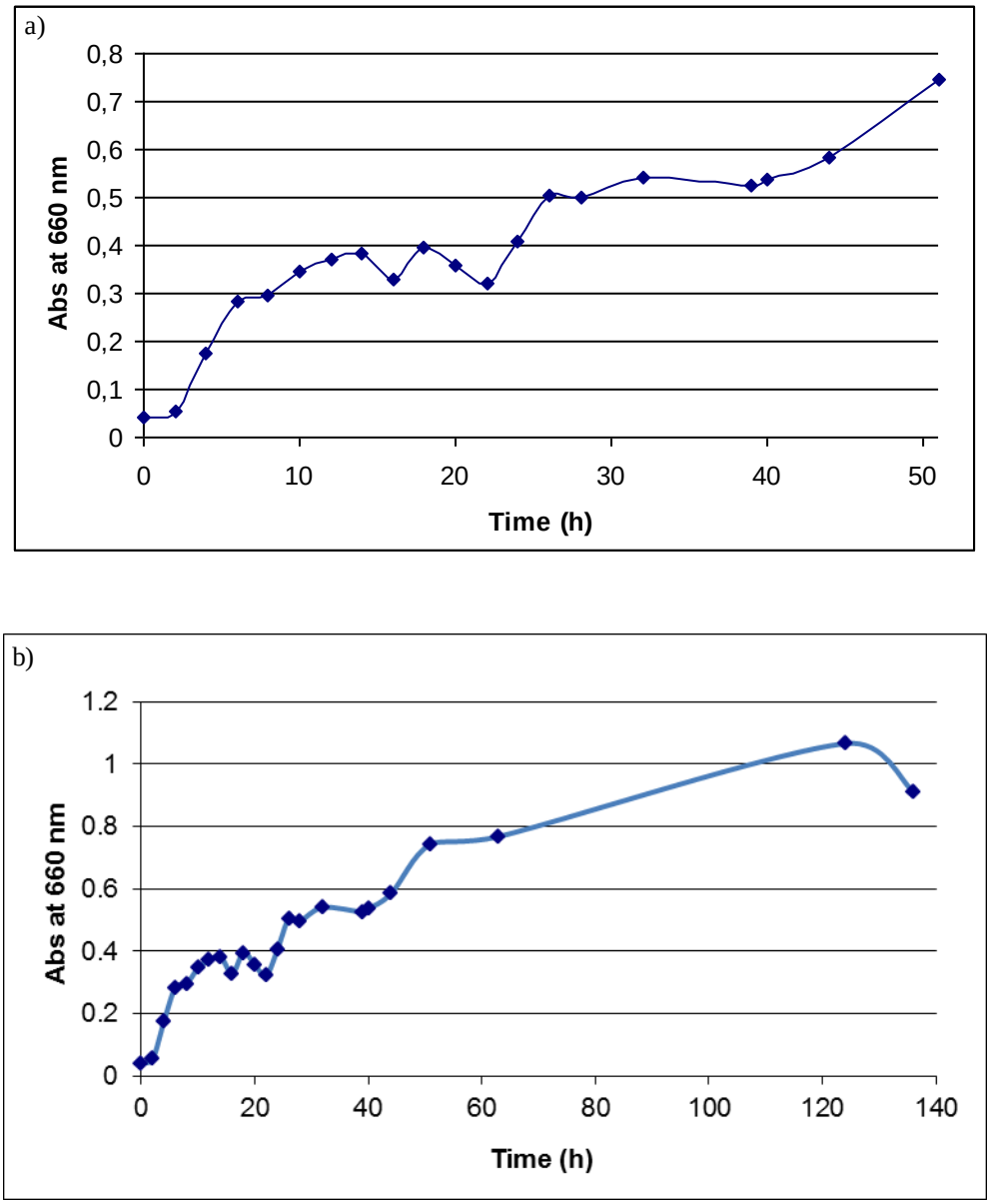


Figure 3.45 Inoculum B growth curve at 30°C. a) up to 50 hours; b) up to 140 hours.

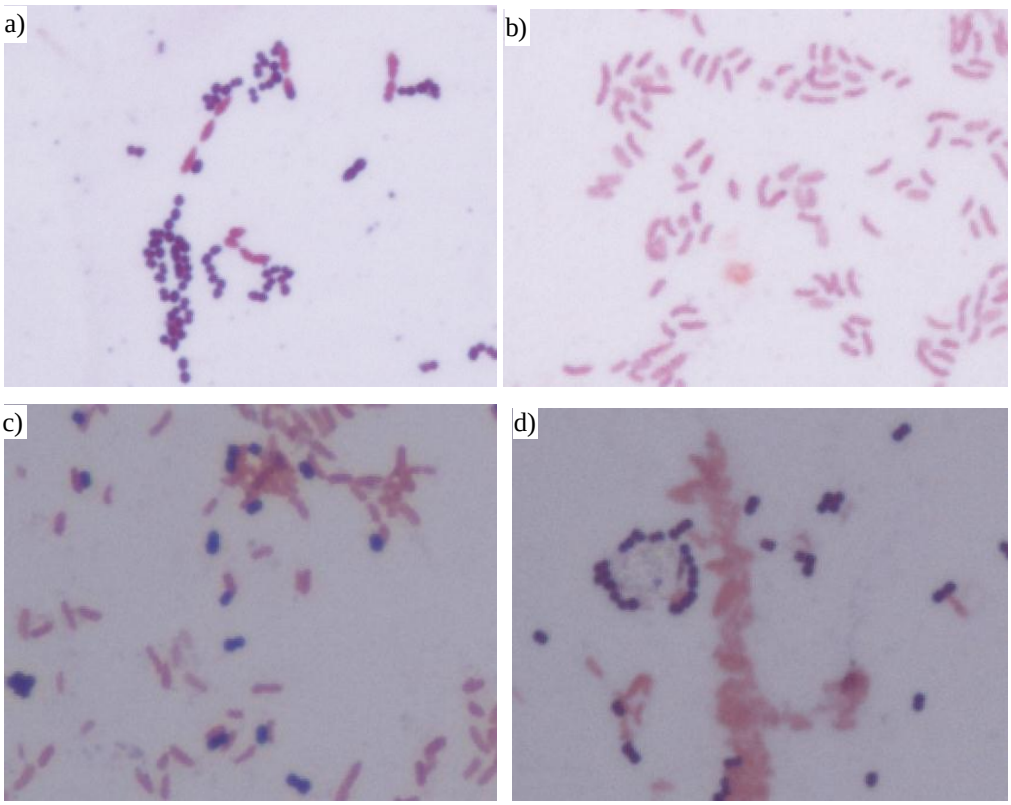


Figure 3.46 Inoculum B gram stained under the microscope (x1000). Different types of microorganisms: a) gram positive cocci and negative rods; b) gram negative rods; c) and d) gram positive cocci and gram negative rods collected at different stages of a 48 hour incubation.

3.6.3.3 Preliminary colony studies

From 10^{-6} and 10^{-7}dilutions of inoculum B, different colonies were selected, isolated and described. It was quite difficult to differentiate colonies with the naked eye, as most were white, circular and small-medium in size. Three initial types of colonies (A, B and C) were isolated in duplicate or triplicate (A1, A2, A3, B1, B2, C1, C2 and C3). Some preliminary tests were carried out on each isolated colony after 24 hours of aerobic incubation at 30^{o}C in Q&C agar. Tests included determination of Gram reaction, bacterial shape and motility under a phase contrast microscope. The ability of these colonies to grow in Q&C media prepared with sea-water was also determined. The same tests were carried out after 4 hours of incubation in Q&C broth. The results obtained are shown in Table 3.42. The Gram stain of the isolate A2 was inconclusive, and for further analysis this isolate was considered as if Gram negative.

Table 3.42 Description and characteristics of the different colonies of inoculum B

		Q&C agar (24h)				Q&C media with sea water (4h)				
		Phase Contrast		Gram Stain			Phase Contrast		Gram Stain	
Colony	**Description**	**Shape**	**Motility**	**Shape**	**Gram**	**Growth**	**Shape**	**Motility**	**Shape**	**Gram**
A1	White, circular, convex and entire margin.	Bacilli some in pairs	few very motile	Bacilli	-	yes	bacilli-cocci few in pairs (smaller than A2)	rapid movement	Bacilli	-
A2		Bacilli	no		+/-	yes	bacilli, few in pairs	no	Bacilli	+/-
A3		Bacilli	few very motile	Bacilli	-	ND				
B1	White, small (the smallest), circular raised and entire margin.	cocci-bacilli in pairs	ND	Cocci	+	yes	bacilli, few in pairs, others in very long chains	slow	Bacilli	+
B2		bacilli, some in pairs	ND	Bacilli	-	yes	cocci	quick	Cocci	-
C1	White, medium-size, circular, convex and entire margin.	rods (bacilli)	no	Bacilli	-	yes	bacilli-cocci small in pairs, few in a long chain	medium	Bacilli	-
C2		rods (bacilli)	no, few small	Bacilli	-	yes	small cocci-bacilli in pairs	slow-medium	Bacilli	-
C3		rods (bacilli)	no	Bacilli	-	ND				

ND: Not determined.

These results showed that, although very similar in colony morphology, the isolates were in fact different microorganisms. Colonies A1 and A2, and also B1 and B2, were bacteria of different shapes and different Gram stain classification.

3.6.3.4 API 20 E

To progress the preliminary bacterial characterisation, an API 20E panel-based analysis was carried out in each colony, in duplicate. The results for these tests are shown in Table 3.43 for the gram negative bacteria and Table 3.44 for the gram positive bacterium B1.

Table 3.43 API 20E for gram negative bacteria isolated from inoculum B

TEST	A1	A2	A3	B2	C1	C2	C3
Beta-Galactosidase (ONPG)	+	+	+	+	+	+	+
Arginine Dihydrolase (ADH)	D	-	D	-	-	-	-
Lysine Decarboxylase (LDC)	-	+	-	+	+	+	+
Ornithine Decarboxylase (ODC)	D	+	D	+	+	+	+
Simmons Citrate (CIT)	+	+	+	-	-	-	-
H_2S Production (H_2S)	-	-	-	-	-	-	-
Urease (URE)	-	-	-	-	-	-	-
Tryptophan Deaminase (TDA)	-	-	-	-	-	-	-
Indole (IND)	-	-	-	+	+	+	+
Voges Proskauer (VP)	+	+	+	-	-	-	-
Gelaitne Hydrolysis (GEL)	-	+	-	-	-	-	-
Glucose (GLU)	+	+	+	+	+	+	+
Mannitol (MAN)	+	+	+	+	+	+	+
Inositol (INO)	-	+	-	-	-	-	-
Sorbitol (SOR)	-	D	-	+	+	+	+
Rhamnose (RHA)	+	-	+	+	+	+	+
Sucrose (SAC)	+	+	+	+	+	+	+
Melibiose (MEL)	+	+	+	+	+	+	+
Amygdaline (AMY)	+	-	+	-	-	-	D
Arabinose (ARA)	+	+	+	+	+	+	+
Identification	Enterobacter*	*Serratia liquefaciens*	Enterobacter*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Certainty (%)		76.2		96.5	96.5	96.5	96.5

D: doubtful

*Possible sps: *Eneterobacter amigenus, E. cloaceae, E. cakazakii or E. agglomerans*

Table 3.44 API 20E for gram positive bacterium isolated from inoculum B

TEST	B1
Beta-Galactosidase (ONPG)	+
Arginine Dihydrolase (ADH)	-
Lysine Decarboxylase (LDC)	-
Ornithine Decarboxylase (ODC)	-
Simmons Citrate (CIT)	+
H_2S Production (H_2S)	-
Urease (URE)	+
Tryptophan Deaminase (TDA)	-
Indole (IND)	-
Voges Proskauer (VP)	+
Gelaitne Hydrolysis (GEL)	-
Glucose (GLU)	+
Mannitol (MAN)	+
Inositol (INO)	+
Sorbitol (SOR)	+
Rhamnose (RHA)	+
Sucrose (SAC)	+
Melibiose (MEL)	+
Amygdaline (AMY)	+
Arabinose (ARA)	+

D: doubtful

On examination of the API 20E results, similarities were found between the gram negative bacteria. It seemed that bacteria A1 and A3 are the same or closely microorganisms, because of the agreement of the results in most of the tests. Analysis of these results by following the analytical profile index for API 20E (Biomerieux), gave identification (Table 3.43) to the genus level, such that bacteria A1 and A3 are probably Enterobacter. Several possible species were listed: *Enterobacter amigenus*, *Enterobacter cloaceae*, *Enterobacter cakazakii* or *Enterobacter agglomerans*. A high degree of similarity was also found between bacteria B2, C1, C2 and C3, which also could be the same organism. The identification by the API 20E system proposes *Eschericha coli*, with 96.5 % certainty and a T value of 0.97. Strain A2, with an unclear Gram stain classification, resulted a different microorganism to strains A1 and A3. Analysis of these results by the API 20 E system gave a very good identification to the genus level, proposing *Serratia liquefaciens* with 76.2% certainty (T value of 0.77) or *Serratia marcescens* with 23.8% certainty (T value of 23.8).

For the isolate giving gram positive test results, one bacterium was considered: B1. Although initially B1 was very similar in morphology to the B2 colonies, it was concluded from API 20E analysis that both were different microorganisms. API 20E is not recommended for identification of Gram positive organisms and served simply as a rapid method of showing that the bacteria did have some different characteristics.

While these microbial studies carried out on inoculum B were preliminary, they suggested that different number of gram positive and gram negative (possibly Enterobacteria spp., *E. coli* and *S. liquefaciens*) microorganisms exist in inoculum B and further study was required.

3.6.3.5 Strain identification

Further genetic characterization of inoculum B microorganisms was carried out by colleagues in the Institute of Biotechnology and Food Science, Slovak University of Technology, Bratislava, Slovakia. Isolated DNA from each strain was used for amplification of 16S rDNA by PCR, which was sequenced and compared in a genetic database (GenBank). In this study (Appendix II) 5 strains were successfully identified (note that strain BS is not related to this project).

This report concludes that strains A1 and A3 are the same microorganism and that their 16S rDNA was 100% identical with submitted and published sequences (de Baere *et al.*, 2001; Sarma *et al.*, 2004), being characterised as *Leclercia adecarboxylata*. The 16S rDNA from strain A2, was shown to be 100% identical to submitted and published sequences of *Serratia sp*. (Del Giudice *et al.*, 2008). It also corresponded to other *Serratia* species (*S. proteoamaculans* and *S. liquefaciens* see report, Appendix II), as there are several different species with the same identity of 16S rDNA. Strain A2 was classified as *Serratia sp*. The 16S rDNA from strain B1 was 100% identical with submitted and published sequences of *Klebsiella pneumoniae* (Sajidan *et al.*, 2004; Li *et al.*, 2009). The strain C3 was characterised as *Escherichia coli*.

3.6.4 Inoculum C

The fungus from inoculum C was isolated and cultured in different types of agar (Q&C agar, yeast agar and potato dextrose agar; Section 2.4.5.2). It was incubated at 30^{o}C until spores were produced. Potato dextrose agar was found to be the most appropriate. The fungus presented a green colony, with a white surrounding margine (Figure 3.47 a). A preliminary microscopic examination of the fungus was carried out. A sample

was taken from a mature colony with sellotape and observed under the microscope on a slide. The observed structure of the fungus revealed external conidiospores located on a conidial head, as the example shows in Figure 3. 47 b.

A molecular identification was carried out by CABI (UK; www.cabi.org). The results indicated the mould was *Aspergillus versicolor* (Vuill.) Tirab (Figure 3.47). The morphological features of this strain were described by CABI analysts as typical of this species (for further detail see appendix III). A 100% homology with the authentic strain of *A. versicolor* was found (ITS) and confirmed by calmodulin sequencing. However, only 93% calmodulin sequence homology was found with a separate unnamed taxon, close to *A. versicolor*, described by Peterson (2008).

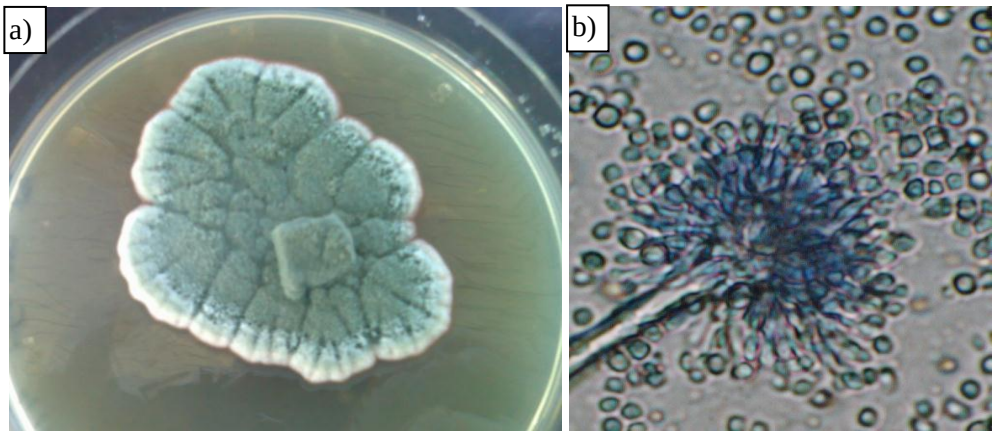


Figure 3.47 *Aspergillus versicolor* (isolated from *A. nodosum* residue). Growing in potato dextrose agar (a) and under the microscope (x100, b; CRCC, 2011).

3.6.5 Microbial studies during the 3-day anaerobic phase

Microorganisms were examined from the anaerobic phase of the microbial digestion of *A. nodosum* residue (batch digestion system, Section 3.4), where the pH was found to decrease. A TVC was carried out and different colonies were isolated. Preliminary tests were carried out before selecting isolates for further identification with the Biolog ID System.

3.6.5.1 Total Viable Count

The TVC was carried out during the 3-day anaerobic phase of the batch digestion system (Section 3.4). Colonies were counted at days 0, 1, 2 and 3 (first and second replicates) or at days 0 and 3 (third replicate; Tables 3.45-46). Different residue samples (employed at the same time for digestion trials BATCH-B+C-2 and BATCH-

WASH 1 and 2, Sections 3.4.2.3 and 3.4.2.4) were used in each replicate to explore the microbial diversity. Each study was carried out in duplicate/triplicate under aerobic (24 h) and anaerobic (48 h) incubation, both at 30^{o}C. The anaerobic phase during the batch digestion system was employed to decrease the initial alkaline pH of the residue (Section 3.4.1.1). The aerobic count was done with the objective of knowing if the aerobic bacteria could survive anaerobic conditions during 3 days, as they may be required during the following aerobic phase. Also, a majority of anaerobic facultative bacteria were shown in the microbial characterization of the residue (Section 3.6.1.3).
The pH during digestion was also recorded and it is shown in Table 3.47 for comparison.

At day 0 of digestion between $1*10^4$ and $5*10^6$ CFU/ml were measured. The numbers increased during digestion finishing with value of between 1 and $8*10^7$ CFU/ml, under aerobic conditions. In the second trial the number of colonies present decreased approximately 5-fold from day 2 ($5.25*10^8$) to day 3 ($9.1*10^7$). Under anaerobic incubation similar results to the aerobic ones were found, which suggest the presence of facultative anaerobic microorganisms. The pH values varied between 8.9 – 9.4 at day 0 and 7.6 – 8.4 at day 3 (Table 3.47).

A succession of microorganisms was observed during the 3 days of digestion, where microorganisms that were present at day 0, were not present or were less abundant at day 2 or 3, and new colonies appeared over this time. Also colonies of different shape and size were observed over the duration of the study time. These natural variations made a reliable total bacterial count very difficult.

Table 3.45 Aerobic TVC during the anaerobic phase of the microbial digestion of *A. nodosum* residue.

	CFU/mL at day			
Replicate	**0**	**1**	**2**	**3**
1	ND	$8.7*10^5 \pm 6.6*10^4$	$9.2*10^6 \pm 5.3*10^5$	$4.1*10^7$
2	$5.3*10^6 \pm 2.1*10^6$	$8.7*10^6 \pm 9.7*10^6$	$5.25*10^8 \pm 4*10^8$	$9.1*10^7 \pm 1.9*10^7$
3	$1.1*10^4$			$5.3*10^7 \pm 1.1*10^7$

ND not determined

Table 3.46 Anaerobic TVC during the anaerobic phase of the microbial digestion of *A. nodosum* residue.

	CFU/mL at day			
Replicate	**0**	**1**	**2**	**3**
1	$6.3*10^5 \pm 2.6*10^5$	$1.6*10^6 \pm 2.7*10^5$	$8.4*10^5 \pm 3.3*10^5$	$8.2*10^7 \pm 5.3*10^6$
2	$1.76*10^6 \pm 1.7*10^5$	$1.4*10^6 \pm 6*10^5$	$3.3*10^7 \pm 4.2*10^6$	$1.05*10^7 \pm 1.7*10^6$
3	ND			$7*10^7$

ND not determined

Table 3.47 pH values during the 3-day anaerobic phase of the microbial digestion of *A. nodosum* residue.

	pH at day			
Replicate	**0**	**1**	**2**	**3**
1	9.38			8.47
2	8.97	8.62	7.98	7.66
3	9.31			7.87

3.6.5.2 Colony isolation and study

A total of eight colonies were observed, photographed (Discovery VMS-001, Veho^{fm}) and isolated from the TVC aerobic agar plates (Figure 3.48). Colonies 1-5 were isolated from day 0 of digestion and colonies 6-8 from day 3. These colonies were tested for the following characteristics: motility, Gram stain, spore stain, acid fast stain, catalase, oxidase and growth on MacConkey agar, (Table 3.48). Tests were carried out in duplicate for each colony. Most of the isolates were Gram negative, with the exception of colony 1, which was Gram positive. Most the isolated were catalase positive, acid-fast stain negative and did not show spore production. Four of the isolates (colonies 3, 4, 5 and 8) showed no growth during incubation under anaerobic conditions (7 days, 30^{o}C) leading to the observation that they are aerobes. The four remaining colonies (1, 2, 6 and 7) showed facultatively anaerobic growth as they grew under both aerobic (24 hours, 30^{o}C) and anaerobic (7days, 30^{o}C) conditions. It was observed that colony 6, changed colour from white (at 24 hours) to orange-yellow after 3-4 days in the agar plate (at both 30^{o}C and 4^{o}C in the fridge). Isolate 8 brought about a change in the colour of the PCA (plate count agar) surrounding the colony. The agar changed from its normal golden/brown colour to red in the area immediately surrounding the colony. This colour change was observed after 2-3 days at both 30^{o}C and 4^{o}C (in the fridge).

Some of the isolates described above correlated with the microbial characterization of *A. nodosum* residue carried out in section 3.6.1.3. Others did not correlate probably due to different residue samples (composition varies as discussed in Section 3.1) and

different time of the year. Also the local company who provided the residue samples, changed location during this project, which could also affect the microbial flora involved with *A. nodosum* residue. Further identification of these colonies is described in the following section.

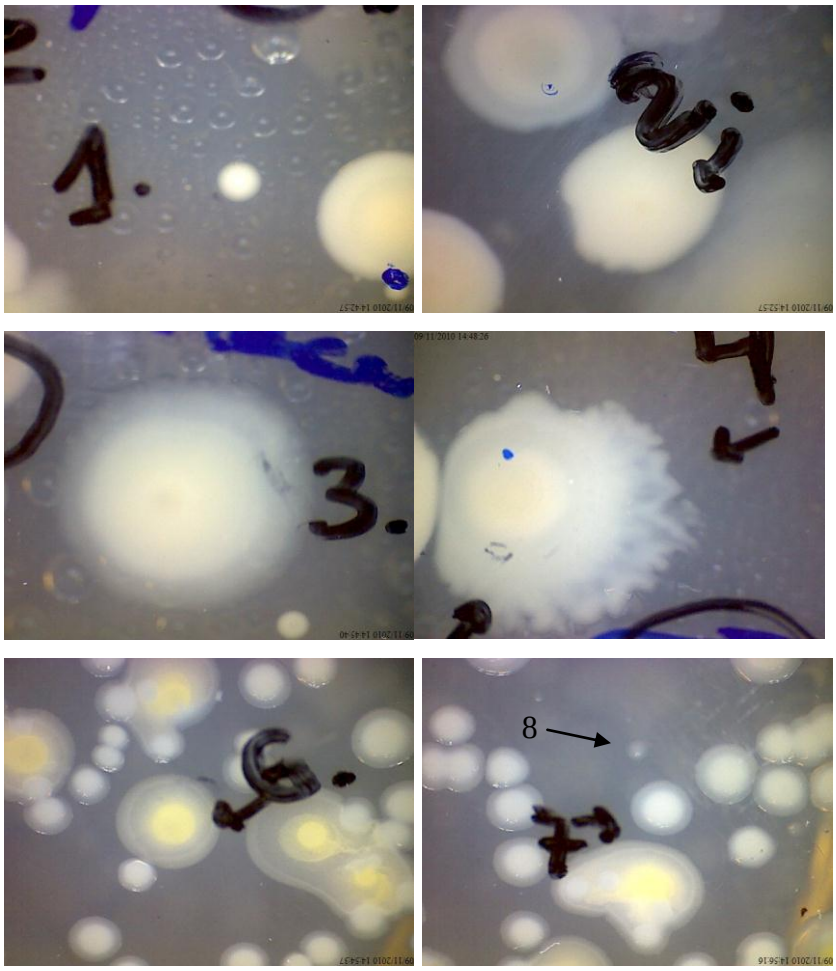


Figure 3.48 Selection of different bacterial colonies during the 3-day anaerobic phase of digestion. Colonies 1 to 8, number 5 not shown.

Table 3.48 Description and characteristics of the different colonies isolated during the anaerobic phase of *A. nodosum* microbial digestion.

	Plate count agar (eye exam)	Nutrient broth (microscope exam)					MacConkey agar					
Colony	Description	Bacterial Shape	Motility	Gram Stain	Catalase	Oxidase	Growth	Lactose	Spore stain	Acid-fast stain	Aerobes	Facultative anaerobes
1	Medium-small circular colony, with 2 white rings: centre darker, outside granulated.	cocci (1, 2, 3, 4 or more together)	medium	+	+	+	+ (very little)	+	ND	ND		+
2	Shiny white, circular, raised, entire margin	rods (some in pairs)		-	+	+	+	+	+(?)	-		+
3	White circular, not entire blurred margin	rods (1 or 2)	rapid	-	+	+	+	+(?)	-	-	+	
4	Blurred white-transparent,	rods (some very long)	very little or none	-	+	+	+	+(?)	-	-	+	
5	Transparent, very small punctiform, difficult to see with the naked eye	Long rods (1 or pairs, some 3)	medium-slow vibration	-	+	+	+	+(?)	-	-	+	
6	"fried egg" type colony, different shades of white, which became orange-yellowish after 3 days	short small rods (mostly individual, some pairs)	rotation	ND	+	-	+	+	-	-		+
7	granula white, circular	Coccobacilli – varying in length from very small to long (or possibly chains)	rapid	-	+	-	+	+	-(?)	-		+
8	Shiny white, circular, very small (grew poorly in broth)	rods, some on chains (no too much growth in the flask)	non	-	+	+	+	+(?)	-	-	+	

ND: not determined

3.6.5.3 Colony identification with Biolog ID System

The colonies isolated and characterised in the previous section (Table 3.48) were identified with the Gen III microplates of the Biolog ID system. Final results of these identifications are shown in Table 3.49 (for further detail see Appendix IV). Biolog identification is acceptable if the similarity index is equal to or greater than 0.5 (Truu et al., 1999). Strains 6 and 8 were not fully identified; however Biolog recognised strain 6 as a Gram negative enteric microorganism, and strain 8 as possible *Pseudomonas* species.

Table 3.49 Identification of bacterial strains according to Biolog Gen III

Strain No.	Spices	Similarity Index
1	*Brochothrix thermosphacta*	0.818
2	*Bradyrhizobium japonicum*	0.667
3	*Pseudomonas tolaasii*	0.549
4	*Pseudomonas maculicola*	0.617
5	*Pseudomonas aeruginosa*	0.596
6	*No ID*	
7	*Enterbacter amnigenus*	0.778
8	*No ID*	

The Gen III microplate is a 96 well plate with different carbon sources and chemical sensitivity tests as explained in section 2.4.6 (Figure 3.50). It also contains a negative and a positive control (wells A-1 and A-10 respectively, Figure 3.50). Growth in a well of the Gen III microplate, utilizing the substrate present, causes reduction of the dye (tetrazolium violet) to a purple colour, indicating a positive result. In Figure 3.49 a sequence of selected pictures of the Gen III microplate with strain 7 giving colour development in certain wells is shown. These pictures were taken by Omnilog incubator-reader. Colour in the positive wells was developed during the 24h incubation.

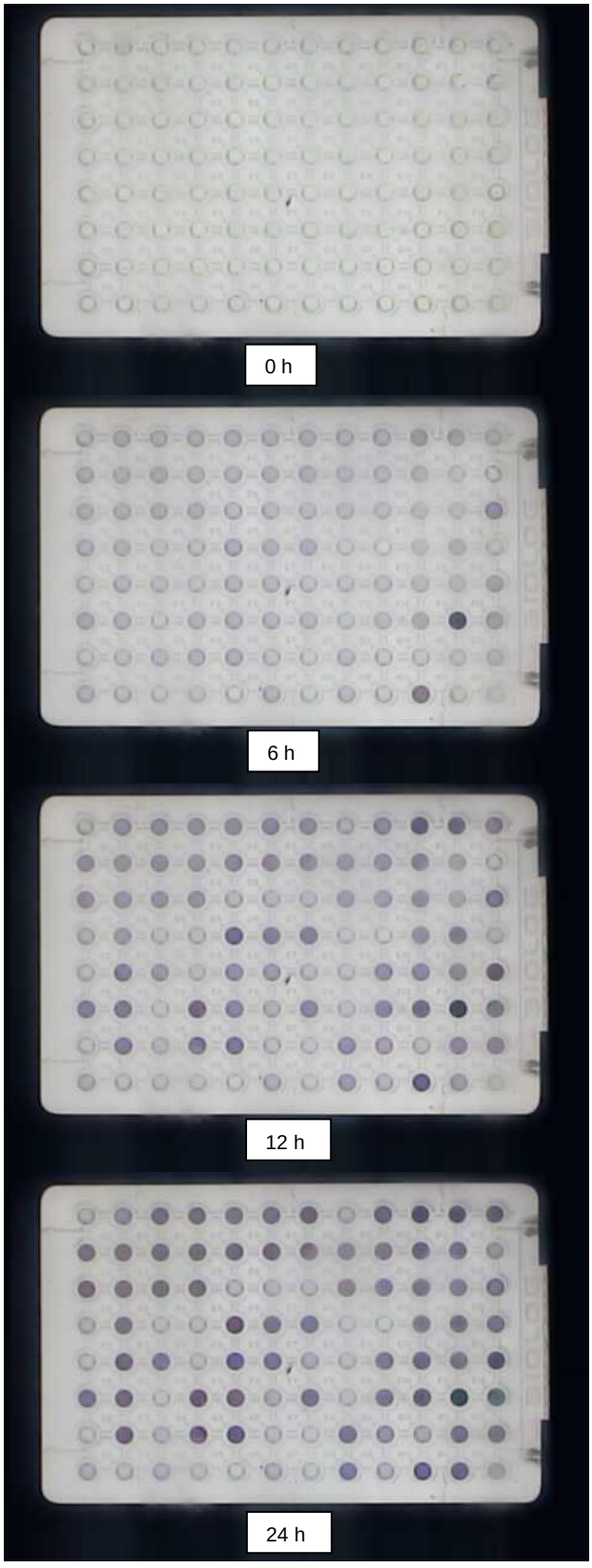


Figure 3.49 Strain 7 growing in Gen III microplate (Biolog) after 0, 6, 12 and 24 h of incubation at 33°C. Pictures were taken by Omnilog (Biolog, US) internal camera.

Absorbance readings are taken every 15 min over the 24 hour incubation period. Omnilog uses these values to plot a growth curve for each well. This allows us to determine how well/rapidly each isolate grows under the conditions in the well, for example specific substrate source, presence of inhibitory compounds, etc. All the data of these tests are collected in Tables 3.50 and 3.51 for carbon sources and chemical sensitivity tests respectively. The growth curves of strains 1-8 are represented for comparison in Figure 3.51. Individual growth curves of each microorganism in each well are presented in Appendix IV (Figures 1 to 8).

Strain 7 was able to utilize the highest number of carbon sources (42 out of 71) in the Gen III microplate, in comparison to the other studied strains (Table 3.50). This suggests that strain 7 (*E. amnigenus*) grows on a wider range of carbon sources. In contrast, strain 8 was only able to utilize L-lactic acid after 24 hours incubation at 33^{o}C. Isolate 5 was able to utilize 15 carbon sources out of 71, strains 1 and 3, 11 out of 71, and strains 4 and 5 used 7 and 5 different carbon sources respectively.

None of the 8 strains tested showed growth when incubated with D- or L-fucose (wells C6 and C7 respectively). Most of the strains (3-8) grew with L-lactic acid (well G4), while only two of them (3 and 7) grew with acetic acid as a main carbon source (well H8). Strains 2 and 5 showed some growth with acetic acid, but it was not enough to be considered positive by the Biolog ID system. None of the 8 strains studied grew on formic acid (well H9), propionic acid (well H7), acetoacetic acid (well H6), or with the butyric acids (wells H2-5). Only strain 4 was B-Hydroxy-D,L-butyric acid positive. Several strains (2-5 and 7) grew in the presence of L-malic acid (well G8) and citric acid (well G5).

Strain 1 was sensitive to all the chemical tests from the Gen III microplate, showing very small growth with tetrazolium violet and blue (F11-12; Table 3.51). The other isolates (2-8) grew in the presence of the majority of chemicals/inhibitory substances, being sensitive to only few of them. Strains 2-8 grew at pH 6, and strains 2-7 also at pH 5. Most of the strains (2-8) grew in the presence of 1% NaCl, while only strains 6 and 8 were tolerant of 8% NaCl (Table 3.51). Most of the studied isolates did not tolerate sodium butyrate (1-5, 8) or sodium bromate (1, 3-8).

Table 3.50 Carbon sources from Biolog Gen III microplate

		Strain							
Well	**Substrate**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A2	Dextrin	-	-	-	-	-	-	-	-
A3	D-Maltose	+++	-	-	-	-	+	+++	-
A4	D-Trehalose	+++	-	-	-	-	+	+++	-
A5	D-Cellobiose	+++	-	-	-	-	+	+++	-
A6	Gentiobiose	+++	-	-	-	-	++	+++	-
A7	Sucrose	+++	-	-	-	-	+	+++	-
A8	D-Turanose	-	-	-	-	-	-	-	-
A9	Stachyose	-	-	-	-	-	+	+++	-
B1	D-Raffinose	-	-	-	-	-	+++	+++	-
B2	α-D-Lactose	-	-	-	-	-	-	+++	-
B3	D-Melibiose	-	-	-	-	-	+	+++	-
B4	B-Methyl-D-Glucoside	+++	-	-	-	-	+	+++	-
B5	D-Salacin	+++	-	-	-	-	++	+++	-
B6	N-Acetyl-D-Glucosamine	+	-	-	-	-	+++	+++	-
B7	N-Acteul-B-D-Mannosamine	-	-	-	-	-	+	+++	-
B8	N-Actetyl-D-Galactosamine	-	-	-	-	-	-	+++	-
B9	N-AcetylNeuraminic acid	-	-	-	-	-	-	+++	-
C1	α-D-Glucose	+++	-	++	+++	-	+++	+++	-
C2	D-Mannose	+++	-	+	-	-	++	+++	-
C3	D-Frustose	+++	-	-	-	-	+	+++	-
C4	D-Galactose	-	-	+++	-	++	+	+++	-
C5	3-Methyl Glucose	-	-	-	-	-	-	-	-
C6	D-Fucose	-	-	-	-	-	-	-	-
C7	L-Fucose	-	-	-	-	-	-	-	-
C8	L-Rhamnose	-	-	-	-	-	-	+++	-
C9	Inosine	-	-	-	-	-	-	+++	-
D1	D-Sorbitol	-	-	-	-	-	-	-	-
D2	D-Mannitol	+++	-	-	-	-	+	+++	-
D3	D-Arabitol	-	-	-	-	-	-	-	-
D4	myo-Inositol	+	-	-	-	-	-	-	-
D5	Glycerol	-	-	-	-	-	++	+++	-
D6	D-Glucose-6-PO4	-	-	-	-	-	-	+++	-
D7	D-Fructose-6-PO4	-	-	-	-	-	-	+++	-
D8	D-Aspartic Acid	-	-	+	-	-	-	-	-
D9	D-Serine	-	-	-	-	-	-	-	-

+++ Positive, ++ positive but with less growth than +++, + very little growth (considered negative by Biolog), - negative.

All wells were compared to the negative control.

Table 3.50 Carbon sources from Biolog Gen III microplate (continuation)

		Strain							
Well	**Substrate**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
E1	Gelatine	-	-	-	-	-	-	-	-
E2	Glycyl-L-Proline	-	-	-	-	-	++	+++	-
E3	L-Alanine	-	-	++	-	-	-	+++	-
E4	L-Arginine	-	-	-	-	-	-	-	-
E5	L-Aspartic Acid	-	-	+	-	-	++	+++	-
E6	L-Glutamic Acid	-	-	+	-	-	-	+++	-
E7	L-Histidine	-	-	+	-	-	-	-	-
E8	L-Pyroglutamic Acid	-	-	-	-	-	-	-	-
E9	L-Serine	-	-	+++	-	-	-	+++	-
F1	Pectin	+	-	-	-	-	+++	+++	-
F2	D-Galacturonic Acid	-	-	+	++	+	+++	+++	-
F3	L-Galactonic Acid Lactone	-	-	-	-	-	-	-	-
F4	D-Gluconic Acid	-	-	++	-	+	+++	+++	-
F5	D-Glucuronic Acid	-	-	+++	+++	-	+++	+++	-
F6	Glucuronamide	-	-	+	-	-	-	-	-
F7	Mucid Acid	-	-	+	+++	-	-	+++	-
F8	Quinic Acid	-	++	+++	+++	++	-	-	-
F9	D-Saccharic Acid	-	-	+++	+	-	-	+++	-
G1	p-Hydroxy-Phenylacetic Acid	-	-	-	+	+	-	-	-
G2	Methyl Pyruvate	-	-	-	-	-	+++	+++	-
G3	D-Lactic Acid Methyl Ester	-	-	-	-	-	-	-	-
G4	L-Lactic Acid	-	-	++	+++	++	++	+++	+++
G5	Citric Acid	-	++	++	+++	++	-	+++	-
G6	α-Keto-Glutaric Acid	-	+	-	-	+	-	++	-
G7	D-Malic Acid	-	-	-	-	-	-	-	-
G8	L-Malic Acid	-	+++	+++	+	+++	-	+++	-
G9	Bromo-Succinic Acid	-	-	-	-	-	-	+	-
H1	Tween 40	-	-	-	-	-	-	-	-
H2	γ-Amino-Butiric Acid	-	-	+	-	-	-	-	-
H3	α-Hydroxy-Butyric Acid	-	-	-	-	-	-	-	-
H4	B-Hydroxy-D,L-Butyric Acid	-	+	-	-	-	-	-	-
H5	α-Keto-Butyric Acid	-	-	-	-	-	-	-	-
H6	Acetoacetic Acid	-	-	-	-	-	-	+	-
H7	Propionic Acid	-	-	-	-	-	-	-	-
H8	Acetic Acid	-	+	++	-	+	-	+++	-
H9	Formic Acid	-	-	-	-	-	-	-	-

+++ Positive, ++ positive but with less growth than +++, + very little growth (considered negative by Biolog), - negative.

All wells were compared to the negative control.

Table 3.51 Chemical sensitivity tests from Biolog Gen III microplate

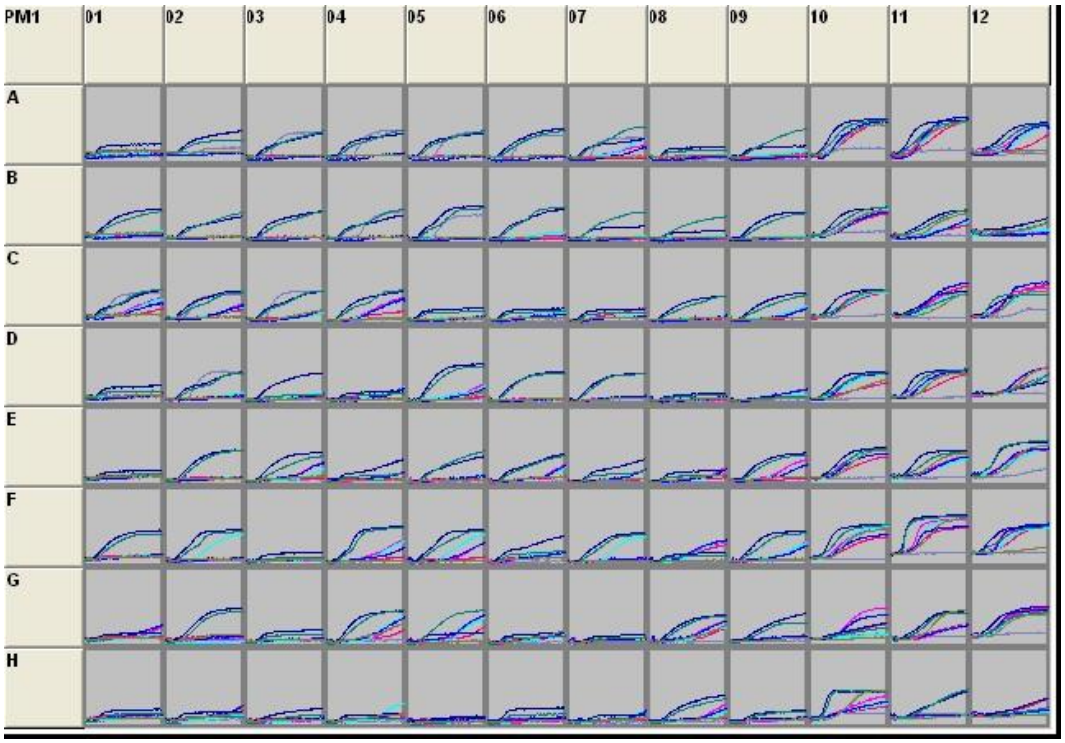
		Strain							
Well	**Conditions**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A11	pH 6	-	+++	+++	+++	+++	+++	+++	+++
A12	pH 5	-	+++	+++	+++	+++	+++	+++	-
B10	1% NaCl	-	+++	+++	+++	+++	+++	+++	+++
B11	4% NaCl	-	+++	++	-	++	+++	+++	+++
B12	8% NaCl	-	-	-	-	-	++	+	+++
C10	1% Sodium Lactate	-	+++	+++	+++	+++	+++	+++	+++
C11	Fusidic Acid	-	+++	+++	+++	+++	+	+++	+++
C12	D-Serine	-	+++	++	+++	++	-	++	+++
D10	Troleandomycin	-	+++	+++	+++	+++	+	+++	+++
D11	Rifamycin SV	-	+++	+++	+++	+++	+++	+++	+++
D12	Minocicline	-	+++	+++	+++	+++	+	+++	+++
E10	Lincomycin	-	+++	+++	+++	+++	+++	+++	+++
E11	Guanidine HCL	-	+++	+++	++	+++	+++	+++	+++
E12	Niaproof 4	-	+++	+++	+++	+++	+++	+++	+++
F10	Vancomycin	-	+++	+++	+++	+++	+++	+++	+++
F11	Tetrazolium violet	+	+++	+++	+++	+++	+++	+++	+++
F12	Tetrazolium Blue	+	+++	+++	+++	+++	+++	+++	-
G10	Nalidixic Acid	-	+++	+++	-	+++	-	-	-
G11	Lithium Chloride	-	+++	-	-	-	+++	+++	+++
G12	Potassium Tellurite	-	++	+++	+++	+++	++	+++	+++
H10	Aztreonam	-	+++	+++	-	++	+++	++	+++
H11	Sodium Butyrate	-	-	-	-	-	+++	+++	-
H12	Sodium Bromate	-	++	-	-	-	-	-	-

+++ Positive, ++ positive but with less growth than +++, + very little growth (considered negative), - negative.

All wells were compared to the positive control.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 PH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 B-Methyl-D-Glucoside	B5 D-Salacin	B6 N-Acetyl-D-Glucosamine	B7 N-Acteul-B-D-Mannosamine	B8 N-Actetyl-D-Galactosamine	B9 N-Acetyl Neuraminic acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Frustose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocicline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCL	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucid Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butiric Acid	H3 α-Hydroxy-Butyric Acid	H4 B-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 3.50 Gen III microplate for bacterial ID (Biolog, US).



1 Strain 1 (*Brochothrix thermosphacta)*
2 Strain 2 (*Bradyrhizobium japonicum*)
3 Strain 3 (*Pseudomonas* tolaasii)
4 Strain 4 (*Pseudomonas maculicola*)
5 Strain 5 (*Pseudomonas aeruginosa*
6 Strain 6 (No ID)
7 Strain 7 (*Enterbacter amnigenus*)
8 Strain 8 (No ID)

Figure 3.51 Strains 1-8 growth profile in Biolog's Gen III microplate during 24 h of incubation at 33^{o}

3.7 Screening for anti-hyaluronidase and anti-elastase activities

The enzymes hyaluronidase and elastase are involved in the turnover of the main components of the skin matrix. *A. nodosum* residue digestion samples and two seaweed species (*A. nodosum* and *L. hyperborea*) were screened for hyaluronidase inhibitors (Section 3.7.1). Fresh seaweed samples, meal and a commercial seaweed extract were treated through a series of experiments (Section 2.5.2) to obtain peptides. The samples were screened for anti-hyaluronidase and anti-elastase activities before and after the treatment (Section 2.7.2). To-date seaweed derived peptides have not been evaluated for this application.

3.7.1 Anti-hyaluronidase activity from *A. nodosum* residue, plant and other samples

Digestate samples (SM) from the residue digestion process and several seaweed samples (*A. nodosum* and *L. hyperborea*) were evaluated for HAase inhibition.

3.7.1.1 *A. nodosum* and *L. hyperborea* seaweeds

Initially, *A. nodosum* and *L. hyperborea* (fresh seaweed samples) were screened for anti-HAase activity (Section 2.5.4). The seaweeds were used in the freeze-dried format and a concentration between 0.25 and 4 mg/ml was studied (Figure 3.52). *L. hyperborea* showed slightly higher inhibition than *A. nodosum* at a concentration between 0.25-2 mg/ml, while there was no difference at 4 mg/ml. *L. hyperborea* extract presented an IC_{50} value of 0.50 mg/ml, in comparison to 0.70 mg/ml, with *A. nodosum* extract.

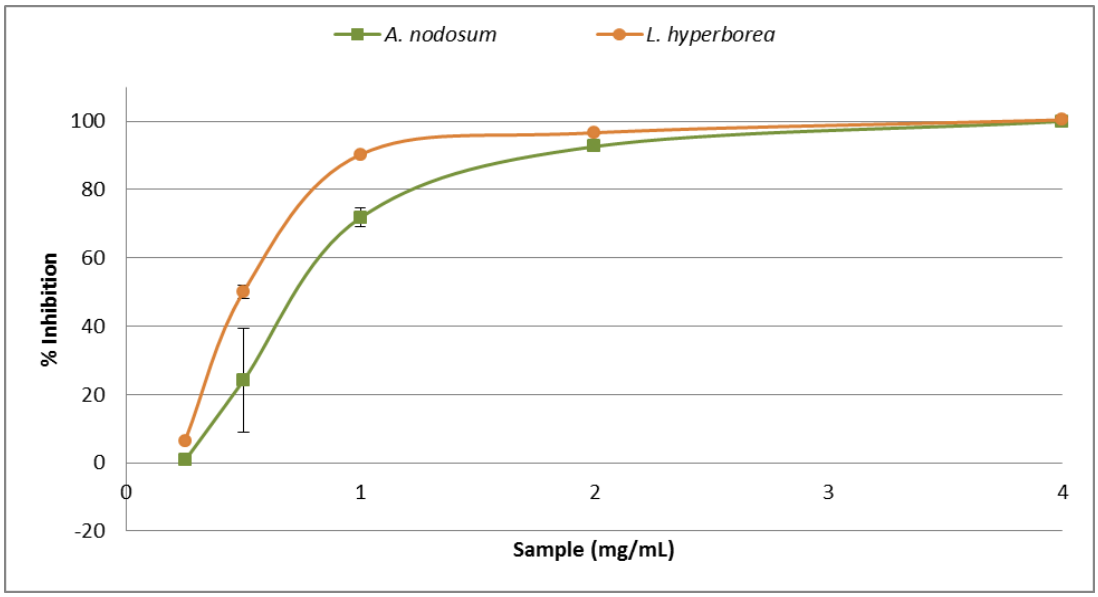


Figure 3.52 Hyaluronidase inhibition with SD by *A. nodosum* and *L. hyperboera* (n = 2).

3.7.1.2 *A. nodosum* residue digestion samples

A. nodosum residue digestion samples, obtained from three different trials (BATCH-6, BATCH-B+C-1 and BATCH-WASH-2), were studied for anti-HAase activity. In each trial an initial amount of 50 g residue and 400 ml water was employed. The SM was separated by centrifugation (Section 2.3.4), freeze-dried and screened for anti-HAase activity. Day 0 of digestion, which is equivalent to an aqueous extract of the residue, showed stronger inhibition than *A. nodosum* and *L. hyperborea* in the three trials tested (Figure 3.53).

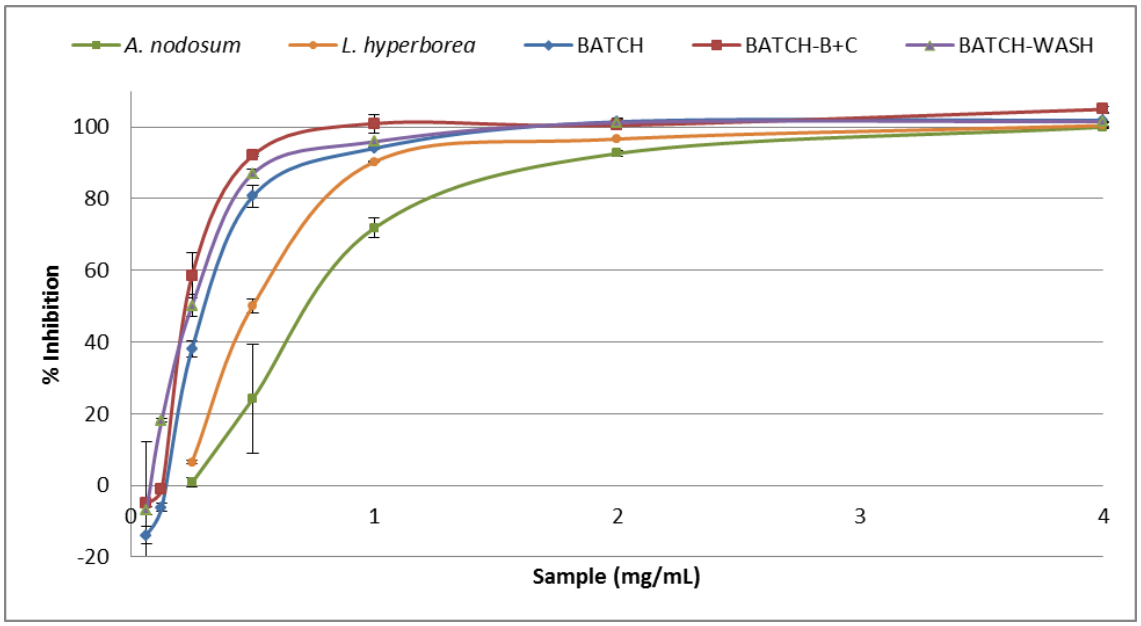


Figure 3.53 Hyaluronidase inhibition by *A. nodosum*, *L. hyperboera* and day 0 of digestion (trials BATCH, BATCH-B+C and BATCH-WASH, prior to wash; n = 2).

For each trial, different digestion times were studied. The BATCH trial consisted of a 3-day anaerobic phase, SM decantation and replacement with water, inoculum B addition, followed by 10-day aerobic phase (Sections 3.4 and 3.4.1). Trial BATCH-B+C, was the same as the BATCH trial, but a mix of inocula B and C was added (Section 3.4.2.3). In trial BATCH-WASH, the residue was washed prior to the digestion process and then run under the same conditions as trial BATCH-B+C (Section 3.4.2.4). Samples studied for anti-HAase activity are presented in Figures 3.54-56 with *A. nodosum* for comparison. In trials BATCH and BATCH B+C, day 0 and 3 presented very similar HAase inhibition values, which were the strongest found over the digestion process. Day 13 presented lower inhibition than days 0 and 3, but which remained more potent than *A. nodosum* (BATCH-B+C, Figure 3.55). In the BATCH trial, day 6 was also

examined and presented very similar inhibition values to fresh *A. nodosum* extract (Figure 3.54). In BATCH-WASH, days 0 (after wash) and 3 presented almost identical inhibition values, lowest at day 0 prior to wash, but still more potent than *A. nodosum* (Figure 3.56). Day 13 of BATCH-WASH presented the lowest HAase inhibition and small activation for a concentration lower than 0.70 mg/ml (Figure 3.56). Trials BATCH and BATCH-B+C presented similar HAase inhibition at the different digestion days, while BATCH-WASH exhibited lower inhibition, probably due to the residue wash prior the digestion process. These data showed that anti-HAase activity persists in digest collected over the 3-day anaerobic phase of the *A. nod*osum residue digestion, while it appears to disappear during the aerobic phase (Figure 3.57).

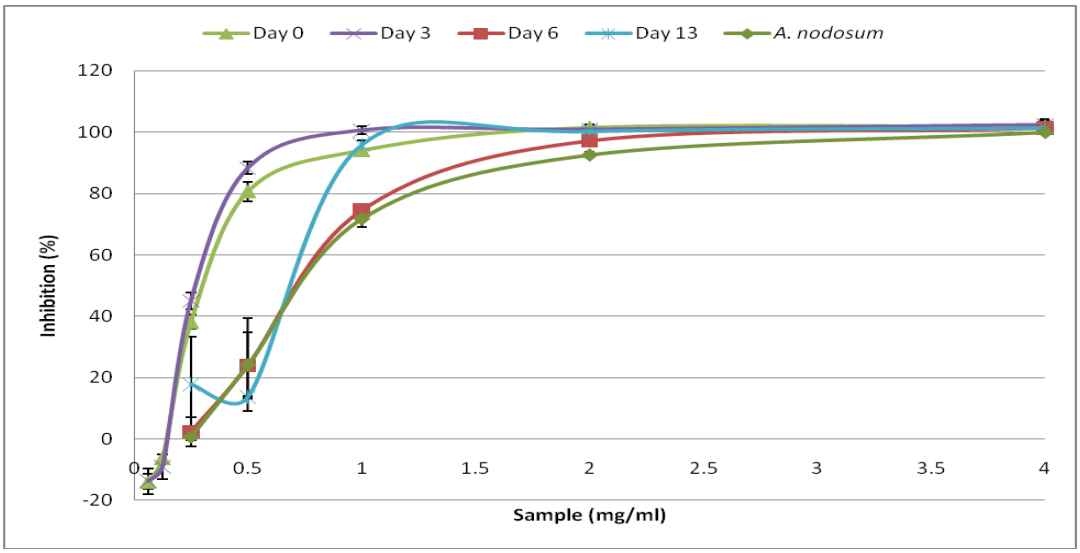


Figure 3.54 Hyaluronidase inhibition by BATCH-6 samples and *A. nodosum* (n = 2).

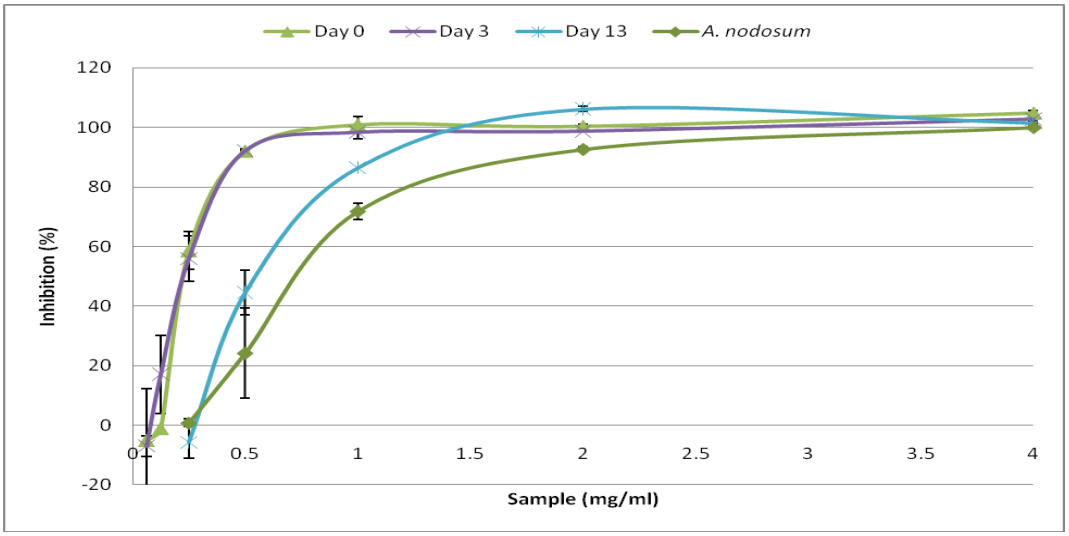


Figure 3.55 Hyaluronidase inhibition by BATCH-B+C-1 samples and *A. nodosum* (n = 2).

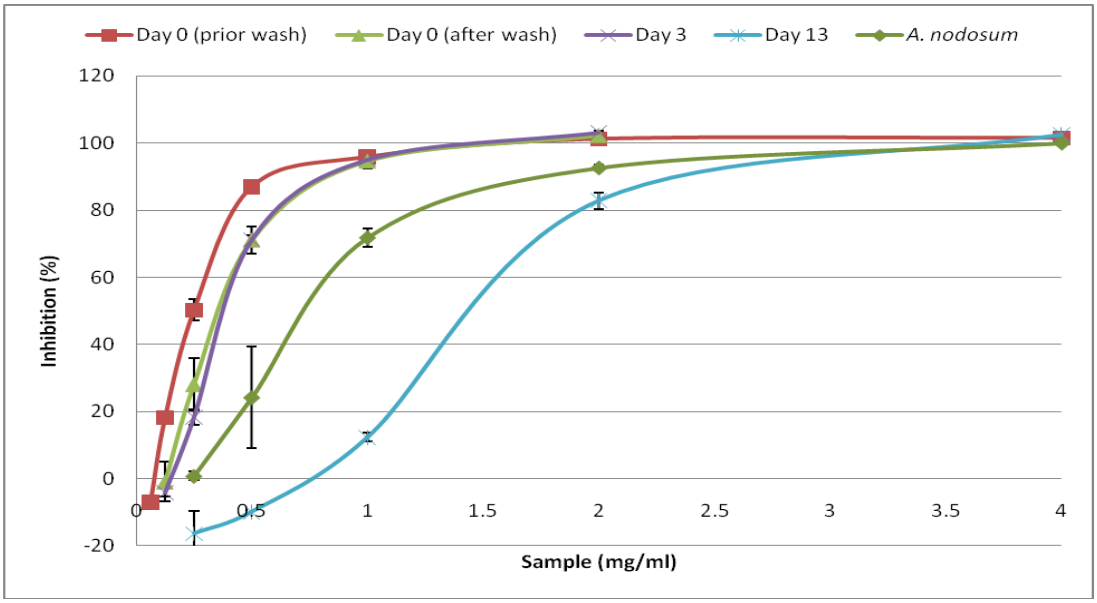


Figure 3.56 Hyaluronidase inhibition by BATCH-WASH-2 samples and *A. nodosum* (SD, n = 2).

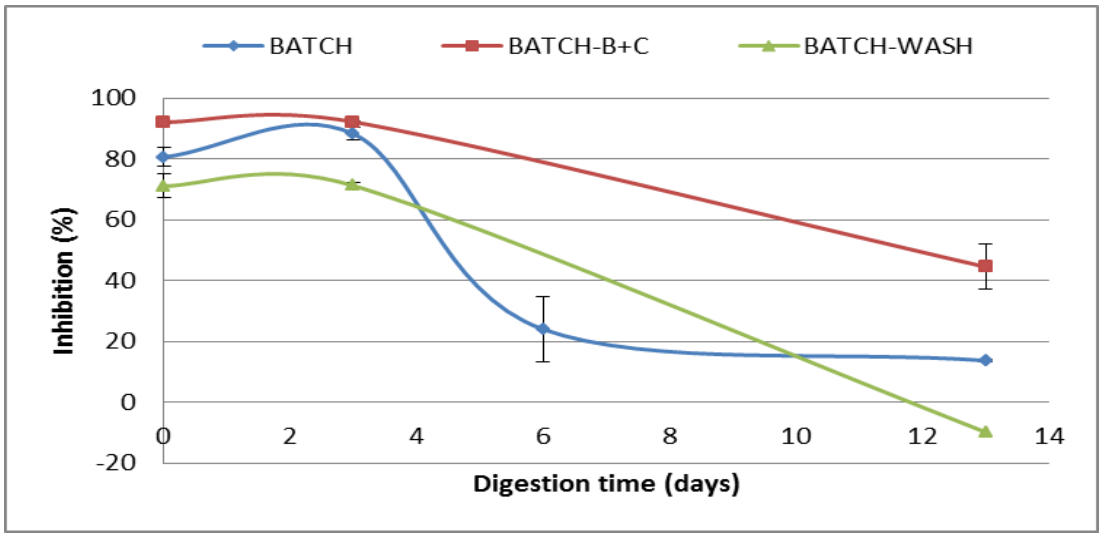


Figure 3.57 Hyaluronidase inhibition of *A. nodosum* residue over digestion time. All samples were assayed at a concentration of 0.5 mg/ml (SD, n = 2).

3.7.1.3 *A. nodosum* methanol extract

A. nodosum methanol extract, which had shown HAase inhibition before (O'Connell, 2010) was also evaluated in this study. Dehydrated *A. nodosum* (10 g) was extracted with 100 mL of 50% (v/v) ethanol over 2 hours and centrifuged (3827.5 xg, 20 min). The extract was freeze dried and assayed for HAase activity. Results showed that a concentration of 6 mg/mL presented 100% inhibition, while a concentration equal or smaller to 1 mg/mL showed less than 20% inhibition (Figure 3.58). The IC_{50} value was

2.5 mg/mL. Based on this graph, it was decided to use a concentration of 4 mg/ml to screen the seaweed samples on HAase activity (Section 3.7.2.3).

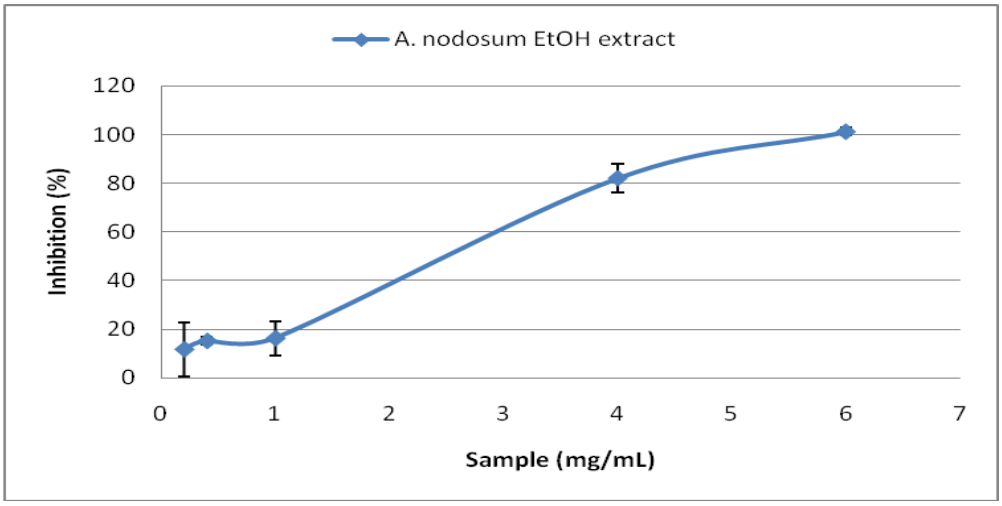


Figure 3.58 Hyaluronidase inhibition by *A. nodosum* methanol extract (SD, n = 2).

3.7.1.4 Sodium heparin

From all the HAase inhibitors presented by the literature, it was decided to use heparin as an inhibitor control. Sodium heparin (ED4071, from Wexport Ltd., Cork) was used for this purpose. A sodium heparin concentration of 50 µg/mL inhibited HAase by over 94% (Figure 3.59). The IC_{50} value for sodium heparin was 43 µg/mL.

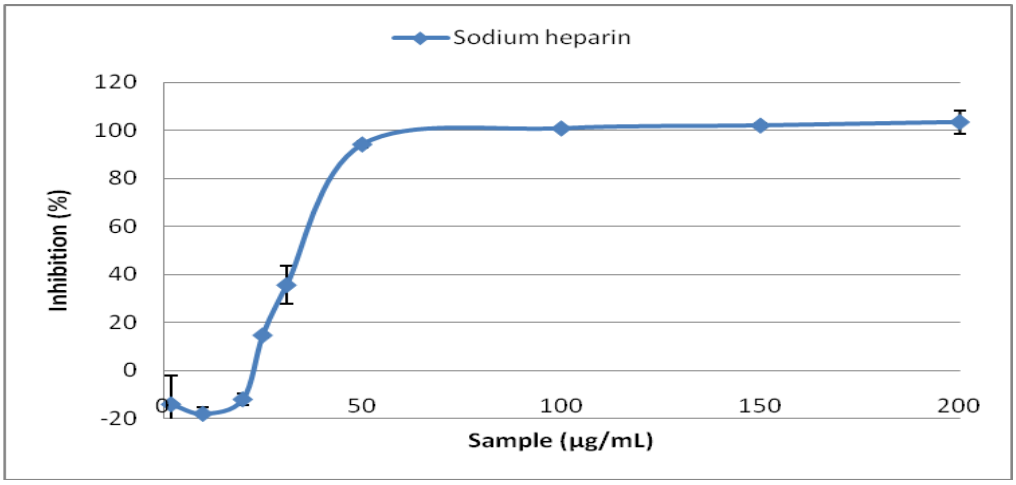


Figure 3.59 Hyaluronidase inhibition by sodium heparin (SD, n = 2).

3.7.1.5 IC_{50} values for Hyaluronidase

IC_{50} values of the crude samples previously studied were calculated according to Figures 3.52-59 (except 3.57), and represented on Table 3.52. *Laminaria hyperborea* presented a lower IC_{50} than *A. nodosum* (500 *vs.*700 µg/ml), while that of *A. nodosum* methanol extract was much higher (2500 µg/ml). *A. nodosum* residues had IC_{50} values between 250 and 270 µg/ml at day 0, which is lower than the IC_{50} value obtained for fresh *A. nodosum* or *L. hyperborea*. This value improved slightly at day 3 in trials BATCH (230 µg/ml) and BATCH-WASH, where the IC_{50} value after washing the residue was 350 µg/ml at day 0 and 340 µg/ml at day 3. In BATCH-B+C, an IC_{50} value of 250 µg/ml persisted in extracts after the 3-day anaerobic phase (Table 3.52). Day 13 of digestion gave IC_{50} values of 650 and 550 µg/ml for trials BATCH and BATCH-B+C, respectively, while trial BATCH-WASH gave the weakest IC_{50} (1400 µg/ml) for a residue digest sample. Sodium heparin gave the strongest IC_{50} (43 µg/ml) for anti-HAase activity. In Table 3.52, HAase IC_{50} values from two Japanese brown seaweeds were also included for comparison.

Table 3.52 IC_{50} values for hyaluronidase inhibition

Sample		IC_{50} (µg/mL)
A. nodosum (crude extract)		700
L. hyperborea (crude extract)		500
A. nodosum methanol extract		2500
Trial BATCH-6	Day 0	270
	Day 3	230
	Day 6	700
	Day 13	650
Trial BATCH-B+C-1	Day 0	250
	Day 3	250
	Day 13	550
Trial BATCH.WASH-2		
	Day 0 (Prior to wash)	250
	Day 0 (after wash)	350
	Day 3	340
	Day 13	1400
Sodium heparin		43
Eisenia bicyclies (crude phlorotannins fraction)[1]		30
Ecklonia kurome (crude phlorotannins fraction)[1]		35

[1] (Shibata *et al.*, 2002)

3.7.1.6 Studying the inhibition type

An experiment was designed to clarify the inhibition type caused by heparin and other samples. For this purpose, sodium heparin and BATCH-B+C (day 0) samples were used at their IC_{50} concentrations (43 and 250 µg/ml respectively). Different concentrations of HA (substrate) from 37.5 to 300 µg/ml were employed. The reciprocal of the absorbance (585 nm) and the HA concentration were graphed (Figure 3.60), following the model of Lineweaver Burk (Macarulla *et al*., 1992). The inhibition type exhibited by heparin seemed to be a competitive type of inhibition. However, the intersection of control and inhibited data suggest that the inhibition type could not be pure competitive (heparin and substrate do not compete equally for the active site).BATCH-B+C (day 0) and control lines appear to be parallel, which suggests an uncompetitive inhibition type. However this is a crude extract and further research (fractionation and identification of the fractions/molecules that inhibit HAase) will be required in order to fully understand the inhibition. It would appear that heparin and the digest samples exhibit different forms of inhibition of HAase.

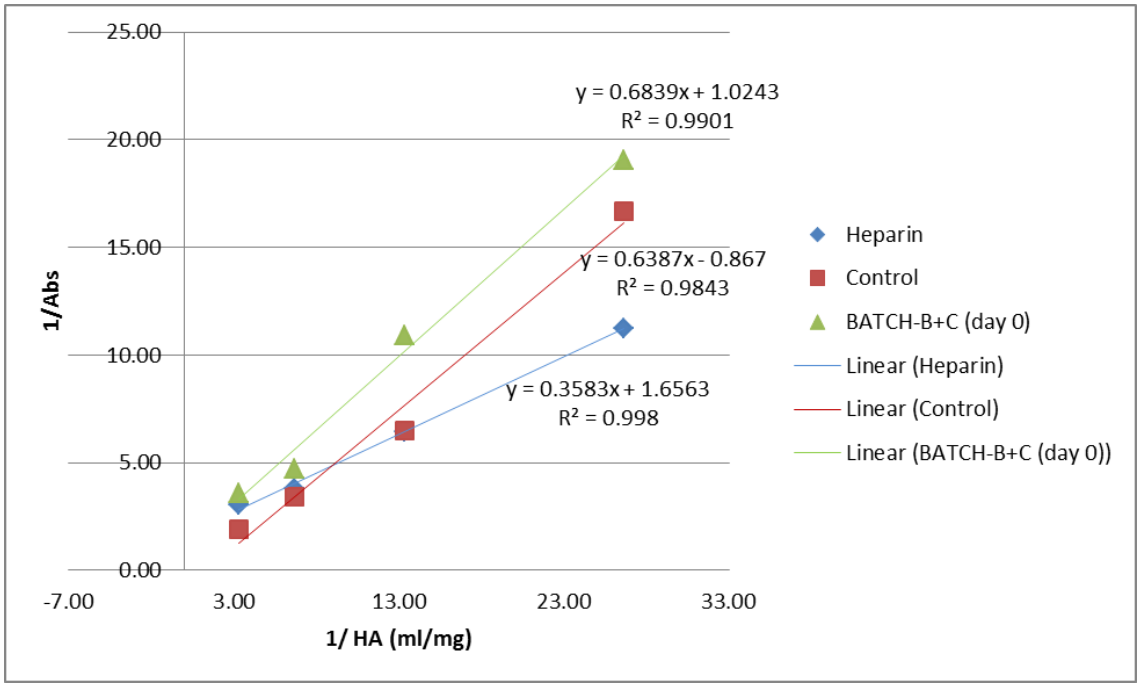


Figure 3.60 Hyaluronidase inhibition type. Heparin and BATCH-B+C samples were assayed at a concentration of 43 and 250 µg/ml respectively.

3.7.2 Samples treated to obtain peptides

Seaweed samples were treated through a series of experiments in order to obtain proteins and generate peptide fractions. The methodology employed and evaluated involved the removal of the lipid content by SFE, breakdown of carbohydrates with a carbohydrases mixture (Viscozyme) liberating proteins and protein hydrolysis with four different types of proteinases (alcalex, protamex, pepsine and bromealin; Section 2.5.2). Fresh and dried seaweed samples, dehydrated *A. nodosum* meal and a commercial aqueous extract (AqE) were treated through these steps to obtain proteins/peptides. Non-SFE samples were also employed for this purpose. The subsamples obtained (supernatant fraction, Section 2.5.2.3) were screened for anti-hyaluronidase and anti-elastase activities.

3.7.2.1 Supercritical Fluid Extraction

SFE was carried out at medium temperature (50^{o}C) to preserve any bioactivity present in the samples. Data recorded for the SFE are presented in Table 3.53, where the amount of sample collected for the extracts and the residues is shown. Fresh *L. hyperborea* showed the heaviest extraction (0.835 g) at 400 bars. This extract was jelly-like in consistency. The AqE (commercial aqueous extract from *A. nodosum*) presented the lower extraction values, probably indicating lower lipid content in comparison with the fresh seaweeds.

Table 3.53 SFE extracts and residual samples' weights

Sample		Initial (g)	Extracts		Residual sample
			Pressure (bars)	g	g
A. nodosum	Fresh	10.053	100	0.008	8.422
			200	0.025	
			400	0.019	
	Dried	10.000	100	0.002	9.829
			200	0.008	
			400	0.025	
	Dehydrated	10.005	100	0.101	9.899
			200	0.013	
			400	0.016	
L. Hyperborea	Fresh	10.011	100	0.063	6.816
			200	0.009	
			400	0.835	
	Dried	10.000	100	0.011	9.690
			200	ND	
			400	0.001	
AqE	Fresh	10.017	100	0.01	9.266
			200	0.002	
			400	0.002	
	Dried	10.050	100	0.002	9.331
			200	0.001	
			400	0.001	

ND no disponible

3.7.2.2 Protein Content

The protein content was measured prior to the SFE with Kjeldahl method and during the protein hydrolysis with BCA assay. Results, shown in Table 3.54, revealed higher total nitrogen content for the dried forms of the seaweeds and AqE than the fresh ones. In *A. nodosum* the higher protein value was found in the dehydrated meal (6.63%). Fresh *L. hyperborea* presented a slightly higher protein content than *A. nodosum* (2.09% *vs.* 1.66%), while these values were equal for the dried forms of both seaweeds (5.18% and 5.17%). The AqE contained lower protein content in its fresh (0.80%) and dried (3.5%) formats. Non-SFE fresh *A. nodosum* and *L. hyperborea*, presented slightly lower protein content than the SFE samples, due to the lipid content making up part of the sample (Table 3.54).

During the protein hydrolysis, the protein contents were found to be high, because viscozyme carbohydrases and proteases were also included in the samples. These protein contents varied between samples and between different proteases in the same sample, but no correlations were found. Samples that presented initial higher protein content with Kjeldahl methodology, did not necessarily showed higher protein content under the BCA protein analysis (Table 3.54). Standard deviation values were high in some cases, this is a question discussed in Section 4.7.2.

Table 3.54 Protein Content

			Initial (Kjeldhal)	During protein Hydrolysis (BCA, n = 2)					
					0 min		30 min		60 min	
			Protein %	Protease	Prot (µg/mL)	SD	Prot (µg/mL)	SD	Prot (µg/mL)	SD
SFE-residue	*A. nodosum*	Fresh	1.66	a	10208	2516	14353	3750	10073	2730
				px	8041	1548	8072	1791	9860	406
				pp	10126	2561	7084	2803	8622	1418
				b	13874	2606	10908	4068	11514	3015
		Dried	5.17	a	7133	460	5897	341	6131	531
				px	5790	52	4930	769	4992	319
				pp	6781	327	5982	53	5950	234
				b	6883	1052	4761	19	6267	223
		Dehydrated	6.63	a	7739	244	7290	1975	6259	813
				px	4871	306	6309	1357	8577	2835
				pp	9281	746	10520	610	10387	52
				b	8796	196	9878	709	11064	3446
	L. Hyperborea	Fresh	2.09	a	1583	244	1199	620	2383	498
				px	2595	919	2087	133	1557	203
				pp	709	199	1629	438	640	745
				b	1699	235	1898	656	2295	263
		Dried	5.18	a	8187	621	7959	30	7310	796
				px	7750	681	5117	254	5307	1277
				pp	7979	519	5302	59	8335	2257
				b	5360	1080	4963	512	6841	97
	AqE	Fresh	0.80	a	3252	287	3151	269	7896	2888
				px	3946	1247	3609	220	2511	946
				pp	2581	301	2402	641	2582	194
				b	5056	1360	3836	1198	4563	147
		Dried	3.50	a	24383	1229	24340	417	20638	420
				px	22139	532	19934	25	21495	786
				pp	26349	1212	22187	744	24333	2349
				b	21461	25	23106	1255	20068	636
non-SFE	*A. nodosum*	Fresh	1.38	a	14054	2620	7234	1840	7407	1023
				px	13608	988	11934	955	10525	2342
				pp	10826	165	8779	659	9534	644
				b	14158	3046	11902	696	12418	291
	L. Hyperborea	Fresh	1.16	a	1649	724	1014	317	1540	59
				px	1951	616	954	97	1554	1361
				pp	1874	436	972	189	1339	385
				b	1039	592	1435	291	2330	185
	AqE	Fresh	0.96	a	3274	1562	4136	1359	6046	1905
				px	6702	2774	10262	1190	10716	5710
				pp	10571	1275	8910	2655	9000	5448
				b	7404	833	3707	2216	8795	2342

3.7.2.3 Hyaluronidase Activity

Seaweed samples were prepared with SFE, carbohydrate digestion and protein hydrolysis. Subsamples collected at each stage were freeze-dried and screened for HAase inhibition. This included original samples (*A. nodosum*, *L. hyperborea* and AqE in their fresh, dried and/or dehydrated format), SFE-residuals, samples collected after carbohydrates digestion (SFE and non-SFE) and samples collected after 60 minutes of protein hydrolysis (with alcalase and protamex).

Two types of viscozyme controls were assayed with HAase, to see how viscozyme affected the enzyme at different stages of the process. The first viscozyme control was used for the samples collected after the carbohydrate digestion step. This control consisted on a viscozyme sample (same amount used for the seaweed samples), dissolved in the digestion buffer (ammonium acetate, section 2.5.2.2), deactivated and freeze-dried. The second type of viscozyme control was also deactivated and hydrolysed with the two proteases alcalase and protamex. This control was used for the samples after the protein hydrolysis. Results of Viscozyme controls are presented on Table 3.55. The three controls were found to activate HAase. Viscozyme (after the carbohydrate digestion) resulted in the highest HAase activation (88.71±6.98%), while viscozyme treated with alcalase or protamex, exhibited lower activation (32.01±4.23% and 39.39±3.21%, respectively). It is important to note that although Viscozyme controls were assayed at the same concentration as the samples (4 mg/ml), its real concentration in a sample will be lower than 4 mg/ml, due to the real sample taking part of the weight.

Table 3.55 Hyaluronidase inhibition by Viscozyme controls (compared to positive control, n = 2)

	% Inhibition	
	Average	**SD**
After carbohydrate digestion	-88.71	6.98
Alcalase	-32.01	4.23
Protamex	-39.39	3.21

Table 3.56 shows the anti-hyaluronidase activity present in the seaweed samples collected at different steps of the peptide-generating process. It was decided to use a concentration of 4 mg/ml for each sample, based on the HAase inhibitory activity showed by *A. nodosum* methanol extract (Figure 3.58, Section 3.7.1.3). Most of the

samples exhibited HAase inhibition close to 100% at a concentration of 4 mg/ml. The lowest inhibition was found in SFE fresh *L. hyperborea* after carbohydrate digestion (76.42 ± 2.62%), followed by the non-SFE version of fresh Laminaria also after carbohydrate digestion (86.38 ± 3.35%). When most of the samples after carbohydrate digestion were compared to Viscozyme control rather than the positive control, the percentage of inhibition slightly increased and the SD values were reduced. This was also observed with the samples treated with alcalase and protamex (Table 3.57).

Table 3.56 Hyaluronidase inhibition by seaweed samples collected at different stages of the process (n=2)

			***vs.* positive control**		***vs.* Viscozyme control**	
Process step	**Sample**		**%Inhib**	**SD**	**%Inhib**	**SD**
1. Original samples	*A. nodosum*	Fresh	98.24	1.38		
		Dried	100.50	0.18		
		Dehydrated	101.39	1.62		
	L. Hypoerborea	Fresh	101.22	1.05		
		Dried	100.96	1.06		
	AqE	Fresh	99.52	0.95		
		Dried	103.99	0.91		
2. SFE-residuals	*A. nodosum*	Fresh	99.60	0.13		
		Dried	100.82	0.12		
		Dehydrated	99.40	0.75		
	L. Hypoerborea	Fresh	100.00	0.00		
		Dried	100.50	0.75		
	AqE	Fresh	95.92	0.42		
		Dried	98.05	1.72		
3. After carbohydrate digestion						
a) SFE-residuals	*A. nodosum*	Fresh	97.89	0.53	98.88	0.28
		Dried	100.45	0.62	100.24	0.33
		Dehydrated	101.80	0.01	100.95	0.00
	L. Hypoerborea	Fresh	76.42	2.62	87.51	1.39
		Dried	100.97	0.31	100.51	0.16
	AqE	Fresh	100.55	0.13	100.29	0.07
		Dried	100.72	0.02	100.38	0.01
b) non-SFE	*A. nodosum*	Fresh	100.19	0.32	100.10	0.17
	L. Hypoerborea	Fresh	86.38	3.35	92.78	1.77
	AqE	Fresh	100.71	0.65	100.38	0.34

Most of the samples after protein hydrolysis exhibited between 95 and 100% inhibition, at a concentration of 4 mg/ml (Table 3.57). Only few samples treated with alcalase exhibited an inhibition lower than 95%. The lowest HAase inhibition was found in fresh *L. hyperborea* treated with alcalase (86.56 ± 1.66% non-SFE and 88.24 ± 0.06% SFE). The non-SFE samples presented lower inhibition in comparison to the SFE-residual samples, because the lipid fraction was included in the total weight of the former and not in the latter. In general, samples treated with protamex, exhibited slightly higher anti-HAase activity than samples treated with alcalase. When comparing each individual sample through the different steps of the process, no dramatic changes on HAase inhibition were found (Table 3.56-57).

Table 3.57 Hyaluronidase inhibition by seaweed samples after protein hydrolysis (n = 2)

				***vs.* positive control**		***vs.* Viscozyme control**	
			Protease	**%Inhib**	**SD**	**%Inhib**	**SD**
SFE-residual	*A. nodosum*	Fresh	a	98.37	5.86	98.76	4.44
			px	101.00	0.29	100.71	0.21
		Dried	a	101.17	6.09	100.89	4.61
			px	101.04	0.14	100.75	0.10
		Dehydrated	a	99.67	1.70	99.75	1.29
			px	101.29	0.07	100.92	0.05
	L. Hyperborea	Fresh	a	88.24	0.06	91.10	0.04
			px	95.94	11.29	97.09	8.10
		Dried	a	91.61	0.72	93.64	0.55
			px	100.68	0.51	100.49	0.37
	AqE	Fresh	a	97.33	1.13	97.98	0.86
			px	103.22	0.93	102.31	0.67
		Dried	a	91.26	2.24	93.38	1.69
			px	101.08	1.06	100.77	0.76
non-SFE	*A. nodosum*	Fresh	a	92.49	1.86	94.31	1.41
			px	100.55	0.26	100.40	0.19
	L. Hyperborea	Fresh	a	86.56	1.66	89.82	1.26
			px	101.49	0.01	101.07	0.01
	AqE	Fresh	a	92.54	2.10	94.35	1.59
			px	100.99	0.47	100.71	0.34

3.7.2.4 Elastase activity

Samples screened for anti-elastase activity are presented in the following sections. A sample concentration of 50.3 µg/ml was chosen, because this was the same concentration (equal to 0.1M) as the inhibitor provided by the kit (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) which caused total inhibition of elastase (Figure 3.61).

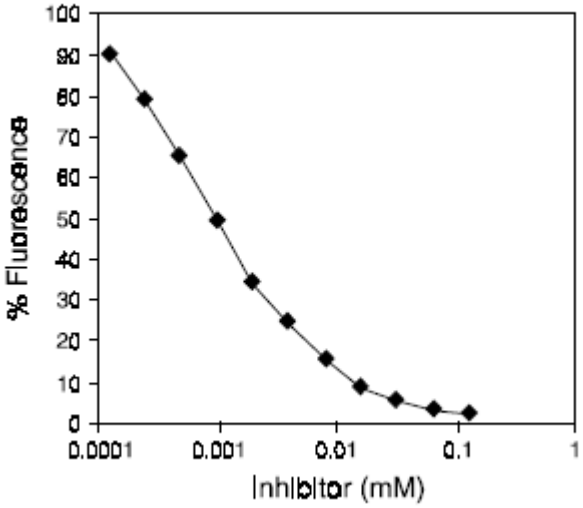


Figure 3.61 Inhibition of elastase by N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. DQ elastin substrate at 25 µg/mL, elastase at 0.5 U/mL and increasing amounts of the inhibitor were incubated together for 2 hours. Fluorescence was measured in a fluorescence multi-well plate reader set for excitation at 485 ± 10 nm and emission detection at 530 ± 15nm. All values were corrected for background fluorescence and expressed relative to the fluorescence obtained in the absence of inhibitor. EnzChek® Elastase Assay Kit (E-12056).

3.7.2.4.1 SFE-extracts

Extracts obtained by SFE were screened directly by elastase assay. Results on Table 3.58 show that most of the samples activated elastase at a concentration of 50.3 µg/ml. The highest activation was found in dried *A. nodosum* (Figure 3.62), where the extract obtained at 200 bar, activated elastase by 151.0 ± 22.6% after 2 hours of incubation, followed by the extract at 100 bar (128.8 ± 4.6%) and 400 bar (95.6 ± 21.1%). These three samples presented a higher initial activation, which was reduced over time (Table 3.58). The AqE samples, both fresh and dried, presented lower activation on elastase than *A. nodosum* and *L. hyperborea*.

Fucoidan samples (Figure 3.63), donated by a local company, were obtained from *A. nodosum* by ethanol extraction (untreated fucoidan) and treated with H_2O_2 (treated fucoidan). The untreated fucoidan showed small elastase inhibition (12.2 ± 31.9%) during the initial 10 minutes, but which changed to activation over time (Table 3.58). The treated fucoidan showed elastase activation, which was constant over time (between 20-33%). Some of these samples presented high SD values, which will be discussed in Section 4.7.2.3.

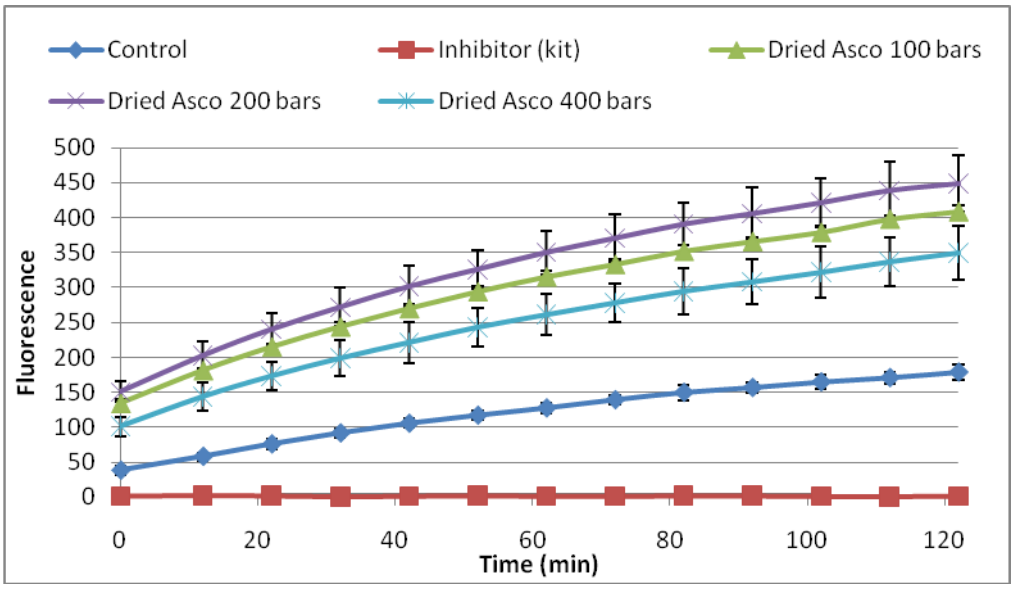


Figure 3.62 Elastase activity and SD of dried *A. nodosum* samples (n = 2). Elastase 0.1U/ml, inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and samples 0.0503 mg/ml, DQ-elastin 0.025 mg/ml.

Table 3.58 Elastase activity on SFE-extracts at different extractions pressures (n = 2)

Sample		Extraction (bars)	10 min		20 min		30 min		60 min		120 min	
			% Inhib.	SD	% Inhib.	SD	% Inhib.	SD	% Inhib.	SD	% Inhib.	SD
A. nodosum	Fresh	100	-47.6	29.8	-37.4	28.7	-33.6	24.0	-33.2	19.1	-31.8	18.4
		200	-66.9	6.1	-60.1	9.4	-51.4	7.6	-51.0	7.3	-47.7	6.9
		400	-67.1	2.9	-62.3	7.4	-57.8	5.0	-57.8	4.2	-53.9	5.8
	Dried	100	-211.5	6.7	-182.7	5.8	-165.8	6.9	-147.3	7.0	-128.8	4.6
		200	-248.1	35.3	-215.9	31.2	-196.2	30.2	-174.7	24.4	-151.0	22.6
		400	-147.5	35.1	-128.2	25.9	-116.9	27.3	-104.9	22.9	-95.6	21.1
	Dehydrated	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		200	-98.4	6.9	-83.2	8.1	-76.0	9.4	-66.7	8.6	-57.3	7.7
		400	-67.7	2.6	-55.4	3.4	-52.7	3.9	-46.0	3.1	-41.7	0.7
L. Hyperborea	Fresh	100	-55.2	0.5	-48.4	1.9	-47.5	1.6	-43.3	2.5	-38.8	2.3
		200	-18.5	20.4	-17.1	22.8	-20.5	21.1	-24.7	21.7	-22.5	21.5
		400	-29.8	34.1	-31.4	33.7	-29.0	31.3	-28.2	32.5	-26.0	30.4
	Dried	100	-77.1	16.1	-67.1	15.0	-59.4	10.6	-55.9	11.7	-49.8	7.5
		200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		400	-125.7	8.0	-110.8	8.5	-100.6	6.6	-90.2	6.8	-79.5	7.5
AqE	Fresh	100	-41.5	6.5	-37.1	1.2	-31.9	5.7	-26.4	1.9	-25.4	3.3
		200	-32.2	27.3	-25.5	25.0	-26.5	21.3	-23.0	18.9	-19.6	18.6
		400	-50.8	1.2	-42.8	1.0	-42.4	5.7	-38.9	5.2	-35.9	5.1
	Dried	100	-23.1	21.7	-24.2	20.0	-25.2	21.9	-27.9	22.1	-25.9	19.2
		200	-25.6	2.8	-25.5	7.8	-26.8	10.6	-29.6	7.8	-29.4	7.2
		400	-43.2	22.6	-36.9	14.1	-37.8	17.8	-41.4	16.4	-38.9	11.7
Fucoidan	Untreated		12.2	31.9	-1.3	33.8	-7.0	30.0	-14.9	27.5	-18.0	22.0
	Treated		-20.4	27.5	-30.4	28.1	-28.5	22.4	-33.3	19.4	-33.4	18.0

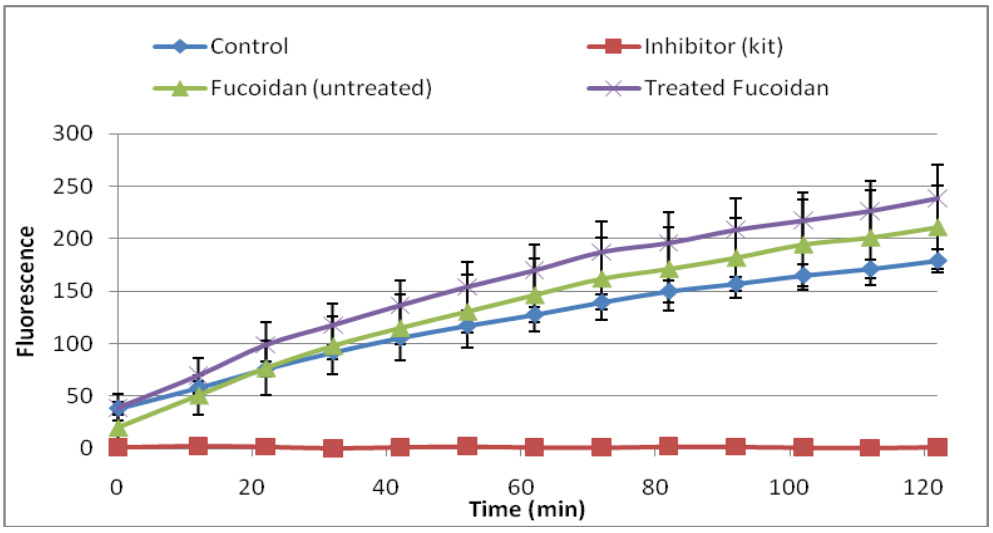


Figure 3.63 Elastase activity and SD of fucoidan samples (n = 2). Elastase 0.1U/ml, inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and samples 0.0503 mg/ml, DQ-elastin 0.025 mg/ml.

3.7.2.4.2 SFE-residual samples and non-SFE samples

SFE-residual samples and non-SFE samples were screened for elastase activity after 60 minutes of protein hydrolysis. Viscozyme carbohydrates mixture was also treated with the four proteases (for 60 min), and assayed with elastase. All the samples were assayed at a concentration of 50.3 µg/ml. Results for Viscozyme are presented in Table 3.59. Viscozyme mixture seemed to activate elastase to small extent (Figure 3.64). The highest activation value (14.29 ± 4.36 %) was found in alcalase (120 minutes). However, some inhibition was found with pepsin (10 minutes), which changed to activation 10 minutes later.

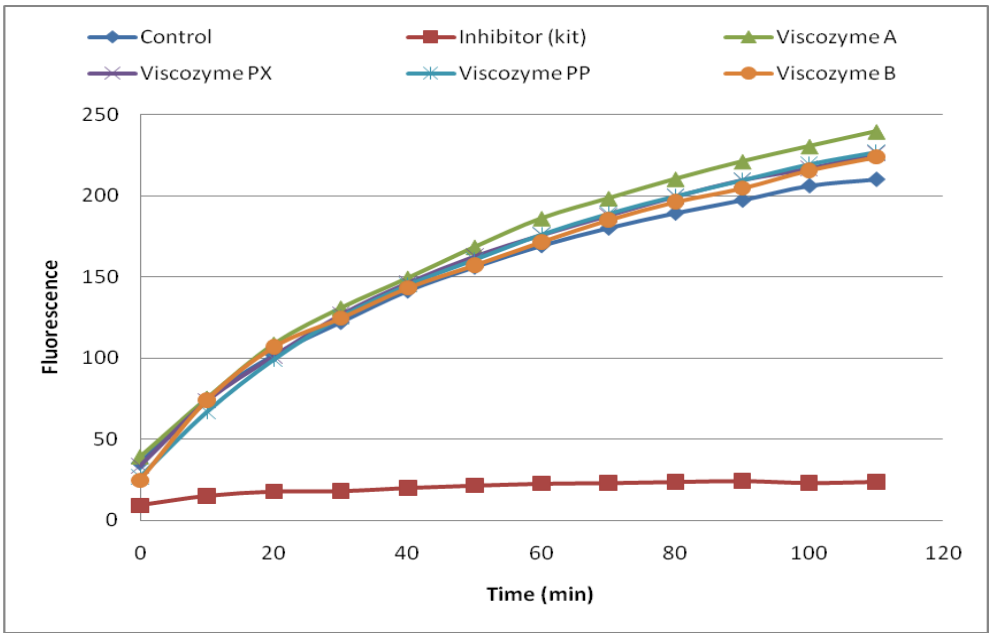


Figure 3.64 Elastase activity with viscozyme control (n = 2). Elastase 0.1U/ml, inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and samples 0.0503 mg/ml, DQ-elastine 0.025 mg/ml.

Table 3.59 Elastase activity on viscozyme carbohydrates mixture after protein hydrolysis (n = 2)

Viscozyme treated with	10 min		20 min		30 min		60 min		120 min	
	% Inhib.	SD	% Inhib.	SD	% Inhib.	SD	% Inhib.	SD	% Inhib.	SD
Alcalase (a)	-2.19	11.5	-8.80	9.2	-9.25	9.3	-11.53	6.9	-14.29	4.36
Protamex (px)	-5.03	29.8	-3.62	19.6	-8.03	19.7	-8.04	19.1	-10.77	16.11
Pepsine (pp)	5.16	20.6	-0.36	13.7	-4.05	7.9	-5.17	6.5	-8.23	4.30
Bromelain (b)	-1.75	11.0	-5.30	3.1	-3.03	4.6	-2.38	5.0	-7.01	3.43

SFE-residual samples and non-SFE samples were compared to the positive control (present in each batch) to calculate the percentage of inhibition. Results for SFE-residual samples are presented in Table 3.60. The highest elastase inhibition was found in fresh *A. nodosum* treated with bromelain (Figure 3.65). This sample, at a concentration of 50.3 µg/ml, presented an inhibition value of 47.9 ± 19.8% at 10 min which decreased to 18.7 ± 4.7% after 120 minutes. Dried *A. nodosum* treated with pepsine and bromelain, did also show some inhibition (15.9 ± 4.7 and 14.0 ± 2.6 %, 10 min) that decreased completely over time (0.5 ± 3.9 and 0.5 ± 3.1 % at 60 min). Dried *A. nodosum* treated with alcalse presented small initial inhibition (Table 3.60). *Laminaria hyperborea* presented elastase activation in both fresh and dried samples treated with the four proteases, at a concentration of 50.3 µg/ml. The highest activation was found in fresh Laminaria treated with alcalase (Figure 3.65), with 45-50% activation for the first 30 minutes, which decreased to 33% after 120 min. Fresh Laminaria treated with protamex, pepsin and bromelain presented very high standard deviation values, which were reduced over time.

The fresh form of the AqE showed a weak initial inhibition (alcalse, protamex and bromelain) which reached low activation values over the incubation time. Dried AqE samples activated elastase, with low initial values, that slightly increased over time. Dried AqE treated with pepsin, presented a final activation value of 20.7 ± 4.3 %.

Non-SFE samples results are shown in Table 3.61. The highest inhibition, as with SFE-residual samples (Table 3.60), was given by the fresh *A. nodosum* treated with bromelain (Figure 3.66). An initial inhibition of 29.0 ± 10.0 % (10 min) decreased slowly over time, disappearing 120 minutes later, which may indicate competitive inhibition. Fresh Laminaria treated with bromelain gave the second highest elastase inhibition: 16.3±0.3% which decreased over time. However, fresh *L. hyperborea* treated with alcalase gave elastase activation (Figure 3.66). Weak initial anti-elastase activity was also found in fresh AqE treated with pepsin and bromelain (Table 3.61). In general, the standard deviation values were quite high and very high in some cases. This matter will be discussed in section 4.

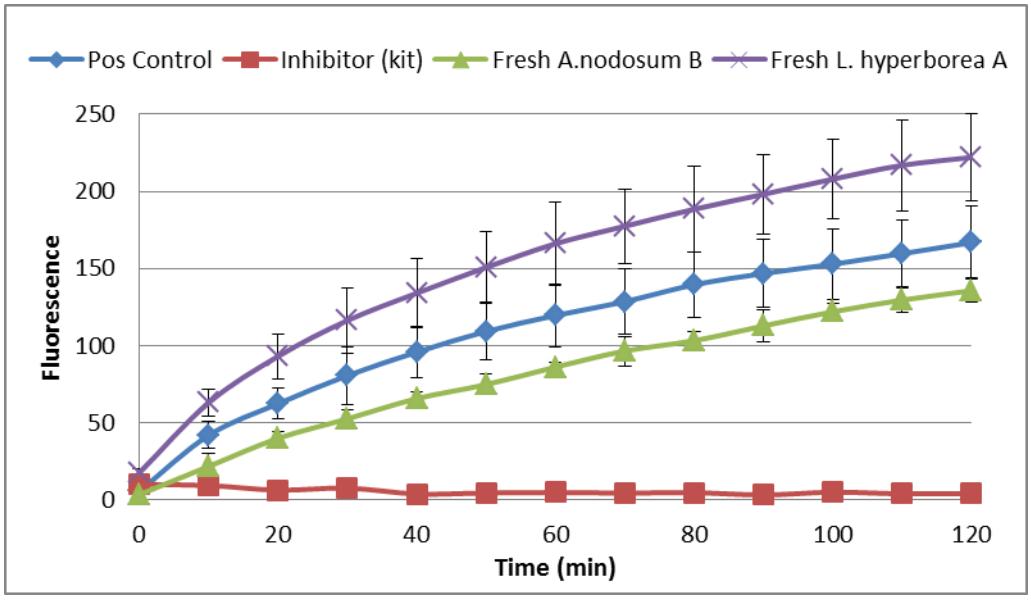


Figure 3.65 Elastase activity with SFE-residual samples: fresh *A. nodosum* bromelain and fresh *L. hyperborea* alcalase (n = 2). Elastase 0.1U/ml, inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and samples 0.0503 mg/ml, DQ-elastine 0.025 mg/ml.

Table 3.60 Elastase activity on SFE-residual samples after protein hydrolysis

Sample		Prot	10 min		20 min		30 min		60 min		120 min	
			% Inhib	SD	% Inhib	SD	% Inhib	SD	% Inhib	SD	% Inhib	SD
A. nodosum	Fresh	a	5.1	73.0	-8.6	38.0	-14.1	20.3	-19.6	18.5	-25.3	17.7
		px	-11.1	20.5	-5.0	27.6	-5.2	18.8	-1.1	16.9	-9.4	18.0
		pp	-1.3	25.9	7.3	23.5	8.6	21.9	8.3	20.2	1.1	19.7
		b	47.9	19.8	35.9	6.9	34.7	7.4	27.9	2.5	18.7	4.7
	Dried	a	10.4	11.2	3.8	4.8	3.8	5.5	1.2	3.9	0.4	3.3
		px	8.4	0.8	0.6	1.1	-0.5	1.6	-2.2	0.6	-6.4	1.7
		pp	15.9	4.7	5.7	4.2	6.8	0.7	0.5	3.9	-2.9	2.0
		b	14.0	2.6	5.3	3.0	5.0	1.7	0.5	3.1	-1.4	4.2
	Dehydrated	a	-5.3	30.5	-15.0	14.0	-13.6	15.2	-14.0	11.5	-15.8	11.3
		px	3.1	32.7	-5.8	30.4	-11.5	16.3	-17.3	2.1	-22.8	0.1
		pp	8.2	11.7	-2.5	12.6	-11.6	6.6	-19.2	2.3	-22.4	2.4
		b	-5.9	15.2	-15.6	7.6	-14.3	1.6	-18.3	1.3	-21.2	0.3
L. Hyperborea	Fresh	a	-50.1	21.1	-49.0	23.1	-44.2	26.3	-38.8	22.3	-33.1	16.8
		px	-37.4	113.4	-41.6	61.4	-38.4	45.8	-34.3	31.3	-31.6	23.4
		pp	-27.0	84.4	-31.3	71.3	-28.2	48.5	-34.9	27.2	-35.9	21.8
		b	-26.6	71.5	-21.6	61.8	-21.9	46.5	-30.5	15.8	-31.8	13.0
	Dried	a	-8.4	25.4	-8.2	20.3	-7.6	18.1	-5.6	20.5	-6.2	17.1
		px	-21.3	13.7	-20.2	8.3	-16.7	6.8	-15.9	6.0	-17.0	4.2
		pp	-41.5	13.4	-31.1	11.4	-25.5	13.2	-21.9	8.5	-22.1	7.5
		b	-39.4	22.7	-26.6	26.8	-21.4	21.4	-19.6	17.8	-21.7	14.0
AqE	Fresh	a	1.1	0.1	3.1	2.6	-2.0	2.9	-3.4	5.4	-5.7	5.6
		px	-14.3	19.4	-10.6	3.8	-8.6	10.0	-6.7	7.0	-11.0	6.9
		pp	5.4	6.9	-8.9	3.1	-9.2	1.2	-6.6	0.2	-12.3	1.6
		b	0.3	2.4	1.3	2.5	-2.2	2.3	0.6	2.3	-5.0	2.8
	Dried	a	-3.4	11.1	-6.9	0.8	-8.3	1.7	-9.3	2.1	-10.1	1.6
		px	-6.6	0.0	-8.2	2.6	-2.9	2.5	-5.3	0.3	-9.4	1.3
		pp	-2.3	8.3	-8.1	5.6	-13.0	1.4	-18.3	4.4	-20.7	4.3
		b	-10.7	19.0	-11.9	16.0	-12.9	18.6	-17.7	20.1	-18.5	14.9

Prot. – protease: a - alcalase, px – protamex, pp – pepsine, b - bromelain; n = 2.

Table 3.61 Elastase activity on non-SFE samples after carbohydrates digestion and protein hydrolysis

Sample		Prot	10 min		20 min		30 min		60 min		120 min	
			% Inhib	SD	% Inhib	SD	% Inhib	SD	% Inhib	SD	% Inhib	SD
A. nodosum	Fresh	a	20.1	11.3	13.5	5.4	6.0	4.0	1.4	2.3	-4.7	2.5
		px	9.7	1.1	4.6	5.9	-2.0	1.9	-4.3	2.6	-13.1	3.9
		pp	17.0	4.2	6.5	4.1	-0.5	4.2	-3.8	3.4	-10.7	3.6
		b	29.0	10.0	21.4	13.3	14.3	13.9	6.2	11.2	-1.8	12.5
L. Hyperborea	Fresh	a	-7.2	9.5	-5.6	7.5	-5.1	8.3	-2.8	7.2	-6.0	8.4
		px	0.2	4.9	0.1	2.1	-2.0	4.3	0.2	2.8	-2.6	1.5
		pp	3.5	0.9	3.2	3.6	-0.6	2.8	1.6	1.0	-0.1	0.0
		b	16.3	0.3	9.4	4.3	4.8	5.3	3.5	7.3	-3.9	7.8
AqE	Fresh	a	0.0	7.5	-6.4	7.8	-12.0	3.1	-10.3	3.2	-13.7	2.4
		px	-5.0	8.2	-7.7	13.2	-11.7	13.4	-5.6	13.8	-10.9	15.0
		pp	4.3	2.1	2.5	4.1	-3.9	8.8	-7.4	5.1	-15.0	2.4
		b	4.0	8.6	0.5	5.6	0.5	1.4	-6.8	3.6	-15.6	1.7

Prot. – protease: a - alcalase, px – protamex, pp – pepsine, b – bromelain; n = 2.

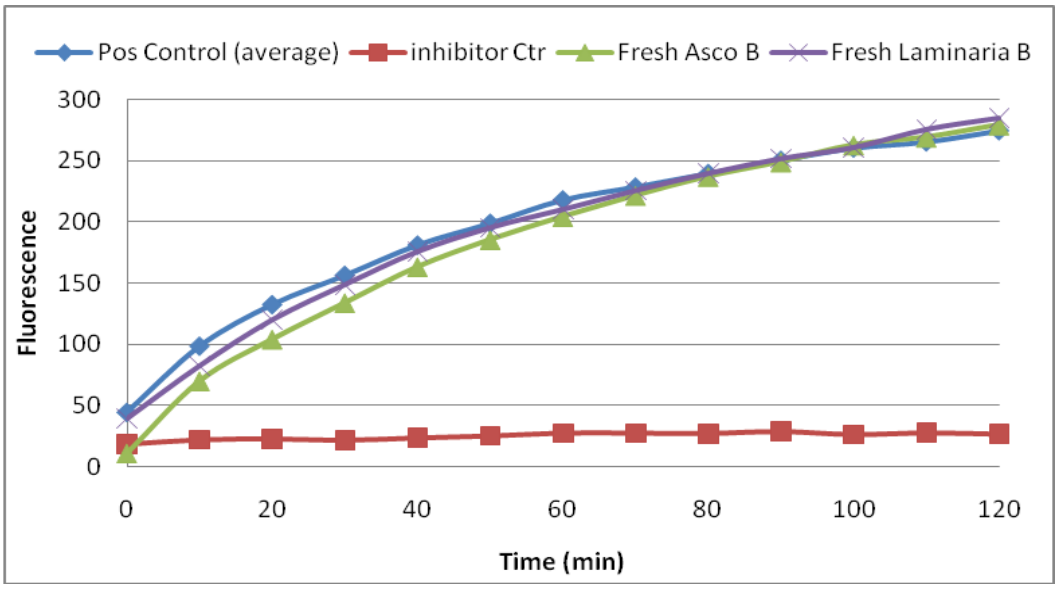


Figure 3.66 Elastase activity with non-SFE samples (n = 2). Elastase 0.1U/ml, inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and samples 0.0503 mg/ml, DQ-elastin 0.025 mg/ml.

4. DISCUSSION

Seaweeds have been used as fertilisers and soil improvers in Europe for centuries (McLaughlin *et al.*, 2006). The interest in using seaweed extracts in horticultural and agricultural crops has increased considerably in the past few decades (Leclerc *et al.*, 2006). The quality and yield of the crops are enhanced by using seaweeds which contribute many nutrients, minerals, trace metals and plant hormones, including cytokinins and substances mimicking cytokinin activity (Canales-López, 1999; Leclerc *et al.*, 2006). *Ascophyllum nodosum* is the most important commercial species of seaweed collected in Ireland for the past 40 years (Morrisey *et al.*, 2001). This brown seaweed is used in soil conditioning and crop spray applications in two forms: dried seaweed meal and/or liquid extracts (Morrisey *et al.*, 2001). Some reports have indicated enhanced plant yield, quality and health in different crops (Abetz and Young, 1983; Hanssen *et al.*, 1987; Norrie *et al.*, 2002; Colapietra and Alexander, 2006; Fan *et al.*, 2011) and fungal disease reduction in carrot crops (Jayaraj *et al.*, 2008) following *A. nodosum* liquid extract application. While studies describe the agricultural applications of the A. *nodosum* extract, little, if any, information is available in the literature concerning the residual waste material.

As explained in Section 1.8, the seaweed residue is currently removed for land-spreading, which is a finite solution because it requires local authority permission and its removal constitutes a production cost that reduces competitiveness. Fifteen hundred kilograms of seaweed residue require remediation for each batch of seaweed extract produced.

4.1 *Ascophyllum nodosum* meal, extract and residue

In the industrial process which features in this work, dry *Ascophyllum nodosum* meal is used. The extraction process is detailed in Figure 1.15, which shows how liquid fertilizer is obtained and the residue remains after the process. An analytical study of these three components, *Ascophyllum nosodum* meal, fertiliser extract and residue from the same batch, was carried out. It was found that the meal presented a very similar composition to the one described for *A. nodosum* by the literature (Figure 3.11; Table 3.6).

When looking at the fertiliser extract and residue compositional results, it was found that fibre and ash were the main components in both the fertiliser extract (45% ash; 46% fibre) and the residue (37% ash; 38% fibre), so it would appear that both are equally distributed during the extraction process. The high percentage of fibre in the residue

presents a difficulty as fibre is more recalcitrant to digestion than other carbohydrate substances. The mineral content in the residue and the fertilizer extract is higher than in the meal and *A. nodosum* plant due, to a large extent, to the addition of potassium carbonate for the extraction process.

There is a significant difference in the lipid content with 18.1% lipid in the residue compared with 3.53% in the fertiliser extract and 1.79% in the original seaweed meal. This indicates that following production lipid was largely retained by filtration on collection of extract, either because it was located on top of the aqueous digest as filtration begins or because it was linked with residue components. It would appear that the majority of the lipid portion of the seaweed has accumulated or remained in the residue. The aqueous nature of the industrial extraction process is the most likely influencing factor in this case. For analysis of crude lipid the method used was Soxhlet extraction. While care was taken, these samples proved difficult to ensure complete extraction of total lipid and other methods may have been more effective. It has been shown that supercritical carbon dioxide extraction results in a greater total lipid content than the Soxhlet method in the brown seaweed *Sargassum hemiphyllum* (Cheung *et al.*, 1998). A similar study in wheat flour also showed that more glycolipid was extracted by supercritical fluid extraction (with carbon dioxide) than by Soxhlet (Hubbard *et al.*, 2004). The fertilizer extraction process degrades the solid material in the seaweed, perhaps allowing better extraction of lipid by Soxhlet method. The further analysis of lipid is discussed in Section 4.2.

There is also a higher protein value left in the residue (5.70%) than in the extract (4.59%) which may represent a source of proteinaceous material, amino acids or peptides of horticultural or other value. A method for the release of this material could be studied in-depth to get a higher value in the fertilizer product, thus improving its quality. It seems this protein could be bound to some other structure in the extraction process, possibly the fibre, and for this reason a higher percentage of protein remains in the residue. One option for the industrial process would be to investigate a re-extraction or modification to the procedure in order to enhance the proteinaceous or nitrogen content in the fertiliser as other comparable products do contain higher nitrogen levels. However the net value increase would have to justify the effort. It was difficult to get information on the extract composition of these other products.

When comparing the polyphenol concentration a higher value was found in the fertilizer product than in the residue (39.07 *vs.* 4.02 mg Gallic acid equivalent/g. dry sample).

This is an almost ten-fold difference. Conversely, a lower concentration was found in the meal (2.26 mg Gallic acid equivalent/g. dry sample). While the polyphenols were not fully extracted, this data shows that were released during the industrial extraction process and subjected to concentration, most appearing in the eventual product, with some residual polyphenols trapped on filter.

The original pH of the meal was 5.25, with pH 8.78 and 9.06 for the fertilizer extract and the residue respectively. The addition of potassium carbonate prior to the extraction process is responsible for this pH change.

4.2 *Ascophyllum nodosum* residue

The composition of the brown seaweed *Ascophyllum nosodum* varies with factors such as the location in which it is found; the water temperature at the time of collection; the salinity; and the season (Haug and Larsen, 1958; Ragan and Jensen, 1978). It has been reported that the moisture content of wet seaweed is 67-82%. Of the 18-33% dry matter, 18-24 % is ash, 4.8-9.8% protein, 1.2-4.8% lipid and 42-64% sugars (Table 3.6). *Ascophyllum nodosum* residue was analysed monthly through a full calendar year to investigate the variability of its composition. While the composition was found to be variable (Section 3.1.6), no conclusions can be drawn in relation to seasonal effects. This is because *A. nodosum* seaweed is collected over several months, most commonly from June to October. It is then dried and pooled for future sale (Kelly *et al.*, 2001). The variations in the composition could be due in part to the natural origin of the residue and the variability associated with the determination of the end point of the industrial extraction process. The end point is currently determined by sight, rather than by any specific parameters.

The moisture content of the residue (84.00%) was regarded as high by the manufacturers in relation to further processing option. This compares with the fresh seaweed at 67-82% (Table 3.6). Removal of water in the residue would be expensive. This poses a problem for future use or transport of the residue. The 16% of total solids compromises 40.66% minerals, 24.80% lipids, 4.9% protein and 29.52% fibre. The residue presents a much higher concentration of minerals than *A. nodosum* (40.66% *vs.* 18-24%; Table 3.6), a much higher lipid content (24.80% *vs*. 1.2-4.8%), similar percentage of protein (4.9% *vs*. 4.8-9.8%) and lower content of fibre (29.52% *vs*. 42-62%). Of all the components, the lipid content showed the most variation (average ± 28.6%) in the residue during the year long study, followed by fibre (± 25.8%) and

protein (± 24.5%), while the mineral content only varied ± 6.6% (based on standard deviation).

The high percentage of water in the residue is problematic from an industrial perspective, especially for transport action after the extraction process. There are a number of options which may improve this, which were outside the remit of this study. These include (1) pressing the residue sample at the last step of the extraction process, obtaining the maximum volume of the fertilizer product and reducing the subsequent cost in transporting the residue, or (2) drying the residue, which would be a very expensive and impractical option. Other possible applications include examining the potential of the residue as a compost ingredient or fertilizer. There have also been some studies in relation to the use of seaweed homogenates as a sorbent in remediation of land or sediment polluted with heavy metals (Murphy *et al.*, 2009). A potential limiting factor to the residue in some applications might be its high content of potassium carbonate.

The pH of the *A. nodosum* residue is alkaline, varying between 8.06 and 9.51 during the year of study. This high pH is due to the addition of 50 kg of potassium carbonate per 750 kg of dry *A. nodosum* meal during the extraction process. Kerner *et al*. (1991), reported pH values between 9 and 10 for two waste sludges arising from an alginate extraction process where the seaweeds used were *Laminaria hyperborea* and a mixture of *L. hyperborea-A. nodosum*. They describe a series of acid and alkaline treatments which were carried out during the extraction process. These treatments are explained in more detail by Carpinter *et al*. (1988). The present alkalinity of the residue was of some concern to this project because microbial digestion usually occurs more rapidly and efficiently at neutral pH. The use of hydrochloric acid, acetic acid, moss peat or horse manure were not effective additives for reducing the pH of the residue. A novel process, based on observations was, therefore, developed using anaerobic incubation to reduce the pH. This formed part of the digestion process (Section 3.3.4).

The polyphenol concentration in *A. nodosum* residue was quite variable during the 12 months of study: from 2.18 to 8.31 mg Gallic acid eq/g dry matter, with an average of 4.87 ± 1.95, which is equivalent to 0.48%. It has been reported that dried *A. nodosum* contains up to 14% of polyphenols (Ragan and Jensen, 1978). They found that the levels showed a minimum concentration in April to May and a maximum during the winter months. This polyphenol content also varies significantly with locality, salinity, light and nutrient concentration (Haug and Larsen, 1958; Connan *et al.*, 2007). Connan

et al. (2007) reported that *A. nodosum* presented a significantly higher polyphenol level than *Bifurcaria bifurcata* or *Pelvetica canaliculata*; and that *A. nosodum* showed greater marked daily variations over the study period (32 hours). The polyphenol content may be a limiting factor in the complete digestion of the seaweed residue. It was noted that during prolonged storage of residue in the presence of what was subsequently described as inoculum C, it was possible for the residue to be digested to near complete liquid (62% IM removal). The duration of this process (28 to 67 days) was not realistic, however, from a commercial perspective. Inoculum C contained a fungus which was isolated and identified as *Aspergillus versicolor* (Section 3.6.4). Scalbert (1991) concluded that filamentous fungi were able to grow in the presence of a higher concentration of phenols than were bacteria. A minimal inhibitory concentration (MIC) higher than 0.5g/l was demonstrated for fungi, while bacteria typically had a MIC of 0.012-1 g/L.

The GC analysis in this study revealed the presence of fatty acids C-20:4, C-20:5 and C-18:3 (Table 3.11). These have been reported to be abundant in brown seaweeds (Jones and Harwood, 1992). Fatty acids in seaweeds generally have an even number of carbon atoms (Sánchez-Machado *et al.*, 2004). However in this work, several fatty acids with an odd number of carbon atoms were also found: C-11:0, C-13:0, C-15:0 and C-17:1 (Table 3.11); while this is unusual, the last two, C-15:0 and C-17:1, have previously been found in *Porphyra* sps, *Undaria pinnatifia*, *Laminaria* sps, *Ulva lactuca* and *Durvillaea antarctica* seaweeds (Ortiz *et al.*, 2006; Dawczynski, 2007). Future work might characterise the fatty acids C-20:4 and C-18:3, check the total concentration of the omega-6 and omega-3 fatty acids in the residue. However it was considered, that the extraction of particular fatty acids of interest would have been better done with fresh meal or seaweed.

The mineral analysis of the residue shows that the Potassium is the most abundant mineral of minerals measured (49.16% of total), due to the addition of potassium carbonate prior to the extraction process as mentioned above. Sodium (17.88% of total), Calcium (16.77% of total) and Magnesium (13.95% of total) are also present in significant amounts. Other studies have reported between 2-3% of Potassium, 3-4% of Sodium, 1-3% of Calcium and 0.5-0.9% of Magnesium in *Ascophyllum nodosum* seaweed (Jensen, 1960; Morrisey *et al.*, 2001). Other minerals, Iron, Phosphorous, Manganese, Zinc, Copper and Cadmium are found in trace amounts in the residue.

4.3 Factors affecting the microbial digestion of *A. nodosum* residue

The microbial digestion system for *Ascophyllum nodosum* residue was developed and optimised through a series of experiments, as explained before in Section 3.3. In the first digestion trials, INIT 1 and 2, an attempt was made to exploit the microbial populations found on the seaweed residue, and centrifugation was shown to be more efficient than filtration to separate the insoluble and soluble material.

Further development included the study of the following digestion variables: incubation type, quantity of water added, duration of incubation, introduction of an inoculum, initial insoluble matter, initial soluble matter and the method of aeration. Added water was varied from a residue: water ratio of 1.2:1 to 1:8. A 1.2:1 ratio of added water gave rise to minimally agitatable consistency. Whereas 1:8 added water was the highest proportion of added water, the industrial partner considered feasible for large scale purposes. Care was taken to avoid excessive water as the removal of water from any digested product would be expensive on an industrial scale. Optimum residue: water ratio was 1:8 as the consistency of the resulting liquid allowed sufficient aeration during incubation. Decanting and separation of solubilised matter was also made easier by the greater volume of water. The duration of incubation was varied from 6 to 16 days, with a final duration of 13 days for most digestions (batch digestion system). This limited duration was indicated by the industrial partner so as to avoid accumulation of batch digestions in a rather restricted commercial area. Inocula A, B and C were studied and it became apparent that each exhibited varying suitability to the task in question. A study of the influence of residues with different initial insoluble matter (84% - 43%) and soluble matter (6%-64%) was undertaken.

As a result of this development an optimised digestion system and several recommendations are suggested for this and similar residues. In the following sections a discussion of the factors that are affecting the microbial digestion of *A. nodosum* residue is undertaken.

4.3.1 Inocula

The nature of the industrial process, which used high pressure (3-5 bar) and temperature (85-95^{o}C), rendered the residue sterile until it emerged from production. In practice the residue was then stored under non-sterile conditions, which allowed airborne and other contaminants to recolonize it. This is in effect a natural selection process as the only organisms which are capable of growth on the residue must be tolerant to the high pH.

A preliminary study of naturally occurring microorganisms on the residue showed a predominance of Gram negative, facultative anaerobes (Sections 3.6.1 and 3.6.5), whose metabolism may have given rise to the observed anaerobic reduction in pH. However, the same microorganisms did not give rise to useful digestion of the residue, with an average reduction of insoluble matter of 11.24% (INIT-1, Table 3.14) or 12.30% (NON-INOCB, Table 3.20). The addition of an inoculum at the beginning of the aerobic phase was considered essential for further progress.

The microorganisms in inoculum A (isolated from decomposing seaweed) seemed to feed in preference on existing soluble matter, reducing it from 63.68% to 46.80% (w/w), with no apparent breakdown of insoluble matter (initial 43.31%; final 46.43%) after a 12-day incubation (INOC-A, Table 3.16). Further investigation showed a lower number of microorganisms present in inoculum A ($7.9*10^6$; Section 3.6.2) in comparison to inoculum B ($2.05*10^9$; Section 3.6.3.1).

In contrast, the use of inoculum B resulted in a marked increase in digestion of insoluble material. In otherwise identical digestions, addition of inoculum B resulted in a decrease of insoluble material of 29.1% (INOCB-3) compared to 12.3% (NON-INOCB) without inoculum (Table 3.20). Inoculum B appeared to have a greater capacity to digest the insoluble matter of the residue, which decreased by 30.3% in trial INOCB-1 (Table 3.18). A preliminary study of this inoculum showed at least four different types of bacteria (Section 3.6.3.4).

The use of inoculum C (Table 3.21, trials INOCC 2 and 3) resulted in the greatest decrease in insoluble matter (62%) with a low ratio of residue to water (1:2), but over an unacceptably long period of aerobic incubation (a minimum of 28 days) from a commercial point of view. A molecular identification of the mould component of this inoculum confirmed it to be a strain of *Aspergillus versicolor*. While the duration of this digestion was considered too long by industrial partners, further research on *A. versicolor* and inoculum C may yield information useful from an industrial perspective, as this species tolerates a relatively low water content, and the alkaline pH of the residue. It could also be less influenced by phenol content, as previously mentioned (Section 4.2).

Inocula B and C were combined together in trials BATCH-B+C 1 and 2 (Section 3.4.2.3). However, no further improvement in digestion yield was found. Both trials presented low initial IM (56%) and high initial SM (41%), a factor discussed in Sections 3.4.3.2 and 4.3.4.

4.3.2 Reduction of residue pH

One of the early issues considered in the development of the digestion process was the need to reduce the initial alkaline pH (8.61±0.39) to facilitate bacterial growth. Aerobic incubation did not promote reduction in pH (pH-AEROB, Figure 3.15). Attempts were made to reduce the pH by addition of HCl (pH-HCL), acetate buffer (pH-ACETBUF) and natural additives like moss peat (pH-MOSSP) and horse manure (pH-HORMA; Section 3.3.3.1). However, it was noted that the pH would gradually return to the original value (8-9) as if buffered, when the residue was allowed to stand (pH-HCL and pH ACETBUF, Figure 3.15) and it was not possible to achieve appreciable degradation of insoluble matter at high pH when using the strategies above. While moss peat did have a neutralising effect (Figure 3.16), the quantity of material required was impractical (20 g of moss peat per 25 g of residue) for use in the digestion process. It was concluded that a significant buffering effect existed in the *A. nodosum* residue, which made the reduction of the initial pH by the methods outlined, very difficult or impractical. In other studies of brown seaweed degradation a continuous addition of HCl or NaOH was employed to maintain a neutral pH (Moen *et al*., 1997b; Hanssen *et al*., 1987). These studies only employed seaweed plants as their raw material for digestion, which naturally presents a lower pH value (6.6; Hanssen *et al*., 1987) than *A. nodosum* residue. A continuous addition of HCl was not considered viable for a large scale application in this project.

With further experimentation the pH was observed to decrease when the digest was incubated under anaerobic conditions (Trials ANAEROB; Figure 3.17). In ANAEROB trials 2 and 3, the pH decreased by an average of 1.95 ± 0.2 within three days. Similar pH decreases for anaerobic digestion were found in the two alkaline sludges from the alginate extraction process previously mentioned (Kerner et al., 1991), where an initial pH of 9-10 decreased sharply to around pH 6.0-6.5 during the first five days. It was found that the pH decreased less (to pH 6.6) in the sludge which contained *L. hyperborea-A. nodosum*, when compared with the *L. hyperborea* sludge (to pH 6.0). In this study, we observed pH decreases to no lower than 7.01 even after 8 days in digestions conducted entirely under anaerobic conditions (ANAEROB trials, Section 3.3.3.2).

As digestion of the residue appeared to be more efficient under aerobic conditions, an initial 3-day anaerobic step to reduce the pH, followed by an aerobic digestion phase was conceived in the batch digestion system (Section 3.4, Figure 3.19). An inoculum

was subsequently added at the beginning of the aerobic phase to support digestion. This basic mixed anaerobic/aerobic incubation resulted in a pH decrease over the 3 first days (Figures 3.18, 3.20 and 3.21), and a subsequent decrease in insoluble matter (Figure 3.22), the efficiency of which was dependent on several other variables. A gradual increase in pH (Figure 3.20) as the aerobic phase progressed was observed, perhaps reflecting the release of complexed potassium carbonate from digested polysaccharide and ultimately preventing complete digestion of insoluble matter due to the elevated pH. The original pH of the residue seemed to prevent effective digestion and did not correlate (R^2=0.11) to digestion products (Figure 3.27). There appeared to be a better correlation (R^2=0.528) between pH after the anaerobic phase and the eventual yield of digested material (Figure 3.28), suggesting the importance of more neutral pH in the initiation of aerobic digestion. It appeared that the lower the pH at day 3, the greater the extent of the subsequent aerobic digestion. The precise mechanism responsible for the pH decrease during the anaerobic phase is not clear. However it is hypothesized that production of certain organic acids (Section 4.5), and liberation of previously complexed fatty acids during the anaerobic phase may have contributed. The gradual reduction in fatty acid content during digestion (Table 3.35) may account in part for the gradual increase in pH during the aerobic phase.

A trial with alternating 3 day anaerobic and 3 day aerobic phases, for a total of 12 days (BATCH-pH, Section 3.4.2.1) resulted in a pH decrease during the anaerobic periods (days 0-3 and 6-9) which tended to increase over the aerobic periods (days 3-6 and 9-12) as shown in Figure 3.24. There was an average decrease of insoluble matter of 30.87% (BATCH-pH), compared to 36.79% (BATCH-CONTROL) with one initial anaerobic phase (Table 3.28). While a repeating pH-reducing effect of the anaerobic digestion seemed desirable, retention of the 10-day aerobic conditions following one 3-day anaerobic phase appeared to be as effective, and technically more convenient.

4.3.3 Potential metabolite inhibition

Residue samples consisted of 24-63% soluble material. The presence of such readily available soluble substrate may inhibit the production of hydrolytic enzymes (Horn and Ostgaard, 2001). As microbial digestion aims to create nutrients, it was considered probable that microbial digestion of insoluble solids would decline when soluble substrate was readily available. The presence of a significant amount of lipid (Table 3.6) presented a convenient substrate for microorganisms. Furthermore long chain fatty acids

may inhibit anaerobic digestion of organic solid (Salminen and Rintala, 2002), but the removal of lipid in advance of microbial digestion was considered to be too expensive for large scale application. Similarly, polyphenols present in the residue are potential inhibitors of brown seaweed degradation. Moen *et al.* (1997b), who studied the biological degradation of *A. nodosum*, found that polyphenols were a limiting factor for conversion of organic matter during anaerobic digestion (alginate lyase activity and methane production were inhibited), while this was less influenced under aerobic conditions. Other work from the same authors showed that the aerobic degradation of organic matter in *Laminaria hyperborea* was depressed when the amount of polyphenols was increased (Moen *et al*., 1997c). In these studies, the addition of formaldehyde has been shown to decrease the polyphenol concentration. However, this option was not tested, as it would be expensive in large scale and potentially valuable digestion products could be affected.

To investigate the influence of metabolite inhibition during the digestion process, two trials were run under the same conditions where one trial (NONDECANT) was a 3-day anaerobic incubation followed by 10 days aerobic digestion, while in the other, 200mL (50% of initial volume) soluble matter was decanted at day 3 and replaced with water following the anaerobic incubation (Trial DECANT, Section 3.3.4). Decanting of soluble matter at day 3 (DECANT) resulted in a greater decrease of the insoluble matter (36.82%) compared to NONDECANT (13.44%; Table 3.23) in 13 days. In general, decanting soluble material resulted in a greater digestion of *A. nodosum* residue.

Incorporation of three decanting steps (one every 3 days; BATCH-DECANT, Section 3.4.2.1) in the batch digestion system resulted in an average 42.48% decrease in insoluble material in the final analysis (Table 3.28). The improved rate of digestion with regular decanting also appeared more independent of initial insoluble matter content, a factor discussed in the next section.

The water content was varied during the development of the digestion (Section 3.3). First trial (INIT-1) had a solid-to-water ratio of 1.2:1 (55 g residue and 45 ml water). For several reasons (pH, inoculum, etc.) this digestion trial was not effective (11.24% IM removal, Table 3.14). Thereafter water content was increased to facilitate adequate aeration by agitation. Addition of water to solid ratio of 1:4.3 (35 g reissue and 150 ml water) did not lead to an improvement in IM digestion (INIT-2, 2.5% IM decrease, Table 3.28). Subsequent digestions, from ANAEROB onwards, employed a standard ratio of 1:8 which allowed effective aeration by agitation and was agreed as feasible by

the industrial partner. Following study of other digestion parameters (pH, inocula, etc.), the ratio of solid to water was reconsidered. Trials BATCH-WATER and WATER 1 and 2 (Section 3.4.2.2) investigated lower volumes of added water (100 ml per 50 g of residue). This change in water content was not considered advantageous as it resulted in low IM digestion possibly due to early metabolite inhibition.

4.3.4 Influence of residue content

For several reasons (natural origin of the residue and determination by sight of the end-point of the industrial extraction process), there was considerable variation in residue content. Extremes of insoluble and soluble matter were observed to cause digestions to accelerate or fail respectively. For example, a residue with an initial 83.5% insoluble content showed a rapid digestion to 41.6% insoluble material (BATCH-2, Table 3.26; a decrease of 50.1%) within 13 days, whereas a residue with 61.7% insoluble content exhibited a reduction to 46.7% insoluble material (BATCH-6, Table 3.26; a decrease of 24.3%) under identical conditions.

To more clearly determine the influence of initial residue composition on digestion, residues of different contents were trialed under the batch digestion system in Section 3.4. During the aerobic phase insoluble matter decreased at a rate in proportion to the initial insoluble matter content and indirectly proportional to the soluble content, but each reached similar final values of insoluble matter (47.82 ± 4.41%) after 10 days of aerobic incubation (Table 3.33). Two correlations were found which explain these relationships. The first one correlates the initial amount of IM with the IM digestion (R^2 = 0.77, Figure 3.29). Residue samples with 67% or higher initial IM exhibited an IM decrease of 35% or higher. However, 65% or lower initial IM showed a 25% or lower IM decrease. In contrast, there was an inverse correlation between final insoluble content and initial soluble content (R^2 = 0.67; Figure 3.30). Residue samples with high initial IM (≥65%) might be preferred for future digestions. On a larger scale, it may be desirable to mix different residues to avoid having to digest residues with low insoluble content. It is also essential that, during production of extract, filter cakes are compressed to ensure complete extraction of soluble material, simultaneously enriching the extracts and facilitating digestion of the remaining insoluble material.

In the absence of readily available, soluble substrate, such as when soluble material was removed by efficient filtration or decanting, it should be possible to establish a high rate of microbial digestion. In trials BATCH-WASH 1 and 2 (Section 3.4.2.4), where the

residue was washed prior to digestion (to decrease the initial amount of SM), the new level of SM increased during the digestion by 339% and 432%, respectively, however the IM did not show the expected corresponding decrease, probably due to some biomass production.

Finally, digestions appeared to be self-limiting at a final insoluble matter of approximately 47-48% with inoculum B in spite of extended incubation. This may be due to the rise in pH evident in all digestions (Figure 3.20), as INOCC trials (Table 3.21) with an apparently alkaline tolerant *A. versicolor* reduced insoluble matter content by 62%, albeit after 28 days of digestion. Also the polyphenol concentration increased towards the end of digestion (Figure 3.32), probably limiting bacterial populations.

4.4 Optimised digestion system

The data obtained determined that certain factors influenced the microbial digestion of an alkaline seaweed residue and suggested an optimised digestion model consisting of an initial 3-day anaerobic phase to reduce pH. At day 3, 200 mL of solubilised material was decanted and replaced with water to delay metabolite inhibition and an inoculum was added. There followed a 10-day aerobic incubation for efficient digestion of insoluble material, during which a systematic decanting regime of solubilised material might be implemented to conserve optimal conditions for digestion. A period of 3 days was evaluated for the systematic SM decantation (BATCH-DECANT); however, further study of a semi-batch or a continuous decantation system might lead to better results.

4.5 Compositional analysis after the digestion

An analysis of the dried insoluble material after 13 days of digestion (batch model) presented 33.51% ± 3.07 of ash, 18.41% ± 0.87 of protein, 2.05% ± 1.39 of lipid and 46.03% ± 1.80 of fibre (Figure 3.34). When comparing these percentages with the initial ones (Table 3.35), the most significant changes were found in the lipid and protein contents (Figure 3.31). In 13 days, the lipid content decreased by 96% suggesting microbial utilisation of the lipid present apparently in preference to polysaccharide, resulting in slow initial digestion of the insoluble content. This also indicates lipase activity is present during the digestion. The extraction of lipases has been proved to be commercially valuable in a wide array of industrial applications: food technology,

detergent, chemical industry, bioremediation, biomedical sciences and cosmetic, textile and paper industries (Houde *et al.*, 2004; Babu *et al.*, 2008). Further investigation in the lipase activity present in the *A. nodosum* residue microbial digestion was attempted following the methodology employed by Lee *et al*., (2003). However, the dark brownish colour of the soluble material was found to interfere with the absorbance measurement (at 405 nm) of the lipase activity assay. Because of this and other reasons (economic) this avenue of the research was not pursued.

Conversely, the protein content increased by 82.36% during the digestion (Figure 3.31), probably due to microbial growth and enzyme production, or retention of the original proteinaceous material in the insoluble fraction. The ash content decreased by 60.0% (Table 3.35). It is likely that ash minerals were transferred to the solubilised material during digestion and lost by decantation. The fibre remained high after the digestion, decreasing only by 24.26% (Table 3.35). This could suggest that the bacteria present did not have the capacity to digest this material, but also that the fibre content may have been added to by the microbial biomass. It is possible that bacterial biomass contributed to both soluble and insoluble content.

The polyphenol concentration was also studied during the batch digestion model. An increase occurred during the 3-day anaerobic phase (0.12 g/L to 0.18 g/L) and decrease during the aerobic phase (to 0.09 g/L at day 10), increasing again during the last 3 days (Figure 3.32). Similar results were published by Moen *et al*., (1997b), who found that the polyphenol concentration increased when digestion proceeded under anaerobic incubation, and conversely it decreased when incubated aerobically.

Organic acid analysis by HPLC showed the presence of lactic, acetic, oxalic and formic acids among others. There was some accumulation of formic (BATCH and BATCH-WASH) and lactic (BATCH, BATCH-B+B and BATCH-WASH) acids during the anaerobic phase, while oxalic was consumed (Figures 3.35 to 3.38). Formic acid was fully consumed during the aerobic phase of BATCH trial, while a concentration lower than 5 mM remained until last day of digestion in trials BATCH-B+C and BATCH-WASH (Figure 3.36), where inoculum C took part. This suggests that *Aspergillus versicolor* or other bacterial strains from inocolum C could be involved with formic acid production. Others, like lactic acid, exhibited some accumulation during the aerobic phase, being fully digested after the digestion process (Figure 3.37). The presence of organic acids in *A. nodosum* residue may be related to the bacterial strains found

growing on the residue. Information on organic acids composition in seaweed was not found in the literature.

4.6 Microbial studies of the residue and the inocula

The microbial studies carried out on this research complemented the determination of *A. nodosum* residue proximal analysis, and its microbial digestion. The objective was to isolate and identify some of the microorganisms involved in the digestion process.

4.6.1 Residue

The preliminary microbial studies carried out in *A. nodosum* residue showed a succession of different microorganisms during 7-day plate incubation, which made it difficult to obtain an accurate colony count. A remarkable 5 to 6-fold difference was found in the total viable numbers when correction for dilution was made, based on colony counts in 10^{-3} and 10^{-4} dilutions ($5.03*10^6$ and $9.17*10^5$ respectively; Table 3.37). This could be due to aggregates of bacteria or to an unusual co-dependent relationship between the microorganisms present in the seaweed residue.

The preliminary studies of these microorganisms showed a predominance of gram negative facultative anaerobes (Table 3.38). Most of which would have come from the surrounding environment because the high temperatures (85-95ºC) and pressure (3-5 bar) employed during the extraction process would ensure sterility of the residue. These microorganisms can tolerate the alkaline pH of the residue, and proliferate as they are involved in decreasing the pH during the 3-day anaerobic phase. This also caused partial digestion of the insoluble material. These results correlated with pH changes presented in trial BATCH-pH, where 3-day anaerobic and 3-day aerobic growth conditions were alternated over 12 days. The pH decreased during the anaerobic periods and increased during the aerobic ones (Figure 3.24).

4.6.2 Inoculum A

The microbial characterization of inoculum A showed a relatively low concentration of microorganisms (of the order of 10^6; Table 3.39) in comparison to inoculum B (of order of 10^9). A succession of microbial dominances during 7-day plate incubation (Section 3.6.2) was found, which made obtaining a reliable colony count difficult. Inoculum A was obtained from seaweed left to decompose over a 4 month period (Section 2.3.3.1).

Using an inoculum gathered in this way ensured that it contained organisms capable of degrading the seaweed. There are several difficulties with an inoculum prepared in this way – firstly, in a batch type of fermentation/digestion, bacteria go through several phases of growth – lag, log, stationary, death or decline and in many cases dormant phase. It is likely that many of the bacteria present were in the later stages of growth rather than actively growing cultures. Secondly, the succession of bacterial colony types seen on plate counts shows that the dominant cultures change over time and the organism present after 4 months of degradation, may not be the ones required to initiate digestion. Finally, the inoculum was stored at -80oC, thawed and added directly to the residue without any additional cultivation.

Inoculum A was used in trial INOC-A (Table 3.17), which had a high initial level of SM (63.6%). During this trial the SM decreased to 46.8%, while the IM increased (from 43.3% at day 0 to 46.4% at day 12). This inoculum would appear to preferentially use the SM rather than IM.

4.6.3 Inoculum B

The studies of inoculum B revealed a higher CFU/ml than inoculum A (of the order of 10^9; Table 3.40). Inoculum B achieved better results for *A. nodosum* residue digestion than Inoculum A (Section 4.3.2). Digestion trials 5.1, 8.3 and the batch digestion model are examples (Tables 3.28 and 3.36). Although inoculum B was shown to digest the insoluble matter of the residue, its growth was influenced by factors such as pH, initial insoluble/soluble material and metabolite inhibition (Section 4.3).

Several previous digestions of brown seaweeds used an incubation temperature of 35^{o}C (Hanssen *et al.*, 1987; Kerner *et al.*, 1991; Moen *et al.*, 1997b). For this reason, it was decided to study the effect, if any, of this higher temperature on inoculum B. It was found that microorganisms in inoculum B grew slightly faster at 35^{o}C than at 30^{o}C (Figure 3.44). However, no significant differences (T-test) were found between both temperatures in the intervals studied (0-8, 8-16 and 16-24 hours; Table 3.41). A temperature of 30ºC was considered more appropriate for the digestion system, because a lower energy requirement would make the digestion more economical as an industrial process.

A preliminary study of the isolates from inoculum B showed the presence of at least four different types of microorganisms (Table 3.42). Strain B1 was Gram positive,

strain A2 was Gram stain variable, with all others Gram negative. All of these isolates grew in Q&C media with seawater, which indicates they tolerated the salt content.

The study of these microorganisms with the API 20E panel based analysis (Tables 3.44-45), confirmed that some of the isolates were the same or closely related microorganisms (strains A1-A3 and B2-C1-C2-C3). API 20E gave the following identifications: strains A1 and A3 *Enterobacter* genus, strains B2 and C1-3 were *E. coli* (96.5% certainty) and strain C2 was *S. liquefaciens* (76.2% certainty).

Further study of these strains with 16S rDNA (Section 3.6.3.5) revealed that strains A1 and A3 were the same microorganism, identified as *Leclercia adecarboxylata*. This identification disagreed with the API 20E, which suggested Enterobacter genus. However, *L. adecarboxylata* strains have characteristics typical of Enterobacteriaceae (Krieg and Holt, 1984). Strain C3 was identified as *E. coli*, confirming the previous identification by the API 20E. Strain A2 was classified as genus Serratia and one of two species (*S. proteoamaculans and S. liquefaciens*). These have the same 16S rDNA identification pattern. *S. liquefaciens* complex can be divided in 3 species and 2 subspecies: *S. liquefaciens* sensu stricto, *S. proteamaculans* subsp. *proteamaculans*, *S. proteamaculans* subsp. *quinovora* and *S. grimesii* (Grimont *et al.*, 1982; Stock *et al.*, 2003).

Strain B1 was identified by 16S rDNA analysis as *Klebsiella pneumoniae*, a gram negative bacterium (Krieg and Holt, 1984). This isolate originally appeared to be Gram positive (Table 3.42). However, the API 20E result also confirmed to be *K. pneumonia*e with 97.1% certainty (T value = 0.93).

4.6.3.1 Species information

Leclercia adecarboxylata is a rare opportunistic human pathogen (Dalamaga *et al.*, 2009), that resembles *Escherichia coli* phenotypically. Strains of *L. adecarboxylata* are Gram-negative, facultative anaerobic, oxidase negative, meshophilic, peritrich-flagelated bacilli. Species of the Leclerciae family are distributed widely in nature. They have been isolated from food, water and other environmental sources, as well as from various clinical specimens (blood, faeces, sputum, urine and wound pus; Stock *et al.*, 2004). According to ATCC culture collection this strain is classified as biosafety level 1.

Serratia sp. occur in the natural environment (soil, water and plant surfaces). They are straight rods, generally motile and facultative anaerobic. Almost all strains can grow at

temperatures between 10-36^{o}C, at pH 5-9 and in the presence of 0-4% (w/v) NaCl (Krieg and Holt, 1984). *S. liquefaciens* can cause bloodstream infections by the use of contaminated clinical equipment (Stock *et al*., 2003).

Klebsiella pneumoniae is a clinically important member of the Enterobacteriaceae family, which exists ubiquitously (Chang *et al.*, 2010). It is normally found in the intestinal tract of man and animals, but in low numbers compared with *E. coli*. It has been isolated in association with several pathological processes in man, like infection of the urinary and respiratory tracts (Krieg and Holt, 1984). *K. pneumoniae* is also common in the environment and can be cultured from soil, water and vegetables According to ATCC culture collection this strain is classified as biosafety level 2.

Escherichia coli occur in the lower part of the intestine of warm-blooded animals (Atlas, 1989). They are straight rods, Gram negative, 76 to 89% motile (peritrichous flagella) and facultative anaerobes (having both respiratory and a fermentative type of metabolism). Their optimum growth temperature is 37^{o}C. Some strains of *E. coli* produce enterotoxins (Krieg and Holt, 1984).

The information obtained from the literature confirms that the four species isolated from inoculum B are Gram negative and facultative anaerobes. Some of them are found in soil, water and plant surfaces (*L. adecarboxylata*, *Serratia sp*., *K. pneumoniae*), while others are found in the intestine of humans and animals (*E. coli* and *K. pneumoniae*). *L. adecarboxylata* and *K. pneumoniae* are opportunistic human pathogens that are considered as biosafety level 1 and 2, respectively. This could cause difficulties for an industrial process and by-products. Some strains of *E. coli* produce enterotoxins (Krieg and Holt, 1984), which could be an obstacle in the digestion process of *A. nodosum* residue and in the possible applications.

4.6.4 Inoculum C

A molecular identification of the mould component of inoculum C confirmed it to be *Aspergilus versicolor*. This species can grow over a broad range of temperatures, as during this research it has been found growing in the fridge at 4^{o}C, at room temperature (20^{o}C approximately) and it also when incubated at 30^{o}C. This could be advantageous from an industrial perspective. Inoculum C showed great potential as it achieved an excellent reduction in the insoluble material (62%, Table 3.27, trials 19.2 and 20), while using much less additional water (100 ml per 50 g of residue).

Aspergillus versicolor is frequently found in stored cereals, hay, cotton, cheese, foods in decomposition and in various types of soil (Torres-Rodríguez *et al.*, 1998). It has also been isolated from the surface of the Caribbean green calcareous alga *Penicillus capitatus* (Belofsky *et al.*, 1998). *A. versicolor* can cause deterioration of paper, artworks, photographic film, polymers and freshly cut wood. Its toxin, sterigmatocystin, was reported in grain from the early 1970s (Liang *et al.*, 2011). *Aspergillus versicolor* can produce polysaccharides called levans, which are natural polymers of the sugar fructose. Levans have considerable potential as thickeners, industrial gums and sweeteners, and also as blood plasma extenders. They have potentially wide application in the food, pharmaceutical, cosmetic and paint industries, where they provide a substitute for gum arabic (Wainwright, 1992). Further research in *A. versicolor*, the polysaccharides produced and the possible applications could be of interest in the microbial digestion of *A. nodosum* residue. This inoculum also contained a bacterial population which was not investigated.

Although many of the microorganisms present in *A. nodosum* residue and the inocula were studied and identified, the relative proportions of each species in the residue or in the inocula is still unknown. A succession of microorganism was found to grow in the residue, during the anaerobic phase of digestion and in the different inocula studied. This shows that the proportion of microorganisms changes as growth conditions change. This is also affected by the different factors that were shown to alter the digestion process: pH, initial insoluble/soluble material, metabolite inhibition, and polyphenol concentration.

Other research on digestion of brown seaweed utilized different types of inocula. Composted or aerobically degraded seaweed tissue (Moen *et al.*, 1997b; Moen *et al.*, 1997c) was used for aerobic degradation, while cattle manure was used for anaerobic conditions (Hanssen *et al.*, 1987; Moen *et al.*, 1997a; Moen *et al.*, 1997b; Horn and Ostgaard, 2001). Degradation of green macro-algae for methane production used a mixed inoculum prepared from sediments, rotting seaweed, sewage sludge and rumen contents (Hansson, 1983). Anaerobic digestion of residues from an alginate extraction process employed marine sediments or algal sludge (Carpentier *et al.*, 1988; Kerner *et al.*, 1991).

Only a few authors provide information concerning the type of microorganisms and the bacterial numbers that are present in the inocula and during digestion. Tang *et al.*,

(2011) studied the composting of *Undaria pinnatifida* seaweed, using *Halomonas* sp. and *Gracilibacillys* sp. (separately) with an initial concentration of $6*10^8$ CFU/g. The inocula employed in *A. nodosum* residue digestion are, however, mixed cultures, and direct comparison is not possible.

4.6.5 Anaerobic phase

Further investigation of the microorganisms present in *A. nodosum* residue was carried out while studying the 3-day anaerobic phase of digestion. An initial number of aerobic bacteria of between $1.1*10^4$ and $5.3*10^6$ CFU/ml (day 0) increased to an average of $6.1*10^7$ CFU/ml at day 3 (replicates 1-3; Table 3.45), while pH decreased an average of 1.22 log values (Table 3.47). This indicates good growth of the bacterial populations present in the residue during the anaerobic phase. A decrease in the bacterial numbers between days 2 ($5.25*10^8$ CFU/ml) and 3 ($9.1*10^7$ CFU/ml) was found in the second replicate (Table 3.45), which shows a change in the bacterial population. When agar plates were incubated under anaerobic conditions, the bacterial numbers found were very similar to the aerobic ones (Tables 3.46 - 3.47). This could indicate the presence of anaerobic facultative microorganisms. The bacterial count was probably affected by the succession of microorganisms observed during the 3-day anaerobic phase, which showed the changing nature of the digestion process.

4.6.5.1 Isolates identification with Biolog System

Eight colonies were isolated from the 3-day anaerobic phase: 7 were Gram negative, 4 aerobes and 4 facultative anaerobes. All of the isolates were acid-fast negative, catalase positive and did not show spore production (Table 3.48). The aerobes were isolated from days 0 (strains 3-5) and 3 (strain 8) of digestion, which shows aerobes are still present after 3 days of anaerobic incubation. Only colonies from the aerobic plates were isolated due to timing and equipment availability. In early work one anaerobic colony was isolated from *A. nodosum* residue (Table 3.38). Further investigation of the anaerobic isolate was not carried out.

Identification of the eight isolates with the Biolog ID System showed the following species: *Brochothrix thermosphacta*, *Bradyrhizobium japonicum*, *Pseudomonas tolaasii*, *Pseudomonas maculicola*, *Pseudomonas aeruginosa* and *Enterobacter amnigenus* (Table 3.49). The Biolog identification was positive for the 6 identified strains, as the similarity index was greater than 0.5 for all of them (Table 3.49; Truu *et*

al., 1999). However, strains 6 and 8 were not identified. Strain 6 was recognized as a Gram negative enteric microorganism and strain 8 as a possible *Pseudomonas sp*. When a strain is not identified by the Biolog system, different options can be considered: the strain is not in Biolog´s data base, a non-pure culture has been used and/or the protocol employed is not the adequate (the microorganism could be a slow grower or it has special requirements). Strain 8 did grow slightly slower than the other strains studied and the option of a slow grower protocol for the Biolog ID System may be considered. Although both strains seemed to be pure cultures (growing in plate count agar and under the microscope) further investigation to identify them employing the Biolog ID System or other techniques (e.g. 16S rDNA) may be required.

4.6.5.2 Species information

Brochothrix thermosphacta is a potent spoilage microorganism in raw and chilled meats and meat products (Gordon Greer *et al.*, 2000), which causes flavour deterioration. It has also been isolated from fish (smoked whiting, frozen cod) and other foods like frozen peas or runner beans, milk, cream, cheese and pre-packed salad (Sneath *et al.*, 1986). These bacteria occur both as coccobacilli as well as long rods, singly and in chains, which makes difficult to quantify its cell numbers (Rattanasomboon *et al.*, 1999). They are Gram positive and facultative anaerobic. They grow within the range of 0-30^{o}C and pH 5.0-9.0, with optimum temperature 20-25^{o}C and pH 7.0 (Sneath *et al.*, 1986). Under anaerobic conditions *B. thermosphacta* produce lactic acid, ethanol and small amounts of short chain fatty acids, causing off-odours (Cayré *et al.*, 2005).

Bradyrhizobium japonicum cells are rod shaped in young cultures and pleomorphic (swollen and elongated) in older cultures or under adverse growth conditions. They are Gram negative, motile (one polar of subpolar flagellum) and aerobic. Their optimum growth temperature is 25-30^{o}C and optimum pH, 6-7. Growth normally does not occur above 42^{o}C or pH 9. *B. japonicum* organisms are able to invade the root hairs of tropical/temperate zone leguminous plants and produce root nodules, where the bacteria occur as intracellular symbionts (Krieg and Holt, 1984). *B. japonicum* forms root nodules on species like *Glycine* (Soybean) and *Macroptilium atropurpureum* (siratro). In these nodules, the bacteria fix atmospheric nitrogen into combined forms, which can be used by the host plant (Godoy *et al.*, 2008). Some strains can fix nitrogen in the free living state. When soybean plants are in symbiotic association with *B. japonicum*, the

need of using nitrogen fertilizer, which is expensive and causes environmental damage, is reduced (Zhang *et al.*, 2003).

The genus Pseudomonas includes Gram negative rod shape microorganisms, motile (one or several polar flagella) and aerobic. They have a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, and in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically (Krieg and Holt, 1984).

Pseudomonas tolaasii is classified in Bergey's manual as isolated from diseased plants and cultivated mushrooms, and fluorescent organisms (Krieg and Holt, 1984). It is the causative agent of the brown blotch disease on the cultivated mushroom *Agaricus bisporus*, causing sunken brown lesions on the cap or on the pileus of the mushroom during cultivation or cold storage (Munsch *et al.*, 2002). This bacterium produces the toxin tolaasin, which is an extracellular lipodepsipeptide (cyclic peptide with an N terminal fatty acid blocking group; Lee *et al.*, 2009)). Tolassin causes the loss of fungal membrane integrity and the posterior release of hyphal contents. It has haemolytic properties and it is phytotoxic and active against a range of basidiomycetes and Gram-positive bacteria (Munsch *et al.*, 2002).

P. maculicola is a pathovar (an infrasubspecific designation for phytopathogenically distinct members of a species) of *Pseudomonas syringae*. *P. syringae* is a common Gram negative bacterium that can be found as a harmless commensal on leaf surfaces or as an important plant pathogen causing blight, canker, speck and spot diseases in agricultural crops. *P. syringae* has approximately 50 different pathovars, which can be distinguished by their pathogenicity on plants and phenotypic characteristics (*Lenz et al.*, 2010). *P. syringae* pv. *maculicola* is an important pathogen of crucifers world-wide, like cabbage or cauliflower (Krieg and Holt, 1984; Bull *et al.*, 2010).

Pseudomonas aeruginosa is a ubiquitous microorganism, which can be found in soil, water, plants, animals and in humans (Atzél *et al*., 2008). It is an opportunistic human pathogen associated with food and water-borne diseases (Casanovas-Massana *et al*., 2010). Normally, it does not cause infection in healthy humans, but it can do it in patients with certain underlying illnesses (Iversen *et al*., 2008). *P. aeruginosa* is important in biodegradation because is able to degrade a large range of pollutants, like aliphatic, aromatic and polyaromatic hydrocarbons (Atzél *et al*., 2008). Occasionally it is pathogenic for plants. It's optimum temperature is 37^{o}C (Krieg and Holt, 1984).

Enterobacter ammigenus are straight rods, Gram negative, motile (peritrichous flagella) and facultative anaerobic. Strains from environmental sources grow better at 20-30^{o}C, in comparison to strains from clinical sources (37^{o}C). *E. ammigenus* can be separated from other *Enterobacter* species by its inability to grow at 41^{o}C (Krieg and Holt, 1984). *E. ammigenus* is found in drinking and surface water and in unpolluted soil. It has also been isolated from raw milk and industrially manufactured Spanish pork sausage (Tamagnini et al., 2008). *E. ammigenus* can cause infection in humans (Capdevila *et al*., 1998).

When comparing results obtained on preliminary testing of the isolates (Table 3.38) there was agreement between 5 of the 6 strains identified by Biolog, and our results (Table 3.48). However, strain 2 identified as *B. japonicum* (similarity index = 0.667; Table 3.49) is classified as aerobe by Bergey´s manual (Krieg and Holt, 1984) but was found to grow under anaerobic incubation (7 days at 30^{o}C) in plate count agar (Table 3.38), which indicates that it might be facultative anaerobe. Also, Strain 1 (*B. thermosphacta*) was Gram positive (Table 3.48) and it is classified as such in Bergey´s manual (Krieg and Holt, 1984). However it showed some slight growth in MacConkey agar.

The Gen III microplate of the Biolog ID System provided information regarding the carbon sources used by the isolates (Table 3.50). The Gen III microplate was only incubated aerobically. Therefore, comments cannot be made on the ability, or otherwise, of these organisms to utilize various carbon sources under anaerobic conditions. None of the 8 strains grew on D- or L- fucose (Table 3.50), which is the main component of fucoidan, an abundant polysaccharide in brown seaweeds (Davis *et al.*, 2003). This suggests that these strains are not able to degrade this polysaccharide, possibly present in *A. nodosum* residue. Most of the strains (3-8) grew with L-lactic acid, while only two of them (3 and 7) grew with acetic acid (Table 3.50). These strains may be responsible for lactic or acetic acid consumption during anaerobic and/or aerobic phases. *B. thermosphacta* produces lactic acid when incubated under anaerobic conditions (Cayré *et al.*, 2005). It has been shown to ferment glucose to L-lactatate, acetate, formate and ethanol (Grau, 1983). *B. thermosphacta* may be one of the species involved in the accumulation of lactic, acetic and formic acids during the anaerobic phase (Figure 3.40). None of the 8 strains grew with formic acid (Table 3.50) as a carbon source. The consumption of formic acid during digestion (Figure 3.39) might be related to the

growth of other strains during the anaerobic phase and/or to microorganisms from inocula B and/or C during the aerobic degradation.

Some of these microorganisms have an optimum temperature between 20 and 30°C (*B. thermosphacta*, *B. japonicum*, *E. amnigenus*), while others grow better at 37°C (*P. aeruginosa*). The optimum pH varies from 6 to 7 for *B. japonicum* and 7 for *B. thermosphacta* although *B. thermosphacta* can grow in a wide pH range (5-9). *B. japonicum* cannot grow at pH higher than 9.The pH of the *A. nodosum* residue would definitely inhibit growth of these organisms prior to the anaerobic phase.

Most of the species identified growing in *A. nodosum* residue seem to come from environmental sources (water, soil or plants). Some of these species are plant pathogens (*P. tolaasii*, *P. maculicola*, *P. aeruginosa*) or produce toxins (*P. tolaasii*). The presence of toxins or plant pathogens might be problematic in a digestion process, depending on application of the residue or digestion by-products.

4.7 Screening for anti-hyaluronidase and anti-elastase activities

The industrial seaweed-based residue was screened for anti-hyaluronidase activity at different times of its microbial digestion. Also, a clean and new methodology was used to obtain peptides from seaweeds and a seaweed extract, which were screened for anti-hyaluronidase and anti- elastase activities.

4.7.1 *A. nodosum* residue anti-hyaluronidase activity

A. nodosum residue provided stronger anti-HAase activity than fresh *A. nodosum* or *L. hyperborea* (Figure 3.53). This anti-HAase activity was maintained (BATCH-B+C) or slightly improved (BATCH and BATCH-WASH) over the 3-day anaerobic digestion phase, while decreased during the 10-day aerobic phase (Figure 3.57). This anti-HAase activity decrease could be influenced by the dilution of the SM at day 3, where solubilised material (200 ml) was decanted and replaced with water (days 0 and 3 for trial BATCH-WASH), and/or the molecules responsible for HAase inhibition were digested by microorganisms of inocula B and/or C under aerobic conditions.

Heparin, a known HAase inhibitior, gave the strongest IC_{50} value (43 µg/ml) in this study (Table 3.52). Toida *et al.* (1999) found that heparin inhibits hyaluronidase activity noncompetitively, while in our study sodium heparin showed a different type of inhibition (competitive, Section 3.7.1.6). Crude phlorotannin fractions of two brown seaweeds from the Japanese coast, *Eisenia bicyclies* and *Ecklonia kurome*, presented

IC_{50} values of 30 and 35 µg/ml respectively (Shibata *et al.*, 2002). The isolated phlorotannins that presented the strongest IC_{50} values were 8,8´-Bieckol (40µM), Dieckol (120µM) and Phlorofucofuroeckol A (140 µM). Three of them appeared to act as competitive inhibitors of HAase. These authors also suggested that polyphenols' higher molecular weight may play an important role in HAase inhibition. A phenolic substance from *Areca catechu* exhibited anti-HAase activity with and IC_{50} value of 210 µg/ml (Lee *et al.*, 2001).

A. nodosum residue exhibited an IC_{50} value (250 µg/ml) in its crude state (Table 3.52), which was between 5 and 6 times higher than sodium heparin or the phlorotannin fractions from the two Japanese brown seaweeds. However, it was only slightly higher than the phenolic substance from *A. catechu* (250 *vs*. 210 µg/ml; Table 3.52). *A. nodosum* residue (aqueous extract) is a crude fraction where there may be different components and molecules that affect HAase in different ways. Phlorotannins from *A. nodosum* could be involved in the anti-HAase activity found. Further research at this point will be needed in order to purify and investigate the compounds that cause HAase inhibition in *A. nodosum* residue.

4.7.2 Samples treated to obtain peptides

A. nodosum, *L. hyperborea* and AqE samples, in their fresh and dried formats, were extracted with SFE, and subjected to carbohydrate digestion and protein hydrolysis, with the aim of extracting the lipid fraction, break down the carbohydrates liberating proteins (especially the ones in the cellular wall), and generating peptides (Figure 2.6).

4.7.2.1 Protein content and protein hydrolysis

Protein analysis determined a total nitrogen content of 5.17% in *A. nodosum*, 5.18% in *L. hyperborea* and 3.50% in AqE in their dried formats after SFE (Table 3.54). BCA-protein analysis during protein hydrolysis, revealed high protein contents in most of the samples, because viscozyme carbohydrases and proteases used were also included in the samples.

One of the aims of studying the protein content during the hydrolysis was to see if proteins were hydrolyzed over this time. During hydrolysis the pH will drop unless buffered due to H+ production when peptide bond is broken. A proper hydrolysis should be carried out by maintaining a constant pH and monitoring the amount of base added during digestion time, in order to ensure that proteases are working well and

calculate the degree of hydrolysis. In our screening study, small volumes (1200 µL) were used for protein hydrolysis and thereby pH could have not been monitored. Protein contents were measured with BCA at time 0, 30 and 60 minutes of hydrolysis. However, was not possible to relate these results to protein hydrolysis, as in the BCA method the Cu^{2+} ion links to the peptide bond, so it is not possible to distinguish between proteins and peptides. This exercise was informative as a preliminary study and future work is required to purify and further characterize extracts that exhibit anti-HAase and/or anti- elastase activity. Methods like the pH-stat, OPA or TNBS (Spellman *et al*., 2003) to measure the degree of hydrolysis are recommended.

4.7.2.2 Anti-hyaluronidase activity

A. nodosum, *L. hyperborea* and AqE samples, were treated by SFE, carbohydrate digestion and protein hydrolysis with alcalase and protamex. The screening showed that most of the samples inhibited HAase between 90-100% at a concentration of 4 mg/ml. *L. hyperborea* samples presented lower HAase inhibition, in comparison with *A. nodosum* or the AqE (Tables 3.57 – 3.58). However, *L. hyperborea* showed stronger anti-HAase activity than *A. nodosum* at lower concentration (0.25-2 mg/ml; Figure 3.52). Samples treated with protamex presented slightly better anti-HAase activity than the samples treated with alcalase (Table 3.57). This small difference could be due to differences in peptide content/type generated by the two proteases.

Viscozyme controls activated HAase by 88.7% (after carbohydrate digestion) and by 32 and 39% after alcalase and protamex (Table 3.55), compared to the positive control. This did not seem to influence the inhibition when this was between 90-100%. However, when samples exhibited inhibition values lower than 90%, this viscozyme effect was more noticed. For example, SFE fresh *L. hyperborea* after carbohydrate digestion (Table 3.56) presented 76.42 ± 2.62% inhibition when compared to the positive control, and 87.51 ± 1.39% inhibition when compared to Viscozyme control. Standard deviation values were lower than the ones obtained in the elastase assay (Section 4.7.2.3). SD values decreased slightly when the inhibition was calculated comparing to the Viscozyme controls instead to the positive controls (Tables 3.57 – 3.58). These results suggest that part of the variation found between duplicates could be due to Viscozyme-derived peptides.

When comparing each sample through the SFE, carbohydrates digestion and protein hydrolysis steps, no significant differences in HAase inhibition were found at the

different stages (Tables 3.57 – 3.58). The concentration used (4mg/ml) for this preliminary screening gave high anti-HAase activity (> 90%) in most of the samples. Although HAase inhibition was found, the results did not provide any clear differences between samples and the different treatments used. The use of Viscozyme to digest the carbohydrates present in the seaweed samples, interfered with HAase activity by activating the enzyme.

In this study, some component(s) from the seaweed plants and the AqE strongly inhibited HAase. This component(s) seem to be present and accessible in the fresh, dried or dehydrated formats of the seaweeds, before and after SFE, carbohydrates digestion and protein hydrolysis. The same or similar components seem to be also present in *A. nodosum* residue, which provided stronger anti-HAase activity than fresh *A. nodosum* or *L. hyperborea* (Figure 3.53).

4.7.2.3 Anti-elastase activity

SFE-extracts (100-400 bars) from all the samples showed elastase activation at a concentration of 50.3 µg/ml (Table 3.58). The highest activation (151.0 ± 22.6% after 2 hours) was exhibited by dried *A. nodosum* extracted at 200 bars (Figure 3.62).

Viscozyme control treated with the four proteases resulted in slight activation of elastase in the four cases (Figure 3.64, Table 3.59). SFE-residual samples and non-SFE samples were digested with viscozyme (carbohydrases mixture) and hydrolysed with four different proteases. Some samples showed elastase activation (fresh and dried *L. hyperborea*, at a concentration of 50.3 µg/ml) and the highest activation was found in fresh *L. hyperborea* treated with alcalase (50.1 ± 21.1% at 10 min).

Fresh *A. nodosum* (SFE-residual and non-SFE) treated with bromelain showed the greatest elastase inhibition (Tables 3.61 - 3.62). This inhibition was more potent in the SFE-residual sample (35.9 ± 6.9% at 20 min) than in the non-SFE sample (21.4 ± 13.3%), probably as the lipid components were not present in the first case. Anti-elastase activity was also found in SFE dried *A. nodosum* (14.0 ± 2.6% after bromelain, 15.9 ± 4.7% after pepsin and 8.4 ± 0.8% after protamex treatment, all at 10 min), non-SFE fresh *Ascophyllum* (20.1 ± 11.3% alcalase, 17.0 ± 4.2% pepsine, 9.7 ± 1.1 protamex at 10 min) and fresh *L. hyperborea* treated with bromelain (16.3 ± 0.3% at 10 minutes). Elastase inhibition was greatest during the first 20 minutes and decreased with time, resulting in elastase activation in some cases (at 60-120 minutes). The AqE

sample exhibited very low elastase inhibition, which was in the early stages of the assay (10-20 minutes; Table 3.61).

Anti-elastase activity was most potent in *A. nodosum* in comparison with *L. hyperborea*. With respect to the four proteases used, samples treated with bromelain showed greater elastase inhibition in some cases, in comparison to the other proteases. Peptides generated from *A. nodosum* by bromelain gave the strongest anti-elastase activity that was not showed by other proteases or other seaweed samples in this study.

The original aim of the methodology used in this study was to generate peptides from seaweed samples. Lipid components were extracted by SFE. However, digested carbohydrates and minerals were also present after the protein hydrolysis. Some of these molecules could have interfered with elastase activity in very different ways.

Previous studies in the literature have shown that few elastase inhibitors have been found in seaweeds. Senni *et al*. (2006) showed that a 16kDa fucoidan fraction extracted from *A. nodosum* inhibited human leucocyte elastase (HLE) in human skin tissue sections. The fucoidan samples used in this study did not show inhibition in porcine pancreatic elastase (PPE; Figure 3.63). The active fraction may not have been present in our samples or was not at the right concentration. Some fucoidan fractions were probably generated in all the samples by viscozyme during the carbohydrates digestion. Although these fractions were present in fresh *A. nodosum* treated with bromelain, they could have not been responsible for elastase inhibition. If these fucoidan fractions or other polysaccharides were responsible of elastase inhibition, the four samples of fresh *A. nodosum* treated with different proteinases would have shown similar inhibition results, which did not happen. This suggests that a protein fraction or peptide from fresh *A. nodosum* treated with bromelain may have been responsible for elastase inhibition.

Bultel-Poncé *et al*. (2002) identified a new ketosteroid (20-hydroxy-5α-cholest-22-ene-3,6-dione) from the red alga *Hypnea musciformis*, which inhibited PPE. Baylac and Racine (2004) reported a *Fucus vesiculosus* resinoid as HLE inhibitor with an IC_{50} between 1.1 and 5 µg/ml. In that study, turmeric oleoresin exhibited the most potent inhibitor of HLE (IC_{50} between 0.04-0.05 µg/ml), among all the essential oils studied. Oils extracted from *A. nodosum, L.hyperborea* and the AqE did not show any anti-elastase activity at a concentration of 50.3 µg/ml (Table 3.58).

Other elastase inhibitors have been found in some plant extracts. An ethanolic extract from *Areca catechu* seed showed both *in vitro* and *in vivo* inhibition of PPE and HLE (Lee and Choi, 1998; Lee and Choi, 1999). A phenolic substance from *A. catechu* plant

inhibited PPE (IC_{50} 26.9 µg/ml) and human neutrophil elastase (IC_{50} 60.8 µg/ml) by competitive inhibition with the substrate (Lee *et al.*, 2001). *A. catechu* seeds assayed with PPE for 20 minutes, exhibited 63% inhibition at 50 µg/ml (Lee and Choi, 1998; Lee and Choi, 1999). In our study, fresh *A. nodosum* (SFE) treated with bromelain presented 36% PPE inhibition at a concentration of 50.3 µg/ml after 20 minutes (Table 3.60). *A. nodosum* sample is a mixture of components, which could probably react with the enzyme in different ways. Further research in this study will include purification and identification of the molecule(s) that is or are responsible for the anti-elastase activity.

Some samples assayed for elastase activity or for protein analysis (BCA) presented high standard deviation values. This could be attributed to a number of reasons. First of all, samples were only assayed in duplicates due to sample availability. Proteases and/or carbohydrases (viscozyme) may not have been properly deactivated at the temperatures and times used (80^{o}C for alcalase, protamex, pepsine and viscozyme and 65^{o}C for bromelain, all for 20 minutes; Table 2.4). If this happened, some bioactivity was still present while samples were incubated for protein analysis (37^{o}C) or elastase activity (25^{o}C). In the case of samples tested for elastase activity, SD values were higher at the beginning of the assay and decreased over time (Table 3.60).

5. CONCLUSIONS AND FUTURE WORK

This was an interesting project, where science and industry came together to study and evaluate a feasible solution for *A. nodosum* residue. Industrial requirements often create challenges which the scientist must incorporate when addressing practical problems. Although several digestions of brown seaweed have been carried out, the *A. nodosum* residue contained added potassium carbonate and fibrous materials that are resistant to chemical and physical digestion, which represented a challenge when pollution is to be avoided.

During this project different methodologies were employed in three main areas of study: analytical sciences, microbiology and enzymology. The techniques employed ranged from simple such as total solid determination, centrifugation, total viable count or microbial staining, to the more advanced such as HPLC, GC, SFE or enzymatic assays, as well as modern techniques like microbial identification by phenotypic pattern. In this research, the analytical composition of an *A. nodosum* residue was studied for first time. A novel microbial digestion system was been developed, posing further research questions and pointing to potential applications. The main findings of this project included the use of a 3-day anaerobic phase to decrease the pH prior to aerobic digestion, factors that affect the microbial digestion process, the identification of the species of microorganisms involved in both residue and inocula, and the anti-hyaluronidase activity of the residue; as well as the anti-hyaluronidase and anti-elastase activities in extracts of *A. nodosum* and *L. hyperborea* plants.

The use of a 3-day anaerobic phase to decrease the pH was developed from an observation during the digestion study. Although the mechanism of action it is not clear, further research in the microorganisms involved during this phase could provide this information. This process could be applied to similar residues or processes, where the pH cannot be reduced by the standard methodologies, or where the use of chemicals needs to be avoided.

Factors affecting the digestion of *A. nosodum* residue are presented in this study. Digestion is strongly affected by the initial relative amounts of insoluble and soluble material present, to a lesser extent by the pH at the beginning of aerobic phase of digestion, but critically by the accumulation of solubilised digestive products. It is suggested that inocula and metabolite inhibition are critical features of such digestions and that similar organic residues require a carefully chosen inoculum, and minimum initial insoluble content of 65-70% and/or maximum soluble content of 25-30% (w/w).

Further research could involve the study of other types of digestion system such as continuous or semi-continuous models. The batch to batch consistency of the industrial seaweed extraction process should also be studied. This research might lead to better reproducibility of the digestion process of *A. nodosum* residue, and future industrial applications.

Knowledge has been provided with regard to which species of microorganisms are able to grow in the residue and in the inocula employed. Care should be taken when selecting these species for further research. Some of them have been shown to release toxins and others are plant or opportunistic human pathogens, which could be problematic depending on the digestion by-product's applications. Further study of these microorganisms might lead to a better understanding and improvement of the digestion process. The role of *A. versicolor*, a microorganism tolerant to high pH, in the solubilisation of the residue warrants further investigation.

A. nodosum residue has shown stronger anti-hyaluronidase activity than the fresh seaweeds *A. nodosum* or *L. hyperborea*. Further research will involve fractionation of the sample and studying the effect of each fraction on HAase activity. Purification and identification of the component(s) responsible for such inhibition, together with an study of the IC_{50} value(s) of this molecule(s) should also be carried out. This information will show how potent are the inhibitor(s) found and it might provide possible future applications of the residue.

The brown seaweeds *A. nodosum* and *L. hyperborea* have been screened for anti-hyaluronidase and anti-elastase activities for first time. Supercritical Fluid Extraction is a clean methodology which does not use organic solvents, protects the environment and provides better quality samples for cosmetic applications. The subsequent step in the extraction, the addition of carbohydrases, produced a solution which activated elastase slightly but cause significant activation of hyaluronidase. For this reason other methodologies to study *A. nodosum* and *L. hyperborea* components for anti-hyaluronidase activity without the use of viscozyme will be required.

A. nodosum treated with bromelain showed potential for anti-elastase activity. Both seaweeds studied and the commercial aqueous extract (AqE) showed potential for anti-hyaluronidase activity, although the effect of concentrations smaller than 4mg/ml should be studied. Further research is proposed to better understand and scale-up the peptide generation methodology. Also, further research will be needed in order to study the type of molecules that cause anti-elastase and/or anti-hyalronidase activities. Further

purification of the active ingredients is required. Using the correct ingredients at the right concentration is very important for cosmetic applications, as other contaminants or inappropriate concentrations may have the opposite to the intended effect in the studied enzymes.

Ascophyllum nodosum residue presents variable conditions which challenge its microbial digestion. Further research is needed in order to develop a feasible and reproducible digestion process for the seaweed industry. *A. nodosum* residue has shown potential for further applications such as anti-hyaluronidase activity. This project has opened new avenues of research, which if fully explored would lead to the fulfilment of the ultimate aim of determining an alternative fate of the by-product of seaweed extraction.

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Appendices

Appendix I

A. nodosum meal, extract and residue composition

The following Tables show the full data for Section 3.2: *Ascophyllum nodosum* meal, extract and residue from the same extraction process. The percentages of moisture, total solids, ash, protein and lipid are shown. The polyphenol concentration is also included.

Table 1 *A. nodosum* meal, extract and residue composition I

	TS (%)	Average	SD	Moisture (%)	Average	SD	Ash (%)	Avergage	SD
	86,92			13,08			23,39		
Meal	86,94	86,82	0,19	13,06	13,18	0,19	23,26	23,23	0,18
	86,60			13,40			23,03		
	9,62			90,38			43,55		
Extract	9,65	9,68	0,08	90,35	90,32	0,08	45,68	46,10	2,79
	9,77			90,23			49,07		
	17,85			82,15			38,42		
Residue	18,09	18,08	0,23	81,91	81,92	0,23	39,35	38,89	0,47
	18,30			81,70			38,90		

Results shown as % (w/w) of dried sample for the ash and % (w/w) of fresh sample for the rest.

Table 2 *A. nodosum* meal, extract and residue composition II

	Protein (%)	Average	SD	Lipid (%)	Average	SD	Polyphenols	Average	SD
	6,31			1,62			2.48		
Meal	6,58	6,46	0,13	1,74	1,79	0,21	2.03	2.26	0.32
	6,47			2,03					
	4,75								
Extract	4,31	4,59	0,24	3,53	3,53		39.07	39.07	
	4,71								
	6,54			20,54			3.91		
Residue	5,29	5,70	0,73	15,36	18,10	2,60	4.13	4.02	0.15
	5,26			18,42					

Results shown as % (w/w) of dried sample for protein and lipid contents and mg gallic acid equivalent/ g dried sample for polyphenols

Appendix II

Identification report of inoculum B isolates

Genetic characterization of microorganisms for Institute of technlogy, Tralee

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Material and methods:

Isolation of DNA was made from 1.5 ml of overnight liquid culture (prepared by Ing. Jimenez). Biomass was centrifuged and chromosomal DNA was extracted by phenol-chloroform method made according to modified protocol (Wilson K., 1997). Quality of isolated DNA was controlled with agarose gel electrophoresis.

Isolated DNA was used for PCR amplification of 16S rDNA with degenerate primers designed in conserved sequences of 16S rDNA subunit (Lane, 1991) with program: 94°C - 3min., 37x (94°C - 45sek., 55°C - 2min., 72°C - 2min.), 72°C - 5min..

Amplified PCR products were purified with SpinPrep PCR Clean-up Kit according to protocol (Novagen). Purified products were sent for sequencing to company Lambda Ltd. and were sequenced on automatic sequencer ABI3100-Avant (Applied Biosystems).

Received sequences were checked and modified in program Chromas Lite and compared to genetic database GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

According to these comparison microorganisms were classified based on highest Query coverage and identity of sequences.

Results and discussions:

Bacterial DNA was successfully isolated from 5 provided strains (A1, A2, A3, B1, C3). Isolation from strain BS was unsuccessful according to standard protocols for G- and also for G+ bacteria (**Fig. 1**). We have tried several modifications of protocols, but without any

success. This strain was therefore not classified. Isolated DNA was then used for amplification of 16S rDNA by PCR (**Fig. 2**).

All sequences of characterized strains are attached at the end of this protocol.

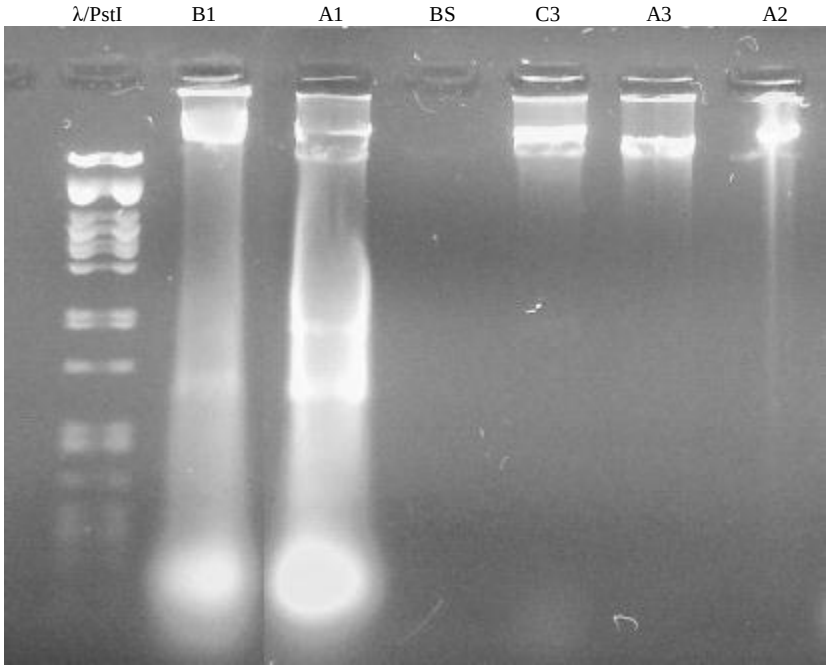

Fig. 1 Electrophoresis of isolated chromosomal DNA. Lane 1: standard of molecular weight λ cleaved with PstI; Lanes 2-7: isolated DNA.

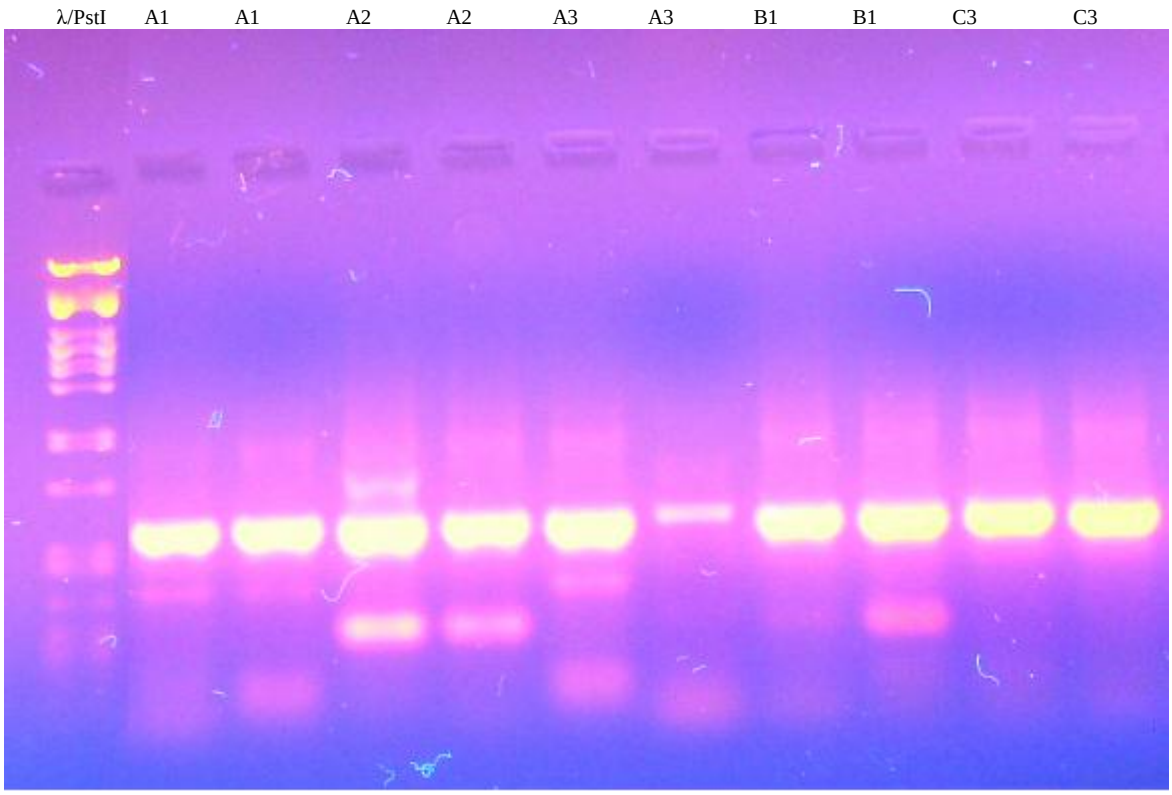

Fig. 2 Electrophoresis of amplified 16S rDNA region. Lane 1: standard of molecular weight λ cleaved with PstI; Lanes 2-10: samples.

Strain A1

The query for 16S rDNA was 100% identical with submitted and published sequences (Sarma et al., 2004; GenBank: AY451327.1) and (Baere et al., 2001; GenBank: AJ277978.1). This microorganism is therefore characterized as ***Leclercia adecarboxylata***.

The full lineage of this microorganism according to Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root) is defined as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Leclercia.

According to ATCC culture collection this strain is classified as Biosafety level 1.

Strain A2

The query for 16S rDNA was 100% identical with submitted and published sequences (Giudice,L et al. 2008, GenBank: EU781737.1) where this microorganism was identified as *Serratia sp.*; (Copeland A, GenBank: CP000826.1) where this microorganism was defined as *Serratia proteamaculans* and (Olsson,C et al. 2003, GenBank: AY243097.1) where this microorganism was defined as *Serratia liquefaciens*. Since there are several different species with the same identity of 16S rDNA sequence, I would suggest to classify this species as ***Serratia sp.***. Further classification of this species would require large systematic studies.

The full lineage of this microorganism according to Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root) is defined as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae, Serratia.

Strain A3

16S rDNA sequence for this microorganism is identical with strain A1. Therefore this isolated culture is also ***Leclercia adecarboxylata***.

Strain B1

The query for 16S rDNA was 100% identical with submitted and published sequences (Li,X.M. et al., 2009; GenBank: EU419756.1) and (Sajidan,A. et al. 2003, GenBank: AF453251.1). This microorganism is therefore characterized as ***Klebsiella pneumoniae***.

The full lineage of this microorganism according to Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root) is defined as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Klebsiella

According to ATCC culture collection this strain is classified in Biosafety level 2.

Strain C3

The query for 16S rDNA was 100% identical with submitted sequence (submitted by genetic company Genoscope, GenBank: CU928162.2). This microorganism is therefore characterized as ***Escherichia coli***.

The full lineage of this microorganism according to Taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root) is defined as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia

References:

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Attachment:

A1

GCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAG
TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA
CATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGC
ATGGCTGTCGTCA

A2

GCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGC
ATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTA
AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAG
TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA
CATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGC
ATGGCTGTCGTCA

A3

GCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAG
TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA
CATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGC
ATGGCTGTCGTCA

B1

GCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA

AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAA
TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGA
CATCCACAGAACTTGCCAGAGATGGCTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGC
ATGGCTGTCGTCA

C3

GCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAG
TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGA
CATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGC
ATGGCTGTCGTCA

Appendix III

Identification report of the mould component of inoculum C (by CABI, UK)

CABI IDENTIFICATION SERVICES

Report: H16/09/YE10

Your ref: PON 122729

Your number	IMI number	Identification and comments
1	397008	**Aspergillus versicolor** (Vuill.) Tirab. A widespread species found in soil and many types of foods, and is also common in indoor environments. The toxin sterigmatocystin is produced by some strains. It is placed in ACDP hazard group 1. Morphological features of your strain are typical of this species and its ITS sequence shows 100% homology with authentic strains of *A. versicolor*. There are however two recently recognized segregate species from *A. versicolor* that can only be separated reliably using calmodulin sequences, which we cannot absolutely rule out as identification of your strain. They have similar ecological requirements. Please let us know if you would like us to undertake further molecular work using calmodulin and we will provide a quote. We will keep the isolate in cool storage, while we await your reply. Report from Dr P.F. Cannon.

CABI IDENTIFICATION SERVICES

Report: H16/09/YE10

Your ref: PON 122729

Your number	IMI number	Identification and comments
1	397008	**Aspergillus versicolor** (Vuill.) Tirab. The identification has been confirmed by calmodulin sequencing. Analysis of this gene sequence shows 100% homology with an authentic strain of *A. versicolor* in its narrowly defined sense. Peterson (*Mycologia* **100**: 205-226, 2008) recognizes a separate, unnamed taxon close to *A. versicolor* but this has only 93% calmodulin sequence homology with your strain. Morphological features of your strain are typical of this species and its ITS sequence also shows 100% homology with authentic strains of *A. versicolor*. This is a widespread species found in soil and many types of foods, and is also common in indoor environments. The toxin sterigmatocystin is produced by some strains. It is placed in ACDP hazard group 1. This material will be discarded. Report from Dr P.F. Cannon.

Appendix IV

Biolog identification results with Gen III microplate

ProgramName	MicroLog 3/5.2.01 33
ProjectName	JUN
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:16 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 1
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:17 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID

ID Result	Species ID: Brochothrix thermosphacta
ID Organism Type	GP-Rod
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.818	0.818	2.598	GP-Rod	Brochothrix thermosphacta
2	0.190	0.190	3.114	GP-Coccus	Streptococcus salivarius
3	0.130	0.130	3.447	GP-Coccus	Enterococcus asini
4	0.127	0.127	3.472	GP-Coccus	Streptococcus macacae

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	< 250	< 250	< 250	< 250	< 250	2	2	2	2	2
B	2	2	2	< 250	< 250	2 +	2	2	2	2	2	2
C	< 250	< 250	< 250	2	2	2	2	2	2	2	2	2
D	2	< 250	2	2 +	2	2	2	2	2	2	2	2
E	2	2	2	2	2	2	2	2	2	2	2	2
F	2 +	2	2	2	2	2	2	2	2	2	2 +	2 +
G	2	2	2	2	2	2	2	2	2	2	2	2
H	2	2	2	2	2	2	2	2	2	2	2	2

Report Date	Feb 23 2011 6:02 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:18 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 2
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:20 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID

ID Result	Species ID: Bradyrhizobium japonicum
ID Organism Type	GN-Nent
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.667	0.667	4.763	GN-Nent	Bradyrhizobium japonicum
2	0.168	0.168	5.035	GN-Nent	Achromobacter xylosoxidans
3	0.138	0.138	5.206	GN-Nent	Pseudomonas fulva
4	0.116	0.116	5.362	GN-Nent	Pseudomonas maculicola

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
B	2	2	2	2	2	2	2	2	2	< 250	< 250	2
C	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
D	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
E	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
F	2	2	2	2	2	2	2	< 250 -	2	< 250	< 250	< 250
G	2	2	2	2	< 250 -	2 +	2	< 250	2	< 250	< 250	< 250 -
H	2	2	2	2 +	2	2	2	2 +	2	< 250	2	+ < 250 -

Report Date	Feb 23 2011 6:01 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:21 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 3
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:23 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID

ID Result	Species ID: Pseudomonas tolaasii
ID Organism Type	GN-Nent
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.549	0.549	6.616	GN-Nent	Pseudomonas tolaasii
2	0.125	0.125	7.400	GN-Nent	Pseudomonas aeruginosa
3	0.095	0.095	7.728	GN-Nent	Pseudomonas synxantha
4	0.058	0.058	8.345	GN-Nent	Burkholderia cepacia

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
B	2	2	2	2	2	2	2	2	2	< 250	< 250 -	2
C	< 250 -	2 +	2	< 250	2	2	2	2	2	< 250	< 250	< 250 -
D	2	2	2	2	2	2	2	2 +	2	< 250	< 250	< 250
E	2	2	< 250 -	2	2 +	2 +	2 +	2	< 250	< 250	< 250	< 250
F	2	2 +	2	< 250 -	< 250	2 +	2 +	< 250	< 250	< 250	< 250	< 250
G	2	2	2	< 250 -	< 250 -	2	2	< 250	2	< 250	2	< 250
H	2	2 +	2	2	2	2	2	< 250 -	2	< 250	2	2

Report Date	Feb 23 2011 6:01 PM

Program	MicroLog 3/5.2.01 33
Project	ML5
File Name	1-8 seaweed strains.D5E
User	biolog
Instrument	Manual Entry
Instrument S/N	0
Incubation Hours	22.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	strain 4
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
Date & Time of Read	Feb 23 2011 5:26 PM
Biolog ID DB	Biolog GEN III 2_5_1_15.I5G

Result	Species ID: Pseudomonas maculicola
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.617	0.617	5.589	GN-Nent	Pseudomonas maculicola
2	0.280	0.280	5.639	GN-Nent	Pseudomonas fluorescens
3	0.012	0.012	7.538	GN-Nent	Pseudomonas aeruginosa
4	0.011	0.011	7.599	GN-Nent	Pseudomonas synxantha

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	< 250	< 250	< 250
B	0	0	0	0	0	0	0	0	0	< 250	0	0
C	< 250	0	0	0	0	0	0	0	0	< 250	< 250	< 250
D	0	0	0	0	0	0	0	0	0	< 250	< 250	< 250
E	0	0	0	0	0	0	0	0	0	< 250	< 250 -	< 250
F	0	< 250 -	0	0	< 250	0	< 250	< 250	0 +	< 250	< 250	< 250
G	0 +	0	0	< 250	< 250	0	0	0 +	0	0 +	0	< 250
H	0	0	0	0	0	0	0	0	0	0	0	0

Report Date May 05 2011 7:10 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:28 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 5
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:30 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID

ID Result	Species ID: Pseudomonas aeruginosa
ID Organism Type	GN-Nent
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.596	0.596	5.843	GN-Nent	Pseudomonas aeruginosa
2	0.148	0.148	6.054	GN-Nent	Burkholderia ambifaria
3	0.133	0.133	6.149	GN-Nent	Pseudomonas alcaligenes
4	0.119	0.119	6.242	GN-Nent	Pseudomonas fluorescens

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
B	2	2	2	2	2	2	2	2	2	< 250	< 250 -	2
C	2	2	2	< 250 -	2	2	2	2	2	< 250	< 250	< 250 -
D	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
E	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
F	2	2 +	2	2 +	2	2	2	< 250 -	2	< 250	< 250	< 250
G	2 +	2	2	< 250 -	< 250 -	2 +	2	< 250	2	< 250	2	< 250
H	2	2	2	2	2	2	2	2 +	2	< 250 -	2	2

Report Date	Feb 23 2011 6:00 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:31 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 6
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:33 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID
ID Result	No ID
ID Organism Type	
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	---	0.107	11.076	GN-Ent	Cronobacter muytjensii
2	---	0.073	11.531	GN-Ent	Pantoea agglomerans bgp 2
3	---	0.062	11.724	GP-Rod-SB	Bacillus licheniformis
4	---	0.059	11.787	GN-Ent	Raoultella terrigena

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2 +	2 +	2 +	< 250 -	2 +	2	2 +	< 250	< 250	< 250
B	< 250	2	2 +	2 +	< 250 -	< 250	2 +	2	2	< 250	< 250	< 250 -
C	< 250	< 250 -	2 +	2 +	2	2	2	2	2	< 250	2 +	2
D	2	2 +	2	2	< 250 -	2	2	2	2	2 +	< 250	2 +
E	2	< 250 -	2	2	2	2	2	2	2	< 250	< 250	< 250
F	< 250	< 250	2	< 250	< 250	2	2	2	2	< 250	< 250	< 250
G	2	< 250	2	< 250 -	2	2	2	2	2	2	< 250	< 250 -
H	2	2	2	2	2	2	2	2	2	< 250	< 250	2

Report Date	Feb 23 2011 6:00 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:33 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 7
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Feb 23 2011 5:37 PM
Biolog ID DB	C:\Program Files (x86)\Biolog\DATABASE\Biolog GEN III 2_5_1_15.I5G
ID State	Final ID

ID Result	Species ID: Enterobacter amnigenus
ID Organism Type	GN-Ent
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.989	0.778	3.015	GN-Ent	Enterobacter amnigenus
2	0.006	0.004	6.265	GN-Ent	Enterobacter turicensis
3	0.004	0.002	6.577	GN-Ent	Enterobacter nimipressuralis
4	0.001	0.001	7.333	GN-Ent	Enterobacter cloacae ss cloacae

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	< 250	< 250	< 250	< 250	< 250	2	< 250	< 250	< 250	< 250
B	< 250	< 250	< 250	< 250	< 250	< 250	< 250	< 250	< 250	< 250	< 250	2 +
C	< 250	< 250	< 250	< 250	2	2	2	< 250	< 250	< 250	< 250	< 250 -
D	2	< 250	2	2	< 250	< 250	< 250	2	2	< 250	< 250	< 250
E	2	< 250	< 250	2	< 250	< 250	2	2	< 250	< 250	< 250	< 250
F	< 250	< 250	2	< 250	< 250	2	< 250	2	< 250	< 250	< 250	< 250
G	2	< 250	2	< 250	< 250	< 250 -	2	< 250	2 +	2	< 250	< 250
H	2	2	2	2	2	2 +	2	< 250	2	< 250 -	< 250	2

Report Date	Feb 23 2011 5:59 PM

ProgramName	MicroLog 3/5.2.01 33
ProjectName	ML5
DataFileName	1-8 seaweed strains.D5E
DataPath	C:\Program Files (x86)\Biolog\ML_52\
WSOperator	
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Feb 22 2011 7:37 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	0
Position	1-A
PlateType Int	GEN III
Protocol Int	A
Sample ID	strain 8
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	ID Not Searched
ID State	Final ID
ID Result	No ID Yet
ID Organism Type	
ID Comment	
ID Notice	Continue Incubation

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	< 250	< 250	2
B	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
C	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
D	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
E	2	2	2	2	2	2	2	2	2	< 250	< 250	< 250
F	2	2	2	2	2	2	2	2	2	< 250	< 250	2
G	2	2	2	< 250	2	2	2	2	2	2	< 250	< 250
H	2	2	2	2	2	2	2	2	2	< 250	2	2

Report Date Feb 23 2011 5:58 PM

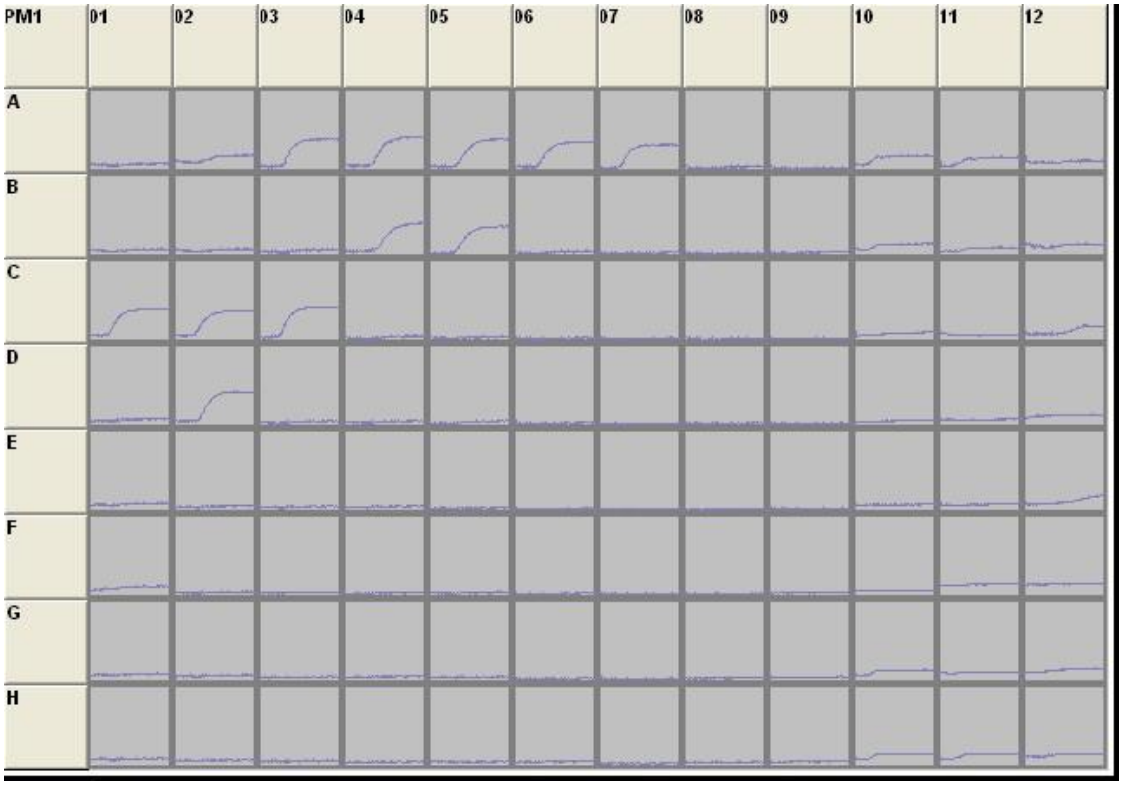

Figure 1 Strain 1 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

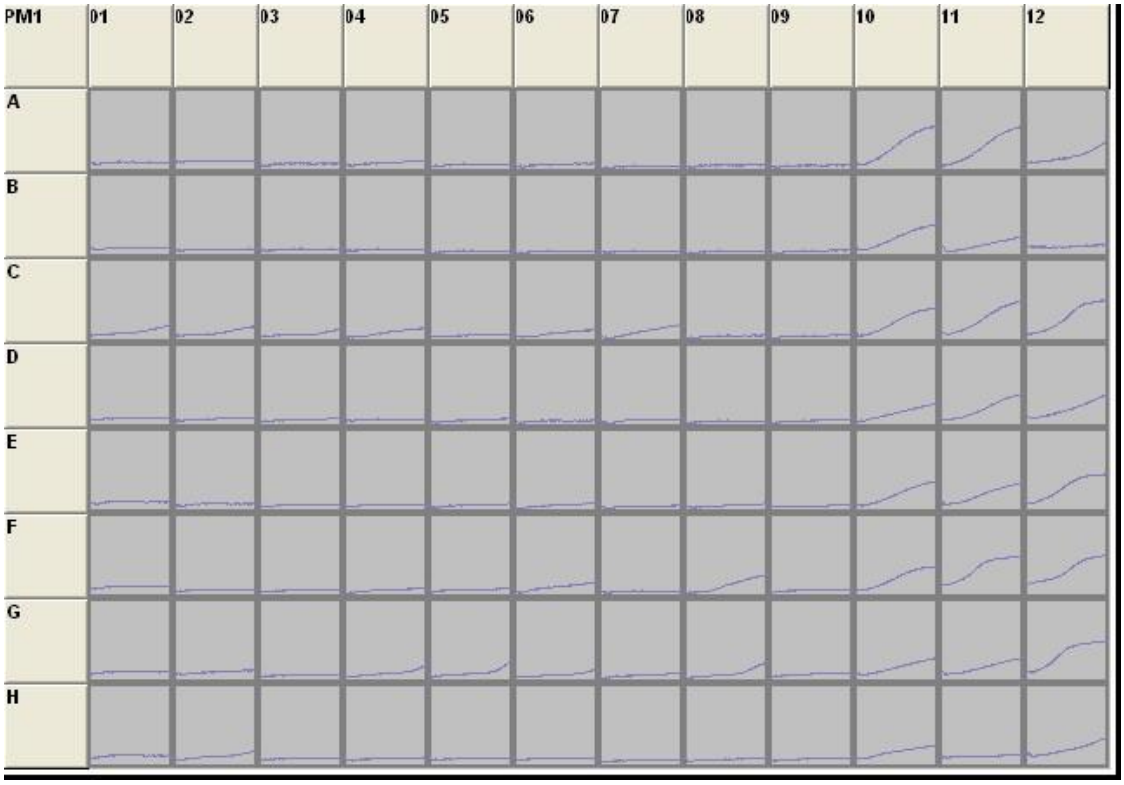

Figure 2 Strain 2 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

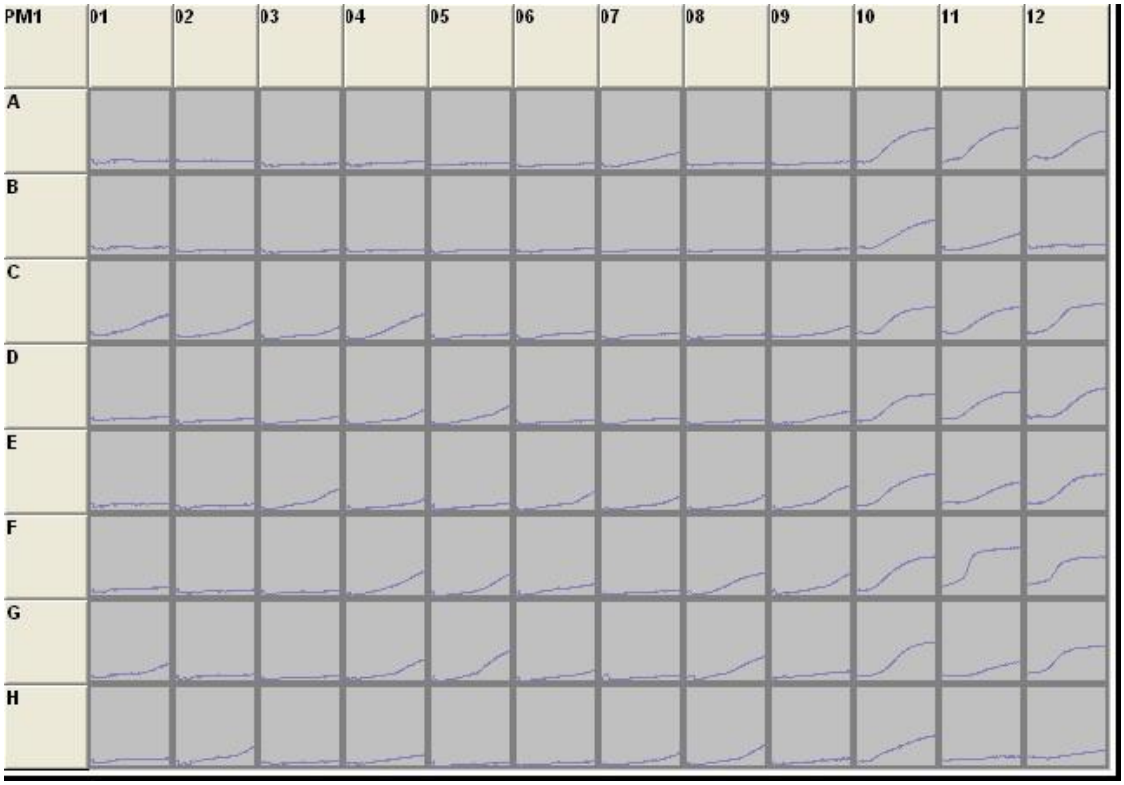

Figure 3 Strain 3 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

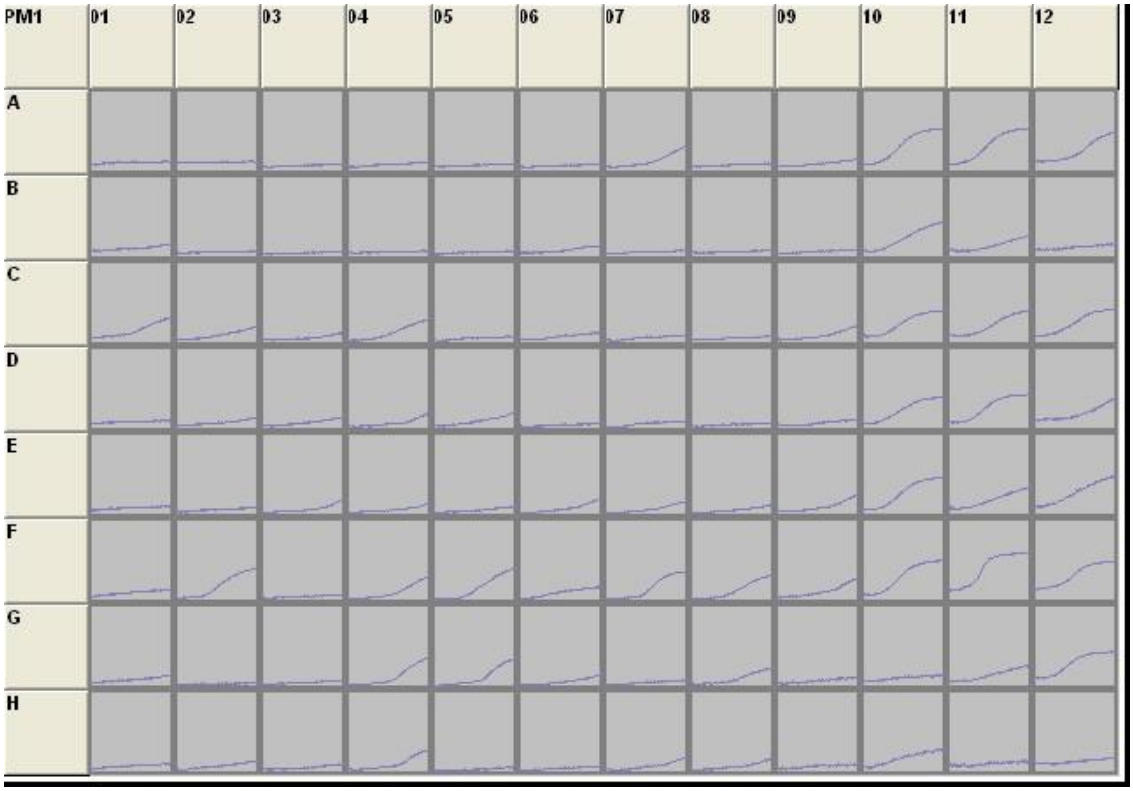

Figure 4 Strain 4 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

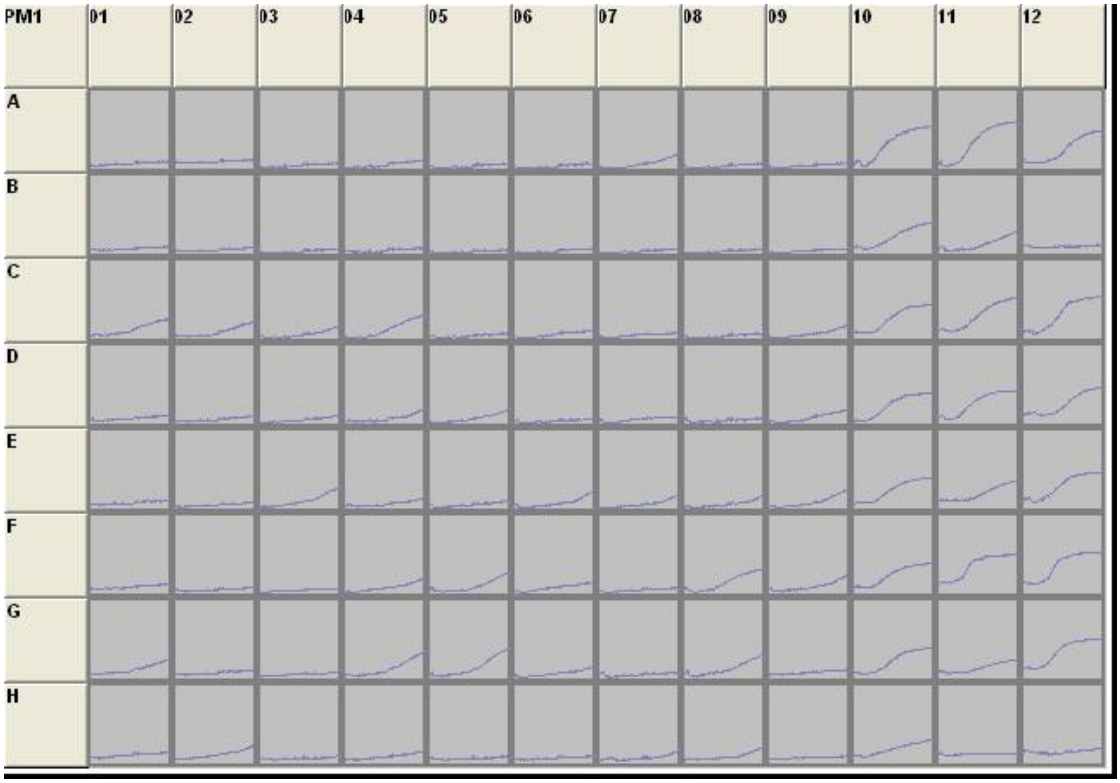

Figure 5 Strain 5 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

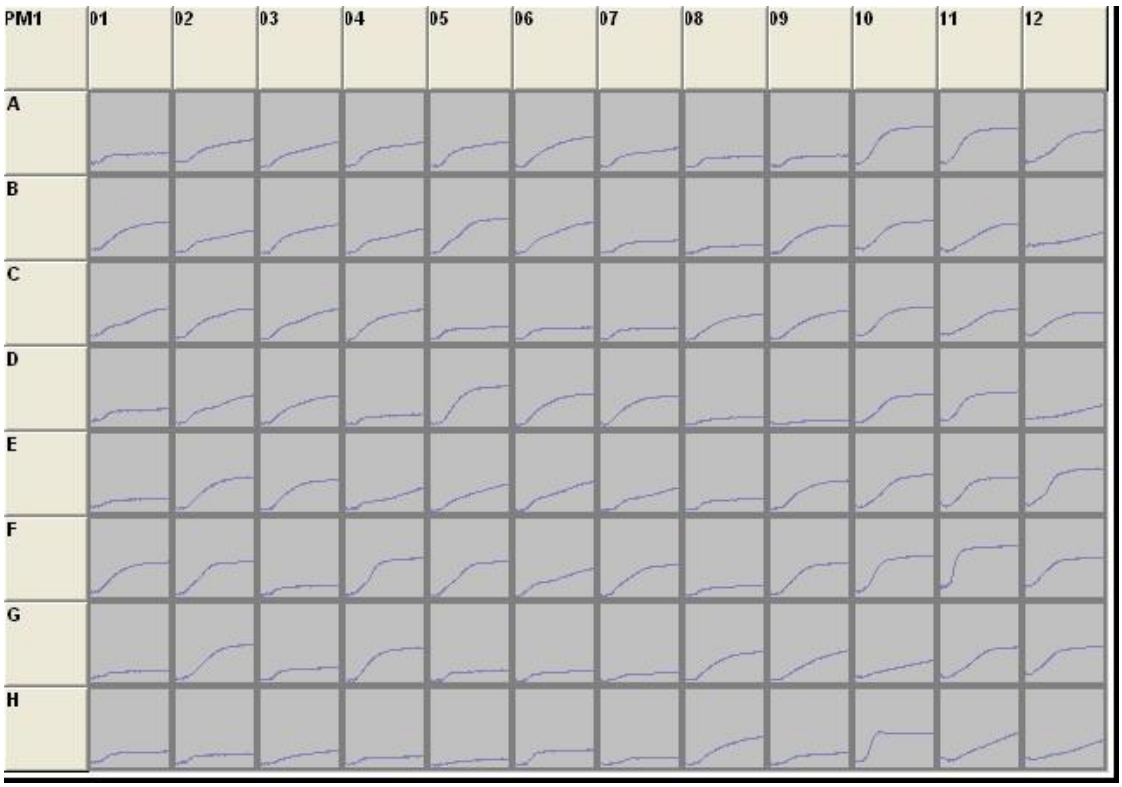

Figure 6 Strain 6 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

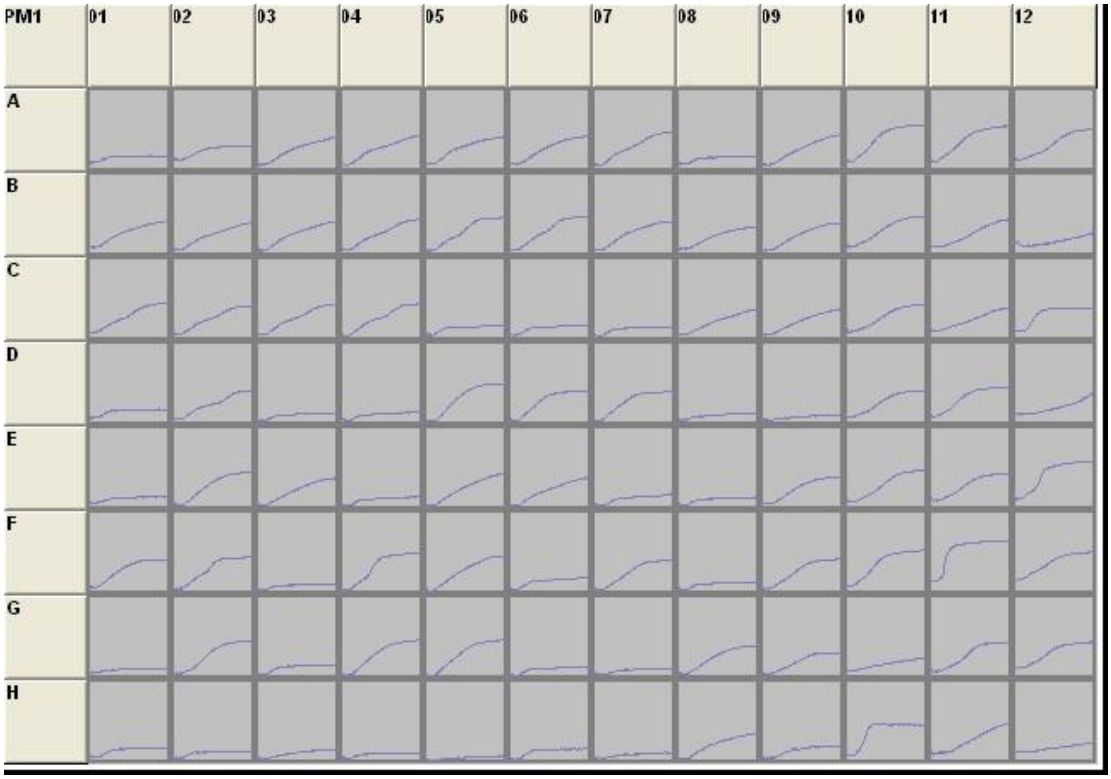

Figure 7 Strain 7 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

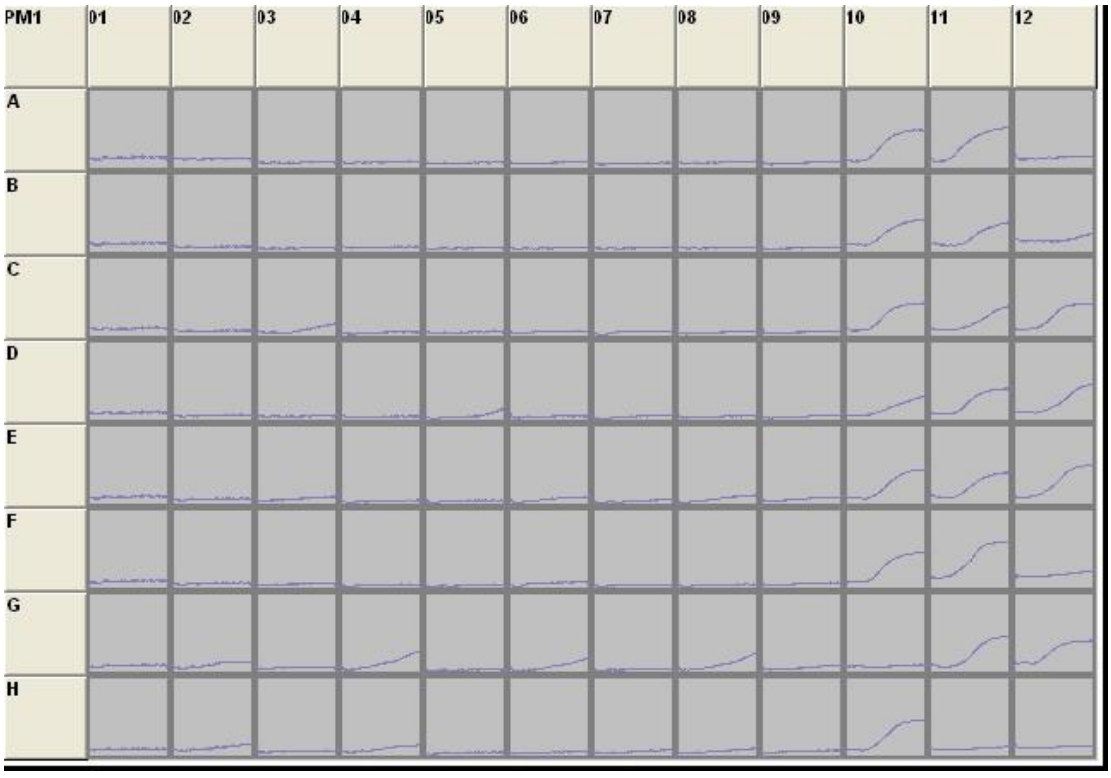

Figure 8 Strain 8 growth profiles in Biolog's Gen III microplate during 24 h of incubation at 33°C.

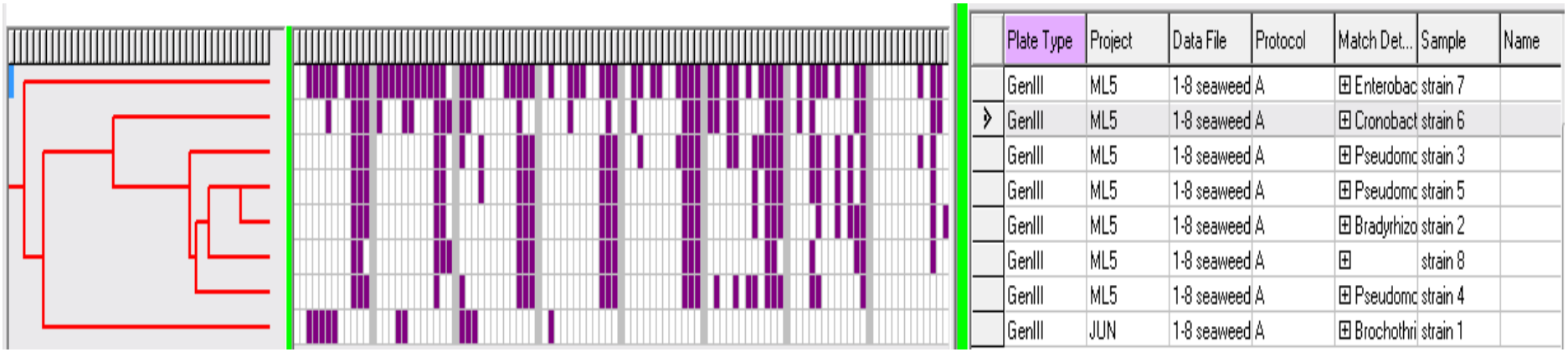

	Plate Type	Project	Data File	Protocol	Match Det...	Sample	Name
	GenIII	ML5	1-8 seaweed	A	⊞ Enterobac	strain 7	
▶	GenIII	ML5	1-8 seaweed	A	⊞ Cronobact	strain 6	
	GenIII	ML5	1-8 seaweed	A	⊞ Pseudomc	strain 3	
	GenIII	ML5	1-8 seaweed	A	⊞ Pseudomc	strain 5	
	GenIII	ML5	1-8 seaweed	A	⊞ Bradyrhizo	strain 2	
	GenIII	ML5	1-8 seaweed	A	⊞	strain 8	
	GenIII	ML5	1-8 seaweed	A	⊞ Pseudomc	strain 4	
	GenIII	JUN	1-8 seaweed	A	⊞ Brochothri	strain 1	

Figure 9 Clustering of dendrogram based on pearson distances, calculated using linearised dataset derived from biolog GenIII plate.