Master's Thesis Report

Optimization of Bacterial Strains and Development of Fermented Seaweed

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ELENI MILOPOULOU

LUNDS UNIVERSITET

Examiner: Dr. Magnus Carlquist
Supervisor: Associate Prof. Ed van Niel
Assistant Supervisor: Dr. Supansa Westman

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Preface

This master thesis was performed in collaboration between the company Aventure AB and the Division of Biotechnology, LTH, Lund University in Sweden. The project spanned over 20 weeks, between January 2022 and June 2022, and served as the completion of the Master of Science in Food Technology and Nutrition program at LTH, Lund University.

I would like to thank my supervisor from Lund University Associate Prof. Ed Van Niel, who helped me with this project and provided me with supportive feedback in addition to the important discussions throughout the project. I also want to thank my examiner, Dr. Magnus Carlquist for his thoughtful recommendations and comments.

Lastly, I truly want to express my heartfelt gratitude toward my assistant supervisor Dr. Supansa Westman at Aventure AB, for her guidance and assistance with the laboratory procedures and important advice pertaining to the project.

Abstract

This study aimed to understand the exploitation of bacterial strains used in various combinations to assist in the fermentation of seaweeds. The topic stems from the need to consider the valorization of seaweeds due to their abundance in natural habitats as well as their use as an innovative and sustainable food.

Fermentation is a traditional method that has been utilized for food preservation. It offers beneficial properties in addition to constituting a sustainable solution. Seaweed is an aquatic flora that is divided into three categories; brown, red, and green. In this study, the brown seaweed *Alaria esculenta* was considered. The starter cultures contained utilizing commercially procured lactic acid bacteria in their freeze-dried forms. The main species selected for this study were *Lactobacillus* strains*;* mainly involving *L. plantarum, L. casei, L. paracasei,* and *L. rhamnosus.*

Given that glucose and mannitol form some of the main saccharides readily available in brown seaweeds, they were evaluated as substrates to be utilized for the growth of the combined lactobacilli strains. Subsequently, it was seen that all the combinations of the bacterial strains were able to use mannitol and glucose as substrates, by bringing about a pH drop in an industrially relevant timeframe. Analyzing the substrate consumption, it was found that mannitol consumption was over 50% in most of the combinatorial co-cultures. Estimating the short-chain fatty acids in mannitol samples, it was observed that lactic, propionic and butyric acid were the only significant acids detected. Consequently, the bacterial combinations were applied for the fermentation of seaweeds.

Upon analysis of the results, the optimal combination of bacterial strains was not identified due to equal amounts of positive results involving the samples. The seaweed fermentation experiments lasted 6 days although the pH decreased to 4.5 after 1 day. The optical density was closely correlated to the growth of the bacterial strains, which was also increased after 1 day in all of the co-cultures. As a long-term goal, the fermentation process is aimed to be designed to not only help improve the seaweed’s nutritional and storage properties but also to create a commercial product empowered with a consumer-friendly taste and smell.

Popular summary

Exploring synergies – Fermentation and Seaweed!

What if seaweed could become more nutritious and be available in the market for everyone that wants to try innovative food? Seaweed is an aquatic plant that grows in any marine environment. Farmed seaweeds have skyrocketed as many of their applications have been developed a lot in the last years; industrial applications, food sources, fertilizers, and chemical extractions. However, seaweed has been used for many years all around the world, mostly in Asian countries, as its health benefits have been known. It provides high nutritional value; it contains a high amino acid profile and it is rich in protein, vitamins, and minerals. It also contains sugars like glucose and mannitol that can be fermented.

Fermentation is a globally used method that utilizes raw materials, with lactic acid bacteria (LAB) contribution, in order to develop food products that can be preserved longer and offer probiotic health benefits. Although fermentation is a process that has been used in many other food products, like yogurt and sauerkraut, seaweed is a novel product its fermentation conditions are still under research. In this study, four different types of commercial freeze-dried bacterial strains and brown seaweed were mainly used.

A detailed experiment was designed to study how the fermentation process can work for seaweed. Firstly, an experiment was done to evaluate different lactic acid bacterial strains and to acknowledge if the strains could work synergistically when they are combined. Glucose and mannitol were the main carbon sources that combined cultures utilized. It was found that all of the co-cultures are able to ferment, as the bacteria consumed the available carbohydrates. Moreover, at the end of the fermentation process, lactic acid was mainly produced from lactic acid bacteria. Finally, it was found that the seaweed can be fermented in a short time and reach a pH of 4.5 after 2 days.

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Abbreviations

AAB	Acetic acid bacteria
ATP	Adenosine triphosphate
Ca	Calcium
CFU	Colony Forming Unit
DW	Dry weight
EMP	Embden-Meyerhof-Parnas
F6P	fructose-6-phosphate
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration FDA
Fe	Iron
GRAS	Generally Recognized As Safe
HPLC	High-Performance Liquid Chromatography
I	Iodine
IC	High-Performance Ion Exchange Chromatography
K	Potassium
LAB	Lactic acid bacteria
LAF	Lactic acid bacteria fermentation
M1P	mannitol-1-phosphate
M1PDH	mannitol-1-phosphate dehydrogenase
M1PP	M1P phosphatase
M2DH	dependent mannitol – 2- dehydrogenase
Mg	Magnesium
MRS	De Man, Rogosa and, Sharpe agar
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NC	Negative control
OD	Optical density
ODW	Oven Dry Weight
pH	Potential of Hydrogen

1. Theoretical background

1.1. Seaweeds

1.1.1. Definition – Seaweed and Algae

Algae can be defined as aquatic organisms that can convert sunlight and CO_2 into chemicals through photosynthetic activities. Over 200,000 different species of algae have been isolated and categorized based on their color, size and form, chemical properties, cell wall constituents, and intracellular composition. Their sizes range from micrometers to meters, indicating a clear distinction between microalgae and macro-algae. The later, also known as seaweed, are multicellular organisms that grow primarily in the aquatic environment and are alike to plants. Macroalgae do not have need of land to cultivate; their capability to grow in any type of water environment (marine, freshwater, or wastewater) eliminates antagonism with conventional food crops that require land. Regardless of the large number of macro-algal species found in nature, only a few are farmed on an industrial scale, with a global yearly production of around 22 million tons of wet mass in 2011. Although various species of algae are cultivated and collected from wild stocks, macro-algae dominate algal production, which is primarily divided between the groups of brown and red (Rajauria et al., 2015). In comparison to green and brown macroalgae, red macroalgae are the most abundant. Farmed red macroalgae account for 54.4 percent and while farmed brown macroalgae account for 33.6 percent, in comparison with farmed green macroalgae which has only a percentage of 0.08 percent (Nagarajan et al., 2022).

1.1.2. Classification of Seaweeds

Seaweed is a macrophytic algae primitive plant that lacks true roots, stems, and leaves. They are classified as Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). Edible seaweeds are high in protein and contain all essential amino acids, and are currently used as a nutraceutical supplement. Chlorophyta is rich in polysaccharides as it consists mainly of ulvan, cellulose, and starch. Some green algae species, such as Ulva and Caulerpa, contain high levels of arginine and glycine, as well as histidine and taurine, which have pharmacological activity and are required for fetal development (Ramu Ganesan et al., 2020).

Red seaweeds appear to be a potential source of food protein due to their high protein and amino acid composition (Ramu Ganesan et al., 2020). They have also different

types of polysaccharides mostly agar, carrageenan, starch, cellulose, floridean and floridoside. Red seaweed is mainly farmed to extract polysaccharides so they can be used in hydrocolloidal applications. This is the reason that the production of red seaweed is the largest one. Brown seaweed mainly contains polysaccharides like alginate, laminarin, and fucoidan. However, the carbohydrate distribution depends on the species of the brown seaweed (Nagarajan et al., 2022).

1.1.3. Health Claims of Seaweed

Seaweeds, in general, are high in fatty acids, including essential fatty acids and omega fatty acids. Furthermore, polyphenols from seaweeds can help prevent cancer by acting as an antioxidant or as a pro-oxidant on food. Over 30% of dry weight (DW) of macro-algae contains ash which includes minerals such as Fe, I, Ca, Na, and K. Thus, a desirable combination of Ca and K-rich diets are associated with lower risks of hypertension. Seaweeds also contain the appropriate proportion of potassium for blood plasma level essential during pregnancies. It has been observed that the micronutrient requirements often increased during pregnancy which play a role in the physiological function and fetal development. Furthermore, nutraceutical supplementation involving seaweeds is gaining popularity among people of all ages, particularly pregnant women, as seaweeds have been discovered to be an excellent alternative source of supplementation with no negative side effects (Ramu Ganesan et al., 2020).

1.2. Fermentation

1.2.1. Fermentation process

Fermentation is a widely used as a safe method to preserve food using minimal resources. The fermentation process breaks down the edible and inedible raw materials existing in the food components, chemically and enzymatically, following the modification via biotransformation reactions. It is also a beneficial process since it can enrich the macro and micronutrients for example fatty acids, essential amino acids, and vitamins and minerals. The nutritional value of the final product depends on the raw material and the microorganisms and it can be altered by other processes or external environmental conditions (Anis Raihana Mhd Rodzi and Kuan Lee, 2021).

1.2.2. Benefits of Fermentation

Fermentation creates products with a variety of health benefits, including boosting the bioavailability of vitamins and minerals, helping with digestion, reducing the risk of heart disease, and avoiding osteoporosis. Moreover, peptides and peptide fractions of fermented food can provide antioxidant, anticoagulant, and antihypertensive results. Fermented products can balance the composition of the intestinal microbiota due to the innate presence of microorganisms but also because of the bioactive ingredients that exist in the raw materials. The intestinal microbiota has a significant role in shaping the integrity of the intestinal barrier. As a result, it is reasonable to expect that a diet rich in fermented products will help to reduce the negative results of a western diet (dysbiosis) and contribute to preventing and treating inflammatory diseases (Kocot and Wróblewska, 2021).

The interest in fermented products arises through the metabolic activity of microorganisms and the associated biochemical changes that occur, thereby providing the preferred and specific sensory properties. Fermented foods can be produced either through indigenous microbiota activity or through the usage of a starter culture. Both of these final fermented products contain an abundance of bacterial cells but also their metabolites. They also contain bacterial strains with potential probiotic effects. The major amount of microorganisms present are belonging to the family of the lactic acid bacteria (LAB). LABs provide positive effects regarding cardiovascular, immunological, and metabolic health. The bacteria that are existent in fermented food compete with pathogens to gain a site of adhesion on the surface of the intestinal epithelium and they produce metabolites that hamper the growth of pathogens (Kocot and Wróblewska, 2021).

Owing to the bacterium-assisted partial digestion of the food, fermented foods are easily digested, consequently providing vitamins and molecules that are beneficial to the immune and nervous systems. Anti-nutrients are also reduced during the fermentation process, like phytic acid in soy products, which form salts and prevent the optimal absorption of minerals. Microorganisms also lower the amount of fermentable mono-, di-, oligo-saccharides, and polyols; as a result, fermented products can be consumed by people with functional bowel disorders. Lastly, they can be consumed during an anti-inflammatory diet as they are listed as anti-inflammatory products (Kocot and Wróblewska, 2021).

1.2.3. Fermented Foods

Yogurt

Yogurt is one of the most known fermented products that is consumed all over the world. It is a fermented milk product, fermented with the addition of two bacterial strains; *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus (Lactobacillus bulgaricus*). The fermentation process provides a high nutritional value product and with attractive organoleptic characteristics such as flavor and taste. The variants of yogurt are many, such as fruit-flavored, whipped, drinking type, etc. The classic yogurt flavor is a result of lactic acid and carbonyl compound additions as well as non-volatile and volatile acids. At the end of the fermentation, yogurt contains more lactic acid, free amino acids, and fatty acids, in comparison to lactose (Kwon, Nyakudya and Jeong, 2014).

Kombucha

Kombucha is one of the most known fermented products, a tea beverage starting in Asia but appearing in the rest of the world over recent years. It is made from green or black tea, herbs, and sugar, mixed with a microbial community of bacteria (mostly acetic acid bacteria (AAB)) and a variety of yeasts. Depending on the fermentation conditions, it can be consumed after 8-25 days. It has a sweet and sour taste, resembling apple cider, but the taste depends on carbon dioxide, sugar, and organic acid levels. During kombucha fermentation, yeast and bacteria are interacting with the forming compounds and create the final product. Invertase is an enzyme that is responsible for sucrose alternation to monosaccharides. The role of yeasts is to produce ethanol, organic acids, and CO2, while bacteria convert mainly glucose but also fructose, into acetic and gluconic acid, but also ethanol to acetic acid. The production of acetic acid contributes to ethanol production by yeast, therefore more acetic acid is produced, mostly by AAB. Contamination and spoilage of pathogenic organisms are prevented through the accumulation of ethanol and acetic acid in the liquid (Forsvall, 2021).

Sauerkraut

Sauerkraut is another product created from lactic acid fermentation, primarily started with salted white cabbage and it is traditionally from Central and Eastern Europe, the United States, and Asia. The fermentation is based on populations of lactic acid bacteria (LAB) that are indigenous to raw cabbage. Raw cabbage usually contains different

microorganisms, as well as aerobic spoilage bacteria, for example, *Pseudomonas*, *Enterobacteria*, yeasts, and molds. The amount of these aerobic microorganisms is quite higher than in the LAB populations. The dominant aerobic bacterial communities start decreasing during the fermentation, leading to the growth of LAB. Heterofermentative LAB such *as Leuconostoc mesenteroides* and *Leuconostoc fallax* take part during the early stages of the fermentation, dropping the pH by producing high amounts of lactic and acetic acid and creating an anaerobic environment by producing carbon dioxide. After the acid production and the decreasing of pH, more acid tolerant homofermentative LABs start to grow like *L. plantarum* and *L. brevis*. These bacteria are responsible for the major decrease of the pH but also for the last stage of the fermentation. The amount of acids in the final sauerkraut is 2% lactic acid and 1% acetic acid. Its flavor is depended on the composition of the microbial community as well as on the starter cabbage used and its quality (Peñas, Martinez-Villaluenga and Frias, 2017).

Fermented seaweed

Fermented seaweed can have a lot of advantages compared to raw seaweed. Firstly, the growth level and the areal productivity are higher than the terrestrial plants. Seaweed is rich in carbohydrates, and many fermentable sugars – up to 70% of its total mass. Since seaweed is mostly grown either in offshore (sea/ocean) or onshore sites (seaweed farms), they do not pose a direct competition to the food industry dominated by terrestrial products. The lignin content in seaweed is found to be really low, given that the green, red, and brown macro-algae contain 3.3 %, 1.8 %, and 7.3 % respectively. Lastly, fermentation can contribute to a being a viable step towards the valorization of waste products as seaweed blooms are one of the major economic losses for the aquaculture industries (Nagarajan et al., 2022).

1.3.Lactic acid fermentation

Lactic acid bacteria fermentation (LAF) has a huge potential to enhance the functional, sensory, and alimentary aspects of plant and animal foods. It can also contributes to valorization of food waste. Lactic acid bacteria have great diversity, including 30 genera and 300 species, however, the primary usage of LAB species and strains lead to various food functionalities (Khubber et al., 2022).

1.3.1. Lactic acid bacteria

Lactic acid bacteria (LAB) have numerous applications in food, agriculture, and medicine. The bacteria in the group are generally gram-positive, non-sporing, nonrespiring cocci or rods that produce lactic acid as the main product at the end of the carbohydrate fermentation. The principal groups can be divided into four genera: *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus.* Their significance is connected with their ability to use existing sugars to produce organic acids and other metabolites from their metabolic activity in foods. Their widespread presence in foods, combined with their long-term applications, contributes to their acceptance as GRAS (Generally Recognized as Safe) for human consumption, which is a status by the Food and Drug Administration (FDA) for human consumption. There are three main ways to develop the flavor in fermented food products. Firstly, glycolysis (sugar fermentation), secondly lipolysis (fat degradation), and lastly proteolysis (protein degradation). Lactate is the prime organic product of carbohydrate metabolism, and a portion of the intermediate pyruvate can also be converted to diacetyl, acetoin, acetaldehyde, or acetic acid (some of which can have a significant impact on yogurt flavors). Although LAB hav a minor role in lipolysis, proteolysis is the primary biochemical pathway for flavor development in fermented foods. Degradation of these components can lead to the formation of specific alcohols, acids, aldehydes, esters, and sulfur compounds in fermented food products that can play an important role in flavor development. Every fermented food product is made from defined started cultures and carefully selected to be specific and unique (Bintsis, 2018).

LAB is a significant group of probiotic bacteria. As the Food and Agriculture Organization(FAO) suggests, “probiotics are defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host”. *Lactobacillus spp., Bifidobacterium spp*., and *Propionibacterium* spp. are the most common commercial cultures used in food applications. *Lactobacillus acidophilus, L. casei, L reuteri, L. rhamnosus,* and *L.s. plantarum* are the most commonly used LAB in probiotic-containing functional foods (Bintsis, 2018).

Lactic acid bacteria (LAB) are involved in fermentation processes and they can provide either positive or negative results on the final products. LAB in non-dairy fermented products is known since the 1990s, mostly for possibilities as a probiotic vehicle. Experiments showed that isolated LAB from fermented food is responsible for the

modulation of obesity and type 2 diabetes, immunomodulation, and hypocholesterolemic activity. They also showed antioxidant and antimicrobial activity, bacteriocin production, and anti-inflammatory activity. They are bioactive compounds and are used in a broad range of applications (Anis Raihana Mhd Rodzi and Kuan Lee, 2021).

LAB use mostly carbohydrates but also lipids or proteins, phenolics, certain vitamins, and minerals, to produce metabolites that will contribute to rising the shelf life of foods. The releasing of metabolites is influenced by the LAB strain, the components that existed in the growth medium, and gene expression and enzyme activity regulation. Compounds like organic acids, peptides, volatiles, and free amino acids can come over the microbial or fungal membranes and be collected in the cytoplasm. The accumulation of these compounds and the direct competition of microbes are important methods in LAF that causes antimicrobial activity in various food systems. Several metabolites prevent free radicals to be formed, can lower reactive oxygen species and as a consequence, assist to greater antioxidant activity in fermented products. Bioactive compounds that are synthesized from LAF can be responsible for recovering antifungal, antibacterial, and antioxidant components from food (Khubber et al., 2022).

1.3.1.1. Lactobacillus plantarum

Lactobacillus plantarum is a Gram-positive, non-motile, non-spore-forming, microaerophilic, and mesophilic bacterium that grows at 10–15 to 40 degrees Celsius. It shapes straight rods with rounded ends, with a length of around 0.9–1.2 3.0–8.0 mm, occurring singly, in pairs, or short chains (Mayo and Flórez, 2020). *L. plantarum* is an adjustable lactic acid bacterium that can be found in multiple environments such as meat, dairy, and variable vegetable fermentations. It can also be found in the human gastrointestinal tract (GI tract). Furthermore, *L. plantarum* can cause food spoilage in meat, wine, or orange juice. The presence of a large number of surface-anchored proteins implies that *L. plantarum* can associate with a wide range of surfaces and potential growth substrates. Moreover, there is a high number of genes, which is a reliable indicator that this species can adapt to various conditions (de Vries et al., 2006). *L. plantarum's* remarkable ecological adaptability to such diverse environments reflects its ability to ferment a wide variety of carbohydrates, including monosaccharides,

disaccharides, and polysaccharides, from which lactic acid is produced as the major end product (Mayo and Flórez, 2020).

1.3.1.2. *Lactobacillus casei* group

The taxonomy of the *L. casei* group has been divided into three groups, consisting of *L.casei, L. paracasei,* and *L. rhamnosus*. *L. casei* is a Gram-positive bacterium that is non-motile, does not form spores, and is catalase. Cells shape rods in size of 0.7–1.1 2.0–4.0 m with square ends that can occur singly, in pairs, or chains. *L. casei* group species can be found in milk and dairy products, fermented sausages, wine, vegetables, and sourdough (Gobbetti and Minervini, 2014).

Strains of the *L. casei* group are commonly used as probiotics because they can be isolated from human reproductive and gastrointestinal (GI) tracts and stools and have resistance to low pH. *L. casei* strains are better counted in Man-Rogosa-Sharpe (MRS) agar with 2 g l^{-1} of lithium chloride and 3 g l^{-1} of sodium propionate (MRSLP), incubated at 37 °C or 42 °C. All *L. casei* species are facultative heterofermentative lactobacilli. The Embden–Meyerhof pathway converts hexoses (all strains can use glucose, galactose, and fructose) almost entirely into lactic acid. In addition to lactic acid, carbohydrate-limiting conditions produce acetic acid and ethanol, followed by butyric acid, diacetyl, and formic acid (Gobbetti and Minervini, 2014).

1.4. Aim

This project primarily aims to identify a combination of lactic acid bacterial strains having the greatest potential to bring about fermentation of seaweeds. The single substrate evaluation for commercial strains individually was carried out successfully in unpublished lab studies. The project was based on this confirmation that the strains were further subjected to the combinatorial experiments in this master's thesis.

Since seaweed is a material that is founded in abundance, it is important to find an innovative way to utilize it. The fermentation process was chosen in this project to develop a product with probiotic effects. Fermentation focused on different bacterial strains combined to identify potential synergistic effects, both regarding substrates and during seaweed fermentation. This project was divided into two parts:

- The first part was to determine the ability of the combined bacterial strains to grow using mannitol and glucose. The pH and the OD values were to monitor the fermentation process. Additionally, the mono-sugars, sugar alcohols and fermentation products were analyzed to gain a better understanding of the fermentation process.
- The second part involved the application of the already evaluated combined strains to bring about the fermentation of seaweeds. This part was specifically studied in brown seaweed and the primary goal was the evaluation of the fermentation process. The analysis procedures were similar to the first part, including total solids and ash content analysis.

2. Materials and methods

This study contains two main experiments. The main goal is to understand how lactic acid bacteria (LAB) can grow when are being used in seaweed fermentation. *Alaria esculenta* was chosen and used as the seaweed substrate. The screening of combined commercial bacterial strains was firstly taken place. The experiment was set to understand and confirm the growth of bacteria when the combined bacterial strains are used. Another part of the experiment is to optimize seaweed fermentation conditions. Before the fermentation process started, the total solid and ash of seaweed biomass were analyzed and calculated.

2.1. Materials

2.1.1. Seaweed biomass

Brown seaweed (*Alaria esculenta*) harvested in spring (2021.05.18) in Norway was obtained from the Nordic company "Seaweed solutions" which is located in Trondheim, and was used as the substrate for the second experiment. The seaweed batches were kept in the freezer at -20˚C until the time of the experiment. Frozen seaweed was weighted and ground with a Menuett meat mincer (köttkvarn) while it was still frozen to prevent the loss of targeted monosaccharide during the thawing process. Figure 2 shows the ground seaweed used in the experiment.

Figure 1: Ground seaweed

2.1.2. Chemicals

Chemicals used for the preparation of MRS medium, stock solutions, and standards were obtained from Sigma life sciences and/or Sigma Aldrich. Milli-Q water was purified with a 0.2μm filter. A modified MRS broth was prepared using peptone from casein (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), di-potassium hydrogen phosphate (2 g/L), di-Ammonium hydrogen citrate (2 g/L), sodium acetate (5 g/L) magnesium sulfate heptahydrate (0.2 g/L), manganese sulfate monohydrate (0.04 g/L), and tween 80 (1 g/L). Glucose solution (100 g/L) and mannitol solution (100 g/L) were prepared and used as added carbohydrates to the screening experiment. 0.9% NaCl solution was prepared for pre-inoculum preparation and cell cultivation. Every chemical solution used during this experiment was autoclaved at 121˚C for 20 minutes.

2.1.3. Cultures and pre-culture preparation

Five freeze-dried bacterial culture strains were provided by the Sacco Company (Sweden) and were kept at -20˚C until being used for the experiment.

The pre- inoculum was prepared by mixing a dose (0.1g) of the single freeze-dried culture with 10mL of 0.9%NaCl. Dry powder culture and 0.9% NaCl were then shaken well until the culture is completely dissolved. After shaking and resting for 10 minutes, the cultures were shaken again before being used. The single pre-culture was mixed with another in a ratio of 1:1 and it then was shaken simultaneously. After the combined cultures were mixed well, they were thereafter rested for 10 minutes and they were shaken every time before the inoculation. Each combination of cultures was shown in Table 1.

Table 1: Information on commercial LABs used in the experiment

	Commercial name	Bacterial strain	Combinations
1	Lyoflora V-3®	*L. plantarum*	
2	Lyofast BGP 1®	*L. paracasei*	Lyoflora V-3® + Lyofast BGP 1®
3	Lyofast BGP 93®	*L. casei*	Lyoflora V-3® + Lyofast BGP 93®
4	Lyofast LR B®	*L. rhamnosus*	Lyoflora V-3® + Lyofast LR B®

5	Lyofast SP1®	*L. rhamnosus*	Lyoflora V-3® + Lyofast SP1®

2.1.4. Equipment

During the 24 hours of seaweed sugar fermentation, the pH of seaweed fermented broth was measured using a pH meter. For the seaweed fermentation experiment, a real-time pH and gas production monitoring with the Bioprocess Control - Gas Endeavour® software was employed. Optical Density (OD) at 620nm was measured using a lab bench spectrophotometer. The High-Performance Ion Exchange Chromatography (IC) with Pulsed Amperometric Detection based on NREL/TP – 5100-60957 (HPAC-PAD) was used to quantify the number of mono-sugars in the samples. The High-Performance Liquid Chromatography (HPLC) method was used to detect short-chain fatty acids in the samples, using a Bio-Rad Aminex 87-HPX column, and 0.5mM sulfuric acid as eluent was used to analyze the single substrate samples. All the samples were centrifuged before IC and HPLC analysis (Allahgholi et al., 2020). A Menuett meat mincer (köttkvarn) was used to grind the seaweed in a small size. All the necessary equipment for the experiment (fermenters, bottles), as well as the stock solutions and distilled water, were autoclaved at 121˚C for 20 minutes to prevent contamination.

2.2. Methods

2.2.1. Single substrate fermentation

In this experiment, a sterile MRS medium was cooled down at room temperature and was kept in a refrigerator (4 ˚C) till being used for the experiment. 80 mL of modified MRS media was added to 120 mL of serum glass bottles and they were thereafter autoclaved at 121˚C for 20 minutes. 1% (v/v) of the pre-inoculum (4.1.3) was inoculated in sterile MRS media and was incubated at 37℃ for 24 hours. The experiment was performed in triplicated.

During 24 hours of the fermentation process, 2mL of the sample was collected at 0, 3, 5, 7, 8, 9, 10, 11, 12, and 24 hours. Sample at time 0 hour was taken when the bacteria was firstly inoculated to the fermenter, as can be seen in Figure 3. The fermentation process ended after 24 hours, and the last sample was taken at 24 hours. The pH and

OD were measured immediately at every sampling time. The rest of the samples were kept in the freezer (-10 ˚C) and used for IC and HPLC analysis later.

(A) (B)

Figure 2: (A) samples with mannitol, glucose, and no added carbohydrate at the beginning of the fermentation and (B) samples with mannitol, glucose, and without carbohydrates at the end of fermentation

2.2.2. Seaweed fermentation

Seaweed fermentation experiments were carried out using the Gas Endeavor-Bioprocess control system, shown in Figure 4. 150 g of frozen minced seaweed, *Alaria esculenta*, and 250 mL sterile water were added to the 500mL fermenter. 0.1% of the pre-culture was prepared according to section 3.1.3. was inoculated to the fermenter under aseptic conditions. The temperature of the process was controlled at 37°C using a water bath. Before sampling, the sample inside the fermenter was stirred at 40 rpm for 3-5 minutes to ensure homogeneous and uniform mixing. 5 ml of the sample was collected at 0, 12, 24, 36, 48, 72, and 144 hours. Sample 0 was taken when the pre-combined inoculums were inoculated in the fermenters containing seaweed and water. The fermenters were kept for 6 days when the last sample was taken (144 hours). pH was measured every 15 minutes by the real-time pH monitoring electrodes equipped with the system. OD was checked immediately after sampling.

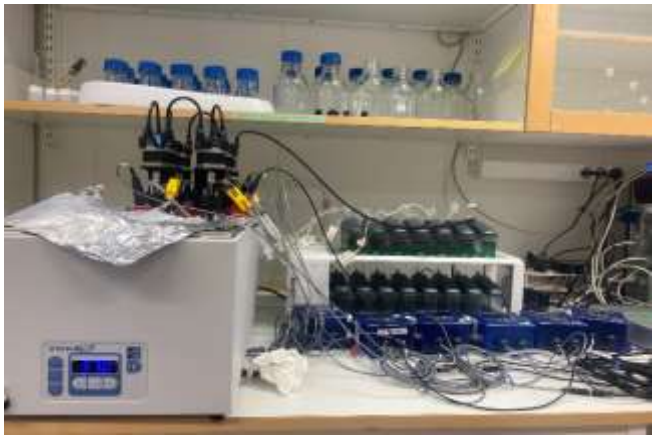


Figure 3: Seaweed fermentation setup. From left to right: Reactors filled with seaweed and waters, equipped with stirring and real-time pH monitoring electrodes. pH display and gas measurement equipment.

2.3. Analysis

2.3.1. pH

The pH was measured to analyze how fast the bacteria produce acids, and therefore the decrease of the pH. It was measured in two different ways. During the screening of combined bacterial strains, a pH meter was used. In the seaweed fermentation experiment, the pH was constantly measured by the Bioprocess Control - Gas Endeavour® software, in which pH monitoring electrodes were included.

2.3.2. OD

OD analysis played an important role in both experiments. It was measured to estimate the growth and the metabolic activity of the bacterial cells. After sampling, every sample was diluted 10 or 20 times with 0.9% NaCl, depending on the time of the sampling. The sample was filtered into cubes and the OD was measured.

2.3.3. Analysis of mono sugars (IC)

The IC method was used to quantify the concentrations of the mono-sugars in the samples. The samples were centrifuged at 13,000 rpm for 10 minutes to remove all the cells from the solution. The clear supernatant was used to prepare the IC sample. Prior to analysis, the samples from serum bottle experiments were diluted 500 times. Then, they were filtered using a 0.2 m filter.

2.3.4. Analysis of Short Chain Fatty Acids (SCFA)

The HPLC method was used to detect single chain fatty acids in the samples. The flow rate was set to 0.5mL/min, and the temperature in the column compartment was kept at 40°C. The Aminex HPX-87H column was used to analyze the bacterial strains' combinations. 5.0M of sulfuric acid was used as eluent with a flow rate of 0.5mL/min and the column compartment temperature was kept at 50°C. The samples from the serum bottle fermentation were diluted 20 times with milli-Q water. The diluted solution (which has a final volume equal to 1mL) was treated with 20μL of 20% (v/v) sulfuric acid and let for at least 30 minutes in a fridge, at 4°C. After the sulfuric acid addition, the samples were centrifuged for 2 minutes at 13,000 rpm to remove any precipitate formation. Lastly, they were then filtered through 0.2 m filters into HPLC vials.

2.3.5. Determination of total solids

To determine the number of total solids that exist in seaweed, a specific method was followed, based on NREL/TP – 5100-60956 (Van Wychen and Laurens, 2015). The procedure started with placing the crucibles in the muffle furnace at 575 °C overnight and after cooling to room temperature, their weight was recorded. Seaweed around 100 ± 5mg was weighted in the crucibles and placed into the oven at around 105°C for 18hours. The samples were allowed to cool at room temperature in a desiccator and the final weight was recorded.

$$\%Total\ solid = 100x\frac{Weight\ crucible + dry\ sample - Wegiht\ empty\ crucible)}{Weight\ of\ algal\ sample\ as\ received}$$

$$\%Moisture = 100 - \%Total\ solids$$

2.3.6. Determination of ash content

The determination of ash content was performed according to the NREL/TP – 5100-60956 (Van Wychen and Laurens, 2015) method. The samples were heated according to a ramp program, in which the starting temperature was 105 ℃ up to 575 ℃ for 180 minutes. The ash content can be calculated according to the following formulas:

$$ODW\ sample = \frac{(Weight\ air\ dried\ sample\ \times \%Total\ solids}{100}$$

$$\%Ash = \ 100 \times \frac{Weight\ crucible + ash - Weight\ crucible}{ODW\ sample}$$

3. Results

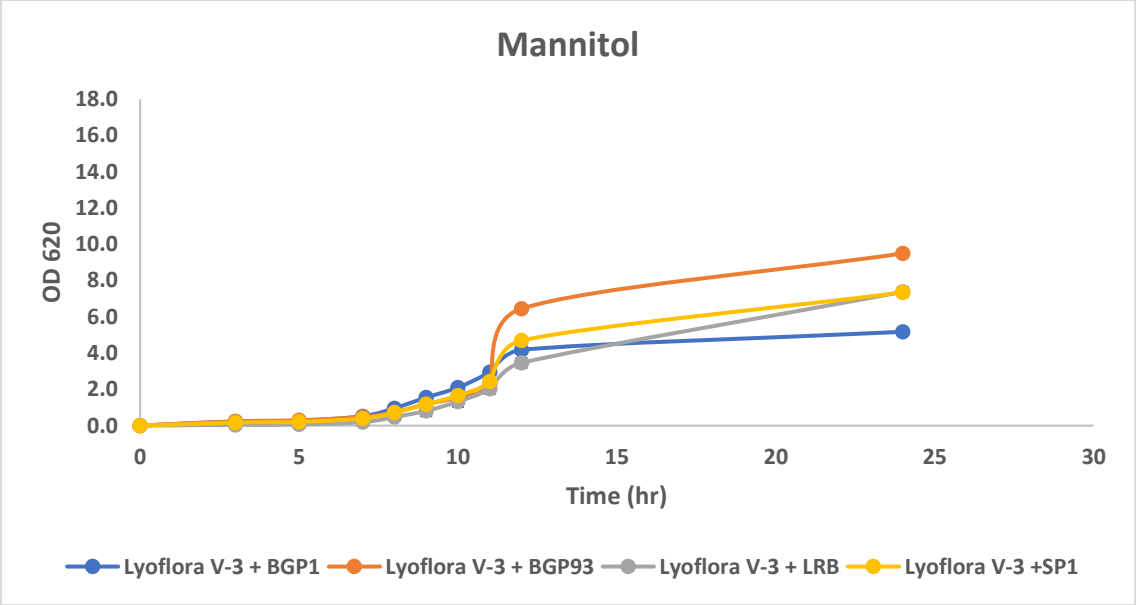
3.1. Screening of combination of commercial bacterial strains

The experiments were carried out using 120 mL serum glass bottles closed with rubber stopper and aluminum caps, with the addition of combined bacterial cultures (0.1 % v/v) in MRS media containing either 20 g/L of mannitol, or 20 g/L glucose, and water as control. This process was monitored by measuring OD and pH at the beginning (time 0hr.), during, and at the end of the fermentation process (time 24hr.).

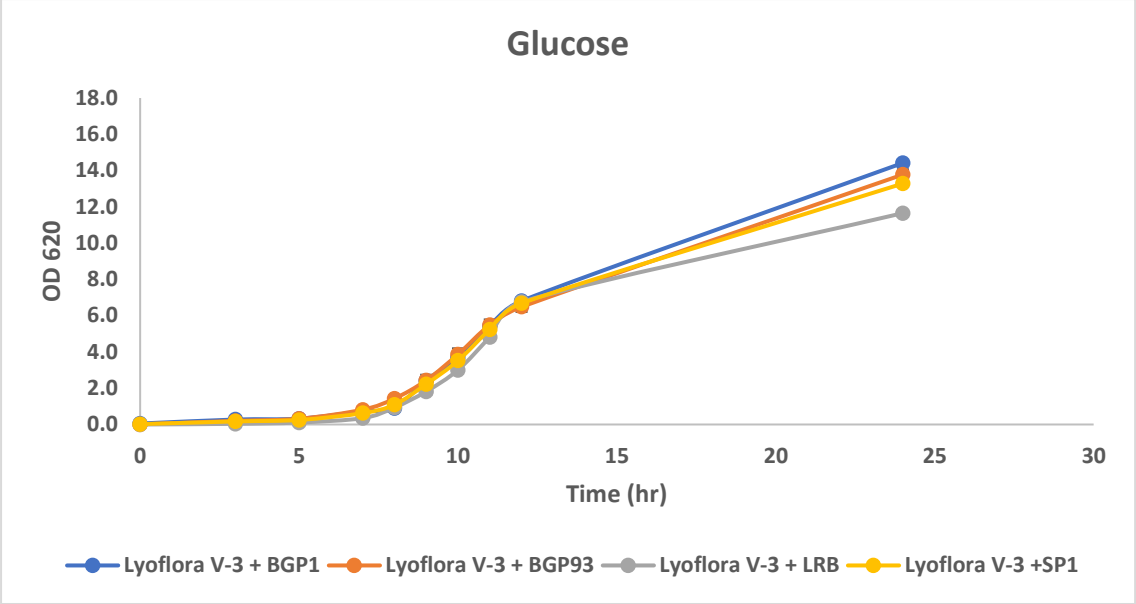
3.1.1. Cell growth

During the fermentation process of 24 hours, the OD of the samples was measured, the results are shown below. Figure 4 shows the OD graphs concerning the bacterial growth in three different media; added glucose, added mannitol, and no added carbohydrates. Negative control (NC), without any carbon source, was studied in the experiment to check if the bacteria can grow solely in MRS media i.e., without any carbohydrate.

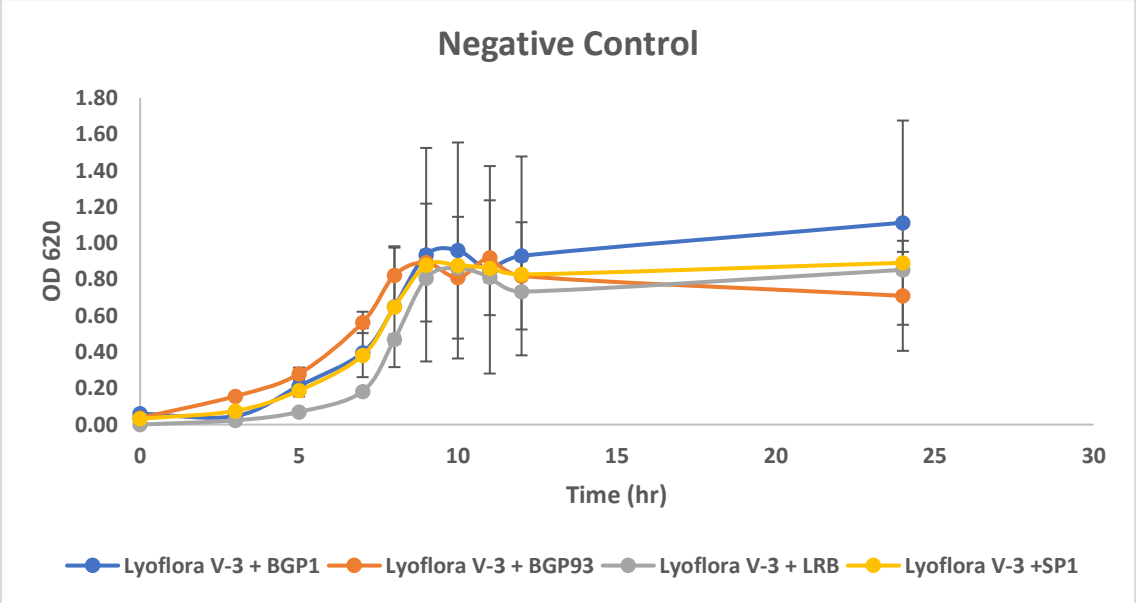
It can be seen that all of the co-cultures were able to grow both in mannitol and glucose. When glucose is used as a substrate, a maximum OD value was measured at 16.13±1.03 while in mannitol as substrate was 9.49±0.33. Negative control shows limited growth of the bacteria. The duration of the lag phase in all the co-cultures in mannitol and glucose is relatively similar (between 7 to 8 hours), but the exponential phase in glucose is accelerated thereby indicating greater growth, as higher OD is obtained.



(A)



(B)



(C)

Figure 4: Triplicates of cultivation of combinations of bacterial species in a) mannitol; b) glucose; c) negative control.

To have a better understanding, Figure 5 shows the bacterial growth in every combination separately, compared with the positive control (added glucose) and negative control (no added carbohydrates). It can be seen that there is a lower growth yield on mannitol compared to glucose as a substrate. All of the co-cultures have higher growth in glucose than in mannitol.

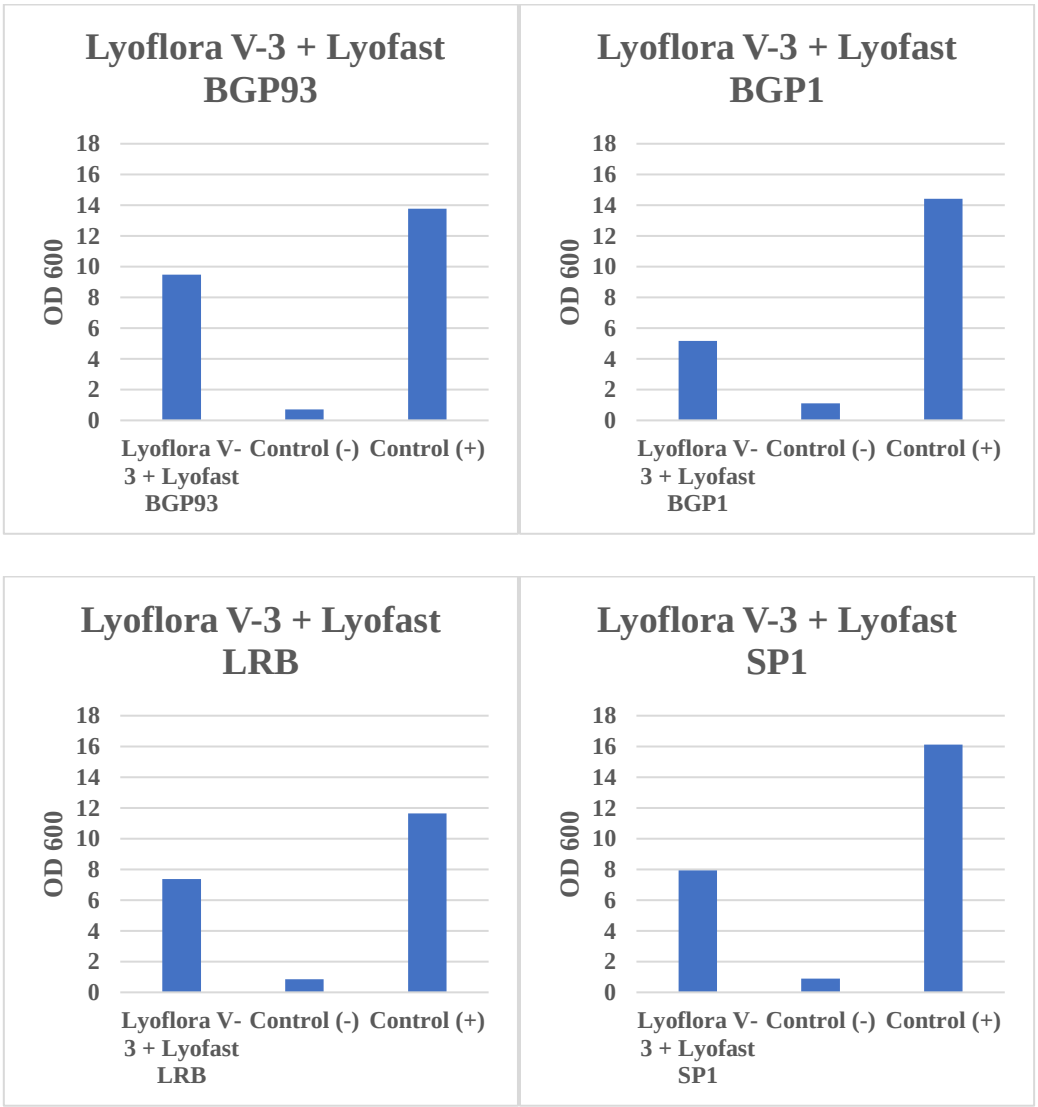
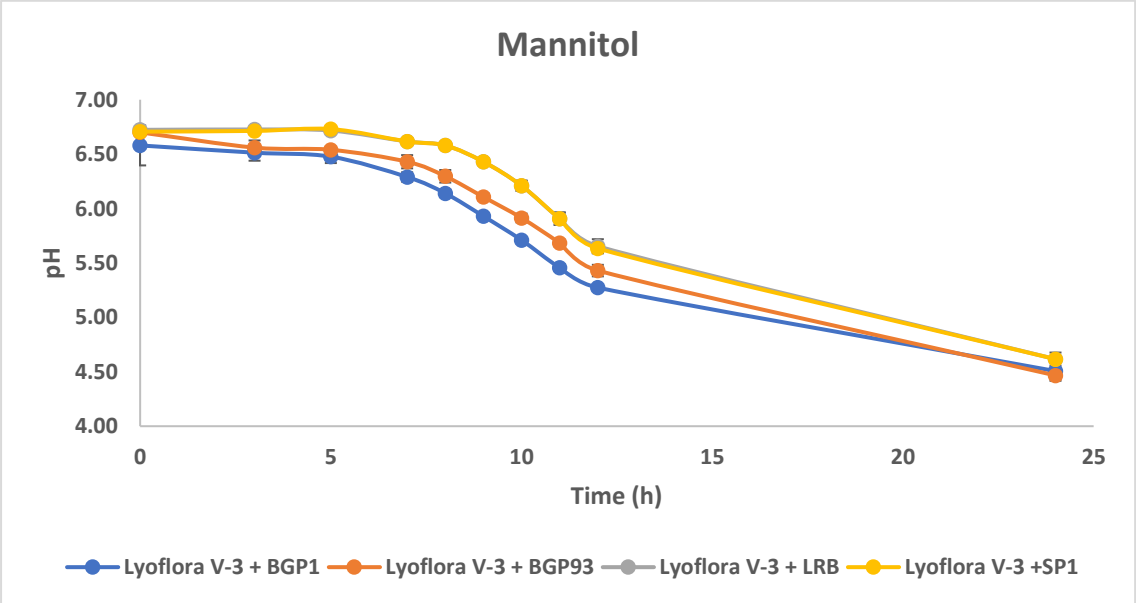


Figure 5: Growth of different co-cultures if commercial LABs on MRS media containing mannitol, compared to control (-) and glucose (control (+))

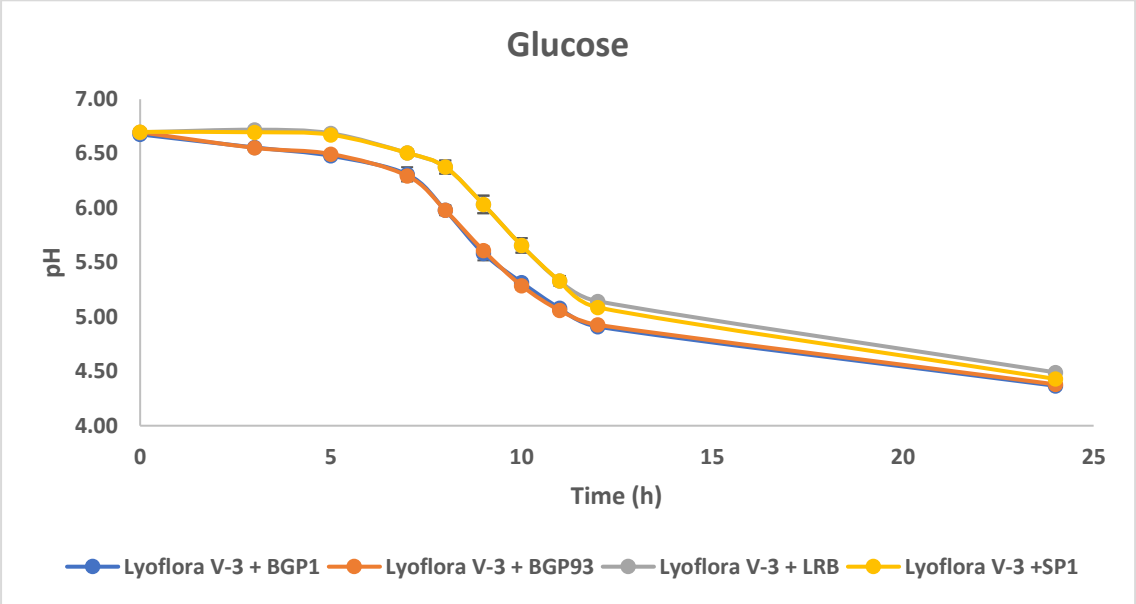
3.1.2. pH value during mannitol or glucose fermentation

The pH was measured during the fermentation experiment and the results are presented below. Figure 6 shows the pH decrease of the four different combined bacterial strains, in three different media: mannitol, glucose, and without carbohydrates. In both substrates, the pH drops to 4.5 after 24 hours due to acid production. Table 2 summarizes the final pH at the end of the fermentation by different commercial combined bacterial strains.

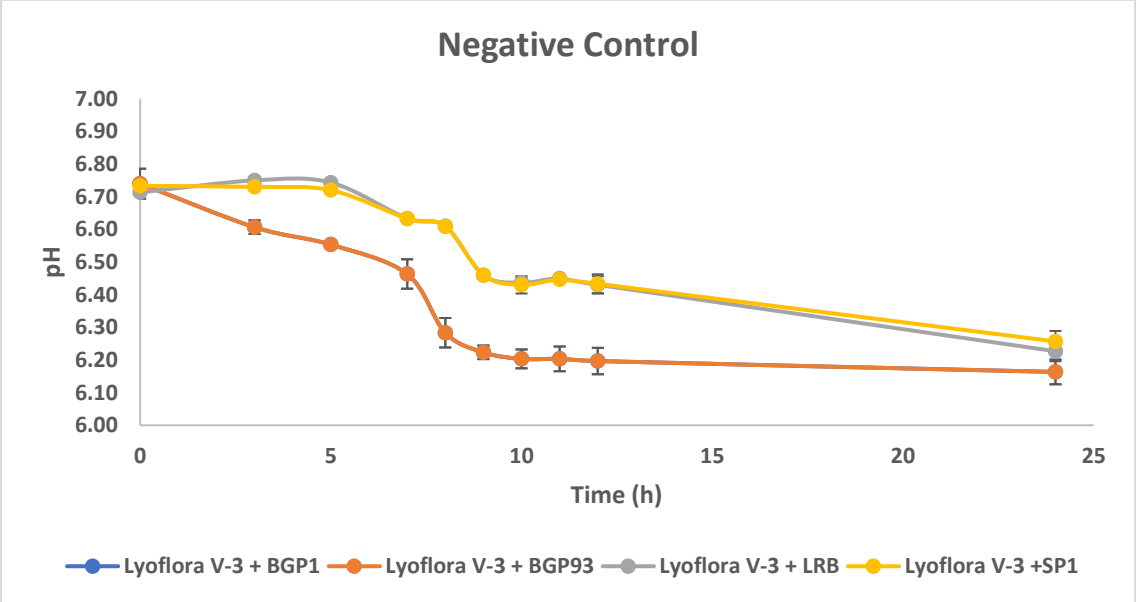
The initial pH was between 6.58-6.73 ± 0.05. In the first few hours, the pH did not decrease, however after 7 hours, pH reduced in all combined cultures; both in glucose and mannitol samples. It is worth mentioning that in the samples with glucose as substrate, the pH decreases faster where at 12 hours, the pH varied between 4.90-5.10, in comparison to when mannitol was used as a substrate where the pH was between 5.27-5.65 at the same time point. The pH of the negative showed a slight drop, with the lowest pH being 6.16± 0.05 when the initial pH for all the samples was 6.71± 0.05. A possible reason is that MRS contains yeast extract, which could have affected some growth.



(A)



(B)



(C)

Figure 6: pH graph with combined bacterial strains during 24 hours in a) mannitol; b) glucose; c) negative control

Table 2: pH profile at the end of the fermentation process by different combinations of bacterial cultures.

N/n	Bacterial sets	pH at the end of fermentation		
		Mannitol	**Glucose (positive control)**	**No added carbohydrate**
1	Lyoflora V-3® + Lyofast BGP1®	4.51±0.01	4.37±0.01	6.17±0.04
2	Lyoflora V-3® + Lyofast BGP93®	4.47±0.05	4.38±0.01	6.25±0.03
3	Lyoflora V-3® + Lyofast LRB®	4.62±0.03	4.49±0.01	6.23±0.03
4	Lyoflora V-3® + Lyofast SP1®	4.62±0.06	4.43±0.03	6.26±0.03

3.1.3. Mannitol Consumption Analysis

After 24 hours, the mannitol consumption of the bacteria using mannitol as the main monosaccharide was analyzed with IC. Figure 8 shows the concentration of mannitol of all the co-cultures, at time 0 and after 24 hours of fermentation. All of the co-cultures consumed similar amounts of mannitol.

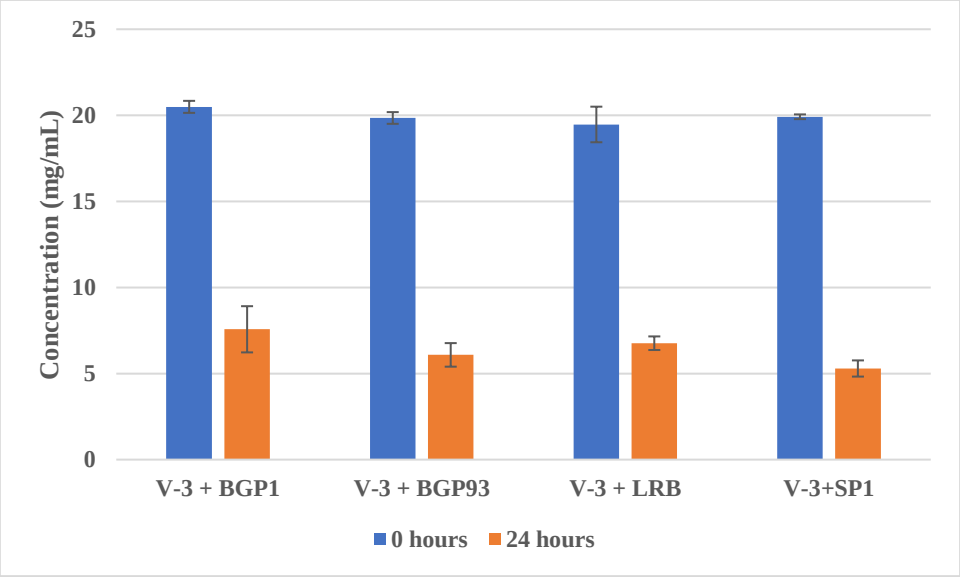


Figure 7: Mannitol concentration during 24 hours in the different co-cultures

3.1.4. Short Chain Fatty Acids (SCFA)

To identify and quantify the amount of the produced single chain fatty acids, the HPLC method was used. Cultures, with mannitol used as a substrate, were measured at four different times; time 0, 7, 12, and after 24 hours of fermentation. In Figure 9, it can be seen that lactic acid production starts after 7 hours and at the end of the fermentation is the acid with the highest concentration for all the co-cultures.

After 7 hours, when the pH was first seen to decrease, the production of lactic acid was observed. After 12 hours the level of lactic acid increased along with the levels of propionic acid. A decrease in acetic acid is observed however, it was not significant. In the last sample after 24 hours, butyric acid production is seen, along with a rapid increase in lactic acid production and decline in acetic acid.

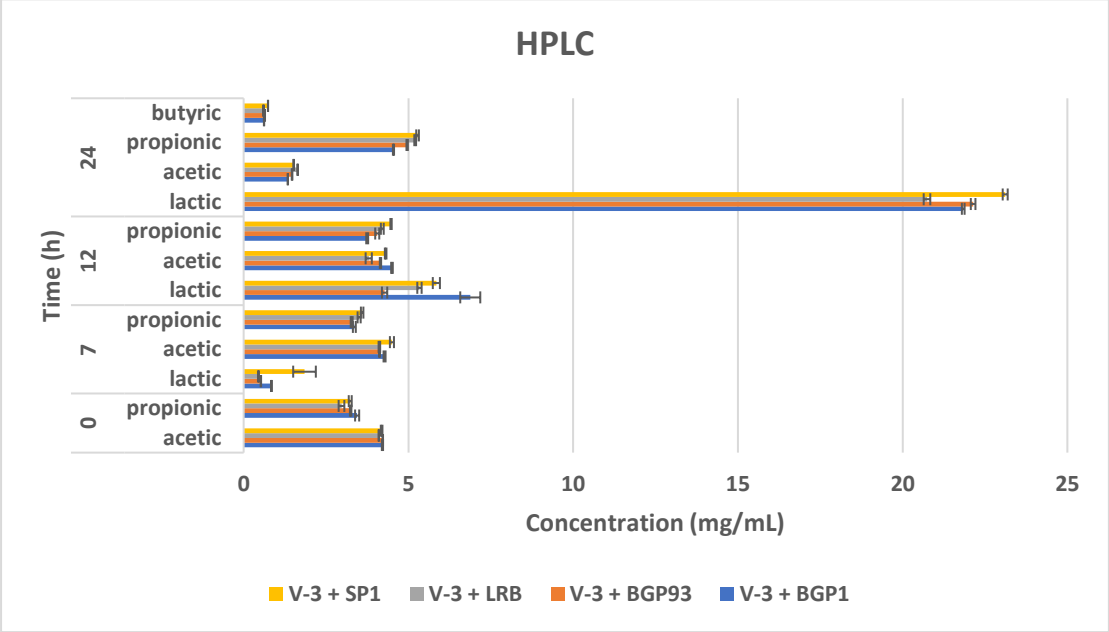


Figure 8: HPLC analysis: the amount of short-chain fatty acids production in cultures with mannitol as substrate.

3.2. Seaweed fermentation experiment

3.2.1. Growth results

To study the effect of bacterial growth during the seaweed fermentation experiment, the OD was measured in all co-cultures. The OD was measured during 144 hours of fermentation (Figure 9). All of the co-cultures, including positive and negative control, showed an increase. The variations in the results might be from algal particles after the filtration. When samples were taken, it was challenging to receive the same seaweed – mixed water ratio, although, before sampling, the samples were stirred for the same amount of time. All the samples were filtered but in some of them, more of the suspended algal particles could be present, giving a higher OD result. Also, the filter that was used to keep the seaweed might be too small, so bacterial cells were trapped in the filter, giving a not-so-accurate result. Therefore, bacterial growth can be detected but using a filter that allows the bacterial cells to flow.

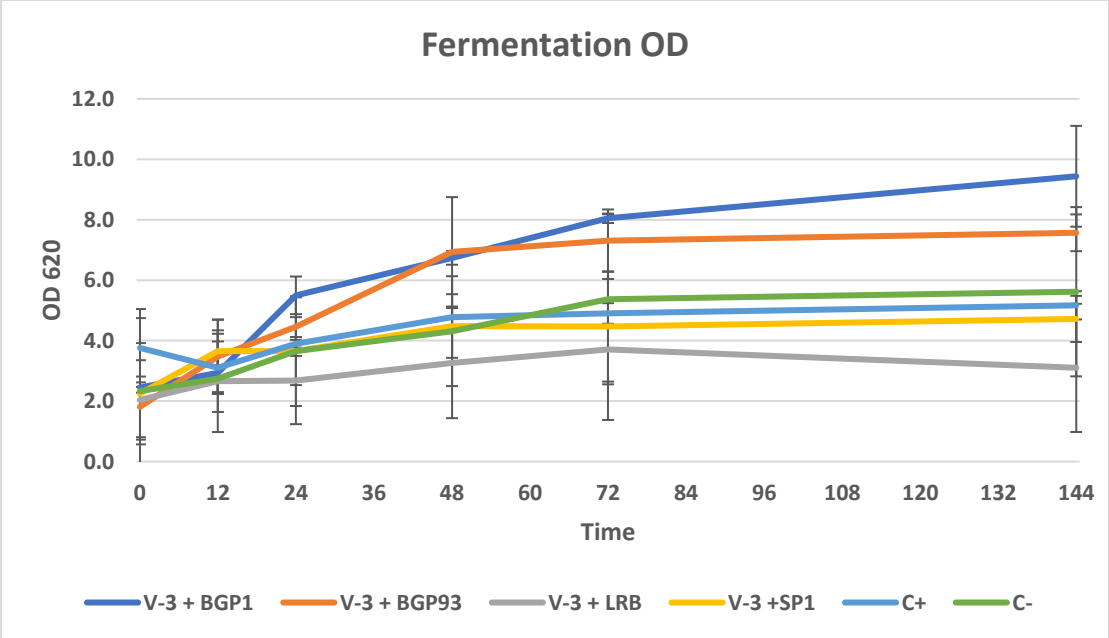


Figure 9: Growth curve during 144 hours of seaweed fermentations

3.2.2. Results of pH

The pH was measured during the seaweed fermentation experiment with pH electrodes that were monitoring it in real-time. Figure 10 shows the pH value measured during 6

days of fermentation. Four of the graphs are the combined bacterial strains, one is the positive control which in this case is Lyoflora V-3, and the negative control (NC)which is seaweed with water and no added bacterial culture. All the combinations of bacterial strains showed a drop in pH to 4.5 after 24 hours. However, the NC dropped after 72 hours due to seaweed-inhabited microorganisms. These microorganisms were not identified in this experiment.

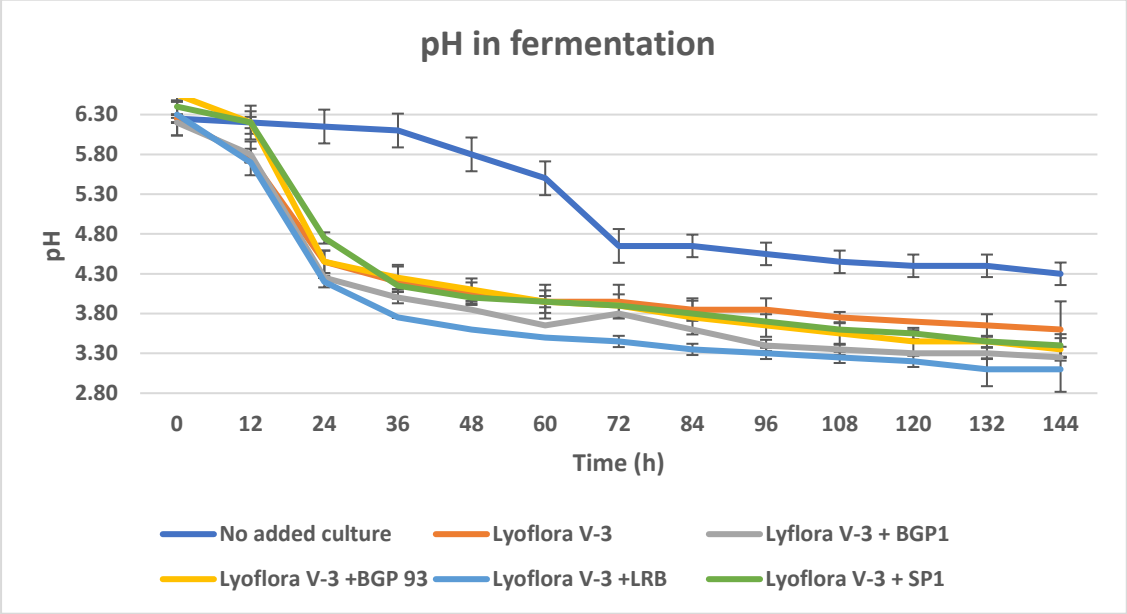


Figure 10: pH drop during 144 hours of seaweed fermentations

3.2.3. Total solids and ash Content of seaweed biomass

Table 3 shows the total solids and ash content estimation results from fermentation experiments performed using the NREL/TP – 5100-60957 protocol (Van Wychen and Laurens, 2015). The samples were freeze-dried before the analysis.

Table 3: Calculated percentage of Total solids, Moisture, Oven Dry Weight (ODW), and Ash.

Freeze-dried sample	
% Total solids	96.85±0.6
% Moisture	3.15±0.6
% ODW sample	7.04±1.44
% Ash	0.51±0.11

4. Discussion

The results were convincing that combined bacterial strains can lead to a successful fermentation process. However, the results were inconclusive regarding the different combinations of the bacterial strains because no significant differences are noticed concerning which combination could be ideal. Therefore, the seaweed fermentation experiment was followed with the same combinations of bacterial strains. This experiment lasted 6 days (144 hours) to analyze and study the trend of bacterial growth. The main goal was to terminate the fermentation process when the pH reaches 4.5. It is a critical point for the bacteria to stop growing to maintain the final product safety. After 24 hours, the recorded pH dropped to the critical point, in the range of 4.20-4.75. The positive control showed higher pH compared to combined V-3 with LRB after 24 hours. Thus, the combination of bacterial strains can contribute to a lower pH in a shorter time.

4.1. Effect of co-cultures fermentations utilizing single substrates

The growth curve indicates the OD values measured for the four different LAB strain co-cultures. It can be noticed (Figure 4) a clear increase in OD for all four combinations, which is indicative of bacterial growth when mannitol is present. An interesting result that showed the highest difference in OD when using mannitol and glucose individually was the combination of Lyoflora V-3 and BGP1. The other three co-cultures had a smaller difference, yet a higher OD was recorded with glucose in comparison to mannitol. This numerical variance shows that the metabolism is adapted to the existed sugar. Glucose has more capabilities compared to mannitol due to Embden-Meyerhof-Parnas (EMP) pathway.

However, mannitol can also be present in the glycolytic pathway, but first, a mannitol metabolism cycle takes place (Figure 11). Mannitol converts to mannitol-1-phosphate and then to fructose-6-phosphate (F6P). This can happen with the assistance of NADH mannitol-1-phosphate dehydrogenase (M1PDH). To maintain the osmotic pressure, M1P phosphatase (M1PP) dephosphorylates M1P to mannitol. During this reaction,

NAD-dependent mannitol–2-dehydrogenase (M2DH) is able to transform mannitol into fructose and then convert it to F6P for energy metabolism (Nguyen et al., 2019).

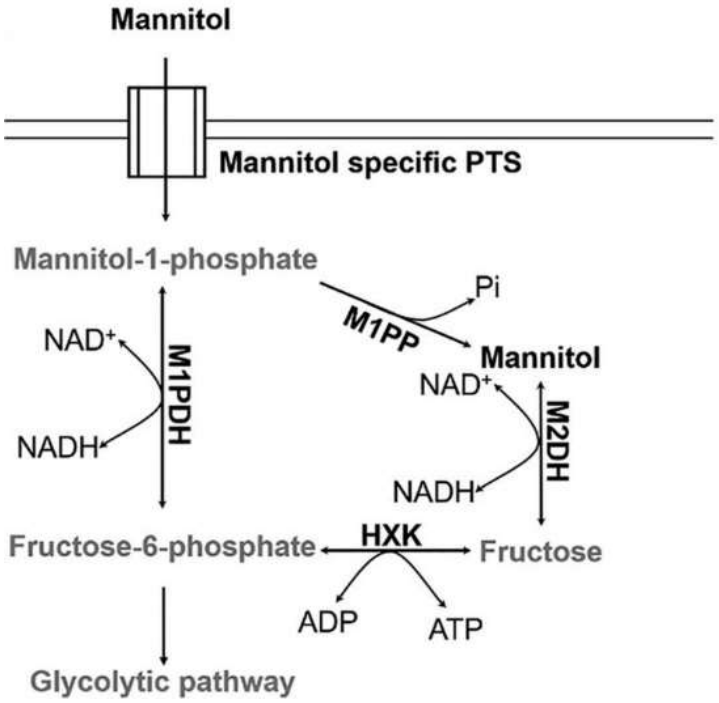


Figure 11: Mannitol metabolic cycle (Nguyen et al., 2019).

Mannitol was used as the target carbon source as it is the largest storage polysaccharide in brown seaweed. Out of the 40.7% of total carbohydrates present in *A. esculenta*, mannitol accounts for 10%. Alginate is the prominent carbohydrate, with 19.7% of total carbohydrates (Stévant et al. 2017). LAB are able to produce ATP during fermentation through the glycolytic pathway EMP, and metabolize 90% of the existing sugars into lactic acid (Papagianni, 2020). As expected (Figure 4), the increase in OD value with time for NC was low, but not zero. This is because the bacteria were inactivated when frozen, but they started activating after being at 37°C for 24 hours.

The declining pH was recorded during the experiments after every sampling. As it is important with LAB to specify the time at which the pH showed a drop to 4.5 because this is the industrially recognized setpoint for indicating the growth of the bacteria and consequently preventing the growth of other microorganisms. Of course, the growth of LAB will decline as the optimum pH of most lactobacilli is around 6, as for instance seen with *L. plantarum* is pH 6.0 (Vera-Peña and Rodriguez-Rodriguez 2020).

The four different co-cultures have similar growth characteristics on mannitol, glucose, or without substrate on (Figure 6). Hence, all the selected combinations are as effective in their fermentation capacity. Lyoflora V-3, which is *L. plantarum*, has been included in all the combinations. The other bacterial strains, *L casei*, *L. paracasei, and L. rhamnosus*, apparently do not have a significant impact on the time at which the pH reduces. *L. plantarum* can provide multiple advantages in seaweed fermentation. *L. plantarum* is used to reduce the fermentation time, develop the production effectiveness, and contribute probiotic effects to the final product (Zhang et al. 2022).

The cultures on mannitol consumed in total up to 75% of this substrate (Figure 7). The highest mannitol consumption occurred with V-3 combined with SP1 bacterial strain. Thus, this proves the utilization of mannitol as a substrate by LAB. However, it would be interesting to evaluate the glucose samples, to compare the consumption trend between the two substrates.

Of the fermentation products detected in the mannitol fermentation, lactic acid and butyric acid are the main acids produced. As expected, after 24 hours of culture, it was found that lactic acid was the highest produced acid during fermentation. Before the start of the fermentation, acetic acid was found in all of the samples. Since everything was autoclaved, the MRS medium is the only possible reason that acetic acid was presented. After 7 hours, when the pH was first seen to decrease, production of lactic acid was observed. After 12 hours the level of lactic acid increased along with the levels of propionic acid. A decrease in acetic acid was observed however, it was not significant. In the last sample after 24 hours, butyric acid production is seen, along with the rapid increase in lactic acid production and decline in acetic acid.

4.2. Seaweed fermentation

The positive control was studied to analyze the Lyoflora V-3 bacterial behavior in comparison with combined bacterial strains. *L. plantarum* can grow in the fermented seaweed but the purpose of the combined bacterial strains was to study if *L. plantarum* combined with other LAB can have a faster and/or better result. Fermented brown seaweed with *Lactobacillus* sp. showed a rise in antioxidant activity and phenolic

compounds. Specifically, L. plantarum has been tested in different seaweeds, giving high antioxidant results (Reboleira et al. 2021).

The pH of V-3 strain decreased at a similar pace as the rest of the combined bacterial strains until 96 hours. After this time, strains combined with V-3 showed a higher decrease in pH. Combinations with SP1, BGP93, and BGP1 showed similar results during the whole time of the experiment while remaining lower than the positive control trend. As expected, V-3 or positive control sample is the dominant culture but the combinations have also an impact on decreasing the pH.

Caper berries fermentation showed that L. plantarum was the most predominant species (Palomino et al. 2015). It can be assumed that the bacterial strains have a synergistic effect when they are combined in co-cultures. During sourdough fermentation, combined *L. plantarum* and *L. casei* can have a good synergy, although *L. plantarum* had a higher growth dynamic compared to *L. casei* (Paucean et al. 2013). Other studies showed that mixed cultures can provide a better result in fermentation. However, the combination between *L. plantarum* and *L. rhamnosus* did not show any significant difference compared to only *L. plantarum*. The bacteria grow faster and the pH decreases more, compared to the strains used individually (Yan et al. 2019).

NC was used to identify if the seaweed contained indigenous microorganisms with fermentation properties. This was possible, because the seaweed used was fresh, and was not subjected to being autoclaved prior to the experiment. Without inoculation, the pH value was stable and it started to drop at 48 hours of incubation (Figure 10). After two days, the decrease in pH value was found to be drastic whereas, at 72 hours, the pH value dropped to 4.60 indicating that indeed seaweed inhabited microorganisms. These microorganisms were not identified in this experiment.

Analysis of total solids and moisture content is critical in the food industry and innovation. Total solids are "the dry matter left after moisture removal." Seaweeds contain a high amount of water and moisture content is important to be calculated as it can have a significant impact on the final product. Moisture content affects the quality and stability of the product as well as food preservation.

Moisture is a vital reason for microbial growth, followed by pH and temperature. It is important to maintain a proper seaweed–water ratio for the final fermented product (Zambrano et al. 2019). Analysis of ash content provides information about the mineral

profile of the food product. The ash content can vary between 0.2-5% wet weight basis (S Suzanne Nielsen 2009). It would be useful in future studies to have the percentage of total solids to have an optimized seaweed–water ratio in the fermentation. The ash content represents the minerals present in the seaweed. Seaweed solutions, which was the exclusive supplier during this project, mentioned that specifically, *A. esculenta* is rich in Ca, vitamin A, Vitamin C, vitamin K, and complex of vitamin B. It also contains metals such as Fe, I, Mg, and K. The ash was analyzed in the raw seaweed but it would be interesting to be analyzed also in the fermented seaweed as well, to study the possible changes in mineral content brought about as a result of fermentation. Ash content variations can be found in different seaweed species depending on the time of harvest and the area of origin of the seaweed. There are also variations in ash content between green, brown, and red seaweed but also between species from the same group (Rohani-Ghadikolaei et al. 2011).

5. Conclusion

This preliminary study was conducted to evaluate the effect of combined lactic acid bacterial strains upon the fermentation of brown seaweed *Alaria esculenta*. The combined bacterial strains subjected to a defined media to evaluate their ability to utilize glucose and mannitol as substrates, in order to have a better understanding for the seaweed fermentation. Subsequently, *A. esculenta* was fermented utilizing the same combination of strains. The strains with *L. plantarum* as the main culture, in combination with *L. casei, L. paracasei, and L. rhamnosus* were optimized to bring about the fermentation through the utilization of saccharide substrates in seaweeds.

The fermentation process decreased the pH to 4.50 within 24 hours supported by the increasing growth curve trends. However, there was no significant difference in the results between the fermentation capabilities of the four combinations of bacterial strains. Thus, fermentation of seaweeds can be viewed as a sustainable and safe way to preserve food, by the development of wet biomass into a product elucidating probiotic benefits.

6. Future work

Further studies related to this project would involve exploring the possibility of subjecting the seaweed to an enzymatic pretreatment as the cell wall of the seaweeds mainly contains cellulose, which could be broken down to ease their utilization by the LAB for fermentation.

Another aspect that would be interesting to analyze is the final nutritional concentration of the fermented seaweed and how the presence of heavy metals in seaweed is reduced. This could help to better understand the impact of the LAB on fermentation. Additionally, a sensory evaluation would be beneficial for this project, to evaluate the taste and the texture obtained after the fermentation process and its implications in the final food project.

7. References

1. Allahgholi, L., Sardari, R.R.R., Hakvåg, S., Ara, K.Z.G., Kristjansdottir, T., Aasen, I.M., Fridjonsson, O.H., Brautaset, T., Hreggvidsson, G.O. and Karlsson, E.N. (2020). Composition analysis and minimal treatments to solubilize polysaccharides from the brown seaweed Laminaria digitata for microbial growth of thermophiles. *Journal of Applied Phycology*, 32(3), pp.1933–1947. doi:10.1007/s10811-020-02103-6.

2. Anis Raihana Mhd Rodzi, N. and Kuan Lee, L. (2021). Traditional Fermented Foods As Vehicle of Non-Dairy Probiotics: Perspectives In South East Asia Countries. *Food Res Int*, vol 150 part B doi:10.1016/j.foodres.2021.110814.

3. de Vries, M.C., Vaughan, E.E., Kleerebezem, M. and de Vos, W.M. (2006). Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract. *Int Dairy Jl*, [online] 16(9), pp.1018–1028. doi:10.1016/j.idairyj.2005.09.003.

4. FAO (2002). *Probiotics in food Health and nutritional properties and guidelines for evaluation*. [online] Available at: https://www.fao.org/3/a0512e/a0512e.pdf.

5. Forsvall, J. (2021). *The Effect of Temperature and Fungal:Bacterial Ratio on Kombucha Culture Fermentation*. [online] Available at: https://lup.lub.lu.se/luur/download?func=downloadFile&recordOId=9057046&fileOId=9057061.

6. Gobbetti, M. and Minervini, F. (2014). LACTOBACILLUS | Lactobacillus casei. *Encyclopedia of Food Microbiology*, pp.432–438. doi:10.1016/b978-0-12-384730-0.00180-4.

7. Khubber, S., Marti-Quijal, F.J., Tomasevic, I., Remize, F. and Barba, F.J. (2022). Lactic acid fermentation as a useful strategy to recover antimicrobial and antioxidant compounds from food and by-products. *Current Opinion in Food Science*, 43, pp.189–198. doi:10.1016/j.cofs.2021.11.013.

8. Kocot, A.M. and Wróblewska, B. (2021). Fermented products and bioactive food compounds as a tool to activate autophagy and promote the maintenance of the

intestinal barrier function. *Trends in Food Science & Technology*. [online] doi:10.1016/j.tifs.2021.11.014.

9. Kwon, D.Y., Nyakudya, E. and Jeong, Y.S. (2014). *Fermentation: Food Products*. [online] ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B9780444525123001558.

10. Mayo, B. and Flórez, A.B. (2020). Lactic Acid Bacteria: Lactobacillus spp.: Lactobacillus plantarum. *www.sciencedirect.com*, [online] 207-216. doi:10.1016/B978-0-08-100596-5.00856-8.

11. Nagarajan, D., Oktarina, N., Chen, P.-T., Chen, C.-Y., Lee, D.-J. and Chang, J.-S. (2022). Fermentative lactic acid production from seaweed hydrolysate using Lactobacillus sp. And Weissella sp. *Bioresource Technology*, 344, p.126166. doi:10.1016/j.biortech.2021.126166.

12. Nguyen, T., Kim, T., Ta, H.M., Yeo, W.S., Choi, J., Mizar, P., Lee, S.S., Bae, T., Chaurasia, A.K. and Kim, K.K. (2019). Targeting Mannitol Metabolism as an Alternative Antimicrobial Strategy Based on the Structure-Function Study of Mannitol-1-Phosphate Dehydrogenase in Staphylococcus aureus. *mBio*, [online] 10(4). doi:10.1128/mBio.02660-18.

13. Palomino, J.M., Toledo del Árbol, J., Benomar, N., Abriouel, H., Martínez Cañamero, M., Gálvez, A. and Pérez Pulido, R. (2015). Application of Lactobacillus plantarum Lb9 as starter culture in caper berry fermentation. *LWT - Food Science and Technology*, [online] 60(2, Part 1), pp.788–794. doi:10.1016/j.lwt.2014.09.061.

14. Papagianni, M. (2012). METABOLIC ENGINEERING OF LACTIC ACID BACTERIA FOR THE PRODUCTION OF INDUSTRIALLY IMPORTANT COMPOUNDS. *Computational and Structural Biotechnology Jl*, [online] 3(4), p.e201210003. doi:10.5936/csbj.201210003.

15. Paucean, A., Vodnar, D.-C., Socaci, S.-A. and Socaciu, C. (2013). Carbohydrate metabolic conversions to lactic acid and volatile derivatives, as influenced by Lactobacillus plantarum ATCC 8014 and Lactobacillus casei ATCC 393

efficiency during in vitro and sourdough fermentation. *European Food Res and Technology*, 237(5), pp.679–689. doi:10.1007/s00217-013-2042-6.

16. Peñas, E., Martinez-Villaluenga, C. and Frias, J. (2017). *Chapter 24 - Sauerkraut: Production, Composition, and Health Benefits*. [online] ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B9780128023099000248.

17. Rajauria, G., Cornish, L., Ometto, F., Msuya, F.E. and Villa, R. (2015). *Chapter 12 - Identification and selection of algae for food, feed, and fuel applications*. [online] ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B978012418697200012X#s0010.

18. Ramu Ganesan, A., Subramani, K., Shanmugam, M., Seedevi, P., Park, S., Alfarhan, A.H., Rajagopal, R. and Balasubramanian, B. (2020). A comparison of nutritional value of underexploited edible seaweeds with recommended dietary allowances. *Jl of King Saud University - Science*, 32(1), pp.1206–1211. doi:10.1016/j.jksus.2019.11.009.

19. Reboleira, J., Silva, S., Chatzifragkou, A., Niranjan, K. and Lemos, M.F.L. (2021). Seaweed fermentation within the fields of food and natural products. *Trends in Food Science & Technology*, [online] 116, pp.1056–1073. doi:10.1016/j.tifs.2021.08.018.

20. Rohani-Ghadikolaei, K., Abdulalian, E. and Ng, W.-K. (2011). Evaluation of the proximate, fatty acid and mineral composition of representative green, brown and red seaweeds from the Persian Gulf of Iran as potential food and feed resources. *Jl of Food Science and Technology*, [online] 49(6), pp.774–780. doi:10.1007/s13197-010-0220-0.

21. S Suzanne Nielsen (2009). *Food Analysis*. [online] Available at: https://student.cc.uoc.gr/uploadFiles/184-%CE%A7%CE%97%CE%9C-068/Compositional%20Analysis%20of%20foods%20-%20Food%20Analysis%20-%20S.S.%20Nielsen.pdf.

22. Stévant, P., Marfaing, H., Rustad, T., Sandbakken, I., Fleurence, J. and Chapman, A. (2017). Nutritional value of the kelps Alaria esculenta and Saccharina latissima and effects of short-term storage on biomass quality. *Journal of Applied Phycology*, 29(5), pp.2417–2426. doi:10.1007/s10811-017-1126-2.

23. T, B. (2018). Lactic acid bacteria: their applications in foods. *Journal of Bacteriology & Mycology: Open Access*, 6(2). doi:10.15406/jbmoa.2018.06.00182.

24. Vera-Peña, M.Y. and Rodriguez-Rodriguez, W.L. (2020). Effect of pH on the growth of three lactic acid bacteria strains isolated from sour cream. *Universitas Scientiarum*, [online] 25(2), pp.341–358. doi:10.11144/Javeriana.SC25-2.eopo.

25. Wychen, S. and Laurens, L. (2013). *Determination of Total Solids and Ash in Algal Biomass Laboratory Analytical Procedure (LAP)*. [online] Available at: https://www.nrel.gov/docs/fy16osti/60956.pdf [Accessed 20 May 2022].

26. Yan, Y., Zhang, F., Chai, Z., Liu, M., Battino, M. and Meng, X. (2019). Mixed fermentation of blueberry pomace with L. rhamnosus GG and L. plantarum-1: Enhance the active ingredient, antioxidant activity and health-promoting benefits. *Food and Chemical Toxicology*, 131, p.110541. doi:10.1016/j.fct.2019.05.049.

27. Zambrano, M., Dutta, B., G. Mercerb, D., L.MacLean, H. and F.Touchiead, M. (2019). Assessment of moisture content measurement methods of dried food products in small-scale operations in developing countries: A review. *Trends in Food Science & Technology*, [online] 88, pp.484–496. doi:10.1016/j.tifs.2019.04.006.

28. Zhang, T., Xu, R., Zhao, N., Xu, J., Liu, F., Wei, X. and Fan, M. (2022). Rational design of lycopene emulsion-based nanofood for Lactobacillus plantarum to enhance the growth and flavor production. *Food Hydrocolloids*, [online] 127, p.107518. doi:10.1016/j.foodhyd.2022.107518.