Chapter 1: Introduction

1.1 Background

The morphological characteristic of macromarine algae, often known as "seaweeds," allows them to adhere and settle in the hard substrate of the shallow water zone of the shore, which is ideal for their massive growth (Hoq *et al*., 2016). Seaweeds are regarded as a high-profile commercial marine biota due to their numerous uses as raw materials for biochemicals (agar, agarose, algin, carrageenan), colors, food, feed, enzymes, and medicines (Athithan, 2014). The phrase "seaweed" is a vernacular name for innumerable kinds of microscopic and largely macroscopic, multicellular algae that lack roots or flowers. Instead, they live attached to hard surfaces below the high tide line or drift in the oceans with their leaves, stems, fruits, and seeds (Chapman, 1973; McHugh, 2003; Okazaki, 1971; Round, 1970). There are oceans and seas all over the world where seaweed can be found, and none of them are known to be toxic (Zemke-White and Ohno, 1999). There are 6000 types of red seaweed, 2000 species of brown seaweed, and 1200 species of green seaweed (Robinson, 1980). The south-eastern region of Bangladesh is where reports of natural seaweed abundance originate, and Saint Martin Island has a massive natural seaweed growth. The coastal and estuarine regions of Bangladesh have been home to 200 species of seaweed from 77 genera, whereas St. Martin's Island is home to 1500 metric tons (MT) of red seaweed biomass (Aziz, 2015).

According to estimates, the world's seaweed processing business consumes between 10 and 12 million t (frozen weight) of seaweeds per year, but only 4.5 percent of the total seaweed production in 2010. These seaweeds are either harvested in the wild or grown in offshore and onshore farms. While the amount of wild seaweed harvested has declined, from around 1.2 million t in 2000 to about 0.9 million t in 2010, the amount of cultivated seaweed produced has risen by almost 50% during the past ten years (Nayar and Bott, 2014). At least 221 species of seaweed are utilized commercially worldwide, 101 of which are used to produce phycocolloids and 145 of which are used as food which include 32 chlorophytes, 125 rhodophytes, and 64 phaeophytes (Zemke-White and Ohno, 1999). About 10 species are frequently grown, especially the red algae *Porphyra* spp., *Porphyra tenera*, *Eucheuma* spp., *Kappaphycus alvarezii*, and *Gracilaria* sp. and *Gracilaria verrucosa*, as well as the brown algae *Laminaria*

japonica, *Undaria pinnatifida*, and *Porphyra* spp. (Wikfors and Ohno, 2001). China, the global highest seaweed producer, accounted for roughly 58% production of cultivated seaweed and 45% of its total value. Along with Indonesia, the Philippines, South Korea, Japan, and North Korea are other significant seaweed producers.

In the past, only Japan, China, and the Republic of Korea used seaweeds as food; however, this practice has since become widespread throughout North America, South America, Europe, and Australia (Kılınç *et al*., 2013; McHugh, 2003). Seaweed food products such as burgers, juice, sandwiches, cakes, salads, biscuits, chips, and others are commercially produced in addition to traditional seaweed foods like Korean Wakame and Japanese Nori (Sarkar, 2015). The most remarkable uses of seaweeds are in the phycocolloid or hydrocolloid, cosmetic, biofuel, pharmaceutical, waste water treatment, and bioplastic industries, as well as in the development of medications for Alzheimer's disease, cancer, and gastric ulcers (Burtin, 2003; Gade *et al*., 2013; McHugh, 2003; Wargacki *et al*., 2012;).

Alginates are utilized in dental molds and wound dressings. Agar is used as a culture medium in microbiology as well as other macroalgal polysaccharides, carrageenans, alginates, and agaroses have biomedical uses. *Delisea pulchra* might prevent bacteria from colonizing (Cappitell *et al*., 2008). Red and green algae's sulfated saccharides block some DNA and RNA-enveloped viruses (Kazłowski *et al*., 2012).

A proposal was generated to grow seaweed for eliminating carbon called "ocean afforestation" (Duarte *et al*., 2017). After being harvested, seaweed breaks down in an anaerobic digester to create biogas, which is made up of 60% methane and 40% carbon dioxide. Methane can be utilized as a biofuel while carbon dioxide can be stored to keep it away from the atmosphere. Seaweed grows quickly and doesn't need much area.

According to the Food and Agriculture Organization, Bangladesh has a vast potential for seaweed production due to its beaches, estuaries, and mangroves, which together make up the nation's more than 700 kilometers of coastline and 25,000 square kilometers of coastal territory (FAO).

At Nuniarchara, Inany Beach, and Reju Khal in Cox's Bazar, Bangladesh, some 300 households are involved in the seaweed frame industry. By 2020, they expect to produce 390 tons, with uses in the food, cosmetic, feed, and pharmaceutical industries.

In the coastal areas of Cox's Bazar, the edible seaweed grow such as green genus *Caulerpa, Ulva*, *Enteromorpha*, the red genus *Hypnea, Gracilaria*, *Gelidium*, and the brown genus *Sargassum* etc. Mid-October to mid-April is the ideal time of the year to grow seaweed in Bangladesh's coastal waters. Due to high tides, cultivation is put on hold during the wet season.

1.2 Significance of the Study

As far as the study is going on, there is no sufficient published data on proximate composition, microbial condition, and heavy metals pollution in seaweeds found in Bangladesh. To ensure proper utilization of the natural seaweeds found in the seabed of Cox's Bazar and St. Martin Island, knowing their biochemical composition is necessary. With huge potential, seaweed plays a big role in the blue economy of the country. To establish a successful seaweed industry, analysis of seaweed will contribute in cultivation and increase production which will change the lifestyle of the coastal people economically.

1.3 Objectives

- To learn about proximate composition of seaweeds and their value-added products
- To identify pathogenic bacteria in the collected seaweeds
- To detect presence of heavy metals in the collected seaweed.

Chapter 2: Review of Literature

Seaweeds are renowned for their abundance in minerals, vitamins, and polysaccharides. Brown seaweeds have a relatively low protein content, whereas most red seaweeds are rich in protein. Lipid concentrations are typically modest. Brown seaweeds typically have low proteic fractions (average: 5-15% of the dry weight), whereas green and red seaweeds have greater protein levels (average: 10-30% of the dry weight). The majority of the free amino acids in seaweed include alanine, aminobutyric acid, taurine, omithine, citrulline, and hydroxy-proline, and the amounts vary according on the species. Lipids make up 1-3% of the dry matter of algae, making their contribution as a source of dietary energy appear to be minimal (Arasaki and Arasaki, 1983).

2.1 Proximate Composition of Seaweeds

Over the past two decades, nutritionists and food scientists have placed a significantly greater emphasis on the nutritional evaluation of edible seaweeds (Kumari *et al*., 2010; Ratana-Arporn and Chirapart, 2006). Red seaweeds were the focus of the bulk of studies due to their better nutritional value in comparison to brown and green edible seaweeds (Arasaki and Arasaki, 1983; Marinho-Soriano *et al*., 2006; Wong and Cheung, 2000).

The amino acid composition and essential amino acid score can be used to assess the nutritional quality of protein in seaweeds (FAO/WHO/UNU, 1985; Wong and Cheung, 2000). According to Mabeau and Fleurence (1993), the ash contents of seaweed, which range from 8-40 % dry weight, demonstrate the high element contents of the organisms. Seaweed species, maturities, ambient growth circumstances, and seasonality all affect the nutritional makeup of the species (Ito and Hori, 1989; Ortiz *et al*., 2006). The synthesis of nutrients is affected by changes in ecological conditions, according to research done by Lobban *et al*. (1985).

Marinho-Soriano *et al*. (2006) determined that the dry matter of *Gracilaria cervicornis* consisted of 22.96% protein, 0.43% lipid, 63.12% carbohydrate, 5.65% fiber, 7.72% ash, and 14.33% moisture.

Gracilaria changgi has a dry matter composition of 6.90% protein, 3.30% lipid, 24.70% fiber, and 22.70% ash (Norziah and Ching, 2000).

In the dry matter of *Gracilaria cornea*, Robledo and Freile-Pelegrin (1997) discovered 5.47% protein, 36.29% carbohydrate, 5.21% fiber, and 29.06% ash.

Gracilaria fisheri maintains the following proximate composition according to reports by Benjama and Masniyom (2012): In the dry season, it contains 11.6% protein, 2.7% lipid, 22.9% ash, 5.2% moisture, and 64% total dietary fiber, while in the wet season, it contains 11.6% protein, 1.7% lipid, 21.4% ash, 5.7% moisture, and 57.5% total dietary fiber. The study also showed that *Gracilaria tenuistipitata* has a summertime composition of 20.3% protein, 1.9% lipid, 26% ash, 3.3% moisture, and 60.2% total dietary fiber on a dry basis and in the rainy season it has a total dietary fiber composition of 56.6%, 22.9% protein, 3.6% lipid, 7.9% ash, and 3.9% moisture. Arginine, leucine, and threonine were the essential amino acids found in the highest concentration in the two species. The findings showed that the two species had high levels of K and Cl.

Ulva lactuca was found in the Persian Gulf of Iran, and Rohani-Ghadikolaei *et al*. (2011)'s analysis reveals that it includes 17.11% protein, 3.6% crude fat, 59.1% carbohydrate, 12.41% ash, and 6.8% moisture. *Enteromoroha intestinalis* contains 10.51% protein, 2.9% crude fat, 35.52% carbohydrate, 22.41% ash, and 10.6% moisture. *Gracilaria corticata* has the following composition: 2.19–19.3% protein, 0.46–1.8% crude fat, 5.58–43% carbohydrate, 0.53–23.1% ash, and 0.15–9.2% moisture. In this investigation, *U. lactuca* and *E. intestinalis* had the greatest crude lipid contents (3.6 and 2.9%, respectively), while *G. corticata* had the lowest (1.8%). The study found that *U. lactuca* (66.3%) had the highest relative SFA concentrations in its total lipid composition, which comprised more than half palmitic acid.

According to Azmat *et al*. (2006), seaweeds can store minerals in their thalli by selectively absorbing them from the surrounding seawater. As a result, their mineral content and composition vary depending on the species and locality.

According to Sivaramakrishnan *et al*. (2017), the South Andaman Coast of India's *Enteromorpha* sp. has 78.78% moisture, 19.19% total ash, 2.91% crude fat, 16.56% crude protein, and 3.54% crude fiber. The findings showed that *Enteromorpha* sp. (16.56%) had the highest protein content.

According to Ganesan *et al*. (2014)'s analysis of 3 species from India's northwestern coast, *Enteromorpha tubulosa* had greater levels of sugar (51.05%), protein (19.09%), and fat content (5.56%). The number of macro elements in the green seaweed

Enteromorpha compressa was high (11.42 mg/100g dry wt.), but *Enteromorpha linza* had the highest levels (81.51 mg/100g dry wt.). With the exception of *E. compressa*, all three of the examined species showed relatively high levels of n-3 fatty acids and more unsaturated than saturated fatty acids.

The study found that *E. compressa* contains the following amounts of moisture: 7.63%, ash: 31.21%, total sugar: 44.08%, crude fiber: 2.93%, lipid: 3.56%, and total protein: 17.48%. Alternatively, *E. linza* has the following composition: 28.33% ash, 7.14% moisture, 50.01% total sugar, 7.14% crude fiber, 4.10% lipid, and 12.51% total protein. *E. tubulosa* has 6.28% moisture, 17.01% ash, 51.05% total sugar, 6.28% crude fiber, 5.56% lipid, and 19.09% total protein.

Ratana-Arporn and Chirapart (2006) determined the approximate composition of *Ulva reticulata* on a g/100g sample dry basis as follows: crude protein (n factor = 6.25) 21.06; crude lipid 0.75; crude fiber 4.84; ash 17.58; carbohydrate 55.77; moisture 22.51. According to the study, *Ulva reticulata* has a protein content that is almost three times larger than *Ulva lactuca* of the same genus.

Rasyid (2017) revealed that the dried seaweed *Ulva lactuca* from Pameungpeuk Waters, Indonesia, contained 58.1% carbohydrates, 16.9% moisture, 11.2% ash, 13.6% protein, and 0.19% fat, respectively, and 28.4% dietary fiber.

Using a 100g dry basis sample of *U. lactuca*, Wong and Cheung (2000) noted the approximate composition as follows: crude lipid 1.64, crude protein (N_ 6.25) 7.06, ash 21.3, moisture 10.6, and TDF 55.4.

Debbarma *et al*. (2016) analyzed *Gracilaria edulis* and *Ulva lactuca* and discovered that *G. edulis* had the highest levels of Na (423.33 mg 100 g^{-1}), P (282.5 mg 100 g^{-1}), Ca (223.33 mg 100 g^{-1}), and Fe (65.28 mg 100 g^{-1}) and proximate composition; moisture 87.14% and 84.81%, protein 14.26% and 13.84%, fat 0.93% and 0.86%, ash 7.63% and 12.41%, carbohydrate 32.39% and 43.19%, total dietary fiber (TDF) 63.175% and 53.625% respectively.

According to Nagaraj *et al*. (2019), *G. corticata* (93.55%) has the highest water content, followed by *G. edulis* (89.76%), and *G. salicornia* (86.66%). They showed that *G. edulis* (37.55%) had the highest carbohydrate content, followed by *G. salicornia* (31.25%), and *G. corticata* (25.95%) had the lowest. The research found that Rhodophycean *Gracilaria* species had higher carbohydrate contents than

Chlorophycean species. In contrast to green seaweeds, which may transform soluble carbohydrates into insoluble carbohydrates like fiber and other polysaccharides to store in the cells, *Gracilaria* species create the most carbohydrates through photosynthesis and may also have more phycocolloids in their cell walls. The analysis found *G. edulis* with a greater protein content (14.25%), followed by *G. corticata* (11.99%), and *G. salicornia* to contain lower protein level (7.88%). The seaweed *G. salicornia* had the highest concentration of lipid (3.45%), followed by *G. edulis* (2.47%), and *G. corticata* (1.99%). Ash content was measured in *G. salicornia* (28.87%), *G. edulis* (41.23%), and *G. corticata* (55.45%). *G. salicornia* (21.56%) also had the highest fiber content, followed by *G. edulis* (19.45%) and *G. corticata* (5.67%).

2.2 Proximate Composition of Value-Added Products from Seaweeds

Mamat *et al*. (2018) created a muffin recipe using seaweed powder (*Kappaphycus alvarezii*) and wheat flour, and investigated the textural profile, proximate analysis, and sensory evaluation. When compared to the control sample, they found that adding seaweed powder improved the composition of ash, crude fiber, and moisture content while reducing the levels of protein and carbohydrate. The study's sensory evaluation revealed that up to 6% of seaweed powder may be added to the batter for muffins without significantly altering the color, flavor, or aroma in comparison to the control sample. The study observed the proximate composition as follows when 2% seaweed powder was added: moisture content 29.56%, crude protein 8.47%, crude fat 11.48%, ash 1.05%, crude fiber 0.24%, and carbohydrate content 49.20%. Seaweed powder can help with processing and enhance the strength and structure of bread goods (Guarda *et al*., 2004).

Mamat *et al*. (2016) discussed the impact of seaweed (*Kappaphycus alvarezii*) composite flour on the quality of buns and provided proximate, physical, and sensory studies. The buns' moisture percentage ranged from 27.18% to 29.54%; the sample with the most seaweed powder (8%) had the highest moisture content. When seaweed was added in formulations at a rate more than 4%, the crude fat content dramatically increased. With the level (1–8%) of seaweed powder inclusion, the amount of dietary fiber significantly increased, with a value that ranged from 1.50 to 4.27%. The proximate analysis produced by adding 5% seaweed powder is as follows: moisture

28.14%, ash 1.49%, fat 6.58%, protein 9.54%, carbohydrate 54.25%, and dietary fiber 3.43%.

In a fish burger recipe made with 64% Catla fish flesh, 17% binder (bread crumble), 12% ice, 4% vegetable oil, 1.5% salt, and 1% spices (chili, black pepper, cardamom), Kumarathunge *et al*. (2016) found crude protein to be 16.25%, fat 12.43%, moisture 62.83%, and crude fiber 02.00%. The study found that fish burgers with seaweed had a high protein content (16.25%), as well as greater levels of ash, moisture, fat, and fiber.

Udayangani *et al*. (2019) created nutribars with dried *U. lactuca* powder (moisture content: 15.29%, dry basis) at 5% and 10% (w/w) ratios and assessed the powdered seaweed's proximate composition, crude ulvan content, swelling capacity, water holding capacity (WHC), and oil holding capacity (OHC). The study discovered that 10% *U. lactuca* added nutribars had the highest protein level (8.55%), whereas 0% and 5% seaweed added nutribars had protein contents of 7.54% and 7.89%, respectively. However, nutribars with 10% *U. lactuca* (w/w) added had a higher protein content than the control but they were not deemed to be generally acceptable. The study concluded that nutribars could contain 5% (w/w) of the underutilized green seaweed *U. lactuca*.

Sumana *et al*. (2018) produced a fiber-enriched Khao-Tang, a Thai-style rice cracker, by mixing cassava flour, jasmine rice, and 2.5%, 5.0%, 7.5%, and 10% *Gracilaria gracilis* seaweed powder. The study suggested that rice crackers containing 2.5% powdered *Gracilaria* had the highest overall score for acceptability. Increasing the amount of powdered *Gracilaria* in rice crackers enhanced the fiber content, but the product quality declined slightly in terms of texture, color, and overall acceptance. When 2.5% seaweed powder was added, the study revealed that the approximate composition was moisture 4.58%, protein 7.39%, lipid 16.78%, carbohydrate 68.85%, fiber 1.11%, and ash 1.25%; when 5% was added, the composition was moisture 4.66%, protein 7.81%, lipid 18.62%, carbohydrate 66.27%, fiber 1.22%, and ash 1.40%.

2.3 Heavy Metals Analysis

Seaweeds are efficient accumulators of arsenic and other heavy metals because they take up minerals and other vital components from their surroundings (Smith *et al*., 2010). Terrestrial runoff, primarily from industry, agriculture, or other human activities, is the principal source of anthropogenic heavy metals in coastal waters, according to

Morton and Blackmore (2001). Species, collecting time, growth phase, and collection place all affect the kinds and concentration of metals (Hou and Yan, 1998).

Numerous studies have already documented the use of various *Ulva* and *Enteromorpha* species as bioindicators of metal contamination (Ho, 1990; Haritonidis and Malea, 1999). Since metals like Cd, Cr, Pb, and Hg are linked to industrial wastes and are dangerous and cancer-causing when released with industrial water, dumped in rivers, and finally mixed up, they have a negative impact on both aquatic fauna and flora, as well as seaweed that is grown naturally or in the deep sea. Seaweeds were found to be reliable markers for biomonitoring heavy metals in coastal water (Akcali and Kucuksezgin, 2011).

Ganesan *et al*. (2014) investigated the micro- and trace-elements (As, Cd, Co, Cu, Fe, Hg, Mn, Mo, Ni, Zn, Cr, and Pb), which were discovered in *E. linza* in the highest concentration (81.51 mg/100g d wt.), followed by *E. tubulosa* (57.45 mg/100g d wt.) and *E. compressa* (35.29 mg/100g d wt.).

The study also revealed that certain micronutrients (Fe, Zn, Mn, Cu) in *E. compressa*, *E. linza*, and *E. tubulosa* (32.69, 70.07 and 45.49 mg/100g d wt., respectively) were higher than those reported for sweet corn (4.9 mg/100g), as well as in edible seaweeds like *Laminaria* species (5.1 mg/100g d wt.), *Monostroma oxyspermum* (21.2 mg/100g d wt.), *Enteromorpha flexuosa* (11.8 mg/100g d wt.) *Ulva faciata* (11.2 mg/100g d wt.), *Porphyra vietnamensis* (21.3 mg/100g dry wt.), and also comparable with *Porphyra vietnamensis* (45.5–309 mg/100g) from Indian coast. According to the report's recommendations, the daily intake of *E. compressa*, *E. linza*, and *E. tubulosa* should not exceed 14.29, 1.74, 1.62, and 1.74 g, respectively, based on the amounts of the aforementioned elements and their tolerable limitations

According to Kamala-Kannan *et al*. (2008), Pulicat Lake in India had the highest amount of Cd (59.6 µg g^{-1} dry weight) during the monsoon season in *Ulva lactuca* and the lowest level (21.7 µg g^{-1} dry weight) during the pre-monsoon. According to the data, pre-monsoon had the lowest (10.5 µg g^{-1} dry weight) and post-monsoon had the greatest Cr content (45.7 µg g^{-1} dry weight) in algae. The range of the lead concentration in the algae of Pulicat Lake was between 5.9 and 20.3 µg g^{-1} dry weight.

While Shiber (1980) reported Cd <0.8-2.3 µg g-1 and Pb <7.5-37.5 µg g^{-1} from Beirut (Lebanon), Ho (1990) had discovered 9-41 µg g^{-1} Cd and 0.75-10.59 µg g^{-1} Pb in *Ulva*

lactuca from Hong Kong (China). According to a study by Storelli *et al*. (2001), *U. lactuca* from the South Adriatic Sea in Italy contained Cd 0.20 µg g^{-1} and Pb 0.84 µg g^{-1}, while *Enteromorpha prolifera* included Cd 0.72 µg g^{-1} and Pb 1.15 µg g^{-1}. In *Ulva rigida* from Thermaikos Gulf (Greece), Haritonidis and Malea (1995) reported 0.18-10.7 µg g^{-1} Cr and Cd 0.1-2.5 µg g^{-1}.

The greatest heavy metal BCFs were found in the *Gracilaria* species in Nan'ao, China, with values of 2.45×10^5 for Cr, 1.67×10^5 for Zn, 1.14×10^5 for Pb, and 0.16×10^5 for Cd, respectively (Luo *et al*., 2018). According to Luo *et al*. (2020), the quantities of Cr 125.15 µg g^{-1}, Cd 4.58 µg g^{-1}, Pb 8.85 µg g^{-1}, and Zn 109.15 µg g^{-1} in the body of the *Gracilaria* were the highest. The paper also suggested that *Gracilaria* might be employed as a bio remediator or bio monitor for heavy metals due to its enrichment potential to accumulate Cu, Zn, Pb, and Cd, which exceeded the food limits for seaweed.

According to Tonon *et al*. (2011), Cd has no recognized biological function in the metabolism of *Gracilaria*, while Copper (Cu) is a crucial metal for *Gracilaria*, both as an enzymatic co-factor and as an electron transporter in the process of photosynthesis (plastocyanin). The study also showed that *Gracilaria* is a metal-bioaccumulating organism and that numerous harmful heavy metals, including Cd and Pb, can compete for vital metal transporters during absorption.

Ulva Stenophylla had the greatest lead amounts (1.83 mg/kg), according to Smith *et al*. (2010), although it would not produce hazardous levels of heavy metals if consumed daily. *Ulva Stenophylla* contained 1.88 mg/kg As, 0.10 mg/kg Hg, and 1.83 mg/kg Pb, according to the study.

With the exception of I, which was greater in the brown seaweed, Filippini *et al*. (2020) found that red seaweed had the highest concentration of Zn (18.12 mg/kg) and Mn (9.85 mg/kg) and green seaweed had the highest content of Fe macro elements (78.62 mg/kg). All forms of seaweed generally had significant levels of Fe accumulation; *Porphyra* (112.29 mg/kg) and mixed algae (196.19 mg/kg) had the greatest levels. This might be connected to the high rates of photosynthesis found in tropical coastal ecosystems (Chakraborty *et al*., 2014).

According to many research, the majority of the species-specific heavy metal concentration in dried seaweed sold for human consumption (Besada *et al*., 2009).

According to Güven *et al*. (1995), the chemistry of their cell walls plays a major role in the species-specific differences in the accumulation of various heavy metals (such as As, Cd, and Pb).

2.4 Microbial Analysis of Seaweeds

Numerous bacteria found on seaweed surfaces have the ability to enzymatically break down the cell walls of algae, making them important participants in biotransformation and nutrient recycling in the oceans (Goecke *et al*., 2010; Michel *et al*., 2006). Several investigations have showed that seaweed-associated bacteria are significant producers of fixed nitrogen and detoxifying chemicals (Goecke *et al*., 2010; Riquelme *et al*., 1997).

Only 33 bacterial genera, including *Alteromonas*, *Bacillus*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas*, and *Vibrio*, have been reported from green, red, and brown seaweeds, according to Hollants *et al*. (2013)'s research.

Vibrio sp. are found in *Gracilaria* sp. (Lavilla-Pitogo, (1992); *Gracilaria verrucosa* (Beleneva and Zhukova, 2006); and *Cytophaga/Flavobacterium* group (Weinberger *et al*., 1997) in *Gracilaria conferta* were reported. *Erythrobacter longus* in *Enteromorpha linza* was reported by Shiba and Simidu (1982). *Alphaproteobacteria* and *Bacteroidetes,* (Tait *et al*., 2009) were found in *Ulva* sp., *Flavobacterium* group (Bolinches *et al*., 1988) in *Ulva rigida*, *Deltaproteobacteria* and *Actinobacteria* (Longford *et al*., 2007) in *U. australis*; *Gammaproteobacteria* and *Bacteroidetes*, (Patel *et al*., 2003) were found in *Enteromorpha* sp.; *Alphaproteobacteria* and *Bacteroidetes* were noticed in *Gracilaria vermiculophylla* (Lachnit *et al*., 2011).

Goecke *et al*. (2010) state that more than 50 distinct bacterial species that were initially isolated from seaweeds have been legitimately published to date. The study examined the relationship between specific macroalgal species and bacteria, chemical response mechanisms, phytohormone synthesis, macroalgal morphogenesis induced by bacterial products, and harmful macroalgal-bacterial interactions causing or resulting in algal illnesses.

Chapter 3: Materials and Methods

3.1 Sample Collection

Red seaweed *Gracilaria* sp., green seaweed *Enteromorpha* sp. and *Ulva* sp. were collected by hand-picking along the Cox's Bazar beach at Nuniarchora, Cox's Bazar. The samples were then washed with seawater to remove the dirt, pebbles and sands. The seaweeds were delivered to the lab immediately after collection. In order to carry out microbial analysis, some of the fresh seaweeds were put aside and the remaining were oven dried at 55-60˚C and preserved in a zip lock bag at room temperature for proximate analysis of lab dried sample and further use.

Plate 1: *Ulva* sp.

Plate 2: *Enteromorpha* sp.

Plate 3: *Gracilaria* sp.

3.2 Preparation of Seaweeds for Biochemical and Microbial Analysis

Fresh seaweeds were used immediately to test microbial condition of the samples and the sundried samples were ground into fine powder and used for biochemical analysis of seaweeds and their value added products: muffins and biscuits.

3.3 Materials for Producing Value Added Products

The green seaweed species *Ulva* sp. and *Enteromorpha* sp. and red seaweed *Gracilaria* sp. were thoroughly cleaned and sundried immediately after collection. All seaweeds were then ground into fine powder using a blender, sieved by sieve and stored in zip lock bag at room temperature (25°C). Dried ground seaweeds (2.5 % for the muffins and 5% for the biscuits) were mixed with wheat flour to produce seaweed composite flour. High protein wheat flour (12% protein) and other raw materials like sugar, cooking oil, egg, and baking powder for muffin and biscuit production were procured from the local market at Moulovibazar, Kalurghat, Bangladesh.

3.3.1 Muffin Preparation

To prepare the muffin samples, a basic muffin formulation based on flour weight was used: 142.5 g wheat flour/composite flour, 73.76 g cooking oil, 49.6 g egg, 71.25 g sugar, 8.55 g baking powder, and water as needed. The dry and liquid ingredients were combined in a mixing bowl until a homogeneous mixture was obtained. Hand beating was used to smooth out the batter for 10 minutes. Each paper muffin cup was filled with 35 g of batter. The muffins were put in the middle of a standard electric oven (Sebec, China) and baked for 30 minutes at 174°C. The muffins were placed in polyethylene bags, sealed, and kept for a subsequent analysis after cooling for 1 hour at room temperature (27 ± 2°C).

Plate 4: Control

Plate 5: *Gracilaria* muffin

Plate 6: *Enteromorpha* muffin

Plate 7: *Ulva* muffin

3.3.2 Biscuit Preparation

To prepare the biscuit samples, a basic biscuit formulation based on flour weight was used: 285 g wheat flour/composite flour, 73.76 g cooking oil, 49.6 g egg, 71.25 g sugar, and 8.55 g baking powder. A mixing bowl was used to blend the dry and liquid materials until a homogeneous mixture was achieved. The dough was then made into different shapes of biscuits. The biscuits were placed on the center rack of a conventional electric oven (Sebec, China) and baked at 174°C for 12 min. After cooling at room temperature (27 ± 2°C) for 1 h, the biscuits were packed in polyethylene bags that were sealed and stored for further analysis.

Plate 8: *Enteromorpha* biscuits

Plate 9: *Gracilaria* biscuits

Plate 10: *Ulva* biscuits

Plate 11: Control

3.4 Analytical Procedures

Proximate analysis was conducted to determine the percentage of moisture, ash, crude protein, crude fat, and crude fiber, according to the Association of Official Analytical Chemists' procedure (AOAC, 2016). For each analysis of proximate composition, triplicate samples were used.

3.4.1 Protein

Total protein content was determined by Micro kjeldhal apparatus. (Digestion compact system (DK20/26, VELP scientific) and distillation system (Model: UDK 129, VELP scientific).

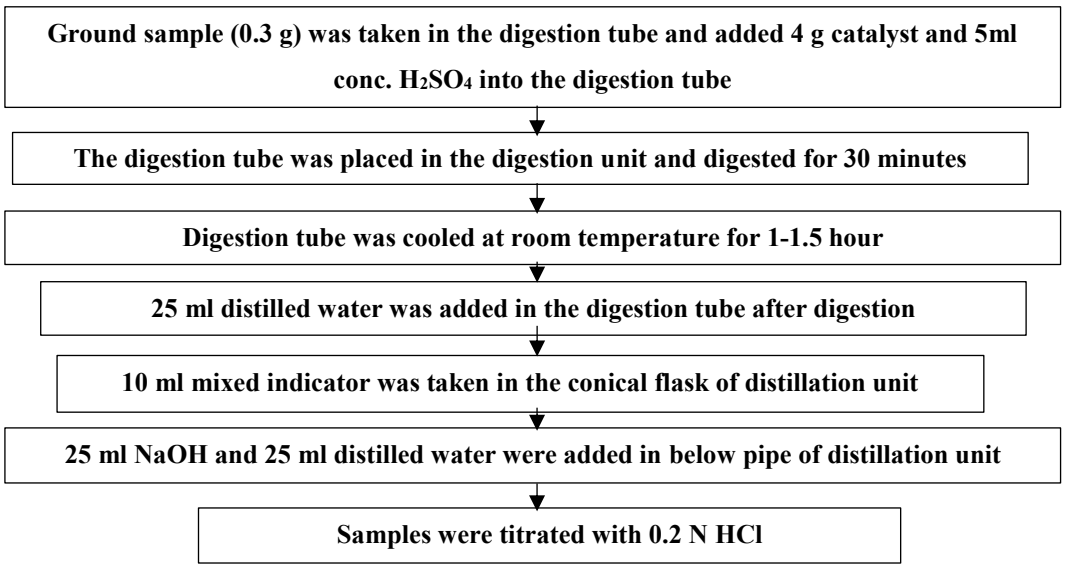


Figure 1: Determination of protein content

Plate 12: Titrated protein sample

Total Nitrogen content was determined by the following formula:

$$\% \text{ of } N = \frac{\text{ml of titrant} \times \text{Strength of HCl (0.2N)} \times \text{Equivalent of Nitrogen (0.014)}}{\text{weight of sample}} \times 100$$

The amount of crude protein was then calculated by the following formula:

$$\% \text{ of Protein} = \% \text{ N} \times 5.85$$

3.4.2 Fiber

Fiber content of the seaweeds was determined by Raw Fiber Extractors (Model: FIWE3, VELP scientific).

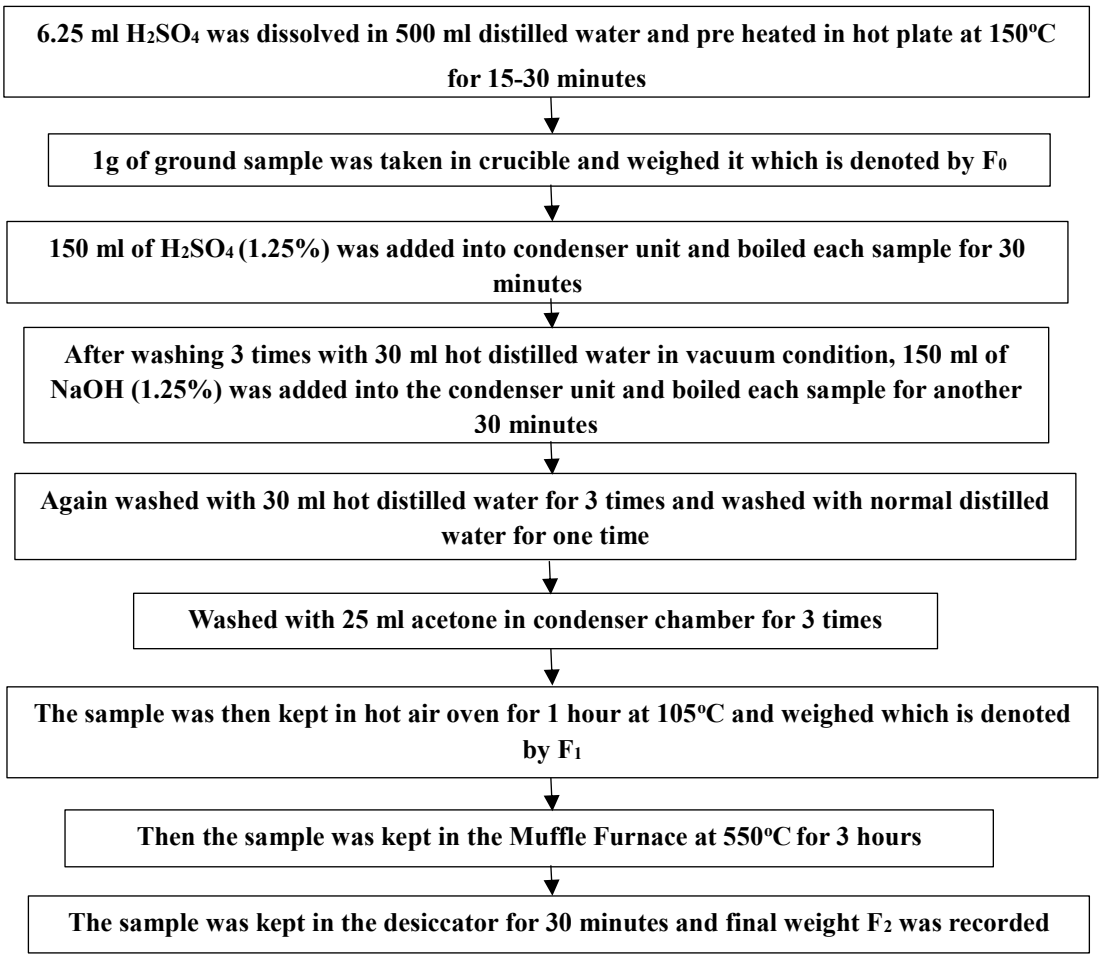


Figure 2: Determination of fiber content

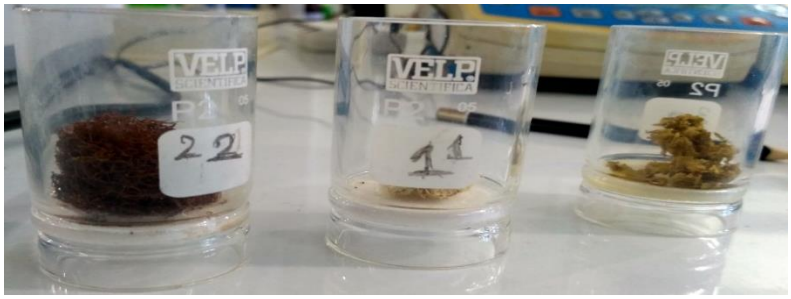


Plate 13: Fiber content after keeping in hot air oven

The Fiber content was determined by the following formula:

$$\textbf{Fiber content} = \frac{F_1 - F_2}{F_0} \times 100$$

3.4.3 Lipid

Lipid content of the sample was determined by Soxhlet Apparatus (Model: RD 40, Food ALYT).

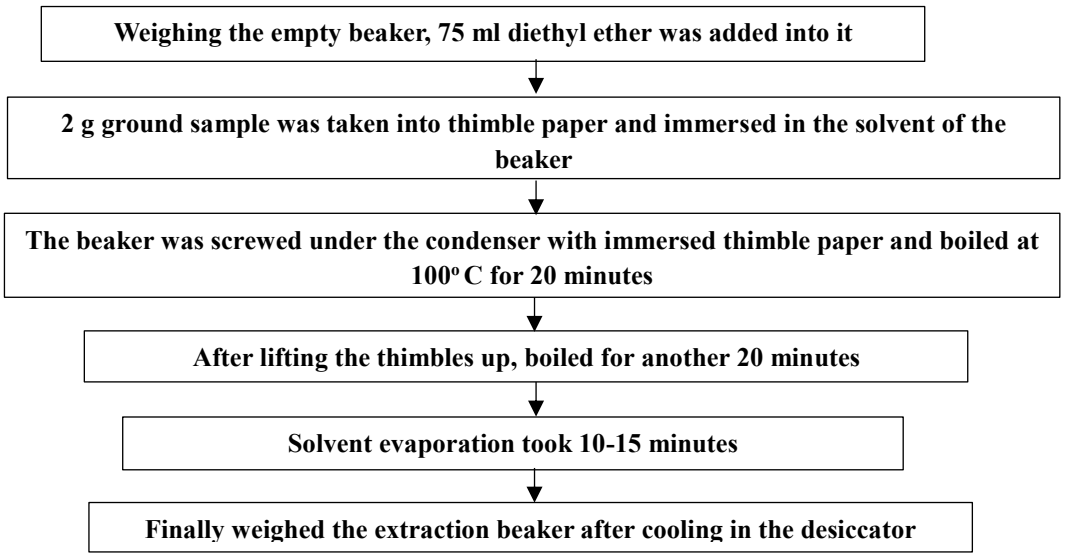


Figure 3: Determination of lipid content

Plate 14: Lipids in dried seaweed

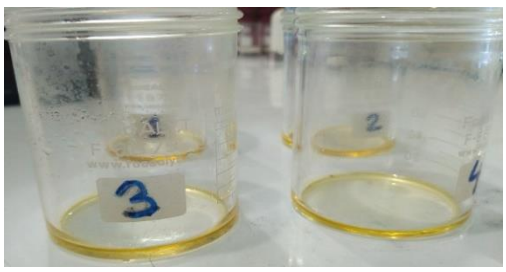


Plate 15: Lipids in value added products

Formula for determination of lipid:

$$\textbf{\% of Lipid} = \frac{\text{weight of lipid}}{\text{weight of sample}} \times 100$$

3.4.4 Ash

Ash content of the sample was determined by Muffle furnace (Model: LHMF 100A, LABNICS Equipment).

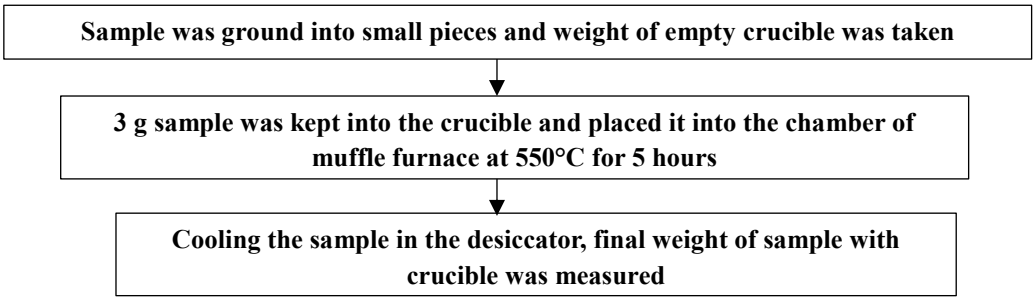


Figure 4: Determination of ash content

Plate 16: Ash content of the products

By using following formula ash content was determined:

$$\% \text{ of Ash} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

3.4.5 Moisture

Moisture content of the sample was determined by Laboratory Drying Oven (Model: BINDER, ED 115).

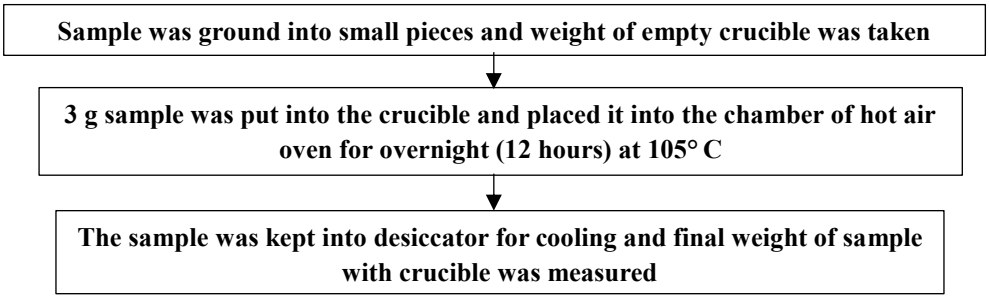


Figure 5: Determination of moisture content

Plate 17: Moisture content of value-added products

Formula for determination of moisture:

$$\textbf{\% of Moisture content} = \frac{\textbf{weight of moisture}}{\textbf{weight of sample}} \times \textbf{100}$$

3.5 Bacteriological Analysis of the Fresh Seaweeds

3.5.1 Analytical Procedures

The media were prepared in the laboratory according to the Association of Official Analytical Chemists' procedure (AOAC, 2016).

3.5.2 Sample Preparation for Standard Plate Count

The term "Standard Plate Count" (SPC) refers to the colony count of mesophilic bacteria growing in an aerobic environment on a standard method of agar (Plate Count Agar). SPC is used as a representative measure to determine the level of food contamination by microbes. By employing a consecutive decimal dilution technique and the pour plate method, the standard plate count of fresh seaweeds was calculated. A sterile blender jar was used to combine 90 ml of sterile physiological saline solution (0.85% NaCl solution) with 10 g of fresh seaweed from each sample. The homogenate was then transferred to a sterile beaker and serial dilution technique was followed. Sterile test tubes were arranged in a sterile rack and marked while 9 ml of 0.85% sterile physiological saline was added in each tube. 1 ml of homogenized sample was taken by sterile micropipette and added with the 9 ml physiological saline in the tube and homogenously mixed by vortex mixture to get a 10^{-1} dilution of original sample solution. Then 1 ml sample from 10^{-1} dilution was taken through micropipette and mixed with 10^{-2} dilution test tube thus serial dilutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were made.

3.5.3 Total Plate Count

1 ml of each diluted sample was pipetted into appropriately marked sterile petri dishes. 15 ml of plate count agar (cooled up to 45 ± 1°C) was poured into each petri plate. By alternatingly rotating and moving the plates back and forth on a level flat surface, the sample dilutions and agar medium were thoroughly and uniformly mixed. Petri dishes were turned upside down after agar solidified and incubated promptly for 24 ± 2 h at 37°C in the incubator. After incubation, colonies developed on the petri dishes were counted following a standard method. For the purpose of counting, petri plates with 30 to 300 colonies were chosen. Plates containing more than 300 colonies are deemed to

have too many to count (TMTC). Too few to count plates are those having fewer than 30 colonies (TFTC). Triplicates of each plate were created. Number of bacteria per gram of the seaweed sample (CFU/g) was calculated by using the following formula:

Number of CFU/g of the sample =

$$\frac{\text{number of colonies} \times \text{dilution factor} \times \text{volume of total sample solution}}{\text{weight of sample plated (g)}}$$

3.5.4 Enumeration of Microorganisms

3.5.4.1 Detection of *Vibrio cholera, V. vulnificus,* and *V. parahaemolyticus*

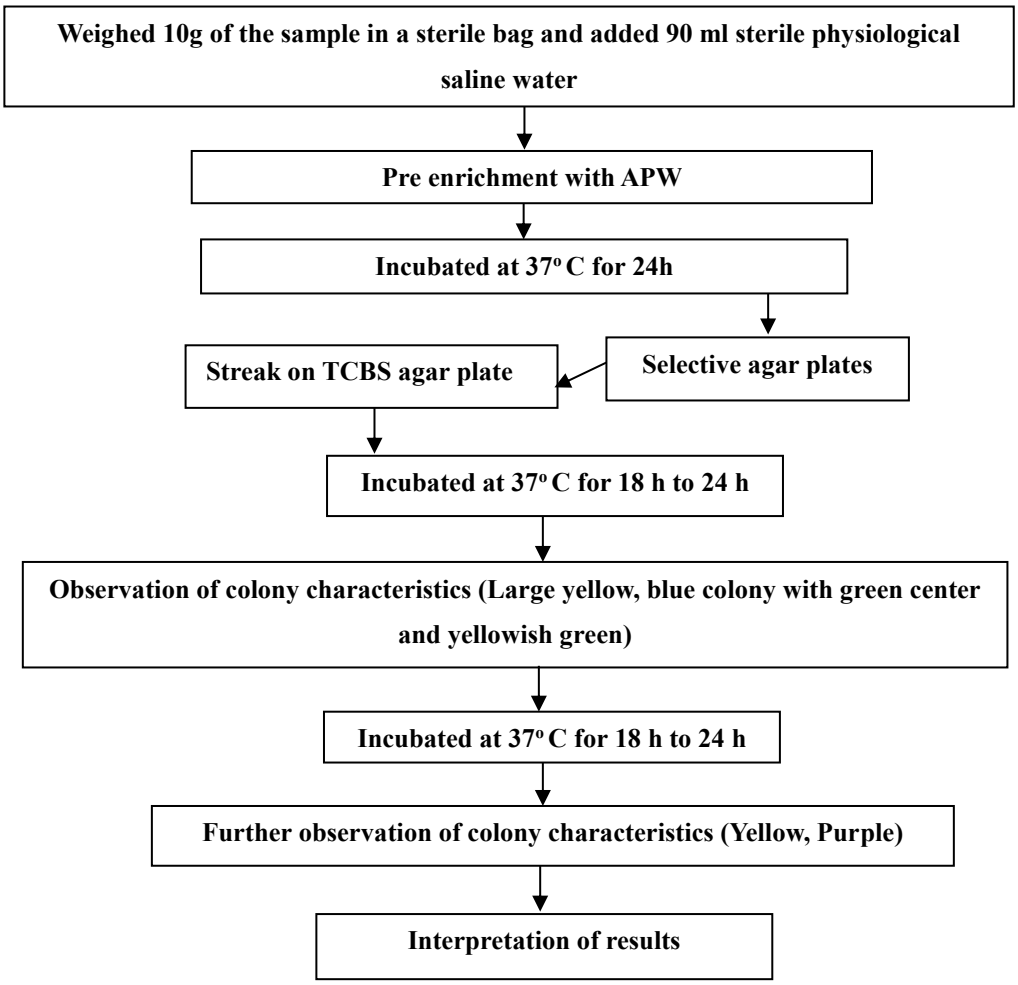


Figure 6: Determination of *Vibrio* sp.

***Vibrio* Spp. Colony Characteristics:**

Vibrio cholera: Large flat yellow colony on TCBS

Vibrio parahaemolyticus: Blue colony with green center on TCBS

3.5.4.2 Detection of *Salmonella* and *Shigella* spp.

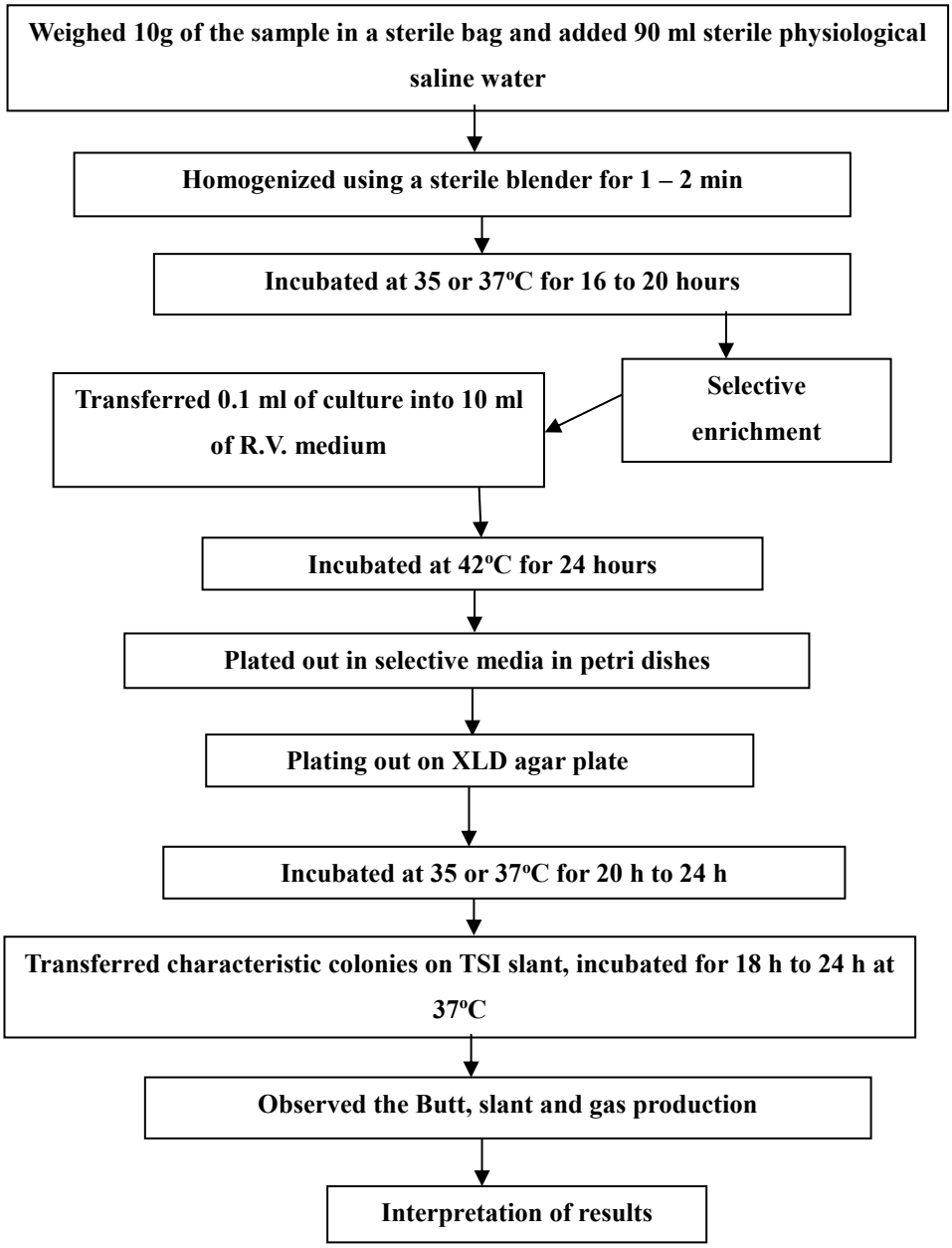


Figure 7: Determination of *Salmonella* spp.

***Salmonella* Spp. characteristics:**

Salmonella enterica: red slant, black-butt (H_2S produced)

Salmonella Typhi: Red slant, yellow butt, H_2S produced

Salmonella Paratyphi A: Red slant, yellow butt, gas production

***Shigella* Spp. characteristics:**

Shigella sonnei: Red slant, yellow butt, no H_2S produced

Shigella flexneri: Red slant, yellow butt, no H_2S produced

3.5.4.3 Detection of *E. coli*

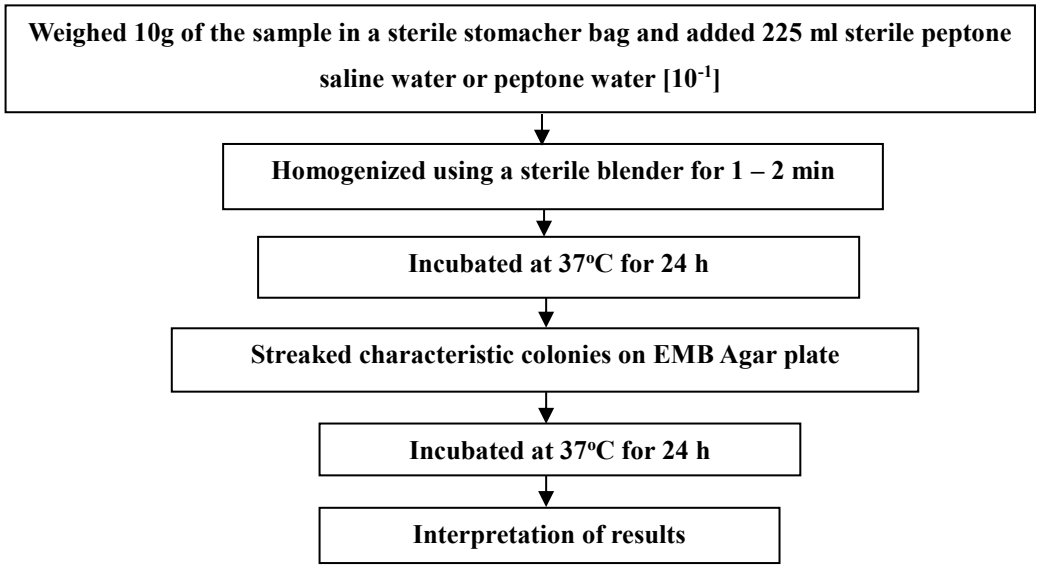


Figure 8: Determination of *E. coli*

***E. coli* Colony Characteristics:**

EMB agar: Greenish Characteristic colony with metallic sheen

3.6 Sample Preparations for Heavy Metal Analysis

The dried seaweed samples were digested for the total heavy metals analysis using the following procedure: 0.20 g of crushed seaweed samples were directly weighed into a digestion tube, and 5 ml of concentrated HNO_3 (trace analytical grade, 70%), procured from Sigma-Aldrich, were added. After that, the mixture was let to stand overnight in a fume hood. The digestion tubes were heated using a temperature-controlled digestion block on the following day with 2 ml of H_2O_2 added. The digestion block was scheduled to gradually ratchet up to 1200°C over 8 hours and then to keep up the temperature for the digestion of seaweed samples. Sample digestion was carried out in each tube until barely any liquid was left behind. Before dilution (50 ml), the tubes from the digesting block were taken out and allowed to cool in the fume cupboard at room temperature. The materials were completely combined before being filtered using filter paper and placed directly into a plastic bottle for storage before analysis. The heavy metal content of seaweed samples were determined using the ICPMS-2030 series inductively coupled plasma mass spectrometry (ICPMS-2030, Shimadzu Company, Kyoto, Japan).

3.7 Statistical Analysis

The experiments were all carried out in triplicates. A one-way analysis of variance (ANOVA) was performed on the data. Tukey's test was used to evaluate the significance level at $p<0.05$ and to calculate the least significant differences. For all data analysis, SPSS for Windows version 26 (SPSS Inc., Chicago, IL, USA) was utilized.

Chapter 4: Results

4.1 Proximate Composition of Seaweeds

Seaweeds are a good source of bioactive substances such as phlorotannins, sulphated polysaccharides, carotenoid pigments, and fucosterol, and with their potential antioxidant, impact in the food sector as functional ingredients and also provide numerous health benefits (Li and Kim, 2011).

Proximate composition analysis of two green seaweeds (*Enteromorpha* sp. and *Ulva* sp.) and one red seaweed (*Gracilaria* sp.) are summarized in Table 1, 2, 3.

The wet samples (Table 1) indicate higher amount of ash and moisture in *Gracilaria* sp. which was not significantly different ($p>0.05$) from other samples. Crude Lipid content observed higher in *Ulva* sp. (0.37%) and lowest in *Gracilaria* sp. (0.17%) and showed significant differences ($p<0.05$) in the values. Crude fiber ranges from 0.94-1.47% where *Gracilaria* sp. contains highest value. Crude protein and carbohydrate value dominates in *Gracilaria* sp. (3.48%) and (6.76%) respectively while *Ulva* sp. holds 4.27% carbohydrate.

Table 1: Proximate composition (% wet weight of sample) of collected wet sample: *Gracilaria* sp., *Enteromorpha* sp., and *Ulva* sp.[a]

Composition	***Gracilaria* sp.**	***Enteromorpha* sp.**	***Ulva* sp.**
Ash	5.31 ± 1.04^{a}	4.37 ± 0.66^{a}	4.41 ± 1.14^{a}
Moisture	84.07 ± 1.38^{a}	91.49 ± 0.94^{a}	87.84 ± 0.71^{a}
Crude Lipid	0.17 ± 0.03^{a}	0.18 ± 0.01^{a}	0.37 ± 0.11^{b}
Crude Fiber	1.47 ± 0.21^{a}	0.94 ± 0.12^{a}	1.23 ± 0.31^{a}
Crude Protein	3.48 ± 0.79^{a}	1.64 ± 0.37^{a}	2.78 ± 0.55^{a}
Carbohydrate[d]	6.76 ± 0.76^{a}	2.75 ± 0.89^{b}	4.27 ± 1.23^{ab}

[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-b) differ considerably ($p< 0.05$, ANOVA, Tukey-HSD).

[d] Calculated by difference [100-% (crude protein + crude lipid + fiber + ash + moisture)]

The lab dried samples (Table 2) denote higher amount of ash and moisture in *Enteromorpha* sp. which was significantly different ($p<0.05$) from other samples. Crude Lipid content tends to be higher in *Ulva* sp. (1.01%) and lowest in *Gracilaria* sp. (0.35%) and showed no significant differences ($p>0.05$) in the values. Crude fiber ranges from 2.98-3.77% where *Ulva* sp. comprises highest value. Crude protein value dominates in *Gracilaria* sp. (20.90%) on the contrary carbohydrate value is the lowest while *Ulva* sp. stores 43.04% carbohydrate.

Table 2: Proximate composition (% dry weight of sample) of lab dried sample: *Gracilaria* sp., *Enteromorpha* sp., and *Ulva* sp.[a]

Composition	***Gracilaria* sp.**	***Enteromorpha* sp.**	***Ulva* sp.**
Ash	30.97 ± 0.24^{a}	42.73 ± 1.52^{b}	31.36 ± 1.17^{a}
Moisture	13.86 ± 0.12^{a}	14.20 ± 3.17^{a}	10.02 ± 0.56^{a}
Crude Lipid	0.35 ± 0.01^{a}	0.39 ± 0.03^{a}	1.01 ± 0.74^{a}
Crude Fiber	2.98 ± 0.26^{a}	2.96 ± 0.09^{a}	3.77 ± 0.14^{b}
Crude Protein	20.90 ± 0.21^{c}	11.78 ± 0.45^{b}	10.80 ± 0.27^{a}
Carbohydrate[d]	30.94 ± 0.17^{b}	27.95 ± 1.47^{a}	43.04 ± 0.88^{c}

[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-c) differ considerably ($p< 0.05$, ANOVA, Tukey-HSD).

[d] Calculated by difference [100-%(crude protein + crude lipid + fiber + ash + moisture)]

Market dried samples (Table 3) demonstrate higher crude protein label in *Gracilaria* sp. (14.60%) and lower in *Enteromorpha* sp. (10.43%) and present significant difference ($p<0.05$). Ash and moisture contents are higher in *Enteromorpha* sp. Carbohydrate values differ from 1-4% revealing highest value in *Ulva* sp. (54.77%). Crude lipid ranges from 0.13-0.24% and lowest content found in *Gracilaria* sp. Crude fiber appears highest in *Gracilaria* sp. (16.67%) and found lowest in *Enteromorpha* sp. (5.66%).

Table 3: Proximate composition (% dry weight of sample) of market dried sample: *Gracilaria* sp., *Enteromorpha* sp., and *Ulva* sp.[a]

Composition	*Gracilaria* sp.	*Enteromorpha* sp.	*Ulva* sp.
Ash	14.29 ± 0.17[a]	23.12 ± 0.77[c]	20.68 ± 0.31[b]
Moisture	3.51 ± 0.08[a]	6.52 ± 2.49[b]	4.08 ± 0.32[a]
Crude Lipid	0.13 ± 0.06[a]	0.19 ± 0.00[a]	0.24 ± 0.19[a]
Crude Fiber	16.67 ± 0.08[c]	5.66 ± 0.18[a]	6.31 ± 0.22[b]
Crude Protein	14.60 ± 0.66[b]	10.43 ± 0.87[a]	13.92 ± 0.40[b]
Carbohydrate[d]	50.80 ± 0.60[b]	51.76 ± 6.05[a]	54.77 ± 0.92[b]

[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-c) differ considerably ($p < 0.05$, ANOVA, Tukey-HSD).

[d] Calculated by difference [100-% (crude protein + crude lipid + fiber + ash + moisture)]

4.2 Proximate Composition of Seaweed Products

The experimental proximate composition results of biscuits produced from seaweeds are shown in Table 4. The moisture content of the biscuits ranged from 7.63% to 17.41% where *Gracilaria* biscuit had the highest content which was significantly different ($p<0.05$) from other samples. Crude lipid ranged from 14.83% to 16.48% and there was no significant differences ($p>0.05$) in the values. Crude Protein ranged from 9.92% to 10.60% and *Ulva* biscuit had the highest amount. Ash content ranged from 0.30% to 1.44%, where *Gracilaria* biscuit had the lowest amount with no significant differences ($p>0.05$) in the values. Carbohydrate is the highest composition in the experiment where *Enteromorpha* biscuit contained the highest amount 65.64%. Crude Fiber ranged from 0.17% to 0.68% and *Gracilaria* biscuit had the highest amount.

The experimental proximate composition results of muffins produced from seaweeds are shown in Table 5. The moisture content of the muffins ranged from 42.93% to 54.16 % where *G.* muffin had the highest content which was significantly different ($p<0.05$) with one sample. Crude lipid ranged from 11.74% to 14.32% and there was no significant differences ($p>0.05$) in the values and *E.* muffin had the highest content. Crude Protein ranged from 7.57% to 8.49% and *U.* muffin had the highest amount. Ash content ranged from 0.39% to 1.70%, where *E*. muffin had the highest amount. Carbohydrate ranged from 22.83% to 32.34% where *G.* muffin contained the lowest

amount. Crude Fiber ranged from 0.18% to 0.32% and *G.* muffin had the highest amount.

Table 4: Proximate composition (% dry weight of sample) of biscuits produced from seaweeds and without seaweed[a]

Composition	Control	*Enteromorpha* biscuit	*Ulva* biscuit	*Gracilaria* biscuit
Ash	1.09 ± 0.08[a]	1.44 ± 0.99[a]	1.19 ± 0.47[a]	0.30 ± 0.22[a]
Moisture	12.20 ± 0.87[b]	7.63 ± 0.56[a]	10.01 ± 0.66[ab]	17.41 ± 2.24[c]
Crude Lipid	15.12 ± 0.07[a]	15.06 ± 0.27[a]	14.83 ± 0.11[a]	16.48 ± 1.40[a]
Crude Fiber	0.17 ± 0.02[a]	0.17 ± 0.02[a]	0.36 ± 0.02[b]	0.68 ± 0.02[c]
Crude Protein	9.92 ± 0.30[a]	10.07 ± 0.23[a]	10.60 ± 0.71[a]	10.04 ± 0.12[a]
Carbohydrate[d]	61.51 ± 0.80[b]	65.64 ± 0.52[c]	63.01 ± 0.46[b]	55.10 ± 1.24[a]

[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-c) differ considerably ($p < 0.05$, ANOVA, Tukey-HSD).

[d] Calculated by difference [100-% (crude protein + crude lipid + fiber + ash + moisture)]

Table 5: Proximate composition (% dry weight of sample) of muffins produced from seaweeds and without seaweed[a]

Composition	Control	*Enteromorpha* muffin	*Ulva* muffin	*Gracilaria* muffin
Ash	0.39 ± 0.11[a]	1.70 ± 0.49[b]	0.76 ± 0.28[ab]	0.62 ± 0.53[a]
Moisture	50.52 ± 3.20[b]	42.93 ± 0.90[a]	52.72 ± 2.20[b]	54.16 ± 0.77[b]
Crude Lipid	11.74 ± 1.63[a]	14.32 ± 0.93[a]	13.39 ± 0.02[a]	13.76 ± 2.93[a]
Crude Fiber	0.18 ± 0.01[a]	0.24 ± 0.04[ab]	0.25 ± 0.03[ab]	0.32 ± 0.05[b]
Crude Protein	7.57 ± 0.11[a]	8.47 ± 0.03[b]	8.49 ± 0.10[b]	8.30 ± 0.16[b]
Carbohydrate[d]	29.59 ± 1.73[bc]	32.34 ± 0.93[c]	24.41 ± 2.05[ab]	22.83 ± 4.13[a]

[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-c) differ considerably ($p < 0.05$, ANOVA, Tukey-HSD).

[d] Calculated by difference [100-% (crude protein + crude lipid + fiber + ash + moisture)]

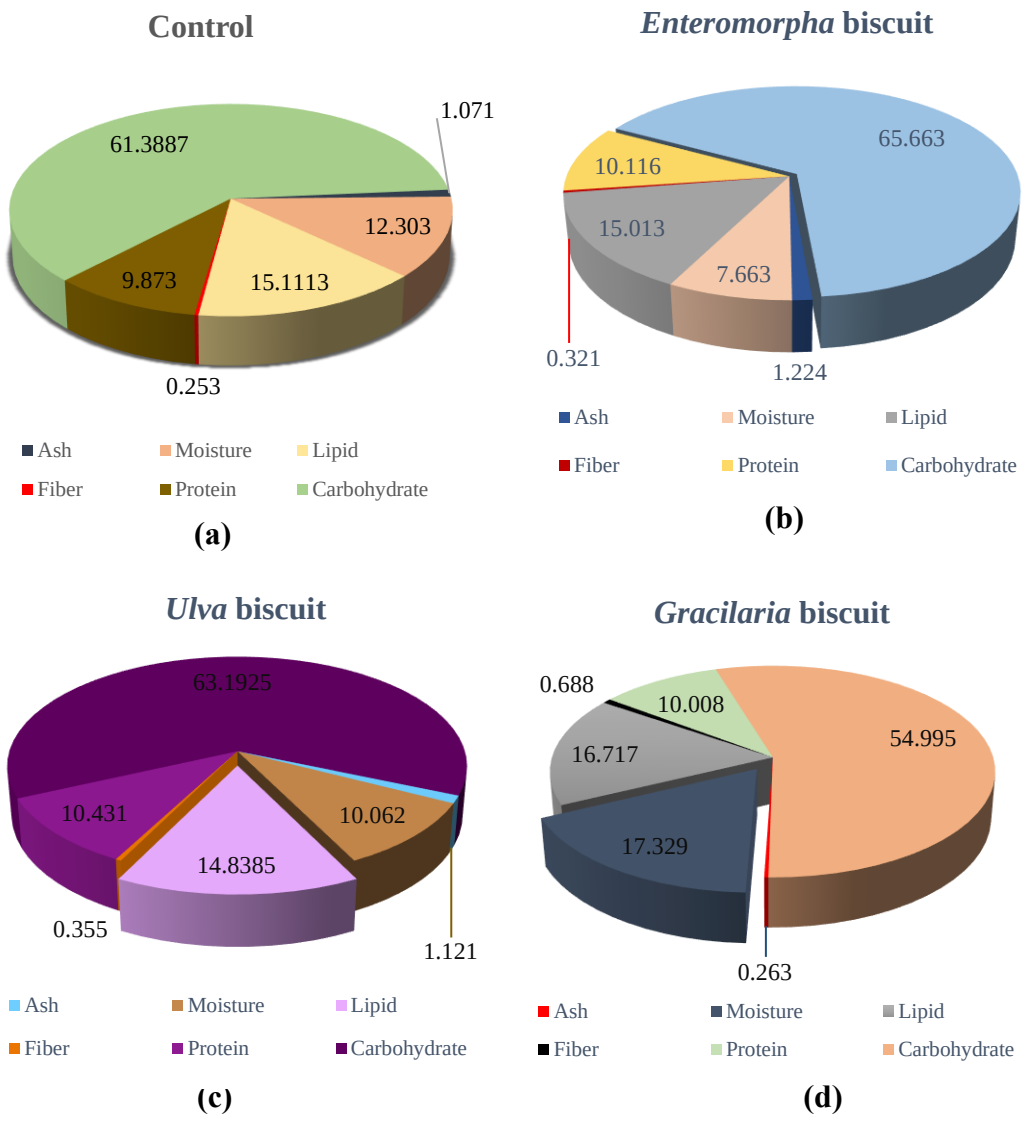
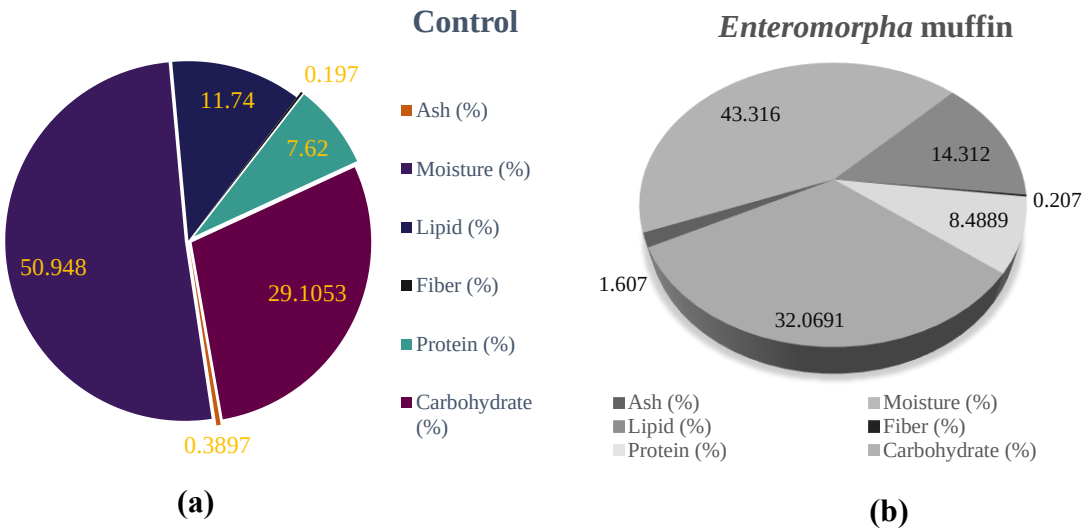
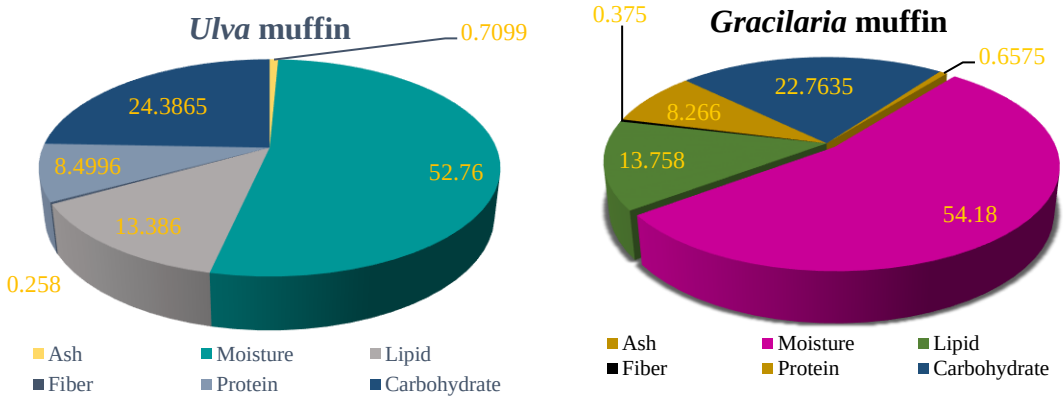


Figure 9: Proximate composition of different biscuits





(c) **(d)**

Figure 10: Proximate composition of different muffins

4.3 Microbial Analysis of Seaweeds

Microbial loads of three seaweeds are outlined in the Table 6. Higher amounts of bacterial load noticed in *Ulva* sp. (13.50×10^6) cfu/g and lowest content in *Enteromorpha* sp. (2.23×10^6) cfu/g.

Table 6: Total bacterial loads in seaweed samples

Seaweed Sample	Replications	Bacterial load (CFU/g)	Mean value	Standard deviation (± sd)
Gracilaria sp.	Replication-1	10.09×10^6	9.43×10^6	1.45×10^6
	Replication-2	8.02×10^6		
	Replication-3	9.14×10^6		
Enteromorpha sp.	Replication-1	3.02×10^6	2.23×10^6	0.85×10^6
	Replication-2	1.26×10^6		
	Replication-3	1.09×10^6		
Ulva sp.	Replication-1	12.01×10^6	13.50×10^6	1.50×10^6
	Replication-2	15.13×10^6		
	Replication-3	13.05×10^6		

Pathogenic bacterial identification was done in the samples and the outcome is presented in the Table 7. In the experiment, *Gracilaria* sp. did not contain pathogenic bacteria *Escherichia coli*, *Vibrio vulnificus*, and *V. parahaemolyticus*. while *Enteromorpha* sp. and *Ulva* sp. possessed all the tested pathogenic bacteria.

Table 7: Pathogenic bacteria in experimented seaweeds[a]

Samples	Replications	*Salmonella* sp.	*Shigella* sp.	*Escherichia coli*	*Vibrio vulnificus* *Vibrio parahaemolyticus*	*Vibrio cholera*
Gracilaria sp.	Replication-1	+	+	-	-	+
	Replication-2	-	-	-	-	-
	Replication-3	+	-	-	-	-
Enteromorpha sp.	Replication-1	-	-	+	+	+
	Replication-2	-	-	-	-	+
	Replication-3	+	-	+	+	-
Ulva sp.	Replication-1	+	+	+	-	+
	Replication-2	-	-	-	+	+
	Replication-3	+	-	+	+	+

[a] Here "+" indicates presence and "-"indicates absence of pathogenic bacteria

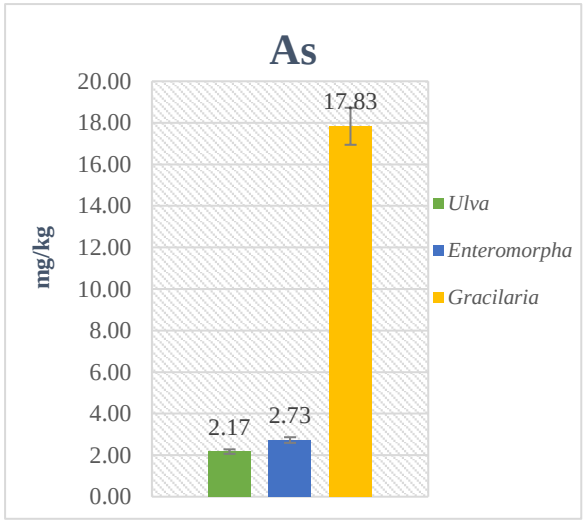
4.4 Analysis of Heavy Metal Concentrations in Seaweeds

The result indicates that Fe is the higher concentrated metal among the three species and *Gracilaria* contains the highest value, followed by Mn (339.14 mg/kg) macro elements, Cu (22.89 mg/kg), and Ni (18.05 mg/kg) but Cr content is higher in *Ulva* (8.36 mg/kg). The result showed significant difference ($p<0.05$) among the samples.

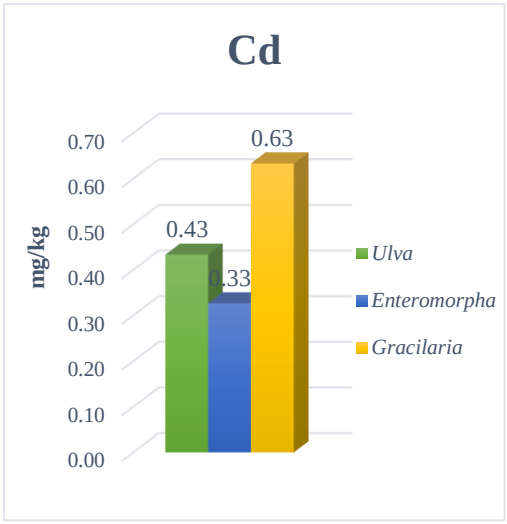
Table 8: Mean metal content ± Standard Deviation (SD) in seaweed samples (mg/kg)

Elements	***Ulva* sp.**	***Enteromoroha* sp.**	***Gracilaria* sp.**
	Chlorophyta (green)	Chlorophyta (green)	Rhodophyta (red)
As	2.17 ± 0.05^{a}	2.73 ± 0.05^{b}	17.83 ± 0.12^{c}
Cd	0.43 ± 0.03^{b}	0.33 ± 0.02^{a}	0.63 ± 0.02^{c}
Co	1.13 ± 0.05^{a}	1.60 ± 0.01^{b}	1.83 ± 0.02^{c}
Cr	8.36 ± 0.02^{c}	6.04 ± 0.01^{a}	6.51 ± 0.02^{b}
Ni	10.02 ± 0.01^{a}	15.47 ± 0.02^{b}	18.05 ± 0.04^{c}
Pb	1.96 ± 0.15^{a}	2.13 ± 0.02^{b}	2.85 ± 0.04^{c}
Mn	123.04 ± 0.04^{a}	177.47 ± 0.02^{b}	339.14 ± 0.01^{c}
Se	1.33 ± 0.01^{a}	1.82 ± 0.01^{b}	1.86 ± 0.02^{c}
Cu	11.19 ± 0.02^{b}	8.53 ± 0.02^{a}	22.89 ± 0.02^{c}
Fe	1352.56 ± 0.02^{a}	6519.57 ± 0.02^{b}	6558.85 ± 0.02^{c}
Zn	5.98 ± 0.02^{a}	6.36 ± 0.02^{b}	17.81 ± 0.01^{c}

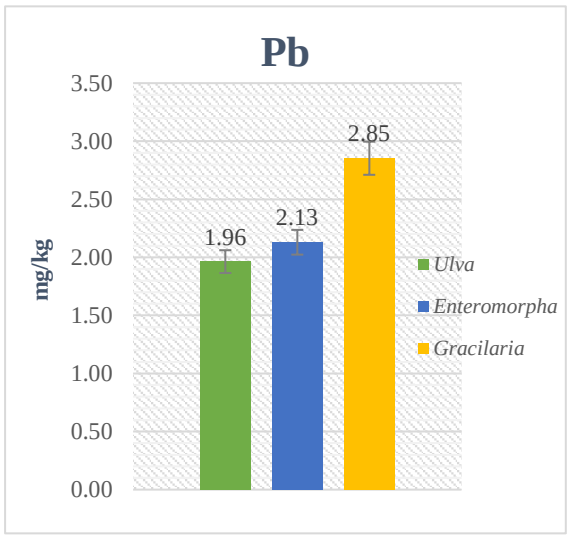
[a] Data are mean values of three determinations ± S.D. Means in identical rows with various letter combinations (a-c) differ considerably ($p< 0.05$, ANOVA, Tukey-HSD).


As
mg/kg
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
17.83
2.17
2.73
Ulva
Enteromorpha
Gracilaria

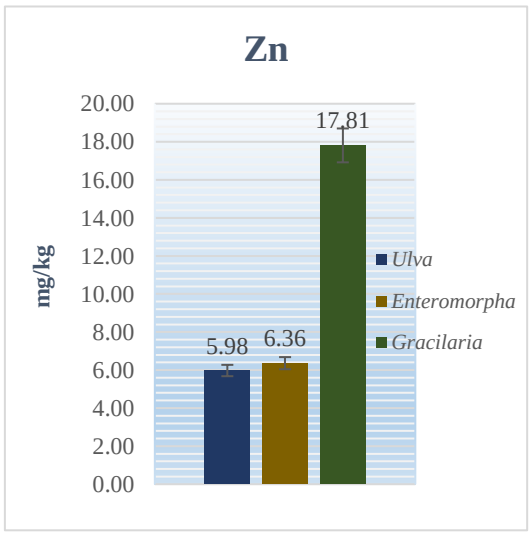
(a)


Cd
mg/kg
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.63
0.43
0.33
Ulva
Enteromorpha
Gracilaria

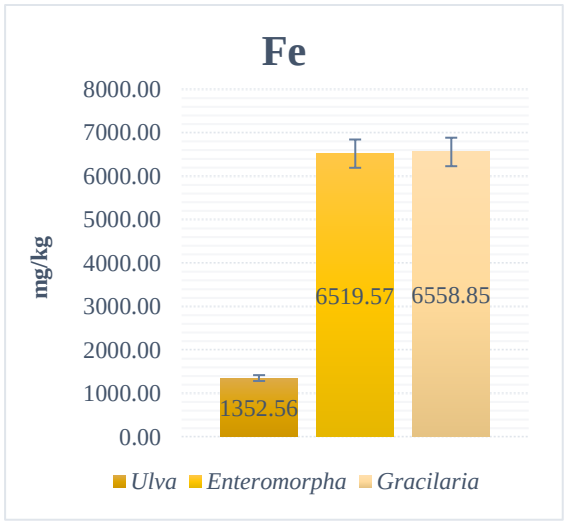
(b)


Pb
mg/kg
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
2.85
2.13
1.96
Ulva
Enteromorpha
Gracilaria

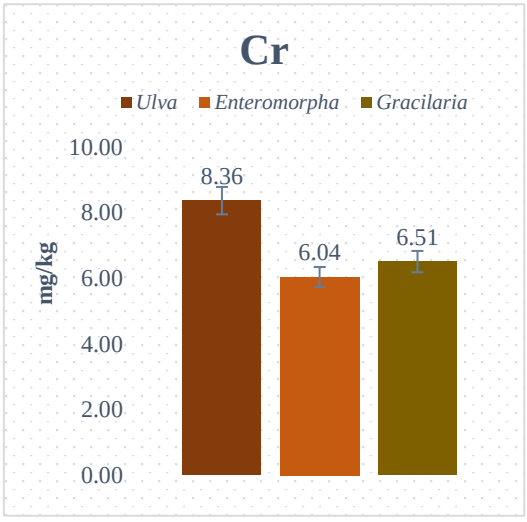
(c)


Zn
mg/kg
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
17.81
5.98
6.36
Ulva
Enteromorpha
Gracilaria

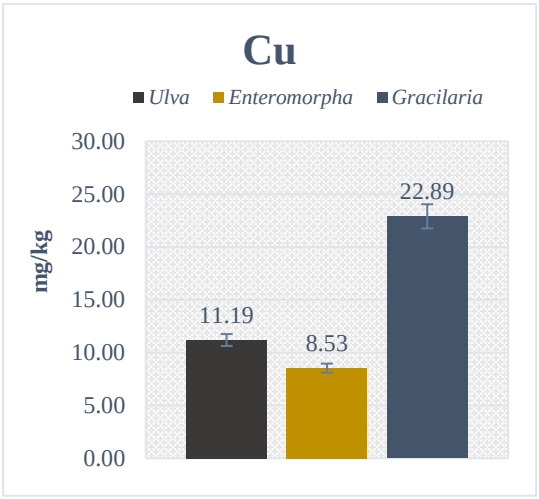
(d)


Fe
mg/kg
8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
6519.57
6558.85
1352.56
Ulva
Enteromorpha
Gracilaria

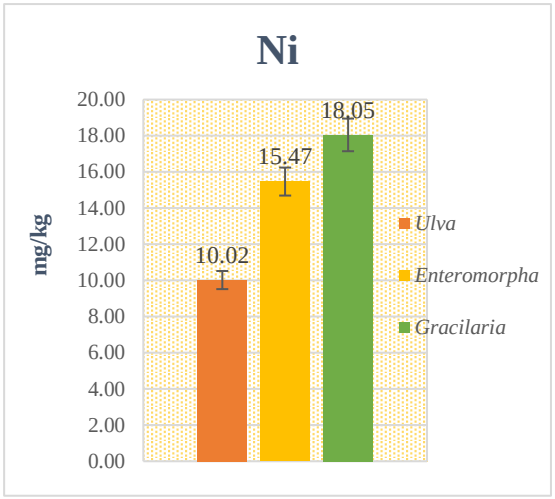
(e)


Cr
Ulva
Enteromorpha
Gracilaria
mg/kg
10.00
8.00
6.00
4.00
2.00
0.00
8.36
6.04
6.51

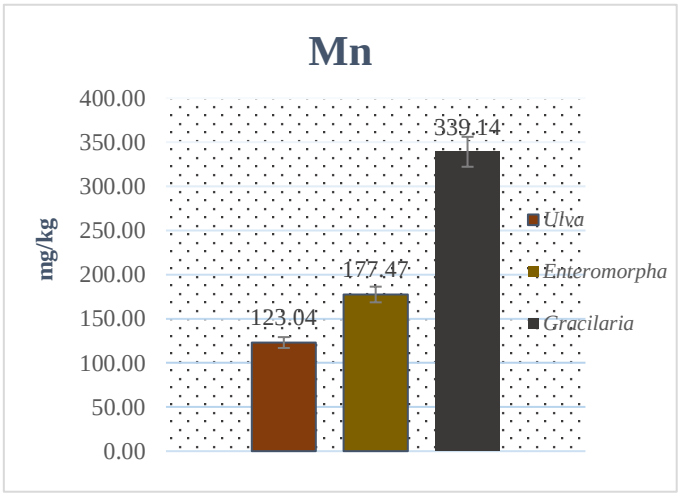
(f)



(g)



(h)



(i)

Figure 11: Graphical presentation of heavy metals

Chapter 5: Discussion

5.1 Proximate Analysis of Seaweeds

Proximate composition of three seaweed species are investigated in the current experiment; wet collected sample, lab dried, and market dried samples. In three conditions the species exposed different ranges of composition.

In wet condition, higher moisture content found in *Enteromorpha* sp. (91.49%), followed by *Ulva* sp. (87.84%), and *Gracilaria* sp. (84.07%). Moisture content found in this study are slightly lower than the literature (Debbarma *et al*., 2016; McDermid and Stuercke, 2003; Nagaraj *et al*., 2019) for the experimented three species. Lab dried samples revealed 10.02-13.86% water content and highest amount in *Enteromorpha* sp. (14.20%). The result can be compared with (Marinho-Soriano *et al.,* 2006; Wong and Cheung, 2000) for *Gracilaria* sp. and *Ulva* sp. whereas the other sample has higher contents mentioned in the literature findings. The samples collected from market contain water 3.51-6.52%, resemble with (Benjama and Masniyom, 2012; Ganesan *et al*., 2014; Rohani-Ghadikolaei *et al*., 2011).

Ash content ranged from 4.41-5.31% which relate with (Debbarma *et al*., 2016; Marinho-Soriano *et al.,* 2006) for *Gracilaria* sp. and other two species contain lower ash value stated in previous reports in case of wet samples. In lab dried sample 30.97-42.73% ash content was found where *Enteromorpha* sp. had the highest value and the results justify the ash content range of 8–40% DW (Mabeau and Fleurence, 1993), and also links with having higher ash content than most of the vegetables (Rupe´rez *et al*., 2002). Market dried sample contain 14.29-23.12% ash which support Rohani-Ghadikolaei *et al*., 2011; Wong and Cheung, 2000) for *Enteromorpha* sp. and *Ulva* sp.

Crude protein content in wet sample ranges from 1.64-3.48%; *Gracilaria* sp. (3.48%), *Enteromorpha* sp. (1.64%), and *Ulva* sp. (2.78%). In lab dried sample highest content found in *Gracilaria* sp. (20.90%) which shows similarities with (Benjama and Masniyom, 2012; Marinho-Soriano *et al.,* 2006; Rohani-Ghadikolaei *et al*., 2011). In market dried sample *Gracilaria* sp. (14.60%), *Enteromorpha* sp. (10.43%), and *Ulva* sp. (13.92%) that keep line with (Rohani-Ghadikolaei *et al*., 2011) for *Enteromorpha* sp.

Crude lipid content in three condition ranged from 0.13-1.01% and *Ulva* sp. possesses highest values than other two samples. The outcomes verify the report of lipid representing 1-3% of algal dry matter by (Arasaki and Arasaki, 1983).

Crude fiber in seaweed samples yielded 0.94-1.47% in wet weight basis and the content ranges from 2.96-16.67% dry weight basis where *Gracilaria* sp. (16.67%) occupies the highest and the amount can be compared with Nagaraj *et al*. (2019). The other observation in this study can be authenticated by the previous reports (Ganesan *et al*., 2014; Ratana-Arporn and Chirapart, 2006; Sivaramakrishnan *et al.,* 2017).

Highest carbohydrate value is observed in *Ulva* sp. (54.77%), that can be evaluated by (Ratana-Arporn and Chirapart, 2006; Rohani-Ghadikolaei *et al*., 2011; Rasyid, 2017). Higher carbohydrate value in *Gracilaria* sp. (50.80%) and in *Enteromorpha* sp. (51.76%).

According to the previous research analysis, factors including species, maturity, environmental growth regulators, and seasonality could be responsible for the broad variation of nutrients in various species (Ito and Hori, 1989; Ortiz *et al*., 2006).

5.2 Proximate Analysis of Seaweed Products

In this experiment, biscuits and muffins are developed incorporating three seaweed powder and their moisture, crude lipid, crude protein, dietary Fiber, ash content and carbohydrate composition analysis are shown in Table 4 and Table 5 respectively.

The ash content in the biscuits did not fluctuate very much in the samples and agrees with (Mamat *et al*., 2016, 2018; Sumana *et al*., 2018). The fiber content found highest in the *G*. biscuits and the current experiment reports 16.72% fiber in the market dried *Gracilaria* sp. Numerous research in the scientific literature have supported the idea that algal fiber is good for human health (Fuller *et al*., 2016). The moisture level varied from 3-10% where the value decreased in *Enteromorpha* and *Ulva* biscuit from the control sample but increased in *Gracilaria* biscuit furthermore the results concur with Zakaria *et al*. (2018). Crude protein improved 1% in other samples than control one though protein content in wheat flour (10-12%) and experimented seaweed species are not vastly diverse (10-20% stated in the lab dried sample of current experiment). The results correspond with (Mamat *et al*., 2016; Sumana *et al*., 2018; Udayangani *et al*., 2019). There are not very distinction in crude lipid content of the samples, only by 1-

2% where the value decreased in *Ulva* biscuit. The findings harmonize with Sumana *et al*., (2018). Carbohydrate value vary from 6-11% and agree with (Mamat *et al*., 2016; Sumana *et al*., 2018).

Because of the well-known ability of seaweed to hold water (hydrocolloids), the moisture level of muffins rose (Mamat *et al.*, 2016, 2018) and *G.* muffin had the highest content. Crude Fiber results indicate that *G.* muffin had the highest amount (0.32%), *E.* muffin valued 0.24% which matches with the results of Mamat *et al.*, (2018). There are little differences in crude protein values between control and seaweed incorporated samples. The results showed similarities with (Mamat *et al*., 2018; Sumana *et al*., 2018; Udayangani *et al*., 2019). The ash content found in the muffins match with the results of (Mamat *et al*., 2016, 2018; Sumana *et al*., 2018). The crude lipid content in the muffins differs from 2-3% with the control sample and the values show similarities with (Kumarathunge *et al*., 2016; Mamat *et al*., 2018). Carbohydrate label almost decreased in the seaweed incorporated muffins than control sample differs from 5-7%. The current findings show that eating seaweed-infused muffins may help the body's requirements for ash and fiber to be met.

5.3 Microbial Analysis of Seaweeds

Ulva sp. presents higher microbial load (13.50×10^6) cfu/g in the current study, followed by *Gracilaria* sp. (9.43×10^6) cfu/g, and *Enteromorpha* sp. (2.23×10^6) cfu/g. According to Shiba and Taga (1980), In the Shizuoka prefecture's Nabeta Inlet, heterotrophic bacteria adhering to the *Emeromorpha linza* had viable numbers between 10^4 and $10^6/cm^2$. Moreover, Table 7, indicates the presence of pathogenic bacteria such as *Salmonella* spp., *Shigella* spp., *Vibrio cholera*, *Vibrio parahaemolyticus*, *Vibrio Vulnificus*, and *Escherichia coli* in different seaweed samples though *Gracilaria* sp. did not contain pathogenic bacteria like *Escherichia coli*, *Vibrio vulnificus*, and *V. parahaemolyticus.* The report tones with (Beleneva and Zhukova, 2006Lavilla-Pitogo, 1992) for *Gracilaria* sp.; Vairappan and Suzuki (2000) found *E. coli* in *U. reticulate.* The reason may include sewage discharge, anthropogenic activities in nearby experimented places etc.

5.4 Heavy Metals Analysis

According to a report by Filippini *et al*. (2020) on macro-elements, France and Spain produced significantly more aluminum than other nations, with values of 110.91 mg/kg and 331.89 mg/kg, respectively. Additionally, France and Spain recorded high values for the macro-elements Mn, Cu, and Zn compared to other nations, reaching greater peaks. Last but not least, the total As value achieved by Korea was 43.90 mg/kg. Toxic metal limits were established for edible French algae by the CEVA (Center d'Etude et devalorization des Algues), which also established thresholds for Cd (0.5 mg/kg dw) and Pb (5 mg/kg dw). The ability of the genus *Ulva* to collect Pb concentrations between 500 and 2200 times has been shown in the literature (Henriques *et al*., 2017), indicating both their excellent capacity to remove heavy metals from the environment and their role as an environmental bio indicator (Shams El-Din *et al*., 2014). In the present study, *Gracilaria* contains Cd 0.64 mg/kg, *Enteromorpha* 0.34 mg/kg, and *Ulva* 0.45 mg/kg while the Pb values found in *Gracilaria, Enteromorpha,* and *Ulva* are 2.87, 2.15, and 1.95 mg/kg respectively. Comparing the present results and considering the 0.5 mg/kg limit for the Cd, only *Gracilaria* exceeded it (0.64 mg/kg), yet none of the samples went over the Pb-specific limit (5 mg/kg).

According to Filippini *et al*. (2020), France, Spain, and China all showed high Fe values: 195.32, 389.58, and 375.04 mg/kg, respectively. The buildup of Fe is typically high in all varieties of seaweed according to Chakraborty *et al*. (2014), however the current study has exceeded all those limitations by over 33 to 16 times. The greatest values were discovered in *Gracilaria* (6558.85 mg/kg) and *Enteromorpha* (6519.58 mg/kg), and the lowest value was discovered in *Ulva* (1352.56 mg/kg), which are substantially greater than the other authors. This could be a result of the high rates of photosynthesis that are typical of subtropical coastal habitats, as well as the ongoing development of Cox's Bazar Airport and the presence of anchored ships that could discharge bilge water into which rust can develop.

As for hazardous metals (Al, Cd, Pb, As, and Hg), *Gracilaria* had the greatest concentrations (17.86 mg/kg) of As, followed by *Enteromorpha* (2.73 mg/kg), and *Ulva* (2.19 mg/kg) had the lowest concentration in the current study. In general, marine organisms exhibit greater As concentrations than terrestrial ones (Phillips, 1990), and inorganic As is more poisonous than organic As (López *et al*., 1994). As most of the

Arsenic is in its organic forms, the "Mixed Commission of the Codex Alimentarius" FAO-WHO, the Food and Agriculture Organization and the World Health Organization, advocated assessing not just the total amount of As present in food but also the concentrations of inorganic As (Tsuda *et al*., 1992). Because of its organic form, it's feasible that seafood with exceptionally high total As concentrations won't be poisonous. Arsenic labels may contain up to 8 g (kg day^{-1}) of arsenic, according to the EFSA CONTAM Panel, 2009. All of the samples surpassed the As level when compared to the EFSA CONTAM Panel. The experimental *Gracilaria* sample (16.72% fiber detected in the present experiment) may contain more As than other samples since high fiber concentration in algae may alter inorganic As bioavailability (Vélez and Montoro, 2001), however, drawing solid conclusions solely from the analytical data is impractical.

Gracilaria has the highest concentration of Copper in the current study (22.89 mg/kg), followed by *Ulva* (11.19 mg/kg), and *Enteromorpha* (8.53 mg/kg). Because Cu and Zn are both frequently found in urban effluents, they point to a common source (González and Torres, 1990). Additionally, because of the nearby presence of human habitations, the experimental sites experienced sewage discharge, which may support the likelihood of higher values for Cu and Zn concentrations. *Ulva* is in accordance with *Ulva rigida*, with Zn concentration 5.61-6.14 mg/kg observed in the most recent report, while Zn values for *Gracilaria*, *Enteromorpha*, and *Ulva* are 17.81 mg/kg, 6.37 mg/kg, and 5.99 mg/kg, respectively in the present study (Besada *et al*., 2009).

The values of Mn, Ni, Cr, and Se in the current study are found to be greater, which may be associated to sewage discharge, construction in the area, and ship bilge water. The greatest Mn label is found in *Gracilaria* (339.15 mg/kg), whereas Ni and Cr values are nearly identical in *Gracilaria* and *Enteromorpha* but somewhat higher in *Ulva* (8.38 mg/kg).

The current investigation shows that *Gracilaria* is the highest heavy metal accumulator among the tested *Gracilaria, Enteromorpha* and *Ulva* samples, in line with Luo *et al*. (2020)'s observation that it has excellent adsorption abilities for heavy metals from seawater.

Chapter 6: Conclusions

Seaweeds are rich in minerals, vital amino acids, and other bioactive components that are great for human health. The growth of aquaculture has been accelerated by the addition of seaweed powder in fish feed. Seaweed is used as food in many countries, mainly Asian countries like China, Japan, South Korea, etc. Seaweed can be eaten raw or cooked. Bangladesh is a new contributor in this industry, thus fundamental knowledge about proximate composition, free fatty acids, minerals, heavy metals, etc. is needed when producing new products. The analysis of seaweeds conducted for this study suggests that *Gracilaria* sp. has higher levels of crude fiber and crude protein, both of which are excellent for health. Seaweed can be employed in food production as it contains a wide variety of poly unsaturated fatty acids, essential amino acids, and high levels of minerals like I, Fe, Ca, and Mg etc., according to prior literature. However, muffins and biscuits made with seaweed powder did not exhibit higher distinction with control sample for proximate composition. The findings reveal that the samples that were tested included dangerous heavy metals, pathogenic microorganisms, and a greater microbial load. To safeguard the consumers' health and safety, these findings must be considered.

Chapter 7: Recommendations

Seaweed culture in Bangladesh is increasing day by day which is a great opportunity for coastal communities as well as for the developing seaweed industry. This research will help to improve different bakery products like bread, cake, biscuits, burgers, buns etc. augmenting their nutritional aspects along with their taste. The following suggestions may be carried out given the constraints of the current study:

- Amino acid profile, fatty acids, and mineral content should be analyzed of the experimented seaweeds
- Water holding capacity, oil holding capacity of the value added products can be measured
- Daily intake dose and health risk assessment based on their heavy metal contents can be measured
- Determination of antibacterial and antioxidant activity to utilize in the pharmaceuticals industry
- Phylogenetic analysis of bacteria associated with these seaweeds through genome sequencing
- Seasonal variation of nutritional value and heavy metal labels in these seaweeds

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PHOTO GALLERY

Plate 18: Collection of samples

Plate 19: Preparation of ingredients for value added products

Plate 20: Microbial analysis of seaweeds

Brief Biography of the Author

Sharmin Jahan Shampa, the eldest child of Md. Jaynal Abedin and Mrs. Sakhina Akter, was born in Chattogram, Bangladesh on November 27, 1998. She completed her SSC from A. L. Khan High School, Chattogram in 2013 and her HSC from Chattogram Cantonment Public College, Chattogram in 2015. She graduated with a Bachelor of Science in Fisheries (Hons.) in 2019 from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. She is currently working toward her Master of Science in the Department of Fishing and Post-Harvest Technology, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University under the guidance of Associate Professor, Dr. Md. Faisal, on the project "Biochemical and Microbial Analysis of Seaweeds and Their Value Added Products". She has a strong desire to strengthen her research skills so that she may advance fishing industry of Bangladesh.