

DEVELOPMENT OF FOOD PRODUCT FROM FERMENTED SEAWEED

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

by

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(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **M. BAGAVATHY DARSHAN (39230005)** and **M. MOHANRAJ (39230027)** who carried out the project "DEVELOPMENT OF FOOD PPRODUCT FROM FERMENTED SEAWEED" under our supervision from August, 2022 to April, 2023.

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I, **M. BAGAVATHY DARSHAN** (39230005) hereby declare that the Project report entitled "**DEVELOPMENT OF FOOD PRODUCT FROM FERMENTED SEAWEED**" done by me under the guidance of **Dr. R. THYAGARAJAN Ph.D., Assistant Professor, Department of Biotechnology** and **Dr. L. STANLEY ABRAHAM, CENTRE FOR OCEAN RESEARCH, Col Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai**, is submitted in partial fulfilment requirements for the award of a Bachelor of Technology degree in biotechnology.

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ABSTRACT

Seaweeds have a significant impact on the marine ecosystem because they are one of the primary producers in marine food chain. They are highly nutrient-dense and rich in antioxidants, which are present in the form of specific vitamins and minerals. A healthy supplement of nutrients such minerals, vitamins and antioxidants helps to protect us to boost our immunity and combat other life style diseases. Seaweeds are one such potent marine bio resource are becoming more popular as nutrient supplements in Indian diet. Because they are easily available source and can be cultivated near shores. This project focusses about seaweed and its major role as nutrient supplement and enhanced its nutrient content through fermentation. The main objective of the project is on development of food products from seaweeds. Selected edible seaweeds such as *Kappaphycus alvarezii*, *Gracilaria Salicornia*, *Gracilaria edulis* and *Sargassum spp.* Were used for product development. Among four species *Gracilaria salicornia* was selected for product development. The aqueous extract of *Gracilaria Salicornia* was used for development of fermented health product using *Lactobacillus spp.* isolated from curd samples. The fermented product will help to be a probiotic drink for various health conditions such as lifestyle diseases and Thyroid deficiency which has to be further validated.

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LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
FAO	FOOD AND AGRICULTURAL ORGANISATION
WHO	WORLD HEALTH ORGANISATION
UNU	UNITED NATIONS UNIVERSITY
CAGR	COMPOUND ANNUAL GROWTH RATE
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
GC-MS	GAS CHROMATOGRAPHY MASS SPECTROMETRY
NMR	NUCLEAR MAGNETIC RESONANCE
IR	INFRA RED
MS	MASS SPECTROMETRY
GADA	GLUTAMIC ACID DECARBOXYLIC AUTOANTIBODIES
SSF	SOLID STATE FERMENTATION
TFA	TRANS FATTY ACIDS
PUFA	POLY UNSATURATED FATTY ACIDS
MUFA	MONO UNSATURATED FATTY ACIDS

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CHAPTER 1

INTRODUCTION

Seaweeds, or seagrass, are the macroalgae present in marine environments and shallow. The three types of seaweed are classified as brown (*Phaeophyta*), red (*Rhodophyta*), and green (*Chlorophyta*). (Parrott *et al.*, 2017). They are able to reproduce both sexually and asexually by means of spores and vegetative growth, respectively. These seaweeds can attach to hard surfaces such as rocks and crustacean shells with the help of specialized structures, called holdfasts. Not only in Asian and European countries, nowadays India is also emerging as one of the top consumers of seaweeds and related exports and imports. (Seaweed for India, www.investindia.gov.in 2021)

1.1 BENEFITS OF SEAWEED

The seaweed compound pheophytin, have biological effects as antioxidants, anti-inflammatory drugs, and neuroprotective substances. One among many kinds of red seaweed (*Rhodophyceae*) with a lot of chlorophyll is *Gracilaria salicornia*. According to the information above, *G. Salicornia* seaweed has the potential to be used as a raw material for functional food products that can offer health benefits in addition to the nutritional benefits they contain. (Lumbessy *et al.*, 2021). High genetic similarity within a community is revealed by molecular analysis, bolstering the theory that dispersal happens by cloning via the fragmentation process (Jennifer *et al.*, 2004).

Numerous seaweed species are used in traditional medicine and recent research has revealed that these plants possess significant biological properties including antioxidant, anti-microbial, anti-proliferative, anti-inflammatory, hypoglycaemic, and cytotoxic activities. In addition, being used in the food and pharmaceutical industries, it can take the place of fossil fuels. Seaweed is a top contender for cutting-edge green energy solutions as well as global initiatives to reclaim land (Cyrus *et al.*, 2022). The nutritional value was determined through a comparison of the amount of essential amino acids in each seaweed to the

suggested pattern for essential amino acid requirements. Numerous essential amino acid levels, particularly in *G. salicornia*, were on par with the FAO/WHO/UNU11 requirement pattern. (Mehdi *et al.*,2012). Geographically widespread and capable of producing commercial quantities of agar, the *Gracilaria* seaweed genera are found in warm-water habitats. A natural marine biomass called seaweed is used to make agar, jellies, plant fodders etc., (Jackline *et al.*, 2019)

1.2 SEAWEED IN DAYTO DAY LIFE

We first became aware of the benefits of seaweed many thousands of years ago, as evidenced by archaeological records, which date seaweed's originated during three and a half billion years ago. (Rose *et al.*,2016). Seaweed has long been used in Japan, China, Hawaii, India, and other places. In 2021, the seaweed market achieved a value of US\$6.73 billion. The forecast by IMARC Group "Seaweed Market Size, Share, Value, Price Trends, Industry Analysis & Forecast (2022-2027" predicts that the market will expand at a CAGR (Compound Annual Growth Rate) of 11.5% between 2022 and 2027. According to the Board of Investments' 2013 report, "Seaweeds: Board of Investments," seaweeds are used in the food industry, pharmaceutical industry, and medical field.

1.3 SEAWEED -A RELIABLE FOOD SOURCE

Seaweeds are rich in potent bioactive compounds for human benefit. (Nagappan T, *et al.*,2013,) (Shah *et al.*,2020). *Gracilaria salicornia* is economically important seaweeds that has thrived the most on reef beds. The native *G. coronipfolia* and *G. parvispora* appear to be in a competitive relationship with it, but the mat form of the alien species allows for a faster growth rate and greater hardness. (Agardh *et al.*,2022) The preferred temperature range for *Gracilaria*, a warm-water seaweed, is typically 20 to 30 °C. Agar, a gel-forming polysaccharide, is especially important in the food and microbiological industries, according to "Seaweed Aquaculture:<https://marine-aquaculture.extension.org> (2019). Not only they are rich in these benefits, but they have also been used commercially to produce many alcoholic beverages with great health benefits, such as beer, rum, wine, and so on. Seaweeds are a highly nutritious food source. Since they resemble

green plants, they can be consumed either raw or cooked. Seaweeds have long been grown and used both as feed for livestock and as a direct source of food for humans. (Nori, www.nori.com). Seaweed abundance, vegan status, and high iron, calcium, and vitamin K content are advantageous. Seaweed can add briny umami flavours to a variety of dishes whether it is served raw, pickled, soaked in broth, used as a thickener, or added to baked goods. (Leahy, *et al.*, 2022) Nori 2.0 is a sauce based food made from seaweed roaming around the famous restaurants for its unique taste and flavour. Nori is a term used to call seaweed traditionally, they are used to make sushi, onigiri in Korean restaurants. Additionally, it is used as a garnish or flavouring in soups and noodle dishes. Most frequently, it is toasted before eating.

1.4 PHYTO AND BIOCHEMICAL PROPERTIES OF FERMENTED SEAWEED

Fermentation of seaweed extracts using *Lactobacillus* strains found that the process resulted in the production of several beneficial compounds, including lactic acid, acetic acid, and mannitol. These compounds have been shown to have potential health benefits, such as reducing inflammation and improving gut health. (Hong *et al.*, 2016) The study also found that the use of *Lactobacillus* strains for seaweed fermentation resulted in higher levels of antioxidant activity compared to unfermented seaweed extracts. This suggests that fermentation with *Lactobacillus* may increase the bioavailability of these beneficial compounds. Physiochemical properties refer to the physical and chemical characteristics of a substance. In the case of *Gracilaria salicornia*, obtaining its physiochemical properties involves analysing its composition, structure, and behaviour under different conditions. This includes studying its moisture content, ash content, protein content, lipid content, carbohydrate content, mineral content, viscosity, colour, and pH, among others. (Gonzalez *et al.*, 2012) Seaweeds are rich in bioactive compounds such as polysaccharides, proteins, lipids, pigments, and minerals. These compounds have been extensively studied for their potential applications in food, pharmaceuticals, and cosmetics. In recent years, the development of advanced analytical techniques has facilitated the identification and quantification of these compounds in seaweed extracts. (Venkatesan *et al.*, 2015). One of the most common methods for the biochemical analysis of seaweeds is High performance liquid chromatography (HPLC). HPLC has been used to separate and quantify various bioactive

compounds in seaweed extracts, including pigments such as chlorophylls, carotenoids, and phycobiliproteins, as well as polysaccharides such as carrageenan, alginate, and fucoidan. HPLC has also been used to identify and quantify various phenolic compounds in seaweed extracts, including phototannins, flavonoids, and hydroxycinnamic acids. (Wijesekara *et al.*,) Another widely used method for the biochemical analysis of seaweeds is gas chromatography-mass spectrometry (GC-MS) (Fitton *et al.*,2016) GC-MS has been used to identify and quantify various volatile and non-volatile compounds in seaweed extracts, including fatty acids, sterols, and terpenes. GC-MS has also been used to identify and quantify various amino acids in seaweed extracts. (Rajauria *et al.*,2014) In addition to HPLC and GC-MS, other analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, and mass spectrometry (MS) have been used for the biochemical analysis of seaweeds. NMR spectroscopy has been used to identify and quantify various metabolites in seaweed extracts, including sugars, organic acids, and amino acids. IR spectroscopy has been used to identify and quantify various polysaccharides in seaweed extracts. MS has been used to identify and quantify various lipids in seaweed extracts. (Ayyad *et al.*,2010) The present project is being focussed with the objective to develop an innovative fermented product using the selected edible seaweeds collected from Mandapam, Ramnad district, Tamil Nadu.

CHAPTER 2

REVIEW OF LITERATURE

LITERATURE SURVEY

2.1 BIOFERMENTATION

Fermentation of seaweed has become an increasingly popular in recent years, as researchers explore its potential for use in various industries. *Gracilaria salicornia* is a red seaweed that has been studied for its potential as a source of bioactive food supplement and functional compounds, including polysaccharides, antioxidants, and antimicrobial agents. (Wang *et al.*, 2019)

The optimal conditions for the fermentation of *Gracilaria salicornia* using a *Lactobacillus plantarum* strain. The researchers found that a fermentation time of 36 hours, at 37°C, with an inoculum size of 5% were the best conditions for the production of a novel polysaccharide with high antioxidant activity. (Zhang *et al.*, 2018)

A Study investigated by the group of scientists (Bai *et al.*, 2022) and (Chiney *et al.*, 2016) of Gram-negative and gram-positive bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*, respectively) were used to test the antibacterial effects of various organic solvent extracts such as acetone, chloroform, diethyl ethanol, and methanol. It was discovered that *Pseudomonas aeruginosa* and *Bacillus subtilis* were both susceptible to the algal ethanol extract also contains a plethora of additional nutrients and minerals for human growth and benefit.

2.2 EDIBLE SEaweeds

Not every species of seaweeds we found in seas were edible because by a wide margin, the seaweed genus *Gracilaria* (including species now recognized as *Polycavernosa*) has been responsible for the greatest number of reports of illnesses in a study made by (Hutlin *et al.*, 2022).

This study investigated the production of fucoidanase, an enzyme that can break down fucoidan, a complex polysaccharide found in brown seaweed. The researchers used *Gracilaria salicornia* as a carbon source for the fermentation of *Bacillus velezensis* NJ-6, and found that the highest fucoidanase activity was achieved after 48 hours of fermentation (Wang *et al.*,2020).

2.3 BIOFERMENTATION OF SEaweEDS

Biofermentation of seaweeds has been evaluated by investigating the production of γ -aminobutyric acid (GABA), an important neurotransmitter, through the fermentation of *Gracilaria salicornia* using *Lactobacillus brevis*. The researchers found that a fermentation time of 24 hours, a temperature of 37°C, and an inoculum size of 5% were the best conditions for the production of GABA. (Lee *et al.*,2014)

This study investigated the potential of seaweed as a feedstock for the production of bioethanol through simultaneous saccharification and fermentation (SSF). The researchers found that a high ethanol yield was achieved using a combination of commercial enzymes and yeast, and that seaweed had great potential as a feedstock.

Overall, these studies demonstrate the potential of *Gracilaria salicornia* as a substrate for fermentation and the production of various bioactive compounds, including polysaccharides, enzymes, GABA, and bioethanol. Further research in this area could lead to the development of new and sustainable methods for the production of these compounds.

Fermentation of seaweed *Gracilaria salicornia* has been a topic of interest in recent years due to its potential as a source of bioactive compounds and functional ingredients. Several studies have investigated the fermentation process of *Gracilaria salicornia* and its effects on the nutritional and functional properties of the seaweed.

One study by Kuda *et al.*, (2013) investigated the effect of fermentation on the antioxidant properties of *Gracilaria salicornia*. The authors found that fermentation increased the total phenolic content and antioxidant activity of the seaweed, which could have potential health benefits.

2.4 LACTIC ACID BACTERIA

Another study by Kim *et al.*, (2016) investigated the fermentation of *Gracilaria salicornia* using lactic acid bacteria. The authors found that fermentation increased the bioavailability of certain minerals, such as calcium and magnesium, and improved the texture and taste of the seaweed.

In a study by Chen *et al.*, (2017), the authors investigated the fermentation of *Gracilaria salicornia* using a mixed culture of lactic acid bacteria and yeast. The authors found that fermentation increased the soluble protein and amino acid content of the seaweed, and also improved its texture and flavor.

Fermentation of seaweed extract from *Gracilaria salicornia* is a process of using microorganisms to convert the complex organic compounds in the extract into simpler substances through metabolic activities. This process has been widely used in various industries, including food, pharmaceutical, and cosmetic industries, due to the many health benefits associated with the extract.

One example of the application of fermentation of seaweed extract in the production of a probiotic drink. In a study published in the Journal of Functional Foods, researchers fermented seaweed extract using *Lactobacillus plantarum* and found that the resulting product had a high antioxidant activity and improved gut health in animal models. (Prathmesh *et al.*, 2017)

In addition, a study by Woo *et al.*, (2018) investigated the potential use of fermented *Gracilaria salicornia* as a functional ingredient in food products. The authors found that the fermented seaweed had potential as a natural preservative and could also enhance the sensory properties of food products.

Overall, these studies suggest that fermentation of *Gracilaria salicornia* can enhance the nutritional and functional properties of the seaweed, making it a promising source of bioactive compounds and functional ingredients. However, further research is needed to fully understand the fermentation process and its effects on the properties of the seaweed.

Fermentation of seaweed, particularly *Gracilaria* and *Salicornia*, has received considerable attention due to their high nutritional value, unique flavor, and potential health benefits. Several studies have been conducted to investigate the fermentation process, microbial diversity, and the impact of fermentation on the physicochemical properties and bioactive compounds of these seaweeds.

In a study conducted by Chung *et al.*, (2019), *Gracilaria verrucosa* was fermented using *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for 3 days. The results showed that the fermented seaweed had higher antioxidant activity, reducing power, and total phenolic content compared to the non-fermented seaweed. Additionally, the fermentation process led to a decrease in the molecular weight of the polysaccharides, which may enhance their bioactivity.

Another study by Kim *et al.*, (2020) investigated the fermentation of *Salicornia herbaceae* using *Lactobacillus plantarum* and *Lactobacillus fermentum*. The fermented seaweed had a higher content of lactic acid and free amino acids, which may contribute to the flavour and nutritional value of the product.

Seaweed farming is a low-cost technology that offers significant returns, which attracted better adoption among the coastal fisher folk. This was revealed by an analysis of the economics and constraints of seaweed farming and collection undertaken in Ramanathapuram District of Tamil Nadu coast in a report made by Johnson *et al.*, (2017)

A study by Cho *et al.*, (2020) explored the microbial diversity during the fermentation of *Gracilaria changii* using a mixed culture of lactic acid bacteria and yeast. The results revealed that the fermentation process increased the abundance of lactic acid bacteria and yeasts, which may contribute to the production of organic acids, enzymes, and other bioactive compounds.

In conclusion, the fermentation of seaweed, has been shown to enhance the nutritional value, bioactivity, and flavour of the products. The use of specific microbial cultures during fermentation can modulate the physicochemical properties and increase the content of bioactive compounds.

CHAPTER 3

AIM AND SCOPE

AIM

1. To collect edible seaweeds from South east coast of South India for development of fermented product.
2. To carry out Molecular characterization of edible seaweeds for identification of the macro algae.
3. To Analyse the physio-chemical characteristics of collected seaweed.
4. To develop an innovative fermented product from selected edible seaweeds.

SCOPE

1. Seaweeds contains essential minerals, dietary fibre, iodine and tyrosine, which act as supplement for several deficiencies.
2. In addition they contain rich source of phytochemicals and serve as reservoir of antioxidants.
3. These properties makes them a suitable marine bio resource for development of food supplement. In this study we tried to develop an innovative fermented food product for addressing the different health condition such as Obesity and other lifestyle diseases.

CHAPTER 4

MATERIALS AND METHODS

4.1 COLLECTION OF SEaweEDS

The Seaweeds, such as *Gracilaria salicornia*, *Gracilaria edulis*, *Kapphycus* and *Sargassum* are a variety of red brown algae that are widely cultivated and available in both East and West Indian coastal lines. We collected four seaweed samples from Mandapam in Ramnad district of Tamil Nadu for the purpose of extraction. Later, the seaweeds were brought to our laboratory in a fresh sterile condition, packed using a clean plastic container tightly and transported without any damage. Seaweed samples were collected to get the seaweed extract. The collected seaweeds were maintained in a sterile environment inside the cold storage at 4°C.

4.2 PROCESSING OF SEaweED

Following collection, the seaweeds were processed through a series of steps which includes washing, grinding, filtration and storage. The seaweeds were taken out from the cold storage and brought to room temperature before being washed. The impurities were removed from the samples. To extract bioactive compounds, there are several combinations of drying and extraction techniques appropriate for each target, as summarized by (Carlos *et al.*, 2015). Then, they were subjected to a process called grinding, in which pulveriser was used to reduce the seaweed's size. Since Seaweeds are rigid and slippery to slice or grind using a mixer or a slicer. Seaweed fragments were manually crushed with a sterile white cloth after being ground. Usually, crushing/ grinding is performed manually or using a pulveriser (Hahn *et al.*, 2015). The extracts were kept in a clean container under refrigeration. Due to their high moisture content, seaweeds are perishable products. (Kadam *et al.*, 2012) They were filtered using filter paper before the seaweed extract was kept in a cold storage facility. About 500 ml of pure seaweed extract was used to characterize the proximate analysis of seaweed.

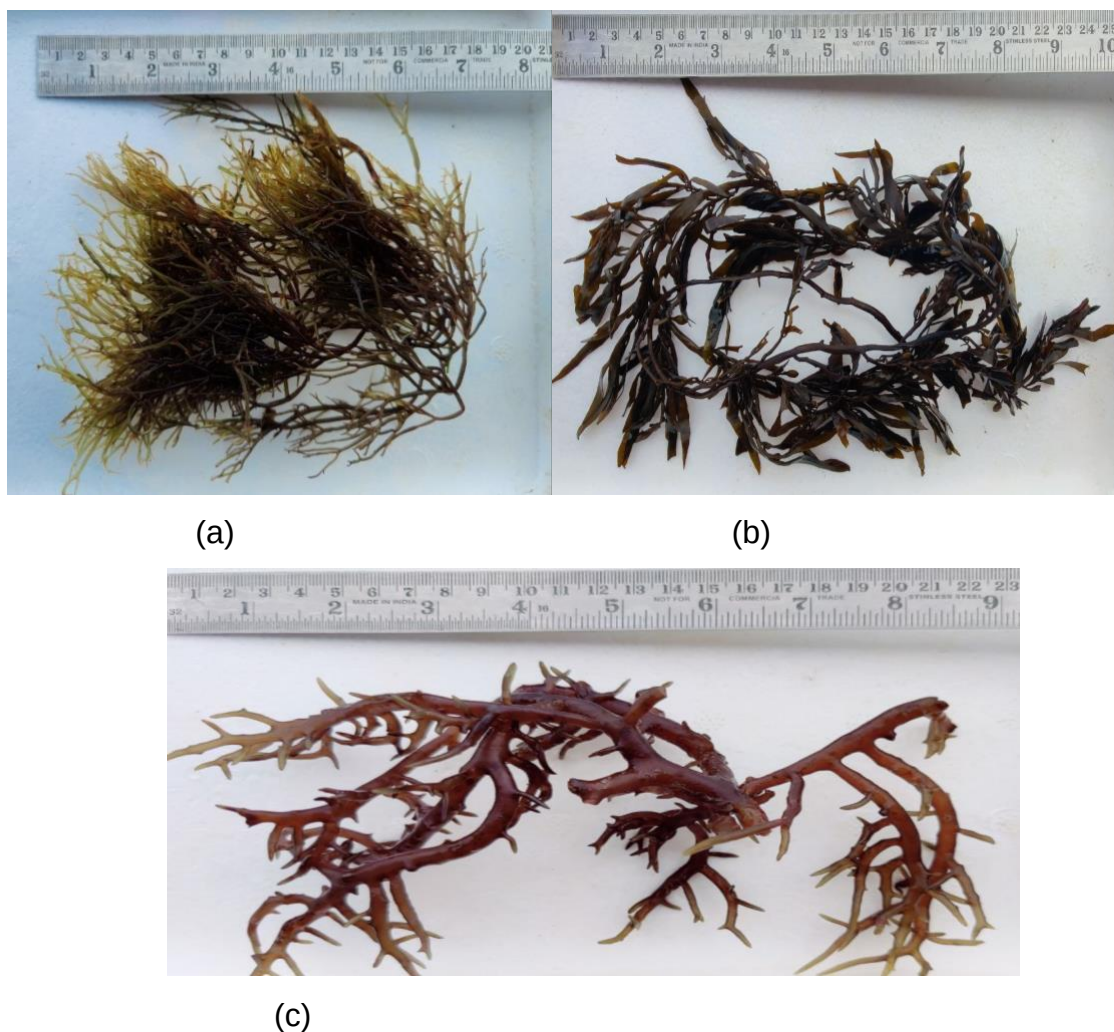


Fig 1.1 Collected Seaweed samples of *Gracilaria salicornia*, *Sargassum* and *Kappaphycus*

4.3 MOLECULAR CHARACTERISATION OF ISOLATED SEAWEED SAMPLES

Among four seaweed samples were subjected to molecular characterisation process. 100 ml of those samples extract were taken from the raw seaweed. The 100 ml of seaweed extract was prepared by grinding the raw seaweed by mortar & pestle and by the gradual addition of hot water. The sample extract was measured using measuring cylinder and filtered using filter paper. They were bottled using a white sterile plastic container.

4.4 DNA EXTRACTION

DNA extraction was carried out using CTAB (cetyltrimethylammonium bromide) method. Fresh seaweed samples were added in CTAB and incubated for 1-2 hours. Followed by Centrifugation of the seaweed mixture at 12,000 rpm for 10 minutes. Later the supernatant and dried DNA pellets were measured the DNA concentration and quality using a nanodrop spectrophotometer. The purified and amplified DNA was stored at -20°C. (Zuccarello & Paul , 2019).

Molecular characterization of seaweed involves the analysis of the genetic material of seaweed species to understand their molecular structure, genetic makeup, and phylogenetic relationships.

4.4.1 METHOD

1. A small amount of dried, or fresh, tissue (approximately 50mg) were added in microcentrifuge tube.
2. 500µl extraction buffer is added to the seaweed in a microcentrifuge tube and mix well.
3. CTAB extraction buffer contains (2% CTAB, 0.1M Tris-HCl (pH 8.0), 1.4M NaCl, 20mM EDTA, 1% polyvinylpyrrolidone – PVP) plus the optional addition of approximately 50µg RNase A and 80µg Proteinase K.
4. Samples are then ground with a microfuge pestle.
5. Incubated the mixture at 55-60°C for 1-2 hours, mixing occasionally.
6. An equal volume of chloroform : isoamyl alcohol (at a ratio 24:1) is added to the mixture and vortex vigorously for 30 seconds.
7. Centrifuged the mixture at 12,000 rpm for 10 minutes to separate the phases.
8. Transferred, the aqueous phase to a fresh microfuge tube and added chloroform : isoamyl alcohol (24:1). Vortex again and centrifuged as before.
9. Transferred, the aqueous phase to a fresh tube and added equal volume of isopropanol. Mix by inverting the tube a few times and incubate for 30 minutes at maximum speed.
10. Centrifuged the tube at 12,000 rpm for 15 minutes to pellet the DNA.
11. Carefully, remove the supernatant and wash the DNA pellet with 70% ethanol.

12. Air-dried the DNA pellet and re suspended it in TE buffer (1mM Tris pH 8, 0.1mM EDTA).
13. Measured the DNA concentration and quality using a spectrophotometer or gel electrophoresis.
14. Stored the DNA at -20°C until further use.

4.4.2. AGAROSE GEL ELECTROPHORESIS

PROCEDURE

1. 0.4g of Agarose powder was added and dissolved in 40ml of 1 × TAE buffer.
2. Then place the mixture in the oven and allow it to mix for few seconds (approximately 30 seconds).
3. After that allowed it to solidify and after that added 1 drop of ETBR (Ethidium Bromide).
4. Sealed the Electrophoresis chamber using Sellotape and check for leakage and then remove the tape.
5. Preparation of 1 × TAE Buffer (500ml) 10ml 50 × TAE Buffer + 490ml of Distilled water
6. Then added 50 × TAE Buffer to the Electrophoresis
7. DNA samples, typically mixed with a loading dye (5µl) that contains a tracking dye to monitor the progress of the electrophoresis, are loaded into wells or slots made in the agarose gel.
8. The DNA samples were loaded at the negative end (cathode) of the gel, and an electric field was applied across the gel by connecting the electrodes to a power supply.
9. When the electric field was applied, DNA molecules migrate through the agarose gel based on their size. Smaller DNA molecules move more quickly through the gel, while larger DNA molecules move more slowly.
10. After the electrophoresis was completed, the DNA molecules are visualised under UV light.

4.4.3. POLYMERASE CHAIN REACTION

1. A specific region of the 18S rRNA gene was amplified using PCR, which involves using short DNA primers that are complementary to conserved regions of the gene.
2. PCR with forward primer A (5'-AACCTGGTTGATCCTGCCAG- 3') and reverse primer SSU- in R1 (5'- CAACCAGATTGCCCTCCA- 3').
3. The PCR-amplified 18S rRNA gene is then exposed to DNA sequencing using methods such as Sanger sequencing or high – throughput sequencing technologies such as illumine sequencing. Sequencing generates a sequence of nucleotides that represents the genetic code of the 18S rRNA gene.
4. The obtained DNA sequence data is then analyzed using databases. The sequences are compared to reference database, such as the National Center for Biotechnology Information (NCBI) database or Ribosomal Database Project (RDP), to identify and classify the organisms present in the seaweed sample.
5. Phylogenetic analysis can also be performed to infer the evolutionary relationship between different organisms based on their 18S rRNA gene sequences.

4.5 ESTIMATION OF BIOCHEMICAL CHARACTERIZATION

protein estimation was carried out by Bradford assay, carbohydrate estimation was carried out by Androne method, energy calculation by FAO method MUFA (Mono unsaturated Fatty Acids), PUFA (Poly Unsaturated Fatty Acids) and TFA (Trans Fatty Acids) estimation by ISSO 5509:2000 method, certain minerals and elements such as arsenic, copper, iron, zinc, calcium and iodine by AOAC and IS methods.

4.5.1 ESTIMATION OF PROTEIN BY BRADFORD METHOD:

The protein value was determined using the Bradford reagent. The 50 ml Bradford reagent was prepared using a mixture of chemicals, including coomassie brilliant

blue 5 mg, orthophosphoric acid 5 μ l, ethanol 75 μ l, and 37.5 ml of distilled water. Eight samples were made in total, along with one control. They were absorbed under 590 nm and the R² value was obtained. They are tabulated in table 1.1 below.



Fig 1.2 Extract of isolated seaweed samples

Table 1.1 Estimation of protein

SAMPLES	BSA (BOVINE SERUM ALBUMIN) (in μ L)	DISTILLED WATER (in μ L)	BRADFORD REAGENT (in μ L)	ABSORBANCE AT 590 NM
S 10	10	990	5	0.677
S 20	20	980	5	0.738
S 40	40	960	5	0.896
S 80	80	920	5	1.044
S 160	160	840	5	1.322
S 320	320	680	5	1.461
S 640	640	360	5	1.612
Blank	0	1	5	0.644

4.5.2 ESTIMATION OF ENERGY BY FAO METHOD

The amount of energy was calculated using FAO method. By this method the sample was burnt, the heat given off was measured by a thermometer. The amount of heat produced were directly proportional to the energy content of the sample. The heat produced by the sample was used to calculate the energy content, typically in units of kilocalories per gram (kcal/g).

4.5.3 ESTIMATION OF NUTRIENTS BY AOAC METHOD

The minerals and elements were calculated by using this method called AOAC with different standards according to the different types of elements. First the seaweed extract sample was dried and ground to a fine powder. The sample was then weighed and placed into a digestion flask. A mixture of concentrated acids, typically nitric and perchloric acid, was added to the sample in the flask. The flask was then heated to break down the organic matter and release the nutrients. After digestion, the mixture was filtered to remove any solid residues calibration curve was prepared using standard solutions of known concentrations of the different elements being analysed. The concentrations of the different elements in the sample were determined by measuring the absorbance of the sample using a spectrophotometer and comparing it to the calibration curve. The concentrations of each element in the sample are calculated based on the absorbance readings and the calibration curve. The results were reported as the concentration of each element in the seaweed extract sample.

4.5.4 ESTIMATION OF CARBOHYDRATE BY ANDRONE METHOD

The estimation of carbohydrate value using Androne method was made and the result was obtained. The seaweed samples were mixed along with chemicals such as hydrochloric acid, sodium hydroxide and distilled water. The coloured compound was formed as a result of reaction. They were measured using spectrophotometer at 490nm and the value was recorded. The Nutritive value of seaweeds was mainly due to their protein, polysaccharide, mineral and vitamin contents. The high levels of non-digestible polysaccharide in their cell walls make seaweeds a rich source of dietary fibre (330-500 g/kg/day on a dry weight basis) (Ruperez *et al.*,2012)

4.6 ISOLATION AND SUBCULTURE OF BACTERIA USING SERIAL DILUTION AND SPREAD PLATE METHODS

Bacterial inoculum subculture involves transferring small amounts of bacteria from one culture to another for the purpose of maintaining the viability of the bacterial strain. By serial dilution method the culture tube with relevant information, such as the bacterial strain, date, and subculture number was labelled. To isolate *Lactobacillus* bacteria, three samples of curds were used. Two of those samples were procured commercially, and the third was a home-made curd. In a sterile environment all of the tubes, including the 50 ml vials, 100 ml measuring cylinder, and the media, were first placed in an autoclave for 15 minutes at 121 degrees Celsius. After autoclave, under aseptic conditions, curd was serially diluted from 10^{-1} , 10^{-2} to 10^{-3} and 10^{-4} where 1 ml of curd sample was added to 9 ml of sterile water. A 100µl of each curd sample, which was then continuously diluted inside each vial before being transferred from one to the next up to four times. The strain was spread to three petri dishes with 50 ml of nutrient media of 1.4g of nutrients and 0.75g of agar (Kale *et al.*, 2014). Using spread plate method, a small amount of bacterial sample was spread over the surface of the agar plate. Through the use of spread plate method, the strain from each curd was isolated. The culture tubes were incubated in a sterile environment. The tests carried out were Gram staining, production of catalase, Indole, Methyl Red, Voges-Proskauer and Citrate utilization. (Somnath *et al.*, 2017)

4.7 IDENTIFICATION OF BACTERIAL STRAIN USING GRAM STAINING METHOD

After incubation at room temperature for up to 24 hrs to allow the growth of *Lactobacillus* bacterial colonies. Additionally, colonies developed on each of those three petri plates. Each colony from those plates were isolated on three different glass slides without disturbing the surrounding colonies. To find the bacteria that are appropriate for fermentation, three smears were prepared for the gram staining procedure. One drop of crystal violet dye was added to the glass smears for the gram staining process, followed by one drop of Gram's iodine and a few drops of 70% ethanol for 5–6 seconds. The smear was then given one minute with one drop of safranin added to it. Each step was completed in one minute, and then the dyes should be eliminated with distilled water. The glass smear was then dried for 5

minutes and examined at 100 times magnification. In order to see the bacteria better, oil was applied to the smear.

4.8 BIOCHEMICAL ANALYSIS USING IMViC

The biochemical analysis of seaweed extract fermentum involves various materials and methods to determine the composition and properties of the extract. The materials used in this analysis typically include chemicals, reagents, and equipment necessary for measuring various parameters such as total soluble solids, protein, carbohydrate, and lipid content. Biochemical identification test was carried out using the IMViC test kit, and the results were compared and evaluated using controls. All three tests—the Indole, Methyl Red, and Voges Proskauer tests—showed the changes visibly. The test kit with the control was exposed to light for up to 24 hours. Following this, the reading from the kit was compared to the result using a control, with the almost positive results obtained.

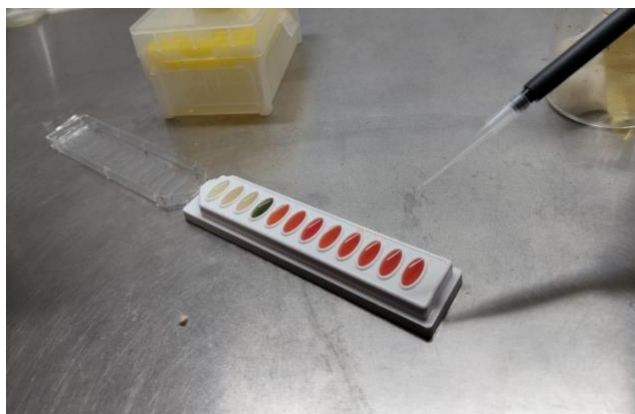


Fig 1.3 Biochemical characterisation of isolates by IMViC test

4.9 OPTIMIZATION OF MEDIA FOR FERMENTATION

The selected strain for fermentation was isolated from the curd sample was inoculated for 48 h at room temperature using fermentation media which had a combination of 2 grams of peptone, 2 grams of sodium chloride, and 1 gram of yeast extract in 200 ml of distilled water. In total of 4 vials including one control, each containing 50ml respectively and then inoculated with bacterial strain in three vials h1,h2,h3 respectively . The isolated *Gracilaria salicornia* seaweed extract measuring 725 ml on addition of 18.25 g of Luria Bertani. These mediums had different amounts of dextrose added to them based on 15 vials, including 5 controls.

Following the addition of mixture, the vials were autoclaved. Later, 2.5 ml of inoculum from h3 fermented media were added to vial 10 vials containing seaweed extract was added in sterile condition. For the fermentation process, the vials were kept at room temperature. The different compositions of media were tabulated below.

Table 1.2: Optimized fermentation medium preparation with varying concentrations of seaweed extract

DEXTROSE %	SAMPLES 1 (SEAWEED EXTRACT + DEXTROSE + INOCULUM)	SAMPLES2 (SEAWEED EXTRACT + DEXTROSE + INOCULUM)	CONTROL (SEAWEED EXTRACT + DEXTROSE)
0.1	(47.5+2.5) ml	(47.5+2.5) ml	47.5 ml
0.5	(47.5+2.5) ml	(47.5+2.5) ml	47.5 ml
1	(47.5+2.5) ml	(47.5+2.5) ml	47.5 ml
1.5	(47.5+2.5) ml	(47.5+2.5) ml	47.5 ml
3	(47.5+2.5) ml	(47.5+2.5) ml	47.5 ml

4.10 DRY WEIGHT METHOD FOR BIOMASS IDENTIFICATION

The dry weight of the biomass in seaweed extract of *Gracilaria Salicornia* was estimated during every interval of 24 hours. Five different samples of 2 ml each were used, along with five controls. Using the Whatman filter, each of them were filtered. A vacuum pump set at 80 degrees for 10 minutes was used to dry all 10 Whatman filters. Analytical balance was used to measure the pre-weight (the weight of the Whatman filter) and the after-weight (after the addition of 2 ml of medias), and the results were computed using the following formula:

$$\text{Final mass} = \frac{\text{pre weight} - \text{after weigh}}{\text{pre weight}}$$

2 m

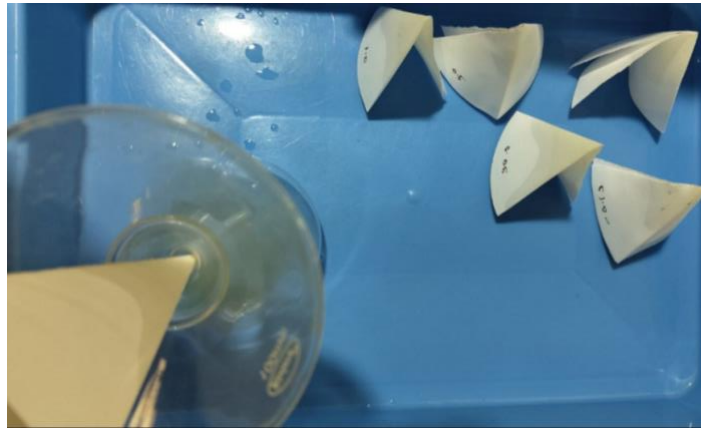


Fig 1.4 Estimation of Dry weight by filtration using whatman filter paper

CHAPTER 5

RESULTS AND DISCUSSION

Seaweeds are valuable resource for human consumption and extraction of economically important phycocolloids. Edible seaweeds are widely consumed as fresh, dried, salted or cooked food as nutrient supplements in several Asian and European nations. (Thilahgavani and Charles ,2014). In general, red and brown macro algal species are commonly used as food condiment and nutritional supplement. Recent reports reveals that *Ulva*les are consumed in China, Japan, USA , France and Chile(Pena-Rodriguez et al.,2011)

5.1 18S r RNA IDENTIFICATION OF SEAWEED SPECIES

The sequencing of the seaweed used for fermentation reveals that around 960 base pairs were showing similarity of 98 % with the seaweed collected in European waters with accession no EU 937743. The seaweed collected was identified as *Gracilaria* spp.

ORGANISM - *Gracilaria* spp.

SEQUENCE

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TATCTGTAGCATCTTCTAAAACTTATTATCGAATGCTGATTTTGCTGTTTCAC
TAATATGATACAGAAGATCAATCAAGGTGTTAATATTAGAAGAGATCAAACGT
ATTTTACAGTTAATTCTTCTATGAATAATAATTATATGTTGCAAATTTTACTGTT
TATAGACAAATTTTCGAAAGGGATTAAATTCCTTTATTATAGGAGATGAATTT
TTTAATTTAGAAAAGGCTTTTATCAATCGACAAATTTGATTAATTCACGGTTTCG
CCAATTAATAGAGAAGCTAGTTTATATGCAAAACAATTTTATCAACCATATCCT
AATACTAAGGTGATTGAATTAGGTACGAAACATTTTTTGGGTAGACTCCTAATA
ATCAAGCTGAAATAAGATATTACAATCAAATTTTAGCATCTCAGGGTTTGGCTT
ACTTTATATCATCTTTGGTTAATAGTAAAGAATATGATATAATTTTTGGTGCTAA
TACTGTACCTTACCGTCGTTTTCCAACATTGCCAGCTGCTAATTTTCCCAATAC
AGAAAAATTGTATAATAGTTTGAATAACAGAGCGTATCAATTGTTATACCTAG
TTTTAAGCCTAGTGCTTTTAATTAGTCAATTTATTTAACTTTCTTGTTTTAGTAC
TTCTTAAATACTATTTTTTCGTACTTATATTTTAAAGCTTTCGAAAATAAAGTTTT
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CAGTTCATAAGTTATAACTATTATTTAAGTAGTATTTTATTATAAATGTTATAGA
TTATAGTATTATTGGAGGAGTATTATTACCAAATCGATTGTTATGCTGATGCAG
AAGTTCCTCTTA

5.2 ANALYSIS OF NUTRITIONAL CHARACTERISTICS OF SEAWEED EXTRACT

The results obtained from the molecular characterisation of various seaweed extract were discussed below. The amount of protein content present in 100 ml of seaweed extract of *Gracilaria Salicornia* was found to be 0.31g/100ml approx. The graph obtained for the value estimation is given below. Similar result was observed using this method for the seaweed extract species. (Bradford *et al.*,1976).

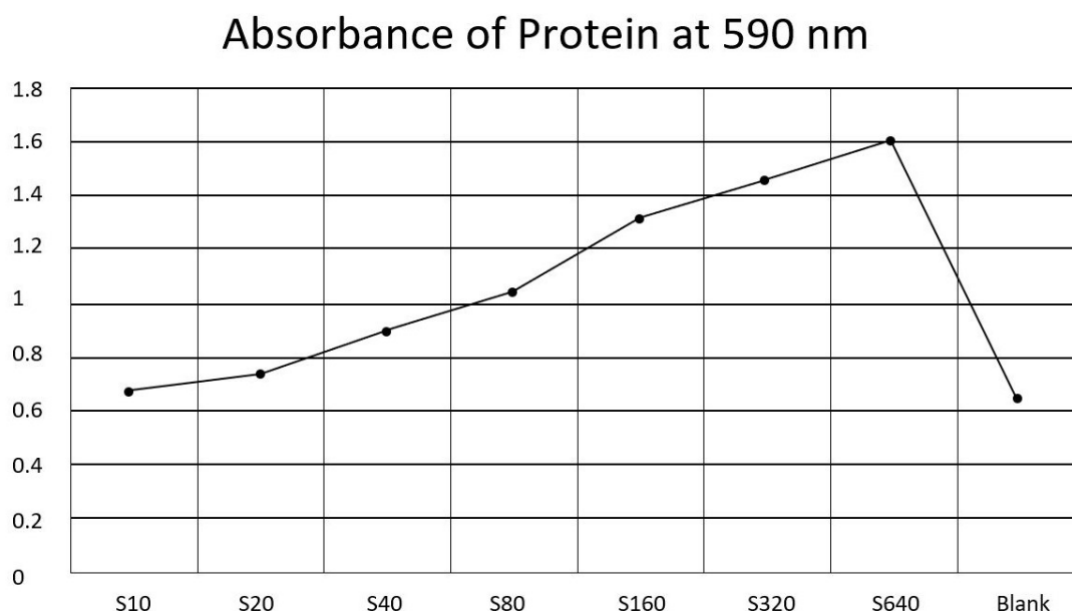


Fig 1.5 Estimation of protein by Bradford method

The carbohydrate value by Androne method was found to be 0.25g/100 ml approx of seaweed extract of *Gracilaria Salicornia* and the energy value was 2.2 kcal by FAO method, present in 100ml of seaweed extract. The values of certain elements were also obtained using AOAC standards and the results with different standards and elements were tabulated below using graph. The similar results were discussed using this method. (Androne, *et al.*,2013) (AOAC International ,2016).

Table 1.3: Physicochemical analysis of *Gracilaria salicornia*

S. No	ELEMENTS	METHOD	UNITS	RESULTS
1	Arsenic	AOAC 20th Edn,2016,986.15	mg/l	DL:0.1
2	Copper	AOAC 20th Edn,2016,999.11	mg/l	0.28
3	Zinc	AOAC 20th Edn,2016,999.11	mg/l	0.59
4	Calcium	IS 5949 :1990(RA.2003)	mg/100ml	1.5
5	Iron	AOAC 20th Edn,2016,999.11	mg/100ml	0.14

Also, the potential contribution of seaweeds to the diet were provided using a sample serving size of 8 g dry weight of seaweed for comparison's sake. (Mac Artain, *et al.*,2008).

5.3 BACTERIAL COLONIES FORMATION FROM SERIAL DILUTION AND SPREAD PLATE METHOD

After those three plates were incubated after serial dilution and spread plate method for upto 24 hrs. The colonies were formed among those plates marked h1, h2 and h3. Among them h1 and h2 were completely free from contamination. The images taken from those plates were shown below.



(d)



(e)



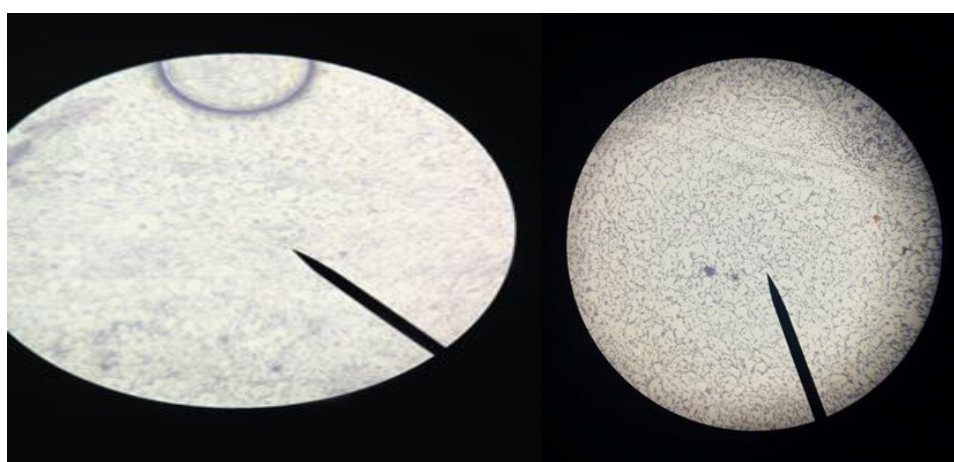
(f)

(g)

Fig 1.6 Optimisation of Inoculum with different media formulation

5.4 IDENTIFICATION OF LACTOBACILLUS STRAIN FROM GRAM STAINING

After observing h1, h2 and h3 smears under microscope at 100x, the strain of bacteria formed was observed, identified and confirmed. From three smears, the one from homemade curd showed positive, rod-shaped bacteria. It was confirmed that observed strain was *Lactobacillus* and the image was showed below. Similar method was carried out for the identification of *Lactobacillus* strain in curd. (Cappuccino, J. *et al.*, 2017).



(h)

(i)

Fig 1.7 Gram staining of bacterial isolates

5.5 RESULTS OF BIOCHEMICAL ANALYSIS BY IMVIC TEST

The test kit was compared with control kit after kept under the light for up to 24 hrs for the result values. From that it was cleared that the fermentation media containing seaweed extract showed the positive colours. This test proved that media

of extract was free from any contamination. The image compared with control was showed below. This method was used to identify gram negative bacterial strain and closely related bacteria in laboratories. (Holt, *et al.*, 1994)



(f)



(g)

Fig 1.8 Imvic test compared with sample and control

5.6 ESTIMATION OF BIOMASS

The biomass weight of final media of five samples with dextrose content and controls were obtained from dry weight method were tabulated below. Similar results were observed using this method in industrial level. (Iftikhar, T *et al.*, 2017)

Table 1.4: Estimation of dry weight

No. of samples	CONTROL at WET STAGE(g/ml)	SAMPLES at WET STAGE(g/ml)	CONTROL at DRY STAGE (g/ml)	SAMPLE S at DRY STAGE (g/ml)
0.1	1.2147	1.1770	0.0495	0.0307
0.5	1.2442	1.1793	0.0643	0.0381
1	1.2147	1.2390	0.0485	0.0617
1.5	1.2494	1.2056	0.0669	0.045

3	1.2484	1.2430	0.0664	0.0637
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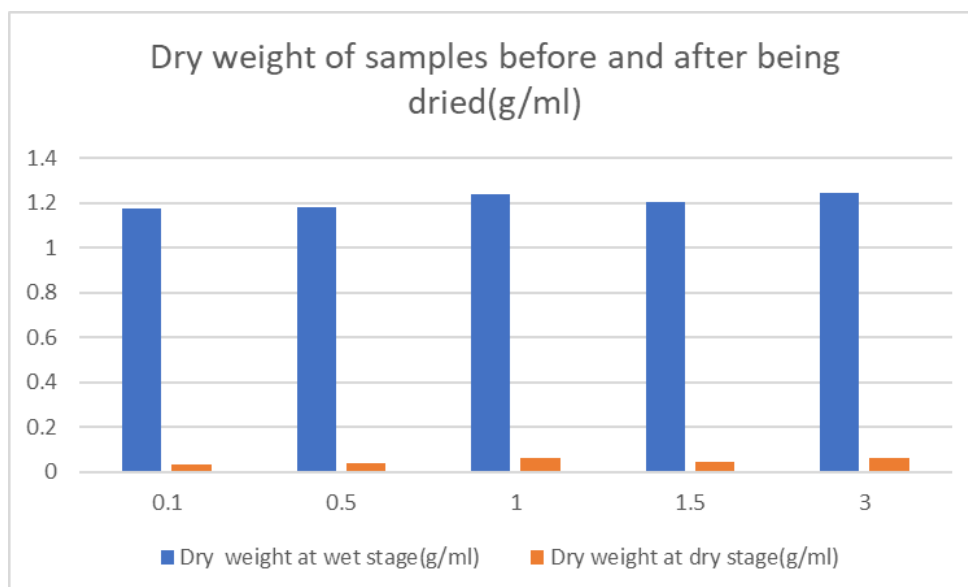


Fig 1.9: Biomass of seaweed extract (samples)

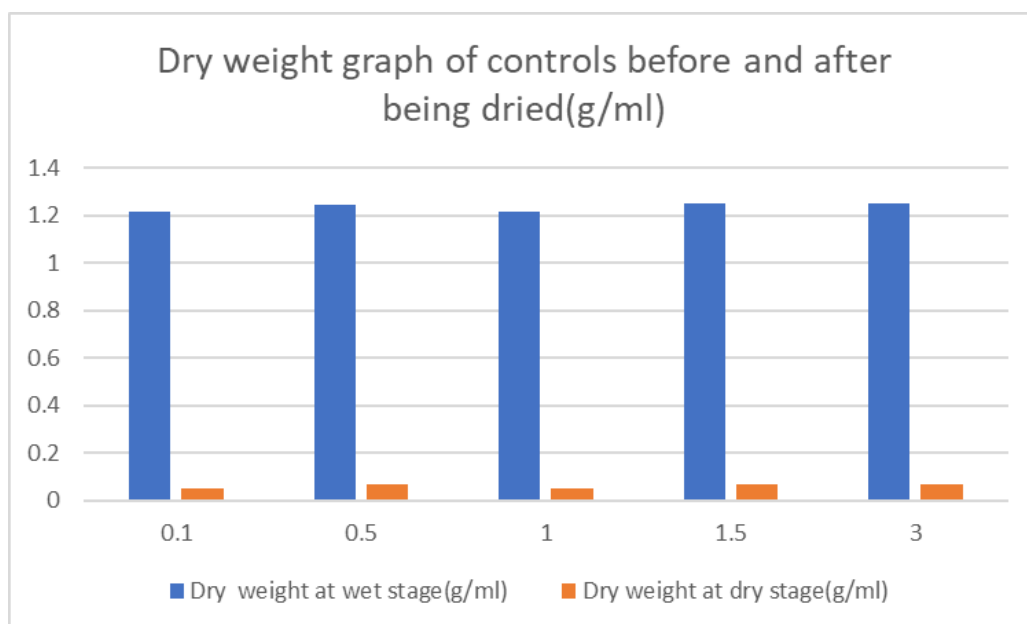


Fig 1.10 Biomass of seaweed extract (control)

5.7 ESTIMATION OF GROWTH DURING FERMENTATION WITH DIFFERENT DEXTROSE CONCENTRATION

After kept under sterile condition the vials were showed bubble formation inside them which indicated that *Lactobacillus* bacteria were growing without contamination. It implied that fermentation was taken place.

Absorbance value at 660 nm:

The OD values were calculated over a mean period of time based on a time interval of 24 hours up to 48 hours, starting from 0 to 48 hours, during the process of media fermentation. 200 μ l of each sample were divided into 96 wells on a micropipette, and the results were calculated. The results were tabulated after the reading at 660 nm was calculated.

Table 1.5: Determination of growth during fermentation at initial stage
At initial stage

DEXTROSE CONCENTRATION IN %	1	2
A 0.1	0.708	0.645
B 0.5	1.192	0.758
C 1.0	0.924	0.856
D 1.5	0.710	0.799
E 3.0	0.688	0.819



Fig 1.11 Production Media during 0hr of fermentation

Table 1.6: Determination of growth during fermentation after 24 h

DEXTROSE CONCENTRATION IN %	1	2
A 0.1	1.128	1.178
B 0.5	1.414	0.750
C 1.0	1.015	0.953
D 1.5	0.791	0.804
E 3.0	0.893	0.925



Fig 1.12 Production Media during 24 hrs of fermentation

Table 1.7: Determination of growth during fermentation after 48 h

Absorbance at 48 HRS

DEXTROSE CONCENTRATION IN %	1	2
A 0.1	0.867	0.549
B 0.5	0.751	0.654
C 1.0	0.413	0.450
D 1.5	0.404	0.537
E 3.0	1.263	0.465

CHAPTER 6

SUMMARY AND CONCLUSION

The present project focusses on collection of edible seaweeds and its major role as nutrient supplement and enhanced nutrient content through fermentation. The main objective of the project is on development of food products from seaweeds. Selected edible seaweeds such as *Kappaphycus alvarezii*, *Gracilaria Salicornia*, *Gracilaria edulis* and *Sargassum spp.* were used for product development. Among four species *Gracilaria salicornia* was selected for product development.

The aqueous extract of *Gracilaria salicornia* was used for development of fermented health product using *Lactobacillus* spp. isolated from curd samples. From the results obtained it was concluded that the seaweed extract can be consumed as a food source on a daily basis.

The fermented health drink produced from the green, red, and brown seaweed species was proven to have its own benefits and was also available for human consumption.

According to our research some of the red seaweed species of *Gracilaria* really beneficial to human beings for consumption. They also help us to lead a good dietary system. The several edible species are useful and found suitable for human consumption. It is clear that seaweed fermented health drink can be consumed as a supplement to overcome un easy health conditions and promotes to facilitate the functions of thyroid.

CHAPTER 7

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