The United Nations University
FISHERIES TRAINING PROGRAMME

P.O. Box 1390, Skulagata 4
120 Reykjavik, Iceland

Final Project 2010

ANTIBACTERIAL EFFECT OF EXTRACTS FROM TWO ICELANDIC ALGAE (*Ascophyllumnodosum* and *Laminariadigitata*)

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ABSTRACT

The in vitro antimicrobial effect of seaweed extracts belonging to *Ascophyllumnodosum* and *Laminariadigitata* were tested against some pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candidaalbicans*). In this study, paper disc diffusion assay, absorbance measurements and growth studies were used to elucidate the effects. The organic solvent extracts of *A. nodosum* and *L. digitata* exhibited high eranti bacterial activity than water or enzyme extracts against all the test microorganisms. Higher inhibitory effect of organic solvent extracts was found against Gram-positive bacteria than Gram-negative bacteria. *Staphylococcus aureus* and methicillin-resistant *S.aureus* (MRSA) were the most sensitive test microorganisms. Acetone extracts of *A. nodosum* showed the strongest antimicrobial activities. The results of the present study indicate the potential use of Icelandic algal extracts as a source of antimicrobial compounds.

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1 INTRODUCTION

1.1 Background

Seaweeds are an important cash crop. They have been used as food stuff in Asian diets for centuries as they contain carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals (Norziah and Ching 2000). Marine algae are also widely exploited as industrial raw material. There are three main phyla, or divisions of algae; brown algae (Phaeophyta), red algae (Rhodophyta), and green algae (Chlorophyta) (Gamal 2010). The chemical composition of seaweed varies and is affected by the species, geographic area, seasons of the year and temperature of the water.

China is the biggest seaweed producing country. In 2005, global seaweed production was 18.6 million tons (FAO 2010). Total seaweed production in China was 14.5 million metric tons, which is 78% of the global output (FAO 2010). In 2009, China exported 50.6 thousand tons of seaweed raw materials and various types of seaweed food, whose export value totalled 165 million US dollars (Li 2010). This accounted for a quarter of the global market. In China, seaweeds are widely used in medicine, food, chemical, spinning and cosmetic industry. Seaweeds are made into seasoning, snack food and additives. Some important industrial raw materials can be extracted from algae, for example iodine, alginate, agar, carrageenan, mannitol and fucoidan. Some seaweed is of medicinal value as components of antibiotics, laxatives, anticoagulants, anti-ulcer products and suspending agents in radiological preparations.

In order to make still a better use of seaweed, people take effort to isolate some high value compounds from it. There is a growing interest to find new natural preservatives for food and other perishable products. Also the increased frequency of pathogenic bacteria resistant to traditional antibiotics has resulted in search for novel antibacterial compounds. As many components of seaweed have potential antimicrobial activity, they are now in the spotlight for natural product discovery. Furthermore, seaweed is a cheap and easy available raw material which is not the case for most other potential source of marine natural products.

Iceland has rich seaweed resources. The situation of Iceland as a boundary between the warm-boreal zones (Atlantic Ocean influence) and the subarctic zones (Arctic influence) provide a good condition for the growth of algae. The seaweed grows in the “photic zone” and they can be very pronounced in the intertidal zone. Iceland has many places favourable for algal growth i.e. Breiðafjördur. However, the commercial utilisation of seaweed in Iceland is very limited and mainly it is harvested for the production of alginate and less for agricultural usage (i.e. feed and fertilizer). It is known that Icelandic seaweed contains some anti-oxidative compounds, however, it is not known whether Icelandic seaweed also contain antibacterial substances.

1.2 Research question

Does Icelandic seaweed contain some antibacterial substances and can they be easily extracted?

1.3 Goal and objectives

The aim of this study is to extract bioactive compounds using different solvents and search for antibacterial activity in the extracts.

The specific objectives are:

-To extract antibacterial substances from algae using organic solvent and investigate its antibacterial activity against pathogenic microbes.

-To extract antibacterial substances from algae using water or commercial enzymes and investigate its antibacterial activity against pathogenic microbes.

2 LITERATURE REVIEW

2.1 Overview of algae

Algae are a large and diverse group of chlorophyll bearing, simple, photosynthetic, thalloid organisms largely aquatic with no differentiation of true roots, stems and leaves, belonging to the division Thallophyta (NCERT 2008). Algae are classified into three classes: Chlorophyceae, Phaeophyceae and Rhodophyceae (Gamal 2010).

Algae are mainly aquatic found in both marine water and fresh water. Algal habitats include terrestrial, such as wet rocks and moist soil and sub aerial, like tree barks; they can flourish at low temperatures. Some algae are found in symbiotic association with fungi (*Lichens*) and animals e.g. sloth bear (NCERT 2008).

Algae may be free floating such as *Chlamydomonas* or they can be attached to substratum e.g. *Ulothrix* and *Sargassum*. Algae have different form and size from unicellular (e.g. *Chlamydomonas*) to multi cellular (e.g. *Laminaria*), colonial (e.g. *Volvox*) to filamentous forms (e.g. *Ulothrix*) and microscopic to massive plant bodies (NCERT 2008). Algae are covered by mucilage just as other aquatic plants. Algae lack vascular tissues and mechanical tissues because being aquatic, they do not require water conduction and buoyancy keeps them upright in water (NCERT 2008).

Reproduction of algae can be vegetative, asexual and sexual. Vegetative reproduction takes place through fragmentation. A sexual reproduction in algae is accomplished by spores called zoospores, which are of two types: mitospores and meiospores. Sexual reproduction involves isogamy, anisogamy and oogamy (NCERT 2008).

Algae can be used as food and food supplement for humans and they are primary producers for aquatic animals. Some marine algae also produce hydrocolloids (water holding substances) such as algin (from *Laminaria*), carrageenan (from *Chondrus*) and agar (from *Gracilaria* and *Gelidium*) which can be used in production of variety of commercial products. Algae can photo synthesize, fix $CO_2$and they release enormous amount of oxygen.

2.2 Biological activity of algae

Seaweeds have been used as food mainly in Asia and crude drugs for the diseases such as iodine deficiency for centuries. Also, some species of seaweeds have been used as a source of

additional vitamins, as medical agents for hyper glycaemia and hypo glycaemia, as vermifuges and insecticides (Gamal 2010).

Marine organisms, including algae, are novel and unexplored sources of potentially useful bio-active compounds that might represent useful leads in the development of new pharmaceutical agents (Iwamoto *et al*., 2001). During the last several decades, many novel compounds have been isolated from seaweeds and some of these substances have been identified to possess interesting biological activities. Some examples of different bio-activities derived from algae are given in the following sections.

2.2.1 Antioxidative activity

Antioxidants can protect human health against damage by reactive oxygen species (ROS) (Matanjun *et al*., 2008). Some compounds from marine algae have antioxidative activity such as polysac charides, polyphenolic compounds. The antioxidative activities of sulphated polysaccharide derived from algae have been determined by several researchers using various methods (Wijesekara *et al*., 2011). The antioxidative activities of polyphenolic compounds from marine macro algae have also been well investigated. Different types of polyphenolic compounds (e. g. catechins, flavonols and flavonol glycosides) have been isolated from organic solvent extracts of several brown and red algae species (Santoso *et al*., 2004; Yoshie-Stark *et al*., 2003). The antioxidant activities of different seaweed species show great variety. Brown algae normally possess better antioxidative activity than green and red algae due to its higher levels of total phlorotann in content (TPC). Some species of brown seaweeds have been reported by several researchers to exhibit superior antioxidant activity in vitro (Wang *et al*., 2009b).

Nakai *et al.,* (2006) investigated the antioxidative activities of phlorotannins derived from 25 common Japanese marine algae species. They reported that 50% ethanol extracts of the brown seaweed *Sargas sumring goldianum* showed the highest radical scavenging activity. Wang *et al.,* (2009a) evaluated antioxidant activities of 10 Icelandic seaweeds using various antioxidant assays. The results showed high correlation between TPC of seaweed extracts and their scavenging capacity against DPPH (1,1-diphenyl-2-picryl hydrazil) and peroxyl radicals, which indicated an important role of seaweed polyphenols as chain-breaking antioxidants.

2.2.2 Cytotoxic activity

The cytotoxic activities of organic solvent extracts of three brown algae species (*Sargassumswartzii*, *Cystoseiramyrica*, *Colpomeniasinuosa*) were investigated by Khanavi *et al.,* (2010). The results showed that extracts had some effects on different cancer cells. Two novel trihydroxylated diterpenes were isolated from the brown algae *Bifurcaria bifurcate* by Culioli *et al.,* (2004). Both compounds were tested in vitro for their cytotoxicity and proved to be active against the NSCLC-N6 cell line. Two novel cyclized merodit erpenoid satomarianones derived from the brown algae *Taoniaatomaria* were found to exhibit significant cytotoxic activity against two lung cancer cell lines (Abates *et al*. 2005). De Inés *et al.,* (2004) investigated the cytotoxic effects of nine halogenated monoterpenes isolated from the red algae *Plocamiumcartilagineum* against different tumour cell lines. The results showed that some compounds had strong and interesting cytotoxic activities.

2.2.3 Antiviral activity

Several different compounds derived from algae show antiviral activities towards some viruses responsible for human infectious diseases. These substances include sulphated polysac charides, fucoidan, sulfoglycolipids, carrageenans, sesquiterpene hydroquinones, etc. As demonstrated by Barbosa *et al*., (2004), three dollabella diene derivatives from the brown algae *Dictyotapfaffi* showed strong anti-HSV-1 activity *in vitro* and one compound also inhibited the reverse transcriptase enzyme of HIV-1. A new sulfolipid has been isolated from marine red algae, *Gigartinatenella* (Ohata *et al*., 1998), as a potent inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1.

According to the findings of Witvrouw *et al*., (1994) and Damonte *et al*., (1994), sulphated polysaccharides from seaweeds *Aghardhiella tenera* and *Nothogenia fastigiata* show antiviral activities against human immunodeficiency virus, Herpes simplex virus, human cytomegalo virus and respiratory syncytial virus. Carlucci *et al*., (1997) reported that carrageenan isolated from the red seaweed *Gigartina skottsbergii* showed antiviral activity against herpes simplex virus types 1 and 2. Feldman *et al.,* (1999) isolated three fractions of fucoidan from the brown seaweed *Leathesiadifformis* and investigated their antiviral activities. They reported that these compounds showed selective antiviral activities against herpes simplex virus types 1 and 2 and human cytomegalo virus.

2.2.4 Anticoagulant activity

Some anticoagulant compounds have been found in algae. Fucoidans isolated from seaweed *Fucusevanescens* and *Laminariacichorioides* kelp can inhibitthrombin and factor Xa of the blood coagulation system (Drozd *et al*., 2006). Pushpamali *et al.,* (2008) isolated an anticoagulant polysac charide from the red algae *Lomentariacatenata* and assayed its anticoagulant activity using the methods of activated partialthromboplast in time, prothrombin time, and thrombin time. The results showed that the isolated compound may act on the intrinsic and/or common pathways of the blood coagulation system. The acidic polysaccharide from the brown algae *Laminariacichorioide* was isolated and tested the anticoagulant activity by Yoon *et al.,* (2007). The result showed that sulfatedfucan from *L. cichorioides* is apromising anticoagulant polysac charide and a possible alternative for an antithrombotic compound due to its preferential heparinco factor II-dependent activity.

2.2.5 Anti-inflammatory activity

Awad (2000) evaluated anti-inflammatory activity of 3-O-beta-D glucopyranosyl-stigmasta-5,25-dien which was isolated from the marine green algae, *Ulvalactuca.* A chlorophyll-related compound, pheophytin a, has been identified from an edible green algae, *Enteromorphaprolifera* by Okai and Higashi-okai (1997). The anti-inflammatory effects of pheophytin a have been analysed using *in vitro* and *in vivo* experiments and the results suggest it has a potent anti-inflammatory activity. A new phlorotannin called phlorofucofuroeckol-B was isolated from the brown algae *Eisenia arborea* by Sugiura *et al.,* (2006). The compound was found to have an inhibitory effect on histamine release from rat basophile leukemia (RBL)-2H3 cells.

2.3 Antibacterial substances from algae

It is well known that the pathogenic microbes (for example *Pseudoalteromonasporphyrae* (Liu *et al*., 2011), *Pseudomonas elongate* (Khambhaty and Mody 2007), *Bacillus polymyxa* (Chesters and Bull 1963), *Deleya marina* (Kraiwattanapong *et al*., 1999) in the oceanic ecosystem can devastate populations of sea weeds. Yet, these sessile organisms suffer remarkably low levels of microbial infection, despite lacking cell-based immune systems. Sea weeds might use targetedanti microbial chemical defence strategies by eliciting secondary metabolites, which are important in ecological interactions between marine macro organisms and microorganisms (Kubanek *et al.,* 2003). Therefore, seaweeds could be a promising source of novel bio-active compounds that can help plant survival by offering protection against stress imposed by the environment.

Algae have recently received significant attention in the search for bioactive compounds to develop new drugs and health foods. Many compounds of marine algae show anti-bacterial activities such as polysaccharide (Laurienzo 2010), lyengaroside (Ali *et al*., 2002), polyhydroxy lated fucophlorethol (Sandsdalen *et al*., 2003), bromophenols (Oh *et al*., 2008), guaianesesquiterpene (Chakraborty *et al*., 2010), lactone malyngolide (Cardelina *et al*., 1979), cycloeudesmol (Sims *et al*., 1975), polyphenolic compound (Devi *et al*., 2008), halogenated compound (Vairappan 2003) and quinone metabolite (Horie *et al*., 2008). Antibacterial activities of compounds derived from algae have also been extensively studied by several other researchers (Salvador *et al*., 2007; Rajasulochana *et al*., 2009; Seenivasan *et al*., 2010).

The antibacterial substances in algae are usually extracted by water (Bansemir *et al*., 2006) or organic solvents such as methanol, ethanol, acetone, ethyl ether, diethyl ether, ethyl acetate, chloroform, dichloromethane, benzyne, hexane, chloroform: methanol (2:1), and chloroform: ethanol (1:1, 2:1). The antibacterial assays are usually done by paper disc diffusion assay, absorbance measurements and growth studies.

2.4 Isolation of algae-lytic bacteria

Seaweed contains a significant amount of proteins, vitamins and minerals essential for human nutrition. In order to get more valuable bio-active substance from algae, enzymes are also used for the degradation of algae. Besides the application of commercial enzymes such as amylase, more and more researchers are seeking novel microbial enzymes.

Alginate has a wide range of applications; further, the degraded low-molecular fragment shows more potential. In recent years, marine microbial alginate lyases have been greatly developed. A novel alginate lyase with high activity on acetylated alginate was isolated from *Pseudomonas* sp. QD03 (Lin *et al.,* 2006).

Agar is a highly heterogeneous polysaccharide. Nowadays, the acid degradation of agar is replaced by enzymatic degradation with the advantages of easy control and mild reaction. In 1902, Gran isolated agar-degrading *Pseudomonas galatica* from seawater. Until now, researchers have found the presence of agar are from species within the genus *Cytophaga, Bacillus, Vibrio, Alteromonas, Pseudoalteromonas, Streptomyces* (Aoki *et al*., 1990; Leon *et al*., 1992; Hosoda *et al*., 2003). Two isolates of agar-degrading bacteria were screened from the coastal water of India (Ghadi *et al*., 1997).

Carrageenan and fucoidan are the main components of marine sulphated polysaccharides. Renner *et al.*, (1998) isolated an extracellular κ-carrageenase with a molecular weight of 30 kD from marine *Cytophaga* MCA-2. A distinct λ-Carrageenan-degrading *Pseudoalteromonas* bacterium (CL19) was isolated from a deep-sea sediment sample by Ohta and Hatada (2006).

3 MATERIALS AND METHODS

3.1 Algae samples

Dried and ground brown seaweed (*Ascophyllumnodosum* and *Laminariadigitata*) were obtained from the company Thorverk, 380 Reykhólar, Iceland.

3.2 Preparation of extracts

The finely ground samples were weighed and 5 g were mixed with 250ml of various solvents (1:50, w/v); 100% ethanol, 100% methanol, 100% acetone and water, respectively. The mixtures were kept for four days at room temperature and mixed at regular intervals. After four days the samples were filtered using Whatman filter paper No. 1 to separate the filtrate and the extracts were freed from solvent by evaporation in a fume hood.

3.3 Degradation of algae by commercial enzymes

500 ml of buffer solution (Table 1) was added to 5 g of dried algae, and then 500mg of the enzyme was added. The enzymatic hydrolysis was performed for 24 hours at 50^{o}C and pH 4.5. Then, each sample was clarified by centrifugation at 5000 × g for 20 minutes (SORVAIL Legend Mach 1.6 R) to remove the unhydrolyzed residue. Enzymatic extracts of seaweed were obtained after filtering the supernatant and lyophilizing (ALPHA 1-4，Christ).

Table 1: Optimum hydrolyzation conditions of particular enzymes.

Enzyme	Optimum condition		Buffer used	Enzyme composition	Commercial sources
	pH	Temp.			
Cellulase	4.5	50	0.1 N AB	Cellulase A “Amano” 3	Amano Enzyme Inc, Japan
Hemicellulase	4.5	50	0.1 N AB	Hemicellulase “Amano” 90	Amano Enzyme Inc, Japan

AB: Acetate buffer;

3.4 Test microorganisms used

The inhibitory effects of extracts were carried out on six species of food pathogenic bacteria, namely:

Gram-positive bacteria
Staphylococcus aureus (DSM 1104 and 2569，SA)
Listeria monocytogenes (LM)
Gram-negative bacteria

Escherichia coli (DSM 1103, EC)
Pseudomonas aeruginosa (PA)

Yeast
Candidaalbicans (CA)

The test bacterial pathogen cultures were obtained from stock cultures maintained in the Microbiology Laboratory, University of Akureyri.

The strains were activated before the antibacterial test. After removal from the refrigerator, strains were incubated overnight in nutrient broth (BD Difco), and then streaked on nutrient agar plate and kept for 24 hours at 37^{o}C. One colony from the plate was picked up for incubating on agar slant tubes at 37^{o}C.

3.5 Antibacterial activity test

The crude extracts were dissolved in the corresponding solvent for the antibacterial activity test. Antibacterial activity was assayed using a paper disc diffusion assay, absorbance measurements and growth studies.

3.5.1 Confirmation of positive control for Listeria monocytogenes

Four common antibacterial agents, Erythromycin, TetracyCline, Rifamycin and Chloramphenicol were compared to confirm the positive control for *Listeria monocytogenes*. *Listeria monocytogenes* was inoculated in nutrient broth and kept overnight at 37^{o}C. Then, 0.1 ml of culture was spread on the surface of Mueller Hinton Agar (DIFCO) plate. Antibioticper paper discs used were Erythromycin 15 µg, TetracyCline 30 µg, Rifamycin 5 µg and Chloramphenicol 30 µg. The plates were incubated for 24 hours at 37^{o}C. The clear zone around the discs was measured to verify the inhibition of the bacteria.

3.5.2 Paper disc diffusion assay

Mueller Hinton Agar Medium (MHA–BD Difco) was prepared and sterilized by autoclaving at 121^{o}C and 15lbs pressure for 15 minutes. 20 ml of the sterilized media was poured into a sterilized Petri dish and allowed to solidify at room temperature.

Each extract was dissolved in 5 ml (30-50 mg/ml) of the corresponding solvent and 0.6-1 mg was applied to sterile filter paper discs (6mm). Absorption of extracts per paper disc was 20µl. The discs were placed on to the agar plates inoculated with an 18 hour culture of the test pathogen (10^6 bacteria/ml) in nutrient broth. A disc load with a certain commercial antibiotic, such as Erythromycin, TetracyCline, Rifamycin and Chloramphenicol, was prepared as a positive control, and a disc load with only corresponding solvent was similarly prepared as a negative control. The plates were incubated for 24 hours at 37^{o}C.

The zone of inhibition of bacteria around the disc was measured and the assay was scored positive (+) if the zone was< 2 mm, doubly positive (++) if ≥ 2 mm, triple positive (+++) if ≥ 7 mm, and negative (–) if no zone was visible.

3.5.3 Microbial growth experiment (absorbance measurements and counting test)

The microbial growth in microplates was studied according to the method used by Eyþórsdóttir A. (2007). Growth studies were carried out in a Bioscreen microplate reader (Growth Curves Ltd, Finland), using 100-wells flat bottom honeycomb microplates. Eight extracts from the two algal species were used with 5 test strains, to find out whether the samples inhibited bacterial growth. Two different final concentrations of extracts were prepared in nutrient broth: 5% and 1%, respectively. Fresh cultures (16 – 20 h) of 5 test strains: *S.aureus* (DSM2569), *L.monocytogenes*, *E. coli*, *P.aeruginosa* and *C.albicans* were used as inoculates, where 0.1 ml of diluted culture was added to each of the extracts supplemented nutrient broth. The dilution was aimed at a final culture density of 10^3– 10^4 cells/ml. These mixtures were used as samples for the microplates. The microplate wells were loaded with 400 µl of samples for each test strain. Corresponding solvents were used as a control for all the concentrations and all the strains in duplicates and a control sample with pure nutrient broth was also run. Cultures of each test strain in nutrient broth served as negative controls (control cultures). The growth studies were carried out at 37^{o}C with optical density (OD) reading at 600nm at 30 minute intervals for 72 hours. The OD readings recorded for pure nutrient broth medium were subtracted from all measurements (stable values of around 0.11 over the whole experiment) and plotted on the growth graphs.

Growth studies on the bacteria *S.aureus* (DSM2569), *L.monocytogenes*, *P.aeruginosa* were also done by plate count method. According to the former results, two samples of acetone extracts were selected for this experiment. Fresh cultures (18 h) of strains were used as inoculates, where 0.1 ml of diluted culture was added to each of the extracts supplemented nutrient broth. The dilution was aimed at a final culture density of 10^3–10^4cells/ml. The concentration of extracts in nutrient broth was 300µg/ml. Control of 1% acetone in nutrient broth was used and cultures of each test strain in nutrient broth served as negative controls (control cultures). At 0, 4, 8, 12, 24, 48, and 72 h, samples were taken and diluted. Then, 0.1 ml samples were spread on the surface of agar and the plates were incubated for 24 hours at 37^{o}C. A spiral system (Eddy-Jet IUL-Instruments, Barcelona) was used to inoculate the plates and an automatic colony counter (Countermat-Flash IUL-Instruments, Barcelona) for counting.

4 RESULTS

4.1 Preparation of extracts

Different methods were tried to extract compounds from algae. The yield of dry material obtained by different extraction methods was compared. In general, the largest amount of dry material in all samples was obtained by the enzyme method. A larger amount of dry material was obtained by water extraction than by organic solvent extraction. Extraction efficiency of enzymes was about 30% (1.5 g dry material from 5 g dry algae material), far more than the 10-12% obtained by water and the 3-7% by organic solvent extraction.

The yield of methanol extracts was highest in all the organic solvent extracts, and acetone extracts had the fastest evaporation and was easiest-dissolve. The extract amounts from *Laminariadigitata* were always greater than from *Ascophyllumno dosum* in all the extractions, probably because of their different particle size.

4.2 Confirmation of positive control for Listeria monocytogenes

The results showed that commercial antibiotics Erythromycin, TetracyCline and Rifamycin can strongly inhibit the growth of *Listeria monocytogenes*. The diameters of the zone of inhibition were about 2 cm. There was no obvious difference among these three commercial antibiotics. Chloramphenicol, however, did not exhibit any inhibitory effect on *L.monocytogenes*. TetracyCline was chosen as the positive control for *L.monocytogenes.*

4.3 Paper disc diffusion assay

Twelve extracts from two algal species, *A.nodosum* and *L.digitata*, were tested for antibacterial activity against the six pathogens. An example of results from the paper disc diffusion test can be seen in Figure 1. Big inhibition zones are seen around the filter paper disc impregnated with organic solvent extracts of *A. nodosum*. Erythromycin was used as positive control. There are small and clear inhibition zones around the paper disc impregnated with organic solvent extracts of *L. digitata* and water solution extracts of both algae species. The enzyme extracts of both algae species and the pure organic solvent used as negative control did not show any antibacterial activities (results not shown in Figure 1). A summary of the results are given in Table 2.

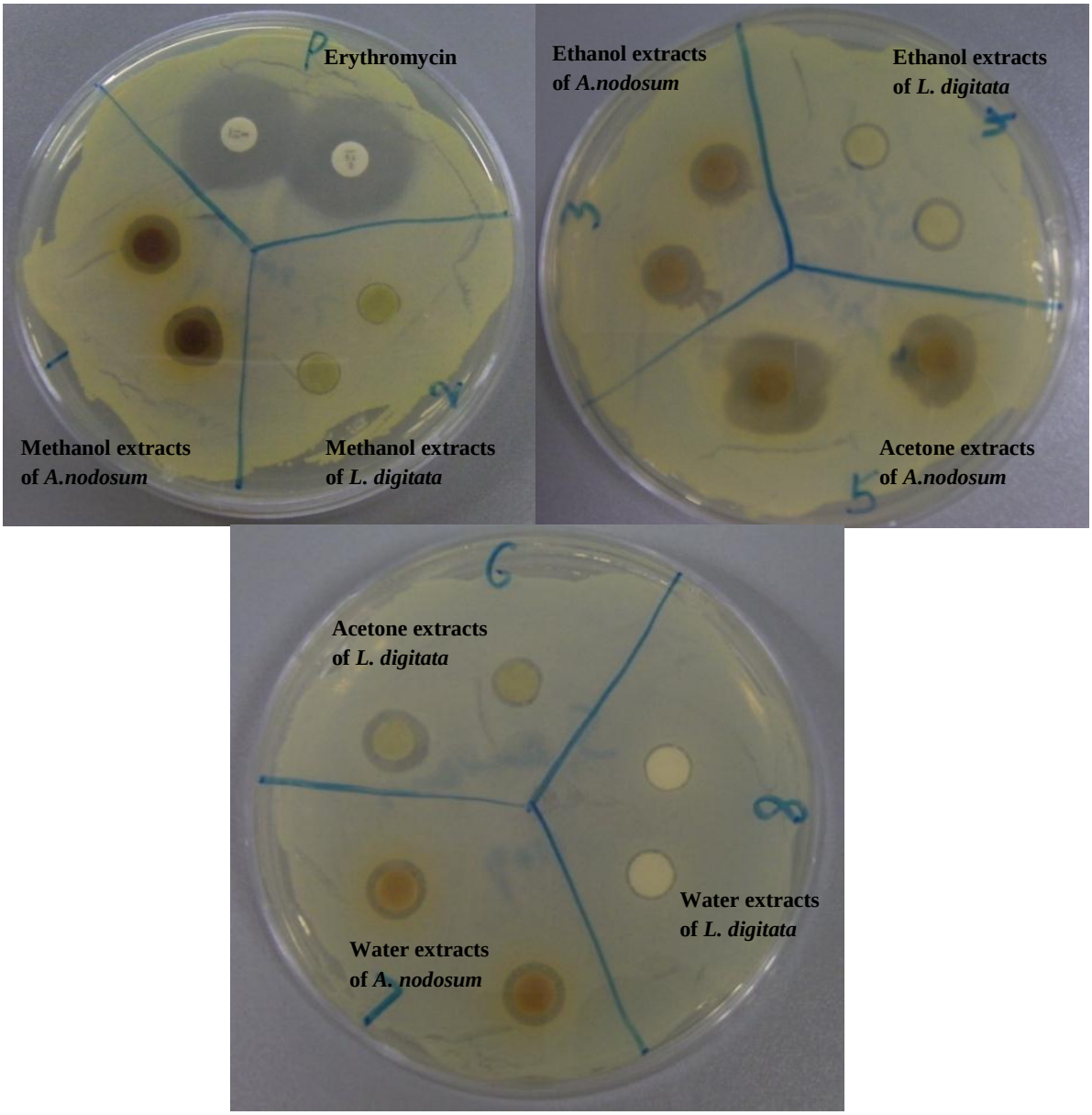


Figure 1: Disc diffusion test of algae extracts on *Staphylococcus aureus* (DSM 2569).

Generally, stronger antibacterial activities were found in *A. nodosum* extracts and the organic extracts exhibited far better antibacterial activity than the aqueous extracts. The algae extracts showed stronger activities against the yeast (*C.albicans*) and Gram-positive bacteria (*S.aureus* and *L.monocytogenes*) than Gram-negative bacteria (*E. coli*and *P.aeruginosa*). The acetone extract of *A. nodosum* exhibited promising inhibition effects against *C.albicans, P.aeruginosa* and *S.aureus*. Significant activity against *L.monocytogenes* was shown by methanol extract of *A. nodosum*.

Water and enzymes (hemicellulase and cellulase) were not found to be suitable for extracting antibacterial materials from the two algal species. Water extracts of *A. nodosum* showed a noticeable inhibition zone against two strains of *S.aureus*, while water extracts of *L. digitata* only exhibited poor activity against *S.aureus* (DSM 2569). The enzyme extracts of both species of algae showed no antimicrobial activity except for a slight inhibition of *S.aureus*.

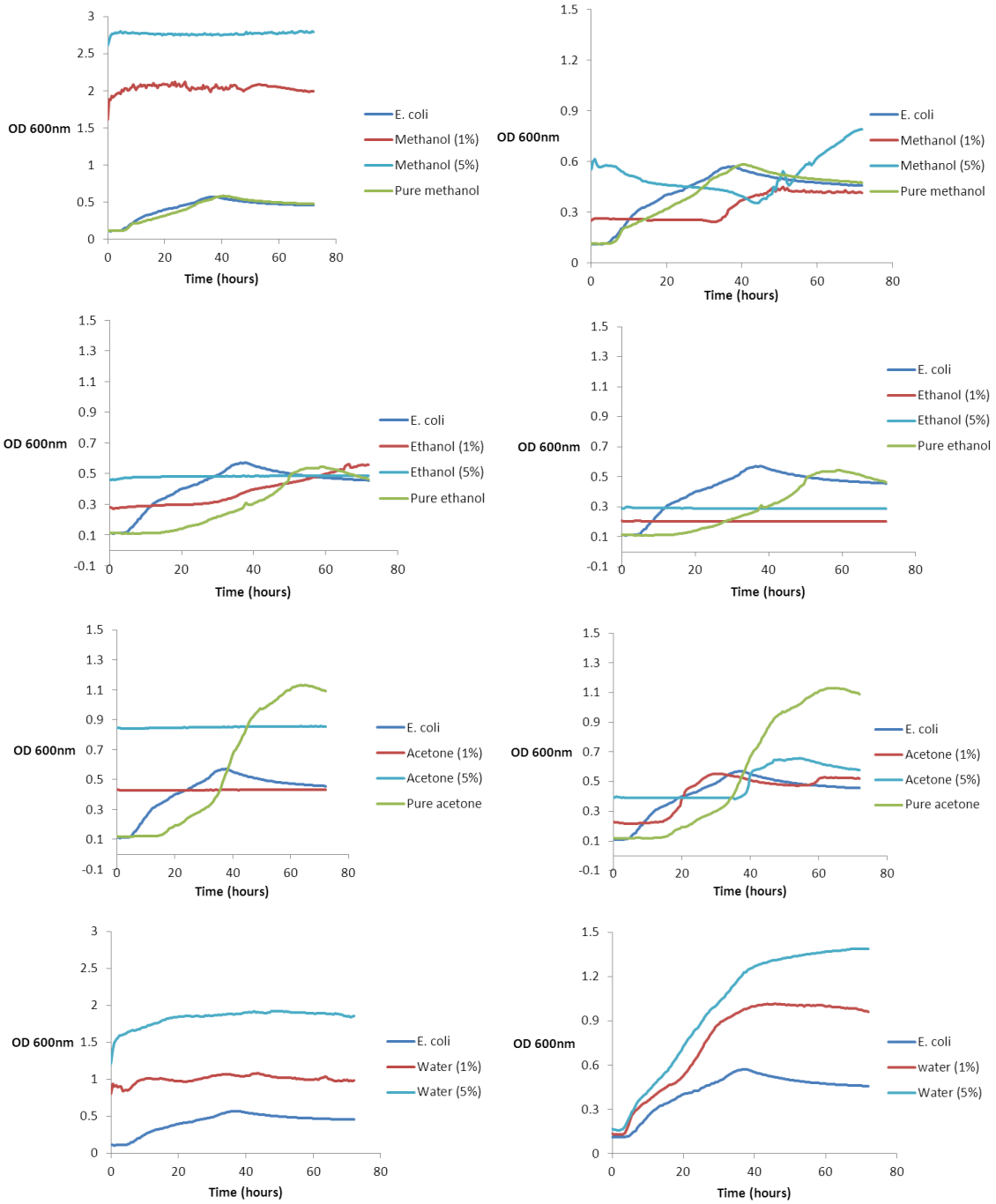
Table 2: Antibacterial activity of crude extracts of algae.

Organism	Solvent, enzyme of extraction/Code	Activity against pathogen					
		SA 2569	SA 1104	LM	EC	PA	CA
Ascophyllumn odosum	Methanol / 1	++	++	+++	+	+	++
	Ethanol / 3	++	++	++	+	+	+
	Acetone / 5	+++	+++	++	+	++	+++
	Water / 7	++	++	—	—	—	—
	Cellulase / 9	+	+	—	—	—	—
	Hemicellulase / 11	+	+	—	—	—	—
Laminariadigitata	Methanol / 2	+	+	+	+	+	+
	Ethanol / 4	+	++	++	+	+	++
	Acetone / 6	++	++	+	+	+	++
	Water / 8	+	—	—	—	—	—
	Cellulase / 10	—	—	—	—	—	—
	Hemicellulase / 12	—	—	—	—	—	—

SA = *Staphylococcus aureus*; LM = *Listeria monocytogenes*; EC = *Escherichia coli*; PA = *Pseudomonas aeruginosa*; CA = *Candida albicans.* (+) equals low activity (<2 mm halo); (++) equals moderate activity (2-7 mm halo); (+++) equals high activity (≥ 7 mm halo);(–)equals no visible zone.

4.4 Absorbance assay

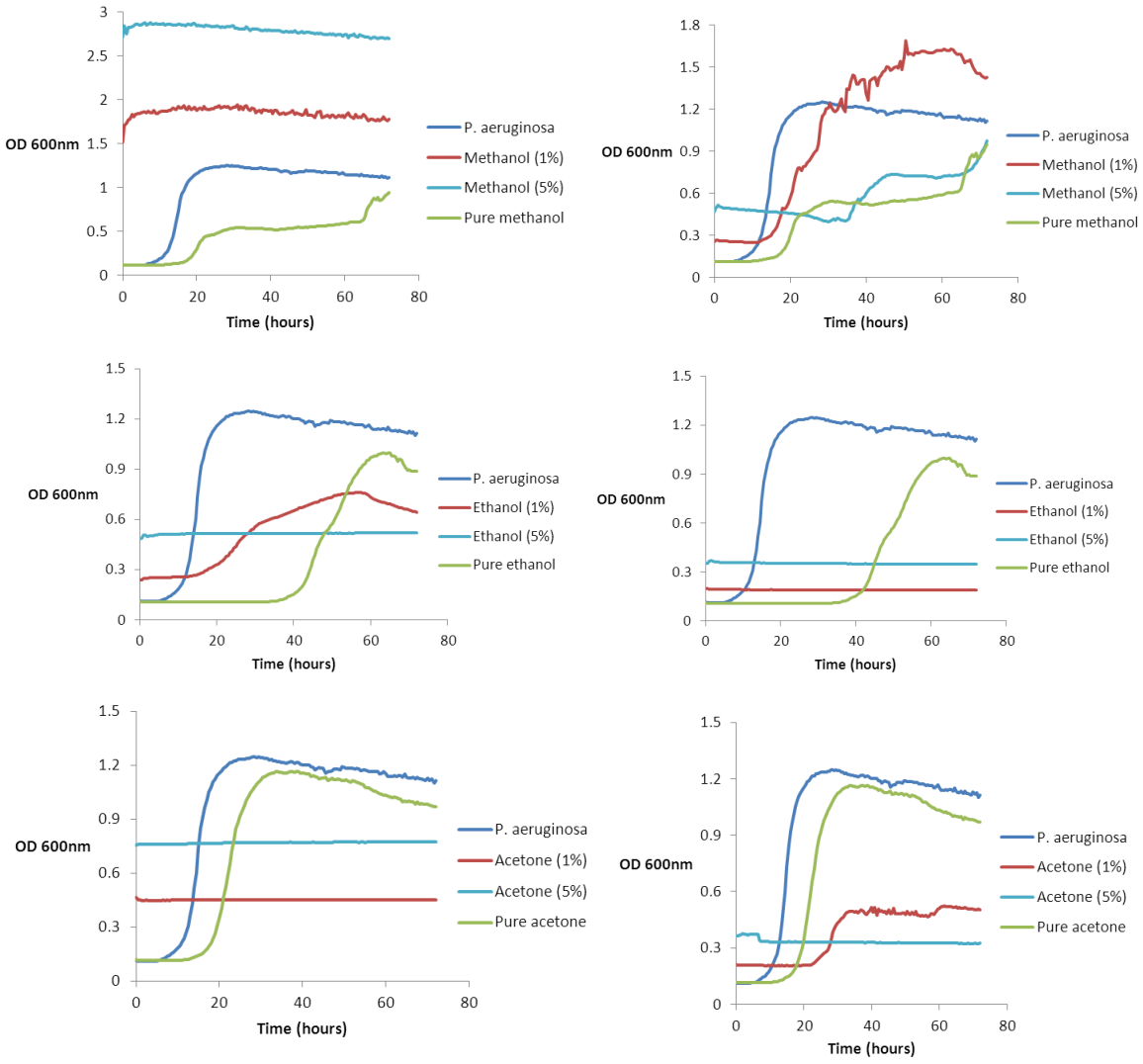
Effects of organic extracts on the growth of test strains are shown in Figures 2 to 5. Growth of the test strains (*S. aureus*, *L. monocytogenes*, *C.albicans*, *E. coli*and *P.aeruginosa*), measured as optical density (OD) at 600 nm in a Bioscreen microplate reader, and plotted against time. Controls consisted of pure culture in nutrient broth without any addition and of culture with added solvent.

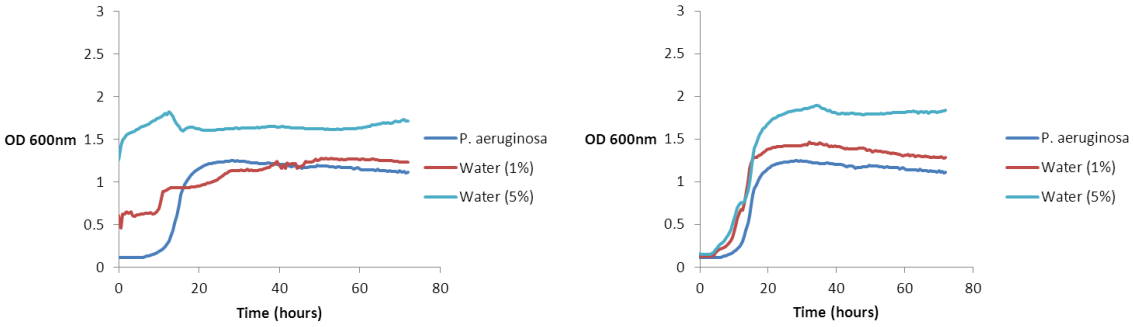


AscophyllumnodosumLaminariadigitata

Figure 2: Effects of algae extracts on the growth of *E. coli* in nutrient broth at 37^{o}C.
(The concentration of 5% equals 1.5 mg/ml; the concentration of 1% equals 300µg/ml.)

The effects of algae extracts on the growth of *E. coli* in nutrient broth at 37^{o}C are shown in Figure 2. Without addition of algae extracts, the bacteria grew well with a short optical density lag phase around 5 h and reached a maximum OD value in 38 h. The solvents alone show no inhibition of the growth of *E. coli*. Because of the strong colour of some of the extracts which can cause great light absorption, the OD value of methanol and water extract of *A. nodosum* was much higher than others. It is therefore difficult to determine the inhibition effects of these two samples. Increasing the algae extracts concentration resulted in greater inhibition. No growth was observed when ethanol extract of *L. digitata*, acetone extract of *A. nodosum*, and 5% ethanol extract of *A. nodosum* were added to the media, whereas slow growth started after 40 h in the cultures with 5% methanol and acetone extract of *L. digitata* and after 30-40 h in the cultures of 1% methanol extract of *L. digitata* and ethanol extract of *A. nodosum*. Water extract of *L. digitata* exhibited no inhibition effects but strongly promoted the growth of the strain.

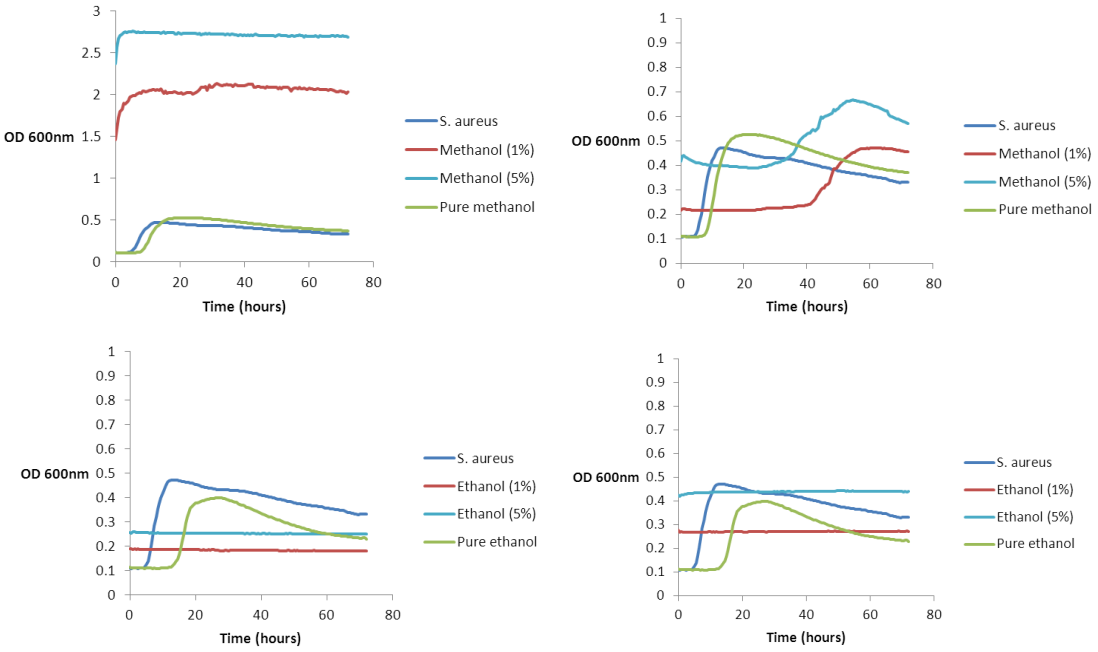


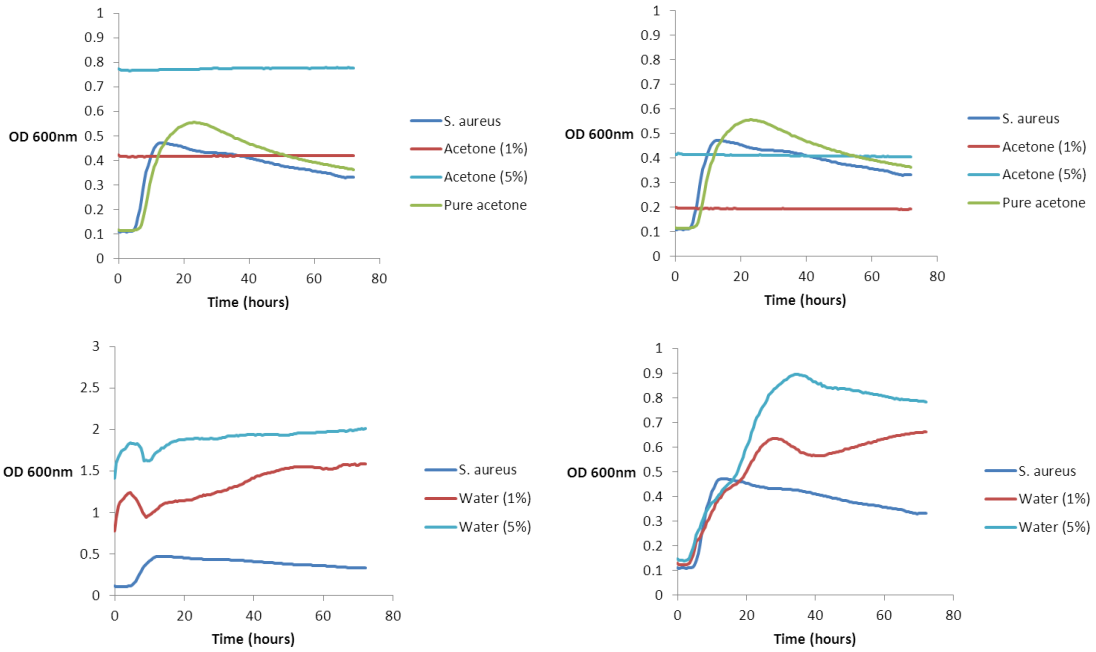


AscophyllumnodosumLaminariadigitata

Figure 3: Effects ofalgae extracts on the growth of *P.aeruginosa* in nutrient broth at 37°C.
(The concentration of 5% equals 1.5 mg/ml; the concentration of 1% equals 300µg/ml.)

The effects of algae extracts on the growth of *P. aeruginosa* in nutrient broth at 37°C are shown in Figure 3. Without addition of algae extracts, the bacteria grew well with a short optical density lag phase around 10 h and reached a maximum OD value in 24 h. The solvents alone showed no inhibition effects on the growth of *P. aeruginosa* except for ethanol. Because of the strong colour of some of the samples which can cause great light absorption, the OD value of methanol and water extract of *A. nodosum* was much higher than others. It is therefore difficult to determine the inhibition effects of these two samples. Increasing the algae extracts concentration resulted in greater inhibition. No growth was observed when ethanol extract of *L. digitata*, acetone extract of *A. nodosum*, and 5% concentration of ethanol extract of *A. nodosum* and acetone extract of *L. digitata*were added in the media, whereas slow growth started after 35 h in the cultures with 5% methanol extract of *L. digitata* added and 20 h in the cultures with 1% concentration of ethanol extract of *A. nodosum* and acetone extract of *L. digitata*. Water extract of *L. digitata* exhibited no inhibition effects but strongly promoted the growth of the strain.





Ascophyllumnodosum Laminariadigitata

Figure 4: Effects of algae extracts on the growth of *S.aureus* in nutrient broth at 37°C.
(The concentration of 5% equals 1.5 mg/ml; the concentration of 1% equals 300µg/ml.)

The effects of algae extracts on the growth of *S. aureus* in nutrient broth at 37°C are shown in Figure 4. Without addition of algae extracts, the bacteria grew well with a short optical density lag phase around 3 h and reached a maximum OD value in 12 h. The solvents alone showed no inhibition of the growth of *S. aureus*. Because of the strong colour of some of the extracts which can cause great light absorption, the OD value of methanol and water extract of *A. nodosum* was much higher than others. It is therefore difficult to determine the inhibition effects of these two samples. Increasing the algae extracts concentration resulted in greater inhibition. No growth was observed when ethanol and acetone extracts were added in the media, whereas slow growth started after 38-42 h in the culture with methanol extracts of *L. digitata*. Water extract of *L. digitata* exhibited no inhibition effects but strongly promoted the growth of the strain.

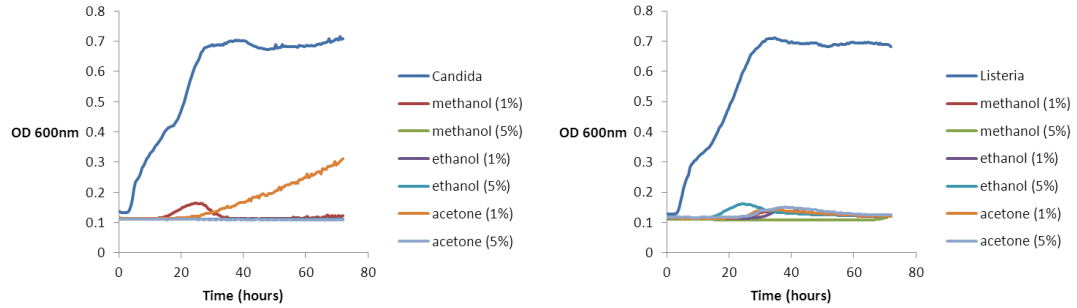


Figure 5: Effects of extracts on the growth of *C.albicans* and *L.monocytogenes*in nutrient broth at 37°C.

The effects of organic solvent on the growth of *C. albicans* and *L.monocytogenes* in nutrient broth at 37°C are shown in Figure 5. All the solvents had a strong ability to inhibit the growth

of *C. albicans* and *L.monocytogenes*. The inhibition of these two bacteria seemed to be the result of the organic solvent, not the algae extracts.

4.5 Growth studies by counting

The effect of acetone extracts of both algae species on the growth of *S. aureus*, *P. aeruginosa* and *L. monocytogenes* is shown in Figure 6. Acetone controls consisting of culture with pure acetone solvent added, but without extracts, were also run to test for interference of the solvent.

Without any addition extract or solvent, the bacteria grew well and reached a maximum value after 24 hours incubation. Pure acetone showed either no inhibition (*S. aureus* and *L. monocytogenes*) or little inhibition (*P. aeruginosa*) on the bacterial growth. The acetone extracts exhibited strong inhibitory effect on *S. aureus* but not on *L. monocytogenes*.

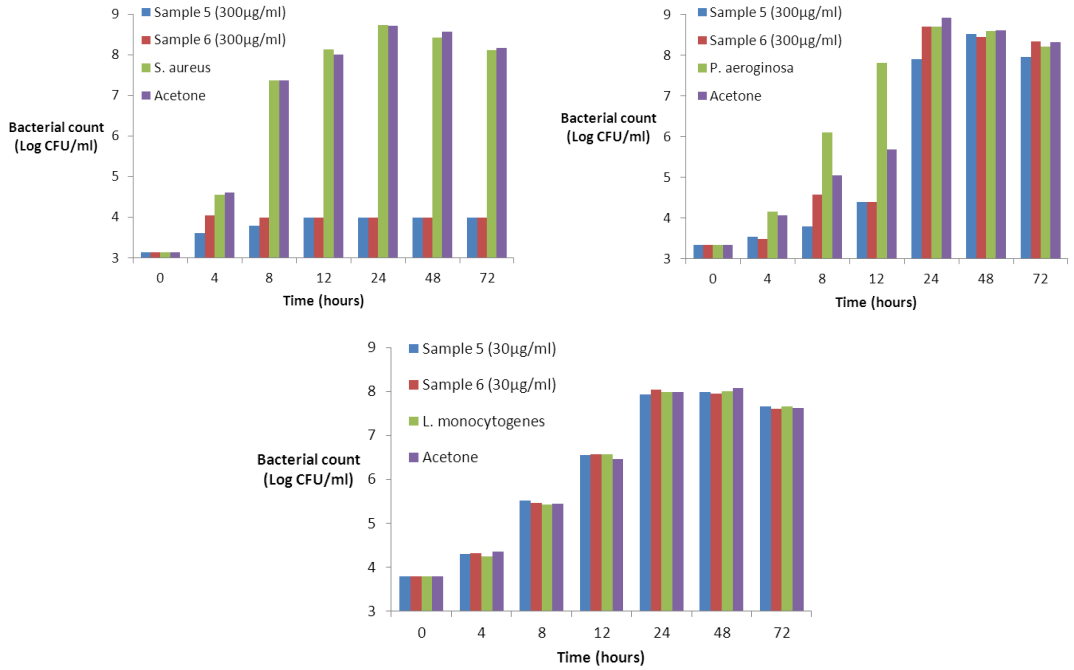


Figure 6: Effects of acetone extracts on the growth of *S.aureus*, *P. aeruginosa*, *L. monocytogenes* at 37°C in nutrient broth.

The effect of acetone extract of *A.nodosum*on the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) is shown in Figure 7. The two concentrations of extracts exhibited different inhibition activities. The 300µg/ml extract showed very strong inhibition effect against MRSA, whereas the extracts of 30µg/ml showed weak inhibition effect.

To see the effect of lowering the incubation temperature (as *L. monocytogenes* can grow at low temperature), the effect of acetone extract from *A.nodosum*on the growth of *L. monocytogenes* at 8°C was also studied and the results are shown in Figure 8.

Both concentrations of the acetone extract exhibited weak inhibition on the growth of *L. monocytogenes*, but 30µg/ml extract did not show any inhibition effect after 144 hours incubation at 8°C.

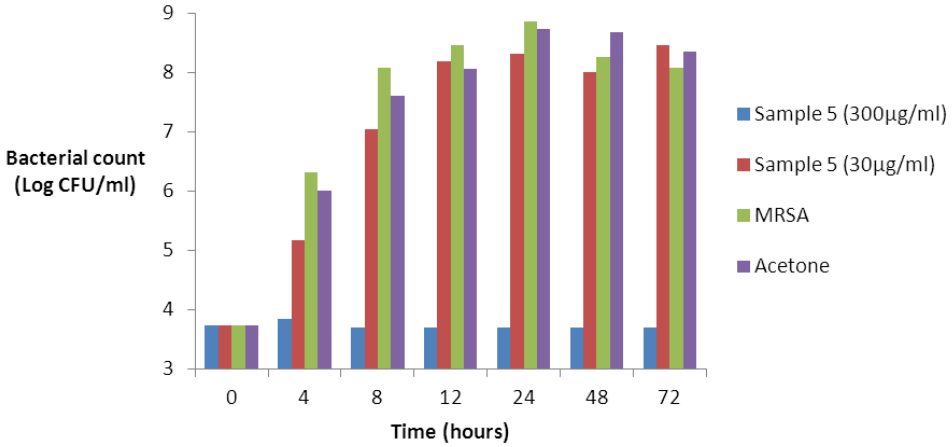


Figure 7: Effect of acetone extract of *A.nodosum* on the growth of methicillin-resistant *Staphylococcus aureus* (MRSA).

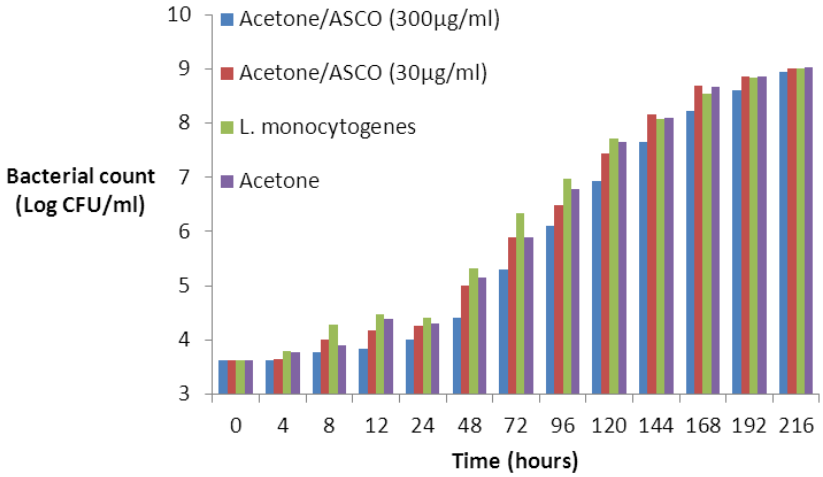


Figure 8: Effect of acetone extract of *A.nodosum* on the growth of *L. monocytogenes* at 8°C.

5 DISCUSSION

This study shows clearly for the first time that Icelandic seaweed contains microbial growth inhibitors. And this is the first time that *A. nodosum* have been shown to contain antibacterial compounds.

5.1 Algae species

Marine seaweeds are known to contain bio-active compounds (e.g. anti-oxidative, cytotoxic, antiviral, anti-inflammatory and antibacterial). They are therefore now considered to be a promising source for bio-actives including antimicrobial agents. Antimicrobial activity depends on both algal species and the efficiency of the extraction. The Icelandic species *A. nodosum* and *L. digitata* have not been studied for their antibacterial activities. The results indicate that organic extracts of *A. nodosum* have higher antibacterial activities than the extracts of *L. digitata*. However, extracts of *L. digitata* have previously been shown to exhibit

antibacterial activity (Dubber and Harder 2008). Dubber and Harder (2008) investigated antibacterial effects of hexane and methanol extracts of the macroalgae *Ceramiumrubrum*, *Mastocarpusstellatus* and *L.digitata*on twelve marine and seven prominent fish pathogenic bacteria. Antibacterial activity was determined with a highly sensitive growth inhibition assay that records the fluorescence intensity of stained bacterial DNA. Among the three algae under investigation, extracts of *L. digitata* and *C.rubrum* were most active and inhibited growth of a variety of marine and fish pathogenic bacteria. Gupta *et al.,* (2010) and Cox *et al.,* (2010) investigated the antimicrobial properties of several Irish edible brown seaweeds using a microtitre method and reported that methanol extracts of *L. digitata* showed good antibacterial activities against *Listeria monocytogenes*.

In this study, the samples were extracted from dry seaweeds. There seems to be a contradiction on whether extraction of bioactive substances is more effective from dry seaweed or fresh seaweed. Some studies reported that the test organisms were more sensitive to extracts of fresh algae (Kolanjinathan and Stella 2009; Shanmughapriya *et al*., 2008; Tuney *et al.,* 2006), whereas Manilal *et al.,* (2009) reported that extracts from dried seaweed exhibited broader and higher antibacterial activity. These studies did not involve *A. nodosum* and *L. digitata*. Further research can be studied to confirm better bioactive extraction from fresh and dried seaweeds.

5.2 Solvent for extraction

As an efficient strategy of investigation, organic solvents have been used to extract the possible active principles from macroalgae (Lima-Filho *et al*., 2002). In this study, acetone yielded the most effective antibacterial extracts. This result is in harmony with that obtained by several researchers. These studies concerning the effectiveness of extraction methods highlight that acetone extraction has higher antimicrobial activity than other organic solvent (Abedin and Taha 2008; Kolanjinathan and Stella 2009; Osman *et al*., 2010). A significant difference in antimicrobial activity was not found between the methanol and ethanol extracts of each algae. The water and enzymes extracts of both species of algae exhibited little or no inhibitory effect against any tested microbial strains. This is not in accordance with reports (e.g. Bansemir *et al.,* (2006), Manilal *et al.,* (2009), Shanmughapriya *et al.,* (2008), Tuney *et al.,* (2006), etc.) for other algal species.

Some studies reported other organic solvent extracts to exhibit stronger antibacterial activity, such as methanol (Choudhury *et al*., 2005; Kannan *et al*., 2010), ethanol (Karthikaidevi *et al*., 2009; Elsie and Dhana Rajan 2010) and diethyl ether (Tun ey *et al*., 2006). It is clear that organic solvents always exhibits higher efficiency in extracting some compounds for antibacterial activities compared to water or enzyme-based methods. According to our experimental results, acetone extracts of *A. nodosum* have the highest antimicrobial activities than other extracts.

5.3 Test microorganisms used

The main objective of this study was to determine the antibacterial activity of extracts from local seaweeds species from Iceland, *A.nodosum* and *L.digitata* against *S.aureus* (DSM 1104 and 2569), *L.monocytogenes*, *E. coli* (DSM 1103), *P.aeruginosa* and *C.albicans*. According to our experimental results, the algae extracts showed better antimicrobial effects on Gram-positive bacteria, especially *S.aureus*. Most organic solvent extracts can inhibit the growth of

S.aureus in both paper disc diffusion test and microbial growth test. Acetone extracts of *A. nodosum* showed strong inhibition effects on both *S.aureus* (DSM 2569) and methicillin-resistant *Staphylococcus aureus* (MRSA) in a concentration of 300µg/ml. Moreover, the acetone solvent itself does not interfere with the measurements. Nowadays, increased resistance to old antibiotics requires seeking of new substitutes. This result may lead to a revelation of some promising antibacterial agents derived from natural material as an alternative to substitute the existing antibiotics, which are already resistant to the pathogens worldwide, especially in the treatment of MRSA.

The bacteria used for testing for the antimicrobial activity, *S.aureus*, *E. coli* and *P.aeruginosa* are commonly used for testing antibacterial compounds especially compounds that are intended for topical applications. *Staphylococcus aureus* has been recognized as an important human pathogen for more than 100 years. It can cause a wide range of life-threatening deep-seated infectious diseases in humans, such as bacteraemia, endocarditis and pneumonia (Kanafan and Fowler 2006). Nowadays, methicillin-resistant *S.aureus* (MRSA) is rapidly becoming rampant no socomial (hospital acquired) infection problem. From this study, it appears that some extracts exhibit a favourable antimicrobial activity against *S. aureus*, such as ethanol and acetone extracts of both algae. Similar results were also reported by Al-Haj *et al.,* (2009). They used red algae *Eucheumadenticulatum* extracts extracted with 60% methanol and tested its antimicrobial activity against two Gram-positive and three Gram-negative bacteria. They reported that the methanol extracts showed inhibitory activity only on the Gram-positive organisms tested including *S. aureus* (both MR and non-MR *S. aureus*) and *Streptococcus pyogenes*. Gram-negative pathogens tested including *E. coli*, *Klebsiellapneumonia* and *P. aeruginosa* showed resistant phenotypic pattern to the extracts.

The antibacterial activities of other algae extracts against *S. aureus* were also studied by several researchers, such as water soluble extract of brown algae *Ecklonia cava* (Kim *et al*., 2008), bromophenols of red algae *Odonthaliacorymbifera* (Oh *et al*., 2008), *Rhodomelaconfervoides* (Xu *et al*., 2002), and halogenated compounds of red algae *Laurenciamajuscule* (Vairappan 2003). Their results showed that these extracts of algae effectively inhibited *S. aureus*.

P.aeruginosa is currently counted among the leading pathogens in ICU pneumonia, no socomial bacteraemia and AIDS primary bacteraemia, drug users endocardit is, exacerbations of cystis fibrosis, malignant external otitis and 'swimmer's ear', and contact lenses keratitis and traumatic endophthalmit is (Giamarellou 2000). In this study, the algae extracts exhibited little inhibition effects against *P.aeruginosa* and *E. coli*, for example, both the acetone extracts only inhibited the growth of *P.aeruginosa* for the first 12 hours. However, there are some reports that indicate algae extracts have antimicrobial activities to *E. coli* and *P.aeruginosa*. Methanol extract of the marine algae *Gracilariachangii* was reported to exhibit a favourable antimicrobial activity against *P.aeruginosa* (Sasidaharan, Darah and Noordin 2010). Seenivasan *et al.,* (2010) studied the antibacterial activity of some marine algae from the southeast coast of India. They reported that acetone, methanol and ethanol extracts of green algae *Ulvafasciata* showed significant antimicrobial activity to *E. coli*.

C.albicans can often be found in antibacterial tests. In this study, the extracts of both algae species showed average inhibition effects on *C.albicans*. Many studies reported that organic solvent extracts of marine seaweeds exhibited inhibition effects against *C.albicans* including methanol, ethanol and acetone (Del Val *et al*., 2001; Osman *et al*., 2010; Afifah *et al*., 2010). In this study, the organic solvent had a strong ability to inhibit the growth of *C. albicans* in

the microbial growth test. However, Oranday *et al.,* (2004) reported that petroleum ether extracts of marine algae *Sargassumfluitans* exhibited high antimicrobial activity against *C.albicans*, with the minimal inhibitory concentration (MIC) 0.16μg/ml.

Listeria monocytogenes is a foodborne pathogen that causes listeriosis (Churchill, Lee and Hall 2006). It is psychotropic organism that can easily adapt and grow under the conditions of most foods. Very poor inhibition effects were detected against *L.monocytogenes* in the microbial growth test and agar plate count test, when the temperature of the incubator was set at 37^{o}C. Acetone extracts of *A. nodosum* exhibited limited antibacterial activity on *L.monocytogenes* when the incubator temperature was set to 8^{o}C. The inhibition effects of algae extracts on *L.monocytogenes* have been reported in several articles. However there is a contradiction between the reported results and the results obtained in this study. The antimicrobial properties of organic solvent extracts of several Irish seaweeds were investigated by Cox *et al.,* (2010) and Gupta *et al.,* (2010). They reported that all the methanolic seaweed extracts of six species of Irish seaweeds, *Laminariadigitata, Laminariasaccharina, Himanthaliaelongata, Palmariapalmata, Chondruscrispus*and *Enteromorphaspirulina* can inhibit *L.monocytogenes*. Different algal species and extraction methods were used in their study as compared to this one. Algae extracts with much higher concentration were tested for antibacterial activity, which may be the reason for this conflict. It could be that *L.monocytogenes*was not as sensitive as *S. aureus*. If acetone extracts of *A. nodosum*with higher concentration were used, maybe better inhibition effects against *L.monocytogenes* can be found. The minimal inhibitory concentration test (MIC test) should be studied in further research.

5.4 Method used for antibacterial test

Three methods were carried out to study the antibacterial activity of algae extracts. They were paper diffusion assay, microbial growth in micro-well plates (growth followed by measuring absorbance) and growth studies by measuring bacterial numbers. Measuring inhibition zones in agar diffusion assays can, under standardised conditions, give both quantitative and qualitative results. From the presented results, it is clear that the diameter of inhibition zone depends mainly on type of algae species, type of solvent used and the test microorganisms. However, some factors might interfere with the results, e.g. nature of the solution, different absorption into the paper disc, evaporation of organic solvents. The extract absorption per paper disc cannot exceed 20 μl, otherwise the solution will overflow. The antimicrobial results can also be studied using microbial growth test and agar plate count test. In the paper diffusion test, the organic solvent extracts showed good inhibition effects on *C. albicans*, especially the acetone extract of *A. nodosum* and all the organic solvent have little influence on the strain. However, the organic solvent itself strongly inhibited *C. albicans* in the growth test. Due to strong colour of the extracts, they might absorb so much light that they mask the OD indicating growth. A control with extract and media without bacteria could have clarified this and should be studied further. For further studies, it is also important to isolate the active compounds from these two crude samples before the growth test is carried out.

The agar plate count method can not only avoid the interference of extract solution colour, but also test the full interaction between the microorganism and algae extracts. Though it requires more work, the agar plate count test is a better method to assay the antimicrobial activity than the other two methods.

The chemical nature of active compounds in these extracts is not so far identified. Our preliminary results suggest that the antibacterial activity observed could be due to more than one chemical compound. The inhibition effects of algae extracts of different concentrations also needs to be studied. Moreover, mechanism of these extracts inhibiting or killing the microorganism is still not clear.

As mentioned in the introduction, algal extracts and isolated compounds from algae show various bio-activities. Some of the activity can be related to the poly-phenol compounds i.e. the anti-oxidant activity and cytotoxic effect. It is well known that compounds having antioxidant activity also have antibacterial activity; however at this stage all speculations on the mode of activity will be very vague. These matters should be investigated further.

6 CONCLUSION

For the first time it has been shown that that Icelandic seaweed contains antibacterial compounds, and this is the first time that *A. nodosum* has been shown to contain antibacterial compounds.

The acetone extract of *A.nodosum* was found to be the most effective and *Staphylococcusaureus* and methicillin-resistant *Staphylococcus aureus* were the most sensitive test microorganisms.

Furthermore, the present study provides some data to show the potential of algal extracts to be used as preservatives.

ACKNOWLEDGEMENTS

This study was carried out at the University of Akureyri. This work was financed by the UNU-FTP (United Nations University-Fisheries Training Programme).

First of all, I would like to express my gratitude to my supervisors, Professor Hjörleifur Einarsson and Arnheiður Eyþórsdóttir, for their wise supervision, encouragement, friendship and motivation at all stages of my dissertation.

I would like to address special thanks to Dr.Tumi Tómasson and Þór H. Ásgeirsson. Their continuous support, guidance and direction were crucial for accomplishing my project.

I would like to express my hearty thanks to Sigríður Kr. Ingvarsdóttir and Bjarni Eiríksson for their warm encouragement and always taking care of me during my stay in Iceland.

The University of Akureyri has provided me excellent environment and facilities to work and learn. All the staff are thanked for their valued contribution in microbial analysis. Special thanks to Guðný Jóhannsdóttir, for her guidance, support and help in the project process.

Finally, I would like to thank all the UNU-FTP fellows for their support, friendship and many enjoyable times during the whole six months in Iceland.

THANK YOU!

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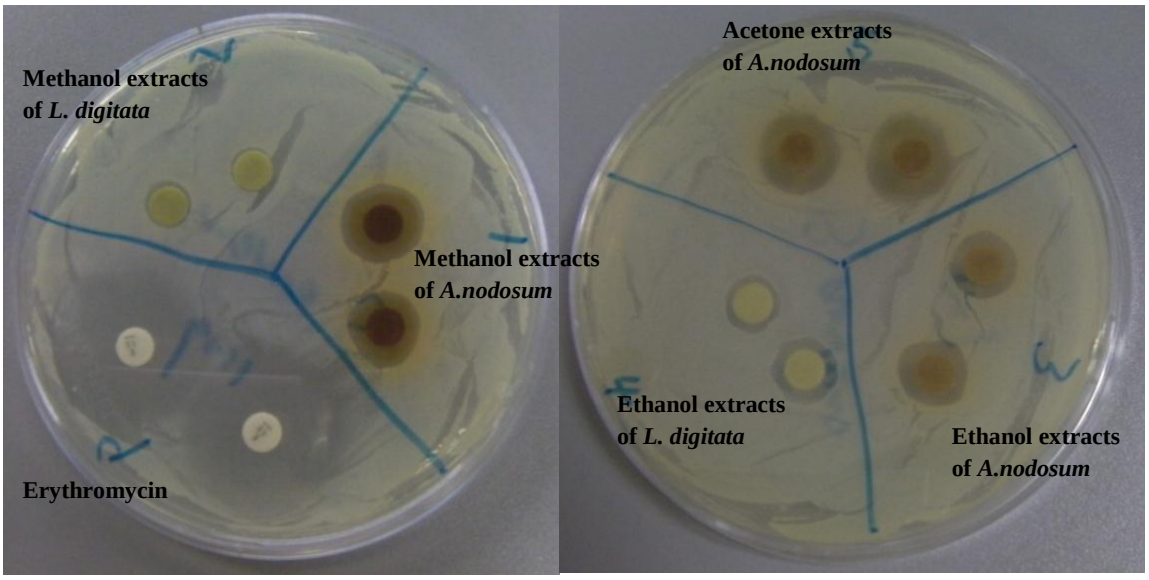
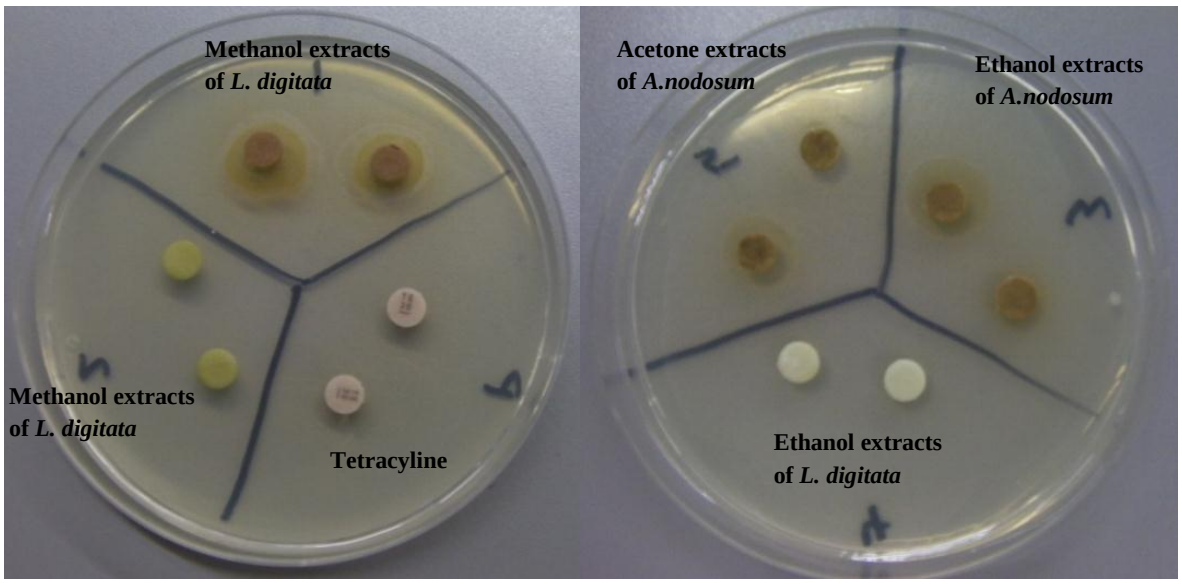
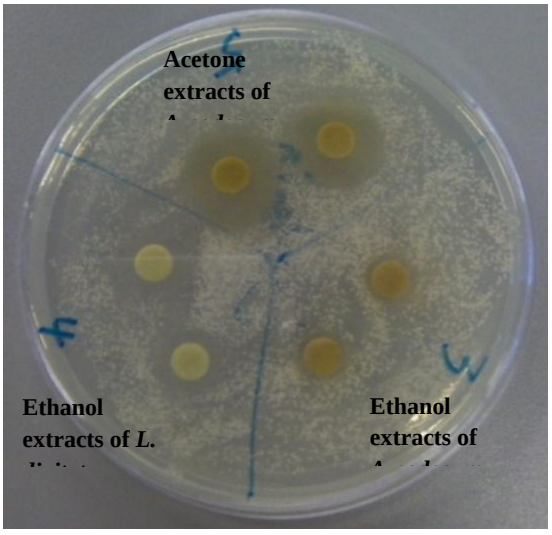
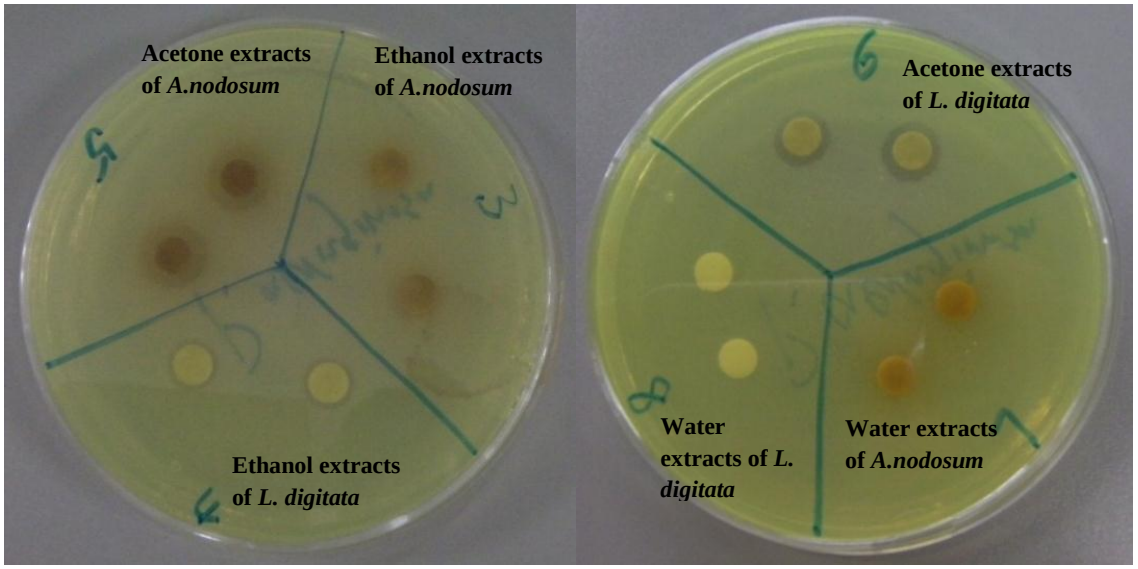
APPENDIX 1**: DISC DIFFUSION TEST OF ALGAE EXTRACTS**

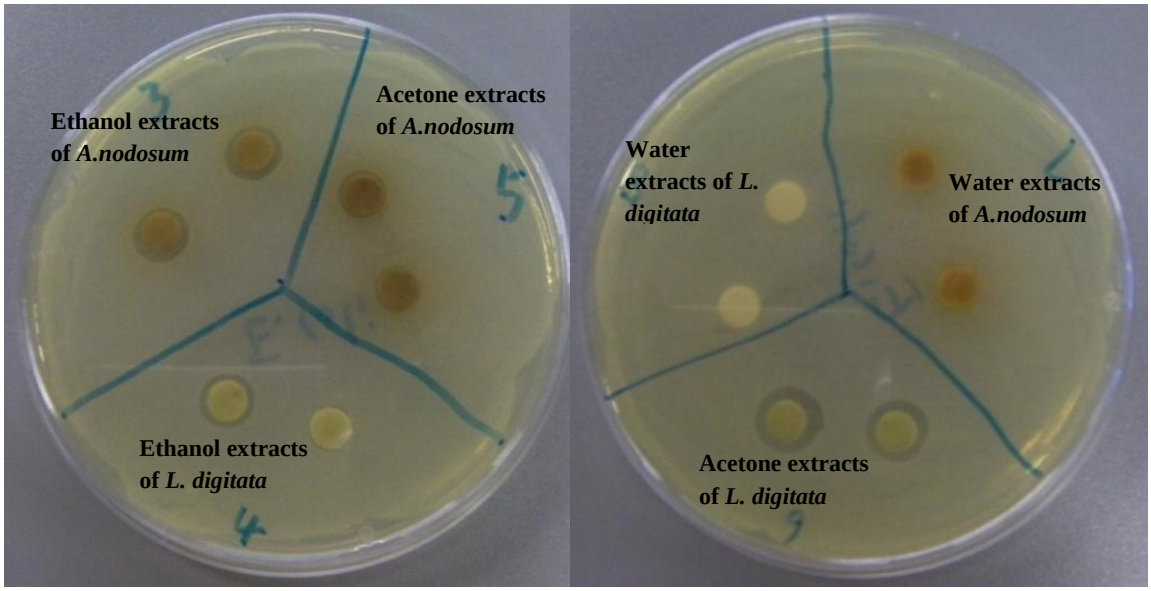




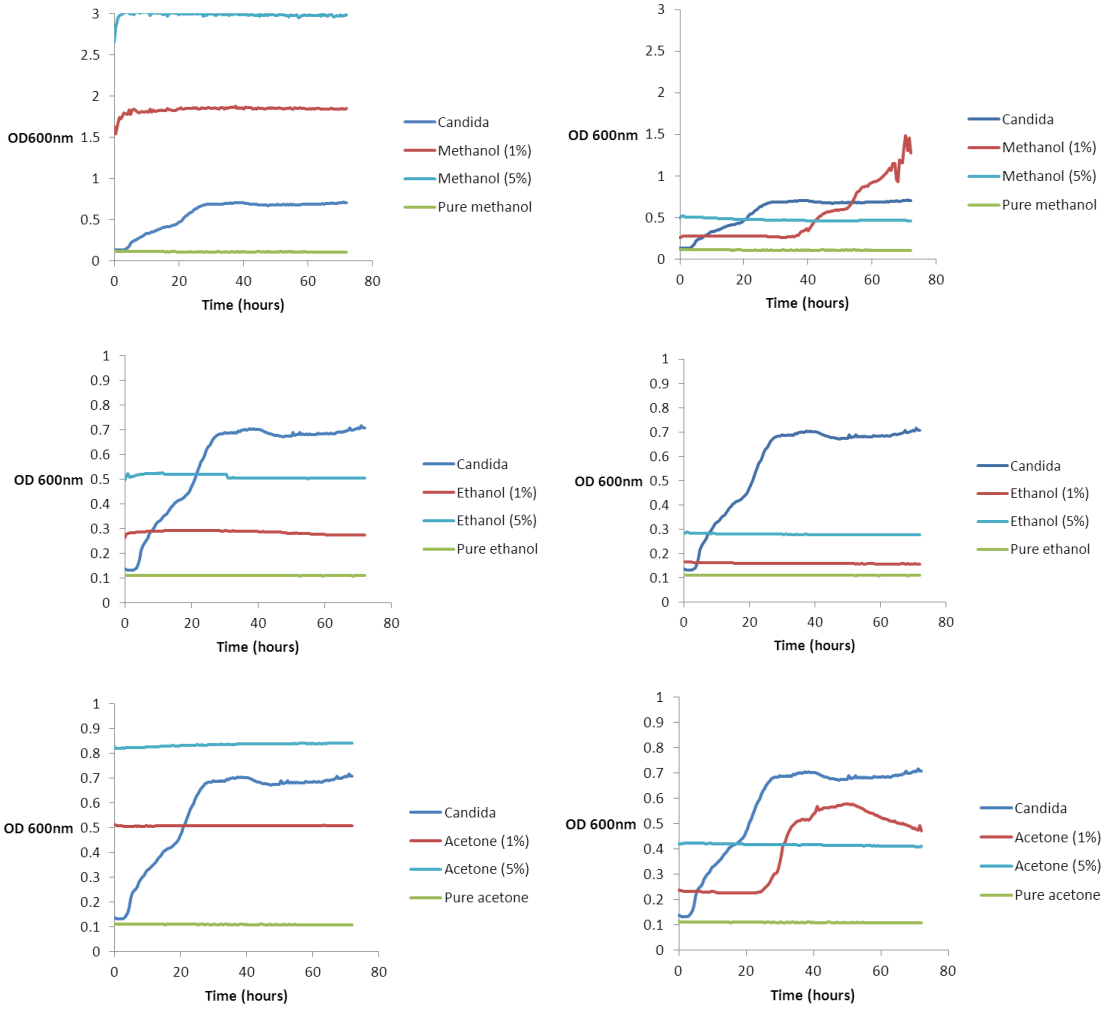


Disc diffusion test of algae extracts on *C.albicans*.





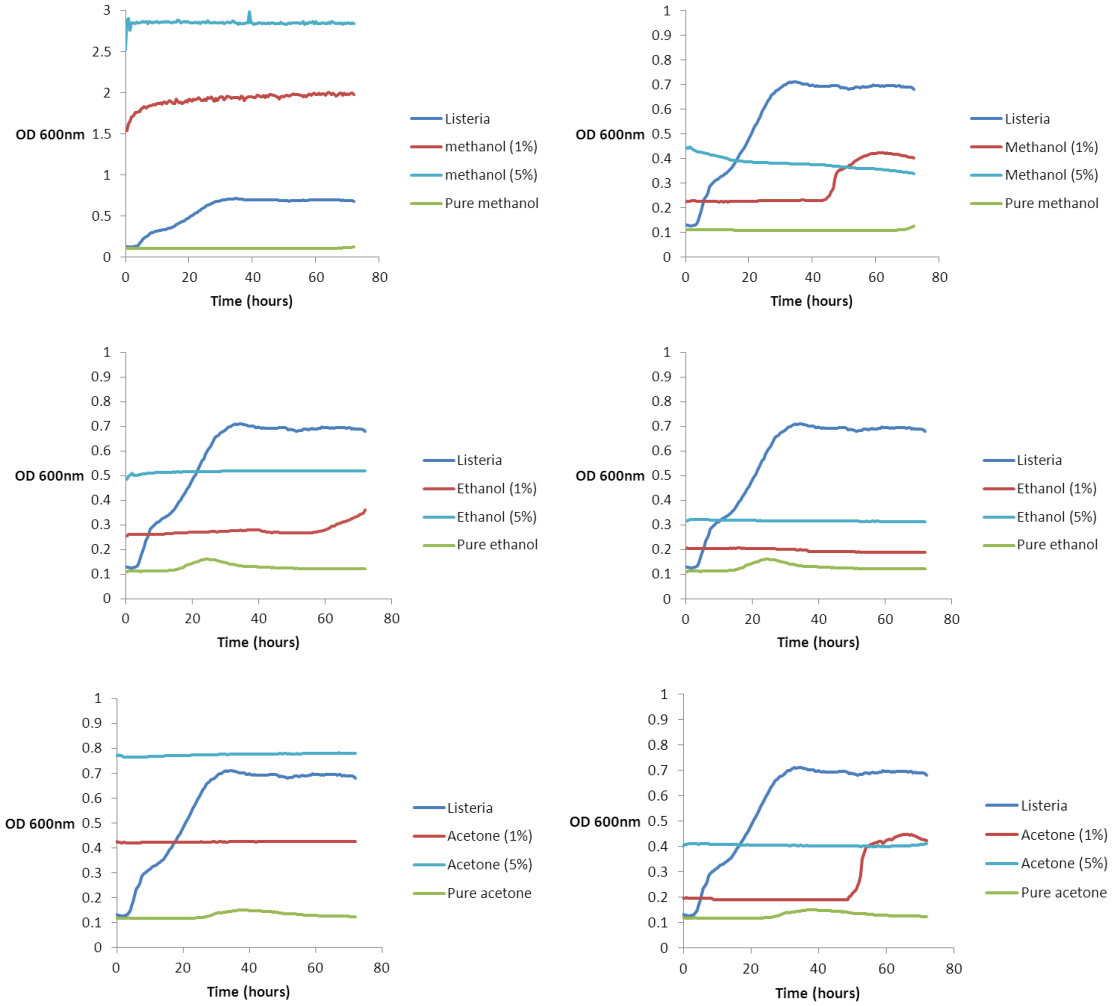
APPENDIX 2: EFFECTS OF EXTRACTS ON THE GROWTH OF *C. ALBICANS* IN NUTRIENT BROTH AT 37^{O}C



AscophyllumnodosumLaminariadigitata

The concentration of 5% is equal to 1.5 mg/ml; the concentration of 1% is equal to 300µg/ml.

APPENDIX 3: EFFECTS OF EXTRACTS ON THE GROWTH OF *L. MONOCYTOGENES* IN NUTRIENT BROTH AT 37^{O}C



Ascophyllumnodosum Laminariadigitata

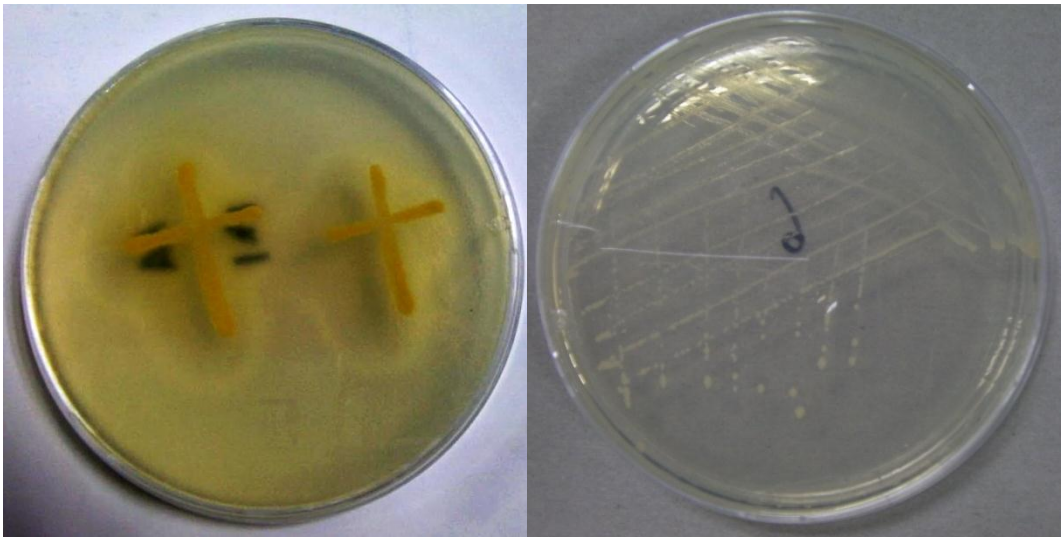
The concentration of 5% equals 1.5 mg/ml; the concentration of 1% equals 300µg/ml.

APPENDIX 4: ISOLATION OF ALGAE-LYSING BACTERIUM

Aim:
To isolate algae-lytic bacteria from the marine environment to use it to make bacterial digest and see if the digest contains antibacterial activity.

Method:
Three kinds of decayed seaweed, *Chondruscrispus, Laminariadigitata* and *Fucusvesiculosus,* were collected at coastal sites in Akureyri, Iceland. About 1gram of each sample was incubated in 20 ml of sterilized nutrition broth and marine broth (BD Difco) with shaking. After 24 hours incubation, the sample was diluted and plated onto nutrition agar and marine agar (BD Difco) and kept at 37^{o}C for two days. Each colony was streaked onto the surface of agar medium with alginate as the sole carbon source, testing for alginate degradation. Another method was also tried: about 1 gram of each sample was directly diluted in buffer, plated onto nutrition agar and marine agar and kept at 37^{o}C for two days. Each colony was streaked onto the surface of alginate agar plate to isolate the alginate-lysing bacteria.

Result:
Samples collected from several sites along the coastal regions of Akureyri, Iceland were screened for algae-lysing bacteria as outlined under *Materials and Methods.* One microorganism producing algae-lysing enzyme showed extensive pit formation or zones of clearance on the agar plate as early as 48 h after inoculation (Figure 9).



Algae-lysing bacterium. Clear alginate–lysing zone can be seen around the colony.