UNITED NATIONS UNIVERSITY
Fisheries Training Programme

unuftp.is Final Project 2016

IN VITRO ANTIBACTERIAL ACTIVITY OF FUCOIDAN ISOLATED FROM *Ascophyllum nodosum* AND *Laminaria digitata*

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ABSTRACT

Both Iceland and China have big seaweed resources. In China the utilization is diversified but in Iceland most of the harvested seaweed go to alginate production. In both countries the products are of relatively low value. However, seaweed is composed of different molecules some of which are of high value e.g. fucoidan. Fucoidan has several interesting bioactivities including antibacterial effect. Increasing resistance of bacteria to available antibiotics calls for new and novel antibacterial compounds. One aim of this study was to extract fucoidan from Icelandic local seaweed species (*Ascophyllum nodosum* and *Laminaria digitate)* and to optimize the extraction conditions. Another objective was to determine the antibacterial activity of the isolated fucoidans. The results showed that the fucoidan content was 4.7-5.6% of dry weigh in *Ascophyllum nodosum* and 2.1-3.6% of dry weight in *Laminaria digitate* depending on reaction conditions. The amount of fucoidan in Icelandic brown seaweed is higher than reported in seaweed harvested in China but lower than reported from the UK. The fractions were isolated through anion-exchange chromatography DEAE-52. The fucoidan from *Ascophyllum nodosum* showed two main fractions whereas fucoidan from *Laminaria digitate* had three main fractions. The fucoidan showed inhibitory effect on *S.aureus* but not on *E.coli* nor *B.subtilis* in nutrient broth without salt. However when 1% NaCl was added, *B.subtilis* was slightly inhibited and the addition of salt enhanced the inhibitory effect on *S. aureus*. The inhibitory effect of fucoidan from *Laminaria digitate* was stronger than fucoidan from *Ascophyllum nodosum*. This is the first study to show that fucoidan extracted from Icelandic seaweed has antibacterial activity.

Keywords: fucoidan, isolated, fraction, Icelandic brown seaweed, antibacterial activity

This paper should be cited as:
He, Y. 2018. *In vitro antibacterial activity of fucoidan islolated from* Ascophyllum nodosum *and* Laminaria digitata. Nations University Fisheries Training Programme, Iceland [final project].
http://www.unuftp.is/static/fellows/document/he16prfa.pdf

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1 INTRODUCTION

1.1 Background

Seaweeds are a group of marine multicellular algae containing dietary fibres, proteins, essential fatty acids, vitamins, carotenoids and minerals. They have been used as food stuff in Asian diets for centuries (Norziah & Ching 2000). They 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). Of those the abundance of brown seaweed is greatest.

China is the biggest seaweed producing country in the world. In 2015, total seaweed production in China was 2.1 million tons, which is about 80% of the global output (Zhao 2016). However, according to FAO statistics (FAO, 2016) the aquatic plant production in China was 13.326.000 tonnes in 2014. Seaweed products steadily grew in recent years. The market share accounted for 4.87% of all aquatic products in 2015, and ranked fourth. In China, the traditional seaweed products are used for food as a "healthy" vegetable, sold on the market only in its crude dried form. Some important industrial raw materials can be extracted from algae, for example alginate, agar, carrageenan, iodine and mannitol. Nowadays seaweeds are widely used in food, chemical, spinning, medicine and cosmetic industry. Seaweeds are made into seasoning, snack food and additives, and small package of shredded and seasoned forms with different flavours have been introduced. But the value of seaweed in China is still low (1.5 dollar per kilogram) compared to the Japanese market.

To make a better use of seaweed, efforts are being made to isolate some high value compounds from it. Brown seaweeds contain high value fucoidans which are complex and heterogeneous, and have various structures. Fucoidan has various biological activities which include antitumor, immunomodulatory, antiviral, antithrombotic, anticoagulant, antithrombotic, antioxidant, antibacterial and antilipidemic activity (Senthilkumar *et al.*, 2013; Lee *et al*., 2013). At the same time, there is a growing interest to find new natural preservatives for food and other perishable products. The increased frequency of pathogenic bacteria resistant to traditional antibiotics has resulted in search for novel antibacterial compounds (Nishiguchi *et al*., 2014). As fucoidan has potential antimicrobial activity, they are now in the spotlight for natural product discovery. Furthermore, seaweed is a cheap and assessable raw material which is not the case for most other potential source of marine natural products.

Iceland has rich seaweed resources. Annual harvest is 20.000-25.000 tonnes (wet weight). The most important economic seaweeds are Rockweed (*Ascophyllum nodosum*) and Kelp (*Laminaria digitata*). Both belong to brown algae.

Rockweed (*Ascophyllum nodosum*) is found growing in the intertidal zone and is common over much of the North Atlantic. It is perennial, growing on large stones and rocks and may reach 1.5 m in length. The species thrive better in areas exposed to air during low tides and are fairly exposed or in sheltered areas. Rockweed is the most common species around Icelandic shores. *A. nodosum* is harvested between the months of April and October using specially designed harvester craft. It is very sensitive to pollution and can be an indicator of environmental cleanliness. Conditions at Breidafjordur are very favourable for this species.

Kelp (*Laminaria digitata*) is one of the largest seaweeds found along the European littoral, with mature plants being 1-2 m in length, and is common over northern Europe. Kelp is composed of a smooth and pliable stipe and a blade of jagged ribbons. The plants are attached to boulders by holdfasts in the lower intertidal and shallow subtidal down to a depth of 20 metres. *L. digitata* is harvested using a specially equipped coaster in late autumn and winter. Kelp flourishes in fairly exposed or in sheltered areas.

The commercial utilisation of seaweed in Iceland is not diversified and mainly it is harvested to produce alginate, less for agricultural usage (i.e. feed and fertilizer) and cosmetic. Therefore, extraction of fuciodan could increase the value of seaweeds. It is known that brown algae growth in the colder waters contain more and pure fucoidan. So Icelandic brown algae may contain more and pure fucoidan than algae harvested in the UK or China. However, the extraction and isolation of fucoidan from Icelandic brown algae have not been studied systematically. Their characterization properties and antibacterial activity are still unknown.

1.2 Research questions

- Does Icelandic *Ascophyllum nodosum* and *Laminaria digitata* contain more fucoidan than brown algae from UK and China?
- What is the difference in characterization of fucoidan extracted from *Ascophyllum nodosum* and *Laminaria digitata*?
- Does the fucoidan from Icelandic seaweed have antibacterial activity?

1.3 Goal and objectives

The aim of this study was to determine the in-vitro antibacterial activity of fucoidan obtained by enzymatic hydrolysis of *Ascophyllum nodosum* and *Laminaria digitate.*

The specific objectives were to:

- Extract fucoidan from *Ascophyllum nodosum* and *Laminaria digitate* using enzymatic hydrolysis
- Characterize, by fractionation, the fucoidan
- Determine the antibacterial activity of the fucoidan.

2 LITERATURE REVIEW

2.1 Bioactive compounds from seaweed

Seaweed contains a variety of bioactive compounds. Brown seaweed, as the largest species of seaweed, contains four kinds of bioactive compounds, respectively alginate, fucoidan, polyphenol and fucoxanthin (Miyashita *et al.,* 2013). Alginate accounted for 15-30% of dry algae. Fucoidan accounted for 1.5-5%. Polyphenol and fucoxanthin content is very small, accounting for less than 0.1%.

Alginate has the highest content of brown algae (Rioux *et al.,* 2007). It is mainly used in chemical and food industry, but its biological activity is not significant. Polyphenols and fucoxanthin have significant antioxidant, antibacterial, antitumor and other biological activities (Pádua *et al.*, 2015). But they are difficult to isolate to obtain pure product, and the final product is very unstable and difficult to preserve. So, until now it has not been used much.

The fucoidan content is much higher than polyphenol and fucoxanthin in brown algae. Fucoidan not only has a variety of significant biological activity, but also is stable (Vo & Kim, 2013). Therefore, many researchers put fucoidan as the focus of their study. However, its antimicrobial activity has not been studied until recently (Lee *et al.*, 2013; Nishiguchi *et al.*, 2014; Shu *et al.*, 2015; Sivaganavelmurugan *et al.,* 2015). These studies provide a scientific basis for the use of fucoidan as a preservative. For the first time in vitro, antibacterial activity of fucoidan isolated from Icelandic seaweed will be studied in this project.

2.2 Overview of Fucoidan

During the last decade, numerous bioactive polysaccharides with interesting functional properties have been discovered from seaweeds. Fucoidan is a sulphated polysaccharide found mainly in various species of brown seaweed such as mozuku, kombu, limu moui, bladderwrack, wakame, and hijiki (variant forms of fucoidan have also been found in animal species, including the sea cucumber). Fucoidan is a term used for a class of sulfated, fucose rich, polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds. FCSPs (Fucoidan containing sulfated polysaccharides) may also contain galactose, mannose, xylose, glucose and/or glucuronic acid, usually in minor amounts (Jiao *et al*., 2011). The polysaccharide was named as “fucoidin” when it was first isolated from marine brown algae by Kylin in 1913 (Kylin *et al*., 1913). Now it is named as “fucoidan” according to IUPAC rules, but also called as fucan, fucosan or sulfated fucan (Berteau *et al*., 2003). Fucoidan is considered as a cell wall-reinforcing molecule and seems to be associated with protection against the effects of desiccation when the seaweed is exposed at low tide. Fucoidans from several species of brown seaweed, for example Fucus vesiculosus, have simple chemical compositions, mainly being composed of fucose and sulfate. But the chemical compositions of most fucoidans are complex. The structure of fucoidan is depicted in Figure 1.

Figure 1: Structure of fucoidan. (a) Basic model structure of fucoidan polysaccharide. (b) A chemical structure of brown seaweed sulfated polysaccharide

The fucoidan content in algae varies between species and different extraction methods can affect the yield. Summary is given in Table 1. The table shows that fucoidan content is reported between 2 and 20%.

2.3 Antibacterial and bio-activity of Fucoidan

Beside antibacterial activity fucoidan have a wide range of biological properties in-vitro, with some of these properties confirmed in-vivo e.g. anti-inflammatory, immunostimulatory, antioxidant, anticoagulant, antiviral, antiproliferative, antiapoptosis and antitumour properties (Gupta & Abu-Ghannam, 2011; Kadam *et al*., 2015; Wijesinghe & Jeon, 2012; Collins *et al*., 2016).

The antibacterial activity of fucoidans have only recently gained attention and overview is summarized in Table 2.

Most of the antimicrobial activity of fucoidan was determined by the inhibition on the growth of different microorganisms (Nishiguchi *et al*., 2014, Shu *et al*., 2015). Only a few polysaccharide antimicrobial activity applications have been studied. Fucoidan may affect bacterial cell wall synthesis and combinations with antibiotics should be investigated further for possible use in antibacterial products (Lee *et al*., 2013). Particularly, these may be useful in the future for the treatment of cariogenic and periodontopathogenic bacteria (Lee *et al*., 2013). The results of the research on Minimum Inhibitory Concentration (MIC) of fucoidan from different macroalgae species are summarized in Table 3.

Table 1: Extraction methods and yield of fucoidan from different macroalgae species.

Macroalgae sp.	Extraction	Fucoidan Yield	References
Silvetia babingtonii	Ethanol (40 °C, 3 h) HCl (60 °C, pH 2–3, 3 h, 2 times) followed by concentration, dialysis and freeze-dry.	4.9%	Anastyuk *et al*. 2012a
Fucus evanescens		5.0%	
Costaria costata		9.1-18.4	Anastyuk *et al*. 2012b
Laminaria cichorioides		12-20%	Anastyuk *et al*. 2010
Sargassum binderi	Methanol: chloroform: water (4:2:1) several times. $CaCl_2$ (2%, 85 °C, 24 h, 6 times). The combined supernatants were treated with 10% $CH_3(CH_2)_{15}$ $N(Br)(CH_3)_3$, followed by centrifugation (3000 g, 10 min) and the pellet washed several times with H2O and ethanol (20%) and dialyzed (2 kDa).	6.16	Lim *et al*. 2014
Ascophyllum nodosum(Bod Ayre Products Ltd Sheland ,UK, October 2011)	Ethanol (80%, room temperature, 18 h) and repeat again at 70 °C (4 h) Microwave treatment (120 °C, 15 min) with biomass in HCl (0.1 M). The mixture was dried (80 °C), re-dissolved $CaCl_2$ (2%, 4 °C, overnight) followed by centrifugation and ethanol precipitation of the supernatant. The new pellet collected after centrifugation was freeze-dried	16.08%	Yuan & Macquarrie 2015
Sargassum swartzii	Soxhlet ethanol-acetone for 24 h HCl (0.05 M, room temperature, 24 h, 2 times). Supernatants mixed with $CaCl_2$ (4%, 4 °C, overnight) followed by centrifugation and ethanol precipitation of supernatant and dialysis of the pellet.	5.96%	Dinesh *et al.* 2016
Coccophora langsdorfii	Ethanol (96%, 40 °C,24 h) and acetone washes HCl (0.1 M, room temperature, 2 times) and the supernatant neutralized with $NaHCO_3$ (3%) to pH 5.7–6.1, followed by concentration, dialysis and freeze-dry.	13.3%	Imbs *et al.* 2016
Ascophyllum nodosum(coast of Aberystwyth)	Ethanol (85%, at room temperature, 12 h) followed by the same treatment at 70 °C. The pellet was treated twice at room temperature and 70 °C with H_2O (7 h). The pulled supernatants were treated with $CaCl_2$ (2 M, 5 h), centrifuged and the pellet dialyzed (1KDa) and freeze-dried	6.5-8.1%	Fletcher *et al.* 2017
Laminaria japonica (China)	enzymatic hydrolyzing technology(.0.06% NovozymesV iscozyme L, at temperature of 40°C, pH 3.5 and 30 min) with hot water(98 °C,3h),then ethanol precipitated and freeze-dry.	1.845%	He *et al.* 2006

Table 2: Antibacterial activity of fucoidan isolated from brown seaweed

Test material	Test organisms	References
Fucoidan with antibiotics	Oral pathogenic bacteria	Lee *et al.* 2013
Fucoidan	*V. alginolyticus* (NBRC15630), *E. coli (JCM5491)* and *S.aureus* (JCM2413)	Nishiguchi *et al.* 2014
Fucoidan-shelled chitosan beads	*Staphylococcus aureus* (*S. aureus*, ATCC 6538) and *Escherichia coli* (*E. coli,* ATCC 11229)	Shu *et al.* 2015
Fucoidan	The shrimp pathogen *V. parahaemolyticus*	Sivaganavelmurugan *et al.* 2015

Table 3: MIC of fucoidan from different macroalgae species.

Macroalgae sp.	MIC (mg/mL) or % of the initial concentration	Bacterial strain	REF
Sargassum wightii	12mg/ml	*Vibrio parahaemolyticus*	Sivaganavelmurugan *et al.* 2015
Sargassum polycystum	12 mg/ml	*Vibrio harveyi*	Chotigeat *et al.* 2004.
	12 mg/ml	*Staphylococcus aureus*	
	6 mg/ml	*Escherichia coli*	
Laurus nobilis	≤60.97%	*Laurus nobilis*	Chmit *et al.* 2014
	>78.39%	*E. faecalis* ATCC29212	
	>78.39%	*S. aureus* ATCC 25923	
	>78.39%	*E. coli* ATCC 35218	
	60.97%<MIC≤78.39%	*P. aeruginosa* ATCC 27853	
	0.25 mg/ml	*S. anginosus* ATCC 31412	
Commercial products(Sigma)	0.5 mg/ml	*A. actinomycetemcomitans ATCC 43717*	Lee *et al.* 2013
	0.25 mg/ml	*F. nucleatum ATCC 51190*	
	0.25 mg/ml	*P. intermedia ATCC 49049*	
	0.125 mg/ml	*P. gingivalis ATCC 33277*	

The in vitro antimicrobial effect of seaweed extracts belonging to *Ascophyllum nodosum* and *Laminaria digitata* were tested against some pathogens (Jin, 2010). It was shown that Icelandic seaweed contains antibacterial compounds. However, it was unclear whether these antibacterial compounds were fucoidan or other components. Therefore, in this project, fucoidan will be isolated from Icelandic *Ascophyllum nodosum* and *Laminaria digitate*. In vitro antibacterial activity of fucoidan will be determined through model system.

3 MATERIALS AND METHODS

3.1 Seaweed samples

Dried and ground brown seaweed (*Ascophyllum nodosum* and *Laminaria digitata*) were obtained from the company Thorverk, 380 Reykhólar, Iceland.

3.2 Fucoidan extraction and fractionation

Dried seaweed was crushed and resuspended in H_2O. Enzymatic hydrolysis was performed as shown in Figure 2; during this hydrolysis, the pH value was adjusted to 4.5 using 1 M HCl, the temperature was 45℃. The hydrolysate was then subjected to an ethanol precipitation protocol (Figure 2) to produce crude fucoidan (Wang *et al*., 2014). Enzyme used was cellulase (Sigma-Aldrich Co. LLC). The temperature range and pH range of the enzymatic reaction are determined by the optimum temperature range (30-70℃) and pH range (4.0-6.0) of cellulose. The enzyme percentage range (0.5-1.5%) and the reaction time range (30-70min) are determined according to the results of the optimization of the enzymatic hydrolysis conditions of other brown algaes, such as *Laminaria japonica* and *Costaria costata* ((He *et al*., 2006; Wang *et al*., 2014).

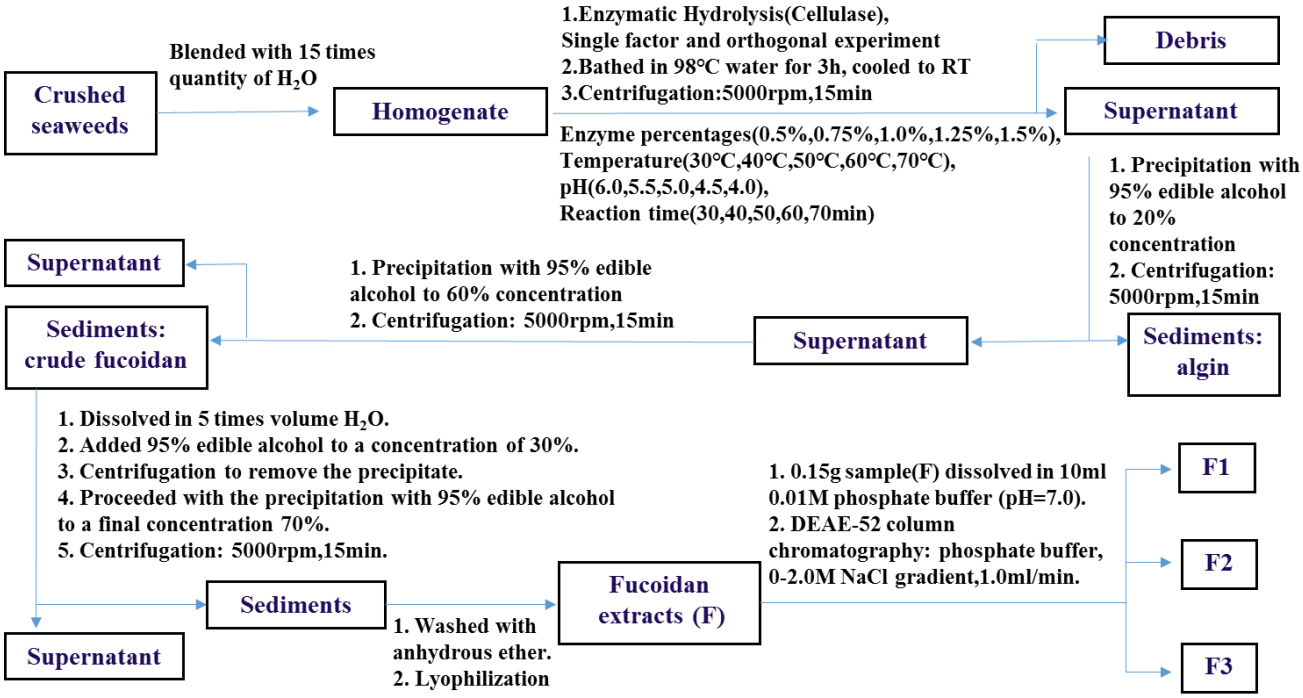


Figure 2: Diagram of the extraction and fractionation of fucoidan from dried seaweeds

The crude extracts were re-dissolved, re-precipitated, washed, and lyophilised to obtain a solid fucoidan mixture (F), which were further fractionated using anion column chromatography.

Sample F was dissolved and applied onto a 300-mm × 10-mm column packed with DEAE-52 (Whatman). The mobile phase parameters were the following: 0–120mL, phosphate buffer (pH

7.0); 121–600mlh, NaCl (0–2.0 M). The flow rate is 1mL/min, and an automatic fraction collector was used to collect the elutions (3 mL/tube). The relevant fractions were dialysed using ultrafiltration, and the samples were subsequently lyophilised.

The extraction and fractionation procedures were executed multiple times to acquire a sufficient amount of fucoidan fractions for the subsequent studies.

3.3 Fucoidan Characterization

The Solid-Phase Colorimetric Method (Lee *et al*., 2012) was used to quantify the fucoidan. This method can detect fucoidan in the range of 1-20μg in 2 μL and can be read specphotometrically at 663nm on a microplate reader or in standard cuvettes (Table 4).

Reagents

Table 4: Preparation of Fucoidan Standards (Sigma-Aldrich Co. LLC)

	Concentration		
Standard	mg/μL	mg/mL	Preparation Directions
A	0.5	0.5	50 μL of Std 1→V_f=1.00mL
B	1	1	100 μL of Std 1→V_f=1.00mL
C	2	2	200 μL of Std 1→V_f=1.00mL
D	3	3	300 μL of Std 1→V_f=1.00mL
E	4	4	400 μL of Std 1→V_f=1.00mL
F	5	5	500 μL of Std 1→V_f=1.00mL
G	6	6	600 μL of Std 1→V_f=1.00mL
H	8	8	800 μL of Std 1→V_f=1.00mL
I(stock)	10	10	10mg of fucoidan per mL

Fucoidan standards were stored in sealed plastic vessels at -20℃.

Twenty mg of fucoidan was carefully weighed and dissolved into a final volume of 2mL of dH_2O and stored at -20℃. Four kinds of solution were prepared respectively: MeOH/Acetone treatment solution (6：4 MeOH/acetone), methylene blue staining solution (0.1% Methylene blue in 50 mM HCL in MeOH/acetone/ dH_2O, 6：4：15), Wash solution (5% HOAc 6% MeOH 4% acetone) and methylene blue elution solution (70% MeOH with 2% sodium dodecyl sulfate).

Procedure

Two μL of standard solution containing fucoidan were put on a 1×1 cm square of Whatman #1 filter paper. The drop was dried under a stream of air. If greater than 2 μL were used the drop was dried directly after each spotting.

The loaded filter paper was treated with Methanol/Acetone Solution (6：4) for 2-3 minutes.
The fucoidan was made visible with Methylene Blue Staining Solution applied for 10 minutes. Excess MB was removed by washing filter paper with Destaining Solution until an appropriate destained background appeared.

3.4 Antibacterial activity test

The antibacterial effect was tested using two different methods: disc diffusion assay and growth inhibition in microplates.

The inhibitory effects of the extracted fucoidan were tested on three species of food related bacteria, namely:

Gram-positive bacteria: *Staphylococcus aureus* (DSM 799)
Bacillus subtilis (DSM 1117)
Gram-negative bacteria: *Escherichia coli* (DSM 1103)

The test bacterial 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) at 35°C.

The fucoidan was dissolved in water for the antibacterial activity test.

3.4.1 Disc diffusion assay

Mueller Hinton Agar medium (MHA–BD Difco) was prepared and sterilized by autoclaving at 121℃ and 15 lbs pressure for 15 minutes. 20 mL of the sterilized media was poured into a sterilized Petri dish and allowed to solidify at room temperature.

The fucoidan was dissolved in 5 mL(100,50,25,10,5,1,0.5,0.1mg/mL) water and 20µLwas applied to sterile filter paper discs (6mm). The discs were placed on to the agar plates that had been inoculated with 0.1mL of a 24 hour culture of the test pathogen (diluted to10^6 bacteria/mL). A disc load with a certain commercial antibiotic (Erythromycin) was used as a positive control. The plates were incubated for 24 hours at 35℃.

The zone of inhibition of bacteria around the disc was measured and the assay was scored positive (+) if the zone was between 0.2-2 mm (from disc edge), doubly positive (++) if $\geqslant$ 2 mm, and negative (–) if no zone was visible.

3.4.2 Microbial growth experiment

The microbial growth in microplates was studied according to the method used by Eyþórsdóttir (2007). Growth studies were carried out in a Bioscreen microplate reader (Growth Curves Ltd, Finland), using 100-wells flat bottom honeycomb microplates. Fucoidan solutions from the two algae were prepared in water: 50, 25, 10, 5, 1 and 0.5mg/mL, respectively. Fresh cultures (16 – 20 h) of three test strains: *E. coli*, *B.subtilis* and S.aureus were used as inoculates, where 10 µL of diluted culture was added to 10µL each of the fucoidan solution and 180µL nutrient broth. The dilution was aimed at a final culture density of 10^4cells/mL. These mixtures were used as samples

for the microplates. Corresponding solvents were used as a control for all the concentrations and all the strains in triplicate 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 35℃ with optical density (OD) reading at 600nm at 15 minute intervals for 48 hours. The OD readings recorded for pure nutrient broth medium and the first value of each well was subtracted from all measurements and plotted on the growth graphs.

3.5 Statistical analysis

All experimental data were carried out in duplicate or triplicates, the mean values and the standard deviation were calculated. One-way analysis of variance (ANOVA) was performed. Statistical analysis was performed with Excel 2016 (Microsoft Co. USA).

4 RESULTS

4.1 Fucoidan extraction

4.1.1 Fucoidan extraction from Ascophyllum nodosum

Enzymatic hydrolysis extract of fucoidan from *Ascophyllum nodosum* was performed as single factor and orthogonal experiment. Single factor experiment was designed according to the following conditions: enzyme percentages 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, temperature 45℃, pH 4.5 and reaction time 40, 50, 60, 70, 80min.

The yield of fucoidan using different enzyme percentages are shown in Figure 3. The yield of fucoidan was highest (5.13%) when enzyme percentage was 1.25%.

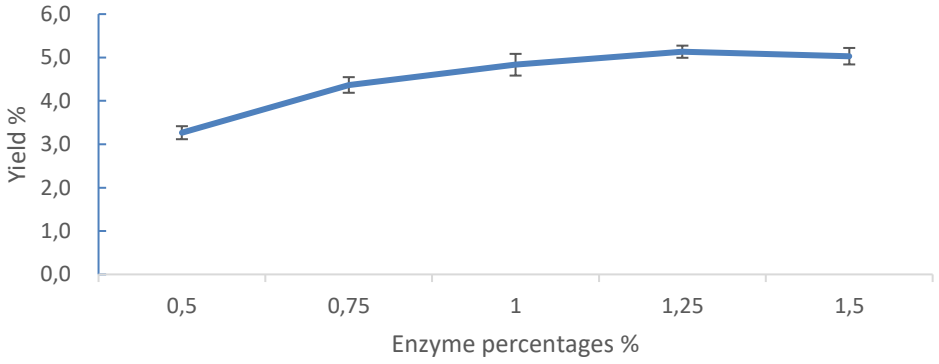


Figure 3: Yield of fucoidan (*Ascophyllum nodosum*) as a function of different enzyme (cellulose) concentration (45℃, pH 4.5, 40min).

The results of reaction time experiment are shown in Figure 4. The yield of fucoidan were highest (5.37%) when reaction time was 60min.

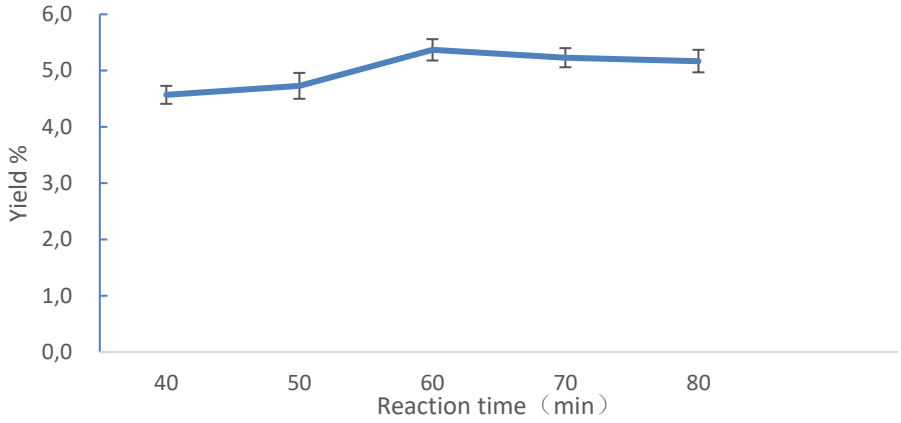


Figure 4: Yield of fucoidan (*Ascophyllum nodosum*) as a function of reaction time (Enzyme percentages1.25 %, 45℃, pH4.5).

Orthogonal experiment was designed (Table 5) according to the results of single factor. The results of orthogonal experiment are shown in Table 6.

Table 5: Orthogonal experiment factor table of fucoidan extraction from *Ascophyllum nodosum*

	Enzyme percentages %	pH	Temperature ℃	Reaction time (min)
level1	1	4	40	50
level2	1.25	4.5	45	60
level3	1.5	5	50	70

Table 6: Orthogonal experiment results of fucoidan extraction from *Ascophyllum nodosum*

	Enzyme percentages % A	pH B	Temperature ℃ C	Reaction time（min） D	Yield %
1	level1	level1	level1	level1	5.6
2	level1	level2	level2	level2	5.0
3	level1	level3	level3	level3	4.9
4	level2	level1	level2	level3	5.5
5	level2	level2	level3	level1	5.2
6	level2	level3	level1	level2	5.1
7	level3	level1	level3	level2	4.9
8	level3	level2	level1	level3	4.7
9	level3	level3	level2	level	5.3
R1	15.5	16	15.4	16.1	
R2	15.8	14.9	15.8	15	
R3	14.9	15.3	15	15.1	
k	0.9	1.1	0.8	1.1	

Note: The value of R1 is the sum of the values of Yield corresponding to level1, The value of R2 is the sum of the values of Yield corresponding to level2, The value of R3 is the sum of the values of Yield corresponding to level3. The value of k is equal to the difference between the maximum and minimum values of the three values R1, R2 and R3.

The optimum technological conditions were determined by orthogonal test and extreme difference analysis. Two groups were experimentally compared; Theoretical optimal group： enzyme percentage 1.25%, pH4, temperature 45℃ and reaction time 50min, and Experimental optimal group: enzyme percentage 1%, pH4, temperature 40℃ and reaction time 50min. The results are shown in Table 7. The yield of fucoidan reached 5.7% (enzyme percentage 1.25%, temperature 45 ℃, pH4 and reaction time 50min).

Table 7: Optimization experiment comparison of fucoidan extraction from *Ascophyllum nodosum*

	Technological conditions	Yield %
Theoretical optimal group	enzyme percentages 1.25%, pH4, Temperature 45℃, reaction time 50min	5.7±0.2
Experimental optimal group	enzyme percentages 1%, pH4, Temperature 40℃, reaction time 50min	5.5±0.1

4.1.2 Fucoidan extraction from Laminaria digitata

Enzymatic hydrolysis extract fucoidan from Laminaria *digitata* was performed as single factor and orthogonal experiment. Single factor experiment was designed according to the following conditions: enzyme percentages 0.75%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, Temperature 45℃, pH4.5 and reaction time 40, 50, 60, 70, 80min.

The results of enzyme percentages experiment are shown in Figure 5. The results of reaction time experiment are shown in Figure 6. The yield of fucoidan were highest (2.9%) when enzyme percentage was 1.5%. The yield of fucoidan was highest (3.2%) when reaction time was 60min.

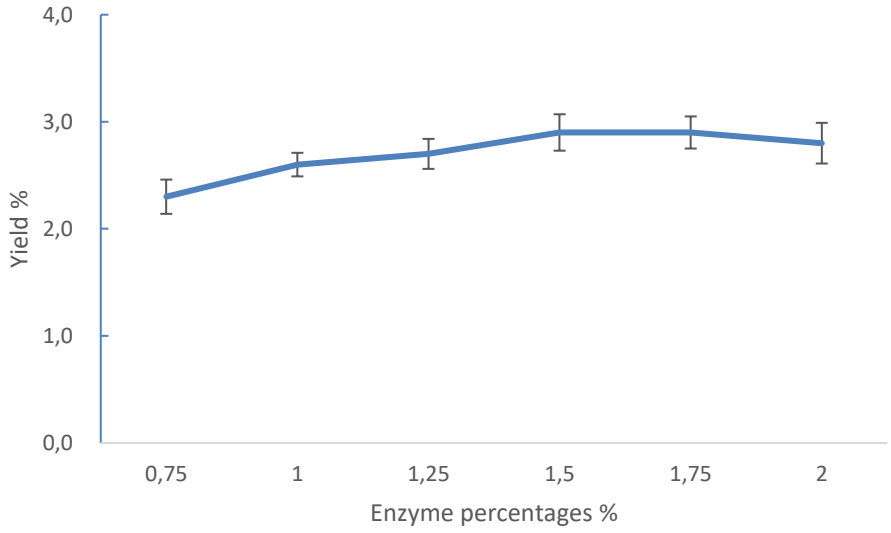


Figure 5: Yield of fucoidan (*Laminaria digitata*) as function of enzyme (cellulose) concentration (45℃, pH4.5,40 min).

Orthogonal experiments were designed (Table 8) according to the results of single factor. The results of orthogonal experiment are shown in Table 9.

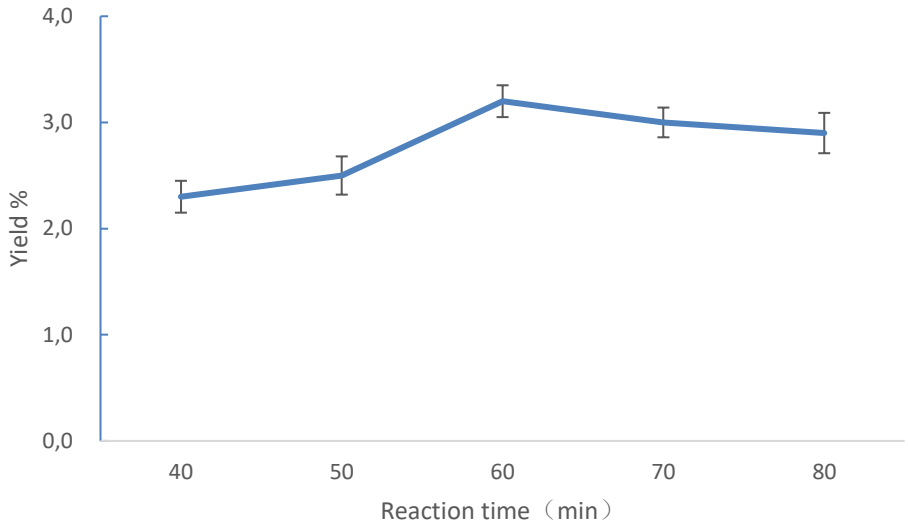


Figure 6: Yield of fucoidan (*Laminaria digitata*) as function of reaction time (Enzyme percentages1.5 %, 45℃, pH4.5).

Table 8: Orthogonal experiment factor table of fucoidan extraction from *Laminaria digitata*

	Enzyme percentages %	pH	Temperature ℃	Reaction time (min)
level1	1.25	4	40	50
level2	1.5	4.5	45	60
level3	1.75	5	50	70

Table 9: Orthogonal experiment results of fucoidan extraction from *Laminaria digitata*

	Enzyme percentages % A	pH B	Temperature ℃ C	Reaction time（min） D	Yield %
1	level1	level1	level1	level1	2.6
2	level1	level2	level2	level2	2.9
3	level1	level3	level3	level3	2.8
4	level2	level1	level2	level3	3.5
5	level2	level2	level3	level1	2.1
6	level2	level3	level1	level2	2.7
7	level3	level1	level3	level2	3.1
8	level3	level2	level1	level3	2.7
9	level3	level3	level2	level	3.6
R1	8.3	9.2	8	8.3	
R2	8.3	7.7	10	8.7	
R3	9.4	9.1	8	9	
k	1.1	1.5	2	0.7	

Note: The value of R1 is the sum of the values of Yield corresponding to level1, The value of R2 is the sum of the values of Yield corresponding to level2, The value of R3 is the sum of the values of Yield corresponding to level3. The value of k is equal to the difference between the maximum and minimum values of the three values R1, R2 and R3.

The optimum technological conditions were determined by orthogonal test and extreme difference analysis. Two groups were experimentally compared; Theoretical optimal group: enzyme percentages1.75%, pH4, temperature 45℃ and reaction time 70min, and Experimental optimal group: enzyme percentages 1.75%, pH5, temperature 45℃ and reaction time 50min. The results are shown in Table 10. The yield of fucoidan reached 3.6% (enzyme percentage 1.75%, temperature 45℃, pH4 and reaction time 70min).

Table 10: Optimization experiment comparison of fucoidan extraction from Laminaria *digitata*

	Technological conditions	Yield %
Theoretical optimal group	enzyme percentages 1.75%, pH4, Temperature 45℃, reaction time 70min	3.6±0. 1
Experimental optimal group	enzyme percentages 1.75%, pH5, Temperature 45℃, reaction time 50min	3.5±0. 1

4.2 Fucoidan fractionation

4.2.1 Fucoidan (extract from Ascophyllum nodosum) fractionation

Figure 7 shows that two fractions (FA1, FA2) were isolated through anion-exchange chromatography DEAE-52. The two fractions appeared before and after gradient elution by sodium chloride, respectively FA-1 and FA-2.

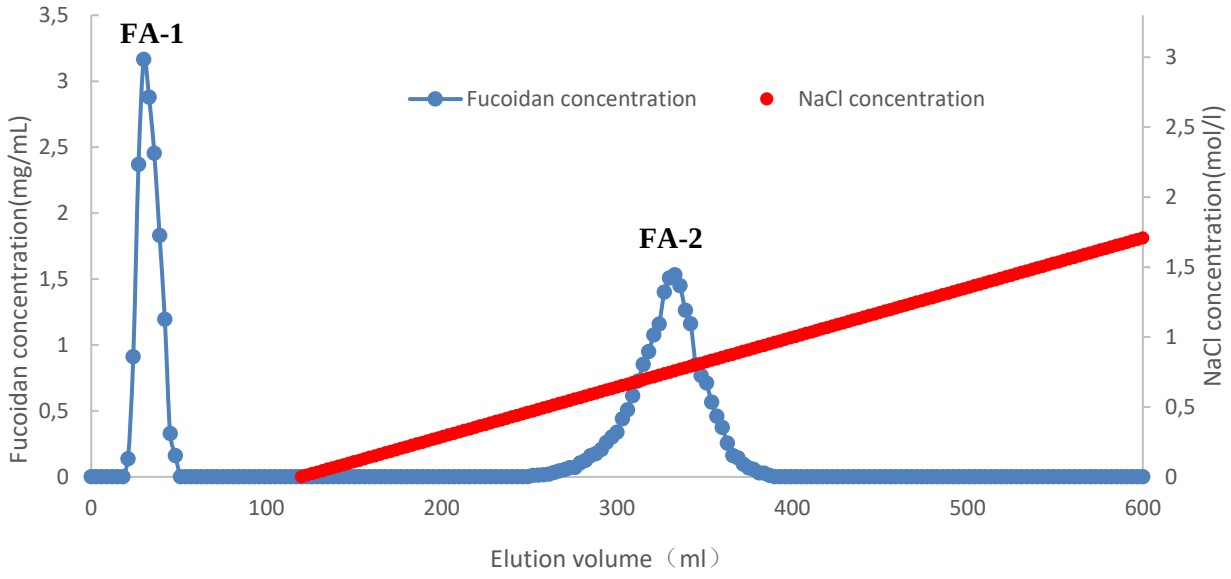


Figure 7: DEAE-52 chromatography elution curve of fucoidan from *Ascophyllum nodosum*

4.2.2 Fucoidan (extract from Laminaria digitata) fractionation

Figure 8 shows that three fractions (FL1, FL2 and FL3) were isolated through anion-exchange chromatography DEAE-52. The three fractions appeared before and after gradient elution by sodium chloride, respectively FL-1, FL-2and FL-3.

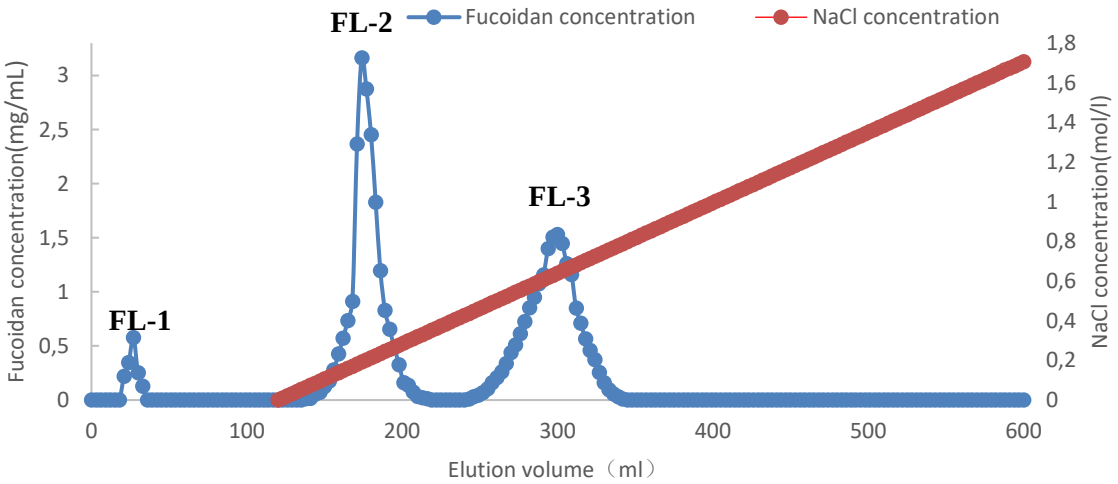


Figure 8: DEAE-52 chromatography elution curve of fucoidan from *Laminaria digitate*

4.3 Antibacterial activity (disc diffusion test)

The fucoidans from two algal species, *A.nodosum* and *L.digitate*, were tested for antibacterial activity against the three pathogens (dilution 100 times). The results from the paper disc diffusion test can be seen in Figure 9. There were small and clear inhibition zones around the paper disc on *Staphylococcus aureus* (DSM 799) and *Bacillus subtilis* (DSM 1117) impregnated with high concentration of fucoidan from both algae species. Fucoidans did not show any antibacterial activities on *Escherichia coli* (DSM 1103). A summary of the results is given in Table 11.

4.4 Microbial growth experiment

Effects of the two fucoidans on the growth of test strains are shown in Figure 10. Growth of the test strains (*Escherichia coli*, *Bacillus subtilis*. *Staphylococcus aureus*), 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 fucoidan solution.

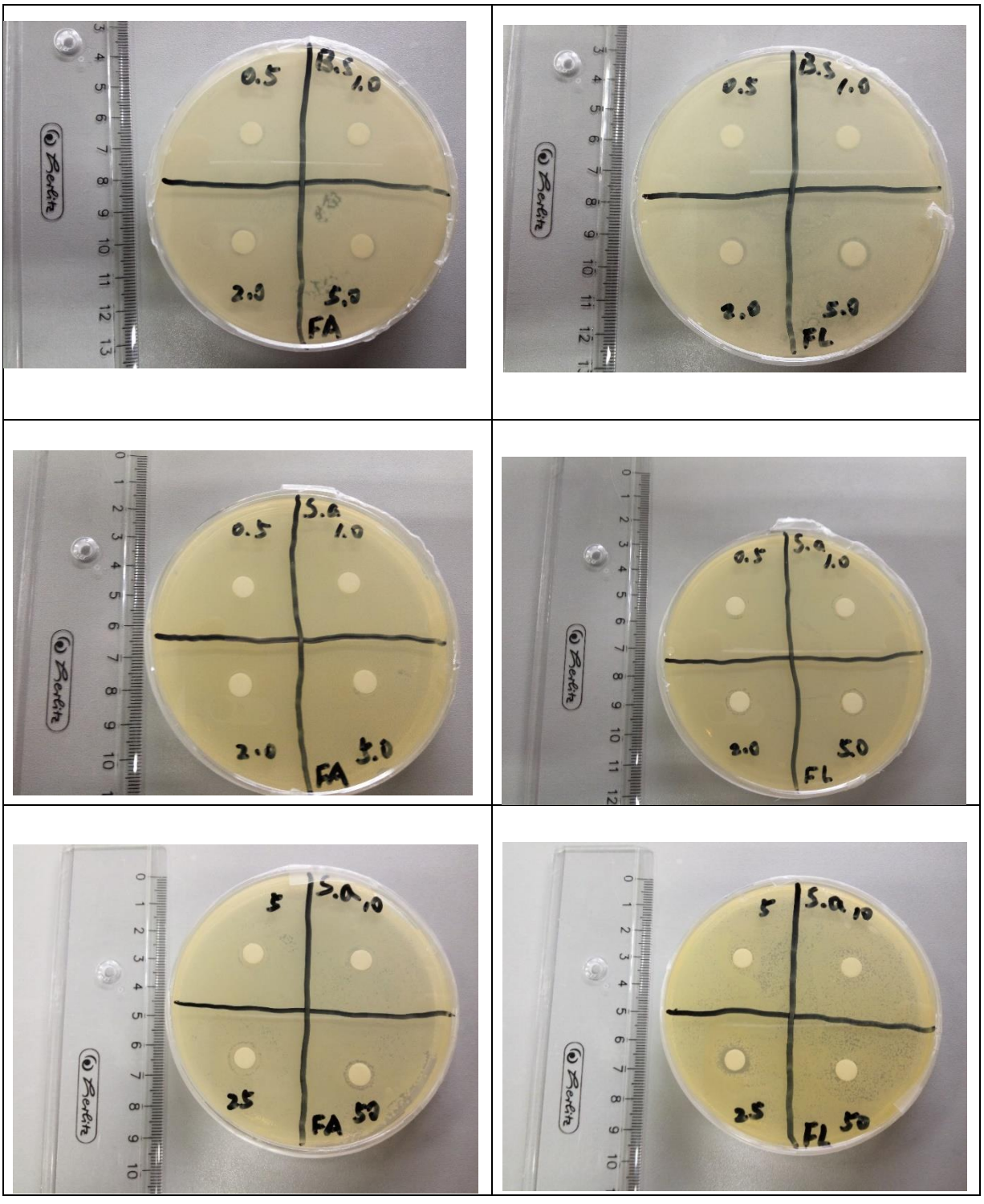


Figure 9: Antibacterial effect of fucoidan (FA: fucoidan from *Ascophyllum*, FL: fucoidan from *Laminarium* (0.5, 1, 2, 5, 10, 25, 50mg/mL) on *Bacillus subtilis* (1117) (BS) and *Staphylococcus aurous* (SA) (DSM 799)

Table 11: The antibacterial effect

		Mg on disc	Activity against pathogen		
			Escherichia coli (DSM 1103)	*Bacillus subtilis* (DSM 1117)	*Staphylococcus aureus* (DSM 799)
FA (Fucoidan extract from *Ascophyllum nodosum*)	0.5mg/ml	0.01	-	-	-
	1.0mg/ml	0.02	-	-	-
	2.0mg/ml	0.04	-	-	-
	5.0mg/ml	0.1	-	-	-
	10mg/ml	0.2	-	-	-
	25mg/ml	0.5	-	-	+
	50mg/ml	1.0	-	-	+
FL (Fucoidan extract from *Laminaria digitata*)	0.5mg/ml	0.01	-	-	-
	1.0mg/ml	0.02	-	-	-
	2.0mg/ml	0.04	-	+	+
	5.0mg/ml	0.1	-	+	+
	10mg/ml	0.2	-	-	-
	25mg/ml	0.5	-	-	+
	50mg/ml	1.0	-	-	-

The fucoidan solutions showed no inhibition of the growth of *E. coli* and *B.subtilis* in nutrient broth without salt. However, *S. aureus* was clearly inhibited by the fucoidan solutions.

When salt (1%) was added to the nutrient broth (Figure 11) *B.subtilis* was inhibited by 5.0mg/mL fucoidan from *Ascophyllum nodosum* and high concentration of fucoidan solutions exhibited significant inhibition effects on *S. aureus*. Addition of salt did not affect the effect of fucoidan on *E.*coli.

From the figures the Minimum Inhibitory Concentration (MIC) of fucoidan on *S. aureus* were estimated at 5.0mg/mL from Ascophyllum *nodosum* and 0.5mg/mL from Laminaria *digitata*.

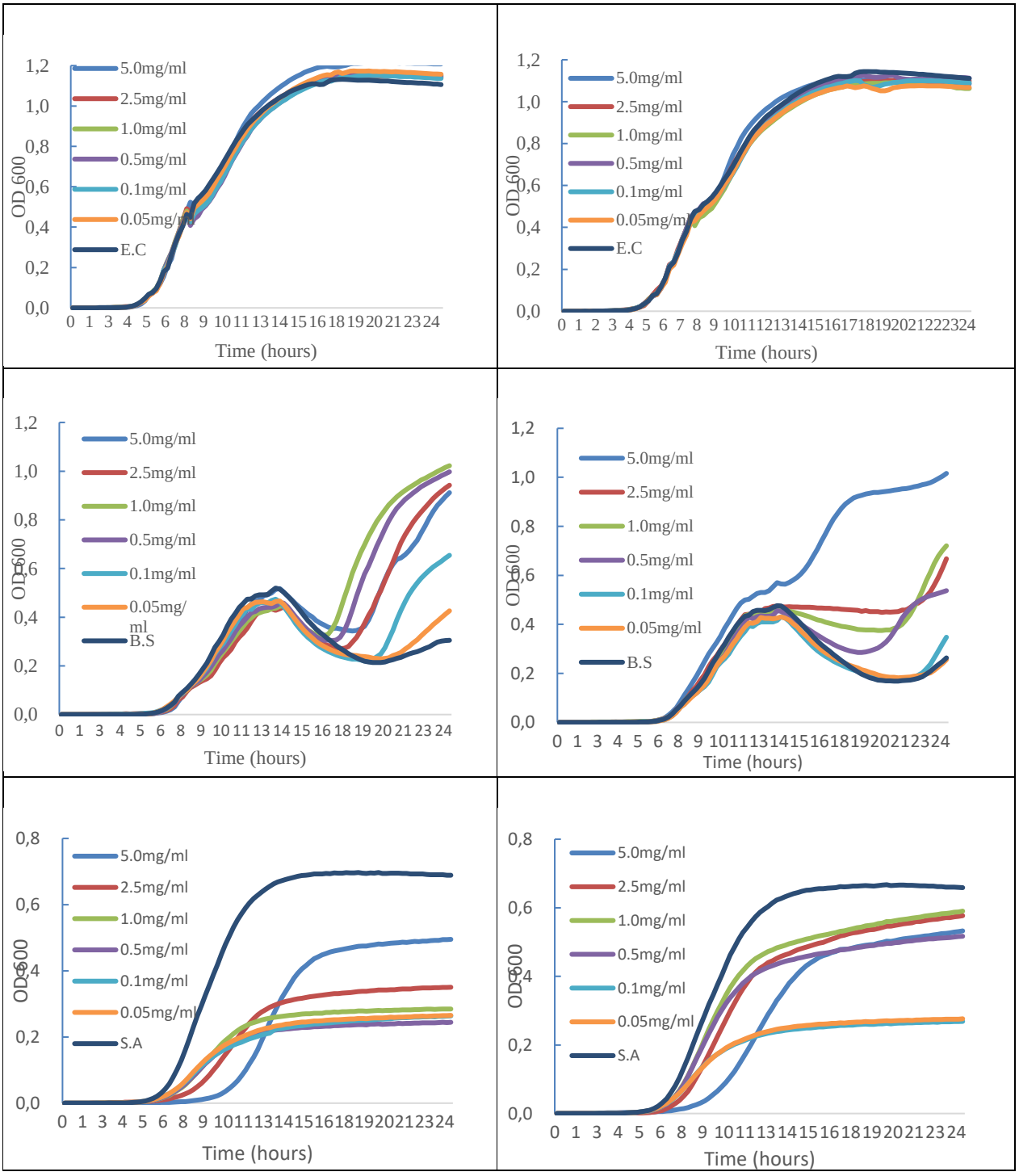


Figure 10: Effect of fucoidan (from *Ascophyllum nodosum* (left), from *Laminaria digitate* (right)) on the growth of *Escherichia coli* (top), *B.subtilis* (middle) and *Staphylococcus aureus(bottom)* in nutrient broth with 0% NaCl

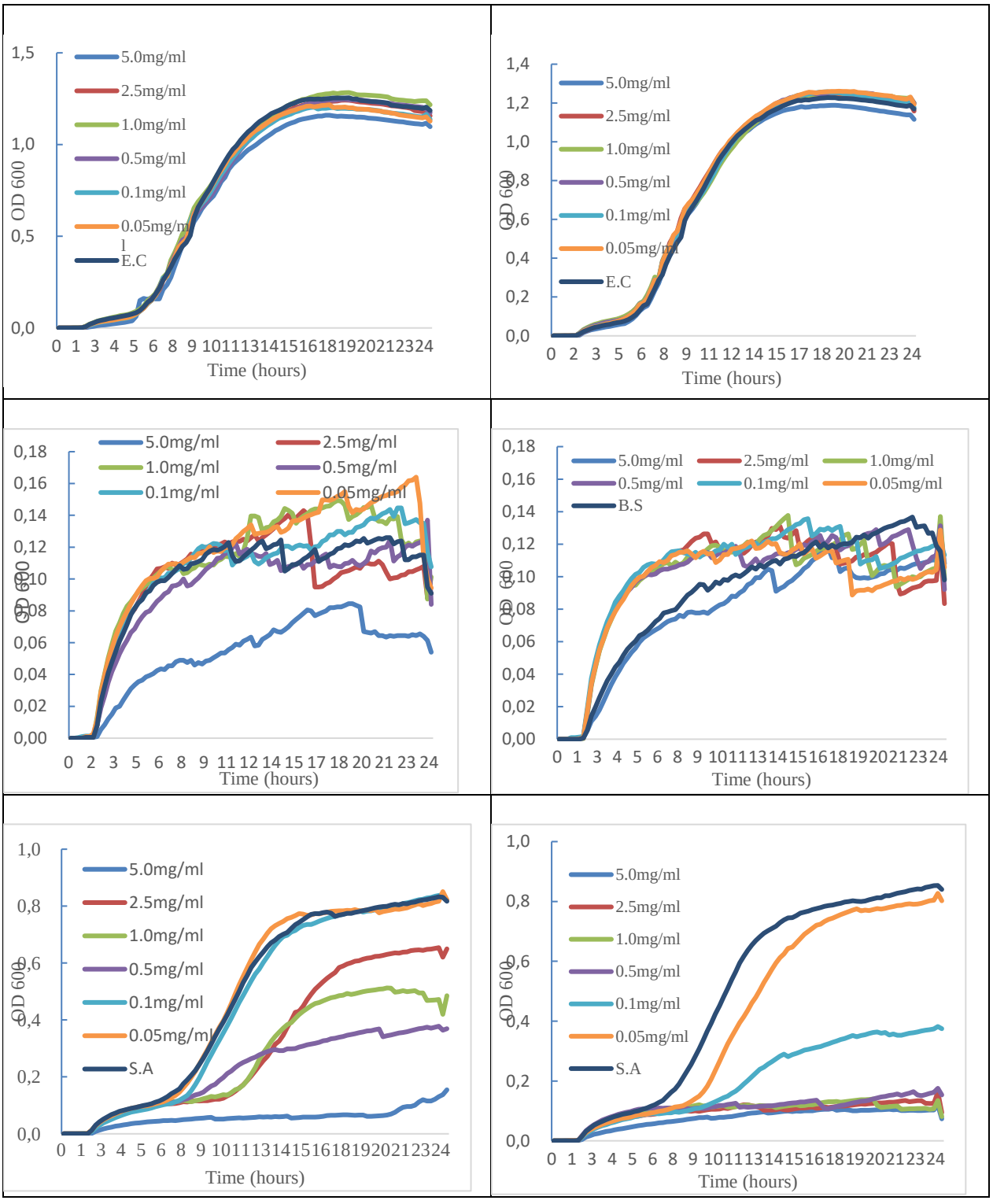


Figure 11: Effect of fucoidan (from *Ascophyllum nodosum* (left), from *Laminaria digitate* (right)) on the growth of *Escherichia coli* (top), *B.subtilis* (middle) and *Staphylococcus aureus(bottom)* in nutrient broth with 1% NaCl

5 DISCUSSION

5.1 Fucoidan extraction

One aim of this study was to extract fucoidan from Icelandic seaweeds and to optimise the extraction conditions. In this study the fucoidan content was found to be 4.7-5.6% of dry weight in *Ascophyllum nodosum* and 2.1-3.6% of dry weight in *Laminaria digitate* depending on reaction conditions. Higher enzyme percentages and more reaction time were needed to extract the fucoidan from *Laminaria digitate* than from *Ascophyllum nodosum*. The cell wall of *Laminaria digitate* are more difficult to crack to release fucoidan because fucoidan were in the intercellular space. *Ascophyllum nodosum* fucoidan extraction yield were higher than *Laminaria digitate*.

When compared to other studies, the fucoidan extracted from *Ascophyllum* growing in different areas was shown that fucoidan extracted from *Ascophyllum nodosum* harvested on the coast of Aberystwyth, Welsh was 6.5-8.1% (Fletcher *et al*., 2017). *Ascophyllum mackaii* (China) fucoidan extraction yield were 4.49% (Liu *et al*., 2013). *Laminaria japonica* (China) fucoidan extraction yield were reached 1.845% (He *et al*., 2006). Therefore, Iceland *Ascophyllum* contained more fucoidan than China, but less than Welsh. And Iceland *Laminaria digitate* also contained more fucoidan than China *Laminaria japonica.* This may be related to the water temperature of the algae growing area, the lower the water temperature, the higher the fucoidan content. However, this requires further validation.

5.2 Fucoidan fractionation

The fucoidan from the two different seaweeds showed marked difference. The fucoidan from *Ascophyllum nodosum* showed two main fractions whereas fucoidan from *Laminaria digitate* had three main fractions. The isolation of fucoidan extracted from China *Laminaria japonica* was studied (He *et al*., 2007). Three fractions were isolated through anion-exchange chromatography DEAE-52. Three fractions appeared after gradient elution by sodium chloride. This result is like that of *Laminaria digitate* in Iceland. Further characterization is pending.

5.3 Antibacterial activity of fucoidan

Another objective of this study was to determine the antibacterial activity of fucoidans from Iceland local seaweeds species, *A.nodosum* and *L.digitata*. The activity was tested against *Escherichia coli* (DSM 1103), *Bacillus subtilis* (DSM 1117) and *Staphylococcus aureus* (DSM 799).

The results showed that the tested fucoidan inhibitory effect against gram-positive bacteria, especially on *S.aureus.*

Two methods were carried out to study the antibacterial activity of fucoidan. In the paper diffusion test the fucoidan showed slight inhibition effects on *S.aureus*, especially the fucoidan from *Laminaria digitate.* The fucoidans did not show any antibacterial activities on *E. coli* and *B. subtilis.*

In the growth studies in micro-well plates the results were consistent with the results of paper diffusion test, however when salt was added B.*subtilis*was slightly inhibited. The addition of salt

enhanced the inhibitory effect of fucoidan on *S. aureus*. And the inhibition effect of fucoidan from *Laminaria digitate* were stronger than fucoidan from *Ascophyllum nodosum*.

The MIC of the isolated fucoidan is higher than reported MIC of fucoidan isolated from *Sargassum polycystum* (Chotigeat *et al.,* 2004). However, that fucoidan had a broader spectrum of inhibition as it inhibited also gram – bacteria.

6 CONCLUSION

The amount of fucoidan in Icelandic brown seaweed is higher than reported in seaweed harvested in China, but lower than reported from the UK.

The isolated fucoidan had an antibacterial effect.

This is the first study to show that fucoidan extracted from Icelandic seaweed has antibacterial activity.

In the future, the bacteria inhibitory activity of fucoidan extracted from Icelandic seaweed under refrigeration (0-6℃) and different salinity conditions (0-22%) will be studied. The structure and monosaccharide composition of fucoidan from analysis will also be researched.

ACKNOWLEDGEMENTS

This study was carried out in laboratory of the University of Akureyri and the programme was financed by the UNU-FTP (United Nations University-Fisheries Training Programme).

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

I am deeply grateful to Dr. Tumi Tómasson, Þór Ásgeirsson and Mary France for giving me the opportunity to participate in the programme and their continued assistance, support and guidance during the six months period of the training programme. Also thanks to Sigríður Kr. Ingvarsdóttir for her considerate care to my life in Reykjavik and Akureyri of 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 during my research work. Special thanks to Arnheiður Eyþórsdóttir, Sean M. Scully and Guðný Jóhannsdóttir, for their hearty 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.

LIST OF REFERENCES

Anastyuk S. D., Imbs T. I., Shevchenko N. M., Dmitrenok P. S., Zvyagintseva T. N. (2012b). ESIMS analysis of fucoidan preparations from *Costaria costata*, extracted from alga at different life-stages. *Carbohydrate Polymers* 90(2): 993-1002.

Anastyuk S. D., Shevchenko N. M., Dmitrenok P. S., Zvyagintseva T. N. (2012a). Structural similarities of fucoidans from brown algae *Silvetia babingtonii* and *Fucus evanescens*, determined by tandem MALDI-TOF mass spectrometry. *Carbohydrate Research* 358: 78-81.

Anastyuk S. D., Shevchenko N. M. Nazarenko E. L., Imbs T. I., Zvyagintseva T. N. (2010). Structural analysis of a highly sulfated fucan from the brown alga *Laminaria cichorioides* by tandem MALDI and ESI mass spectrometry. *Carbohydrate Research* 15: 2206-2212.

Berteau O. and Mulloy B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* 13: 29–40.

Chmit M., Kanaan H., Habib J., Abbass M., Mcheik A., Chokr A. (2014). Antibacterial and antibiofilm activities of polysaccharides, essential oil and fatty oil extracted from *Laurus nobilis* growing in Lebanon. *Asian Pac J Trop Med* 7(Suppl 1): S546-S552.

Chotigeat, W., Tongsupa, S., Supamataya, K. & Phongdara, A. (2004). Effect of fucoidan on disease resistance of black tiger shrimp. *Aquaculture* 233:23-30.

Collins, K. G., Fitzgerald, G. F., Stanton, C., Ross, R. P. (2016). Looking Beyond the Terrestrial: The Potential of Seaweed Derived Bioactives to Treat Non-Communicable Diseases. *Marine drugs* 14(3): 60.

Dinesh S., Menon T., Hanna L. E., Suresh V., Manikannan M. (2016). In vitro anti-HIV-1 activity of fucoidan from *Sargassum swartzii*. *International Journal of Biological Macromolecules* 82: 83-88.

FAO (2016). The State of the World Fisheries and Aquaculture 2016. Rome: Food and Agricultural Organization of the United Nations.

Fletcher H.R., Biller P., Ross A.B., J.M.M. (2017). The seasonal variation of fucoidan within three species of brown microalgae. *Algal Research* 22: 79–86.

Gamal, A. A. E. (2010). Biological importance of marine algae. *Saudi Pharmaceutical Journal* 18: 1-25.

Gupta, S., & Abu-Ghannam, N. (2011). Bio active potential and possible health effects of edible brown seaweeds. *Trends in Food Science and Technology* 22: 315–326.

He Y.H., Wang Q.K., Liu H.H. (2006). Extracting technology of fucoidan from kelp *Laminada japonica* by enzymatic hydrolysis. *Journal of Dalian Fisheries University* 21(1):55-58.

He Y.H., Wang Q.K., Zhang T., Shi Y.F. (2007). Enzymatic Hydrolyzing Technology and Isolation of Fucoidan from *Laminaria japonica*. *Journal of Shenyang Agricultural University* 38(2):215-219.

Imbs T. I., Ermakova S. P., Malyarenko O. S. (Vishchuk), Isakov V. V., Zvyagintseva T. N. (2016). Structural elucidation of polysaccharide fractions from the brown alga *Coccophora langsdorfii* and in vitro investigation of their anticancer activity. *Carbohydrate Polymers* 135: 162-168.

Jiao G.L., Yu G.L., Zhang J.Z., Ewart H. S. (2011). Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae. *Marine Drugs* 9:196–223.

Jin Q. (2010). Antibacterial effect of extracts from two Icelandic algae (*Ascophyllum nodosum* and *Lamina riadigitata*). Final Project. Reykjavik: UNU-Fisheries Training Programme. < http://www.unuftp.is/static/fellows/document/jin-proofread-caitlin-and-ski-mfd.pdf>

Kadam SU, Tiwari BK, Smyth TJ, O'Donnell CP (2015c) Optimization of ultrasound assisted extraction of bioactive components from brown seaweed Ascophyllum nodosum using response surface methodology. *Ultrason Sonochem* 23:308–316.

Kylin, H., (1913). *Hoppe-Seyler' s Zeitschrift für physiologische Chemie* 83:171–197.

Lee J. M., Shin Z.U., Mavlonov G. T., Abdurakhmonov I. Y., Yi T. H. (2012). Solid-Phase Colorimetric Method for the Quantification of Fucoidan. *Appl Biochem Biotechnol* 168:1019–1024.

Lee K. Y., Jeong M. R., Choi S. M., Naa S. S., Cha J. D. (2013). Synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria. A*rchives of oral biology* 58:482–492

Liu X., Qu G.Y., Zhou Y.B., Yi Y., Han L.J. (2013) Ultrasound assisted extraction and in vitro antioxidant activity analysis of fucoidans from *Ascophyllum mackaii* and *Laminaria japonica* residue. *Marine Sciences* 37(12): 34-39.

Miyashita K., Mikami N., Hosokawa M. (2013). Chemical and nutritional characteristics of brown seaweed lipids: A review. *Journal of functional foods* 5: 1507 –1517.

Nishiguchi T., Jiang Z., Ueno M., Takeshita S. Cho K., Roh S. W., Kang K. H., Yamaguchi K., Kim D. and Oda T. (2014). Reevaluation of bactericidal, cytotoxic, and macrophage-stimulating activities of commercially available *Fucus vesiculosus* fucoidan. *Algae* 29(3): 237-247.

Norziah, M. H. and Ching, C. Y. (2000). Nutritional composition of edible seaweed *Gracilariachanggi*. *Food Chemistry* 68: 69-76.

Pádua, D, Rocha, Gargiulo, A.A. Ramos. (2015). Bioactive compounds from brown seaweeds: Phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer. *Phytochemistry Letters* 14: 91–98.

Rioux L.E., Turgeon S.L., Beaulieu M. (2007). Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymers* 69: 530–537

Senthilkumar, K., Manivasagan, P., Venkatesan, J., Kim, S.K. (2013). Brown seaweed fucoidan: Biological activity and apoptosis, growth signaling mechanism in cancer. *Int. J. Biol. Macromol.* 60: 366–374.

Shu, Z. H., Shi, X. Z., Nie, D. Q., & Guan, B. Y. (2015). Low-molecular-weight fucoidan inhibits the viability and invasiveness and triggers apoptosis in IL-1 beta-treated human rheumatoid arthritis fibroblast synoviocytes. *Inflammation* 38(5): 1777–1786.

Sivaganavelmurugan M., Ramnath G. Karthik, Thaddaeus B. Jude, Palavesam A., Immanuel G. (2015). Effect of *Sargassum wightii* fucoidan on growth and disease resistance to *Vibrioc parahaemolyticus* in Penaeus monodon post-larvae. *Aquaculture Nutrition* 21: 960-969

Vo T.S., Kim S.K. (2013). Fucoidans as a natural bioactive ingredient for functional foods. *Journal of functional food* 5: 16 –27.

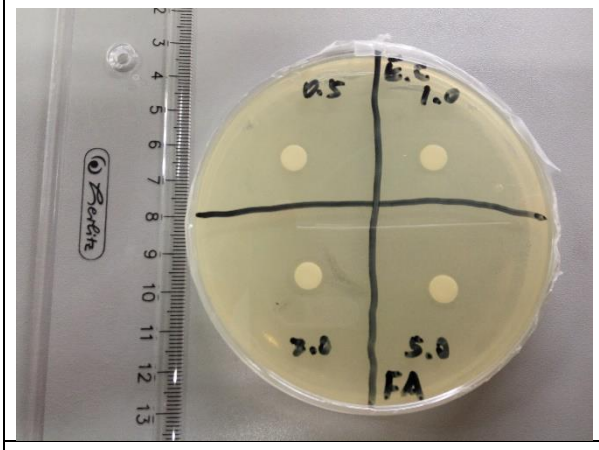
Wang Q.K., Song Y.F., He Y.H., Ren D.D, Kow , Qiao Z.Y., Liu S., Yu X.J. (2014). Structural characterisation of algae *Costaria costata* fucoidan and its effects on CCl_4-induced liver. *Carbohydrate Polymers* 107: 247–254

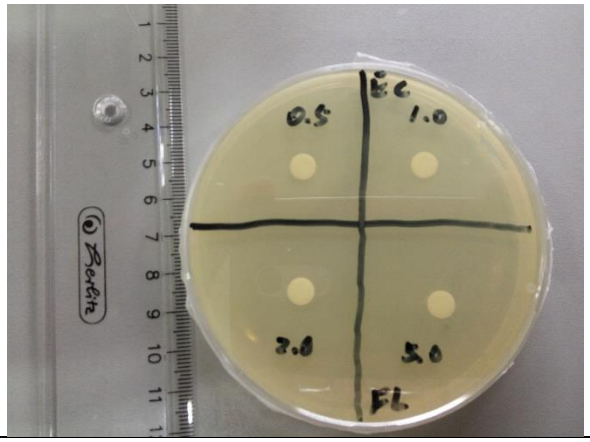
Wijesinghe, W.A.J.P., Jeon, Y.J. (2012). Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review. *Fitoterapia* 83: 6-12.

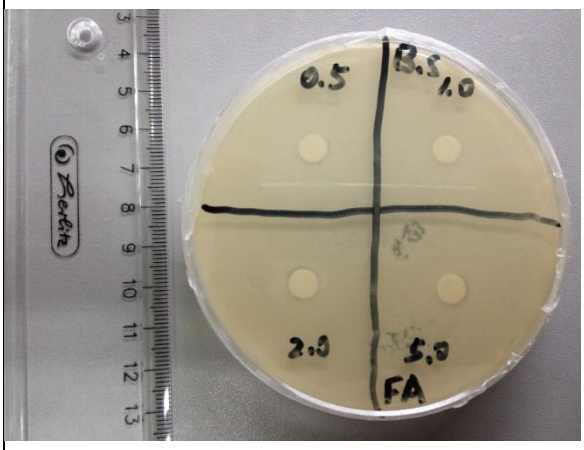
Yuan Y., and Macquarrie D. (2015). Microwave assisted extraction of sulfated polysaccharides (fucoidan)from Ascophyllum nodosum and its antioxidant activity. *Carbohydrate Polymers* 129: 101–107.

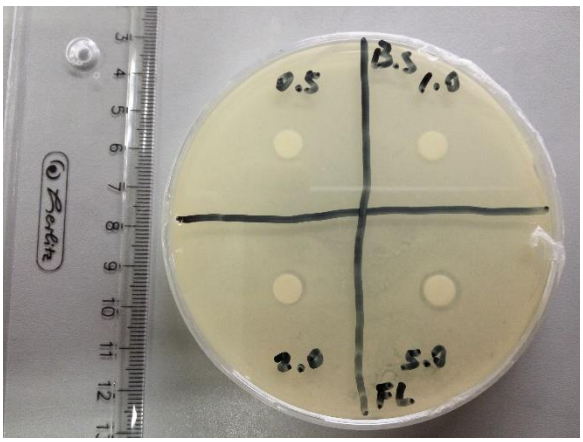
Zhao X.W. (2016). *China fishery statistical yearbook.* Beijing: China Agricultural Publishing House.

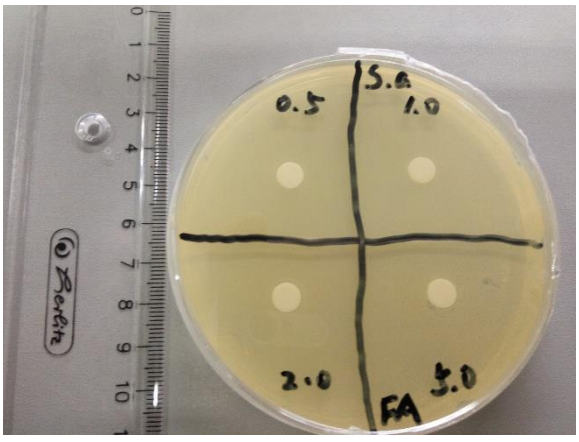
APPENDIX: Disc diffusion test of fucoidan

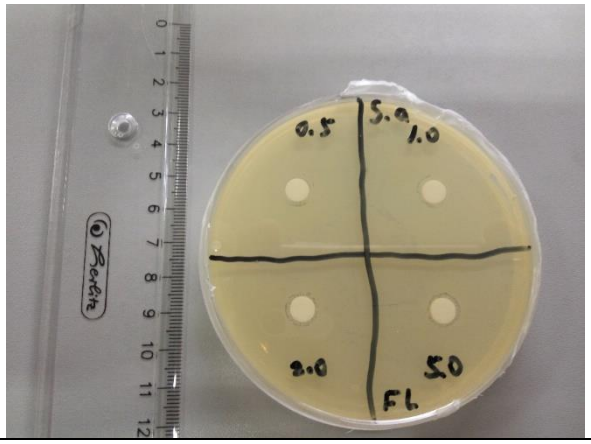












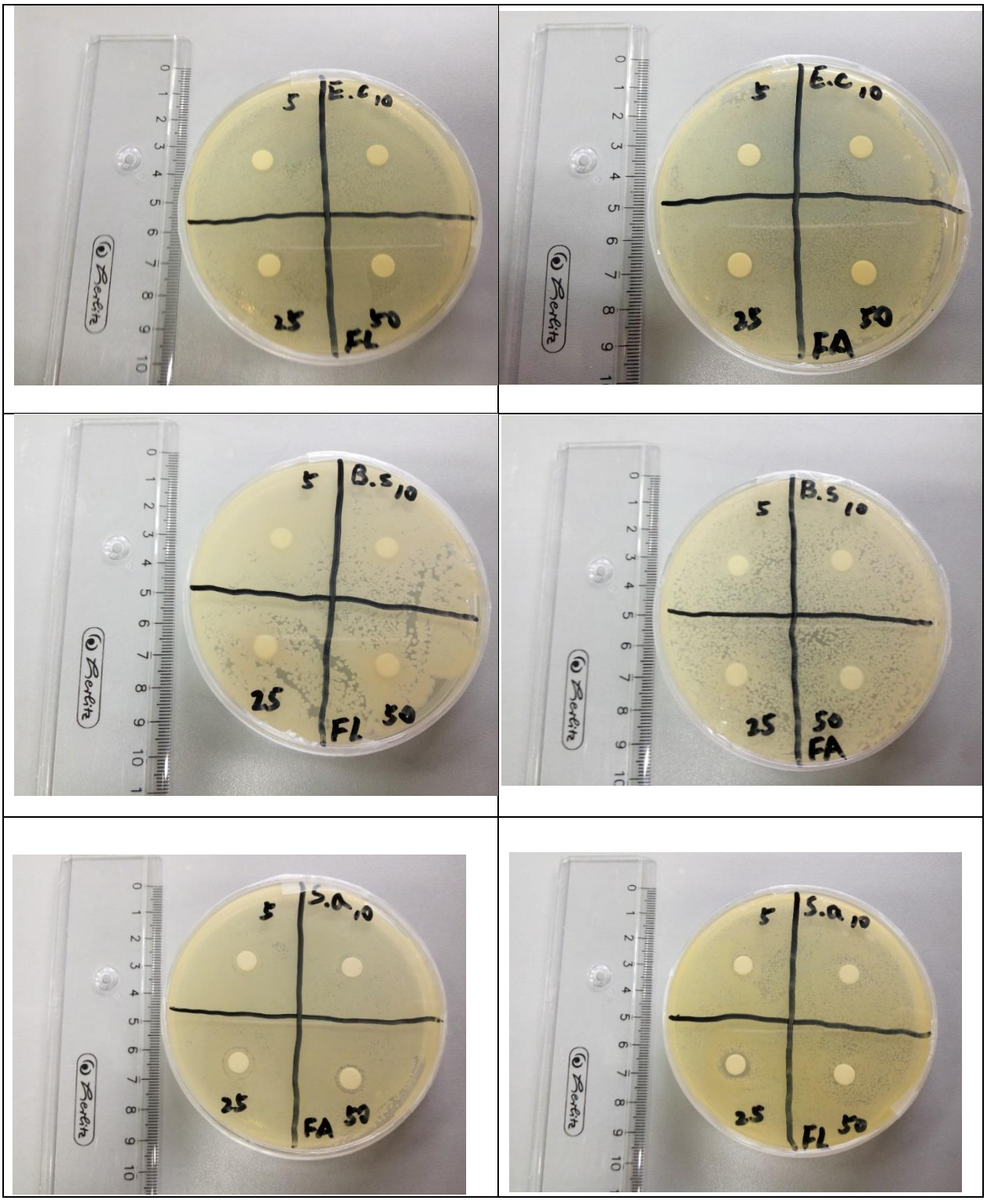


Figure 12: Antibacterial effect of fucoidan (FA: fucoidan from *Ascophyllum*, FL: fucoidan from *Laminarium* (0.5, 1, 2, 5, 10, 25, 50mg/mL) on *Escherichia coli* (DSM 1103), *Bacillus subtilis* (1117) (BS) and *Staphylococcus aurous* (SA) (DSM 799)