Master's Thesis Report
Evaluation of Novel Probiotics Acting on Seaweed Biomass

January 2021 – May 2021

TUSHAR KAUSHIK

LUNDS UNIVERSITET

Primary Supervisor : Madeleine Jönsson
Assistant Supervisor : Leila Allahgholi
Examiner : Eva Nordberg Karlsson

Index

ACKNOWLEDGEMENT

This Master's thesis project was conducted at the Department of Biotechnology at Lund University from January 2021 to May 2021. It has been my pleasure to be able to work and contribute towards this project. Not only has it been truly enriching in terms of being an experience, it has also taught me various aspects of academic research and life skills which I am sure will function as an asset in my future endeavors.

I am greatly thankful to Prof. Eva Nordberg Karlsson for providing me with this invaluable opportunity to be able to work and conduct my thesis work at the Division of Biotechnology despite the ongoing pandemic. My greatest appreciation and thanks to my encouraging supervisors Madeleine Jönsson and Leila Allahgholi for their understanding and guidance without which I would not have been able to accomplish the goals set forth on time. I would also like to thank Roya Sardari for her unforgettable guidance and help with the analytical systems during crucial timepoints.

My deepest salutations to my gurus and my parents without whom this endeavor would not have been possible in the first place. To my friends in Sweden who have been my family away from family. I would like to express my whole-hearted love and gratitude to Virginia, Niharika, Mirac, Lorenzo, Chia-Yin, Sharmeen, Zi Xuan, Yara and Manuel for being my constant pillar of support and comfort during my time here in Lund.

Special thanks to Niharika Singh at the Division of Chemical Engineering for taking the time to review and provide valuable comments on the report as a fellow opponent.

ABBREVIATIONS

LAB **–** Lactic acid bacteria
KS1-4 and CW4 – *Lactobacillus* strains
OD – optical density
HPLC – High Performance Liquid Chromatography
IC or IEC – Ion Exchange Chromatography
SCFA – Single Chain Fatty Acids
GI – Gastrointestinal Tract
NADH – Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
% - Percentage
m – Meter
µm – Micrometer
˚C – Degree Celsius
g/L – Gram per Liter
v/v – Volume by Volume
mL – Milli Liter
µL – Micro Liter
h – Hour
mins – Minutes
HT – Heat-Treated
NHT – Non-Heat-Treated
ODW – Oven Dry Weight
adhE – alcohol dehydrogenase-encoding gene
MRS – De Man, Rogosa and Sharpe
DW – Dry weight
GRAS – Generally Recognized As Safe

ABSTRACT

Fermentation is a technique which has been employed for the preservation of food since time immemorial. With a growing population comes the challenge of providing accessible and nutritious food and at the same time adapting a sustainable approach to achieve the desired target. Seaweeds have been utilized as food in the South-Southeast Asian cuisine for a long time in history. However, in the recent decades, seaweeds - typically green and brown seaweeds have found great scope as a sustainable food alternative in the European market. In this project, the ability to utilize novel lactic acid bacteria (KS1-4 and CW4) for the fermentation of seaweed *A. esculenta* was evaluated. Based on previous (unpublished) studies performed in the lab, it was found that the brown algae *A. esculenta* contain 9.16 ± 0.17 % DW of mannitol and 5.67 ± 0.07 % DW of glucose making them interesting substrates to be used for the cultivation of lactic acid bacteria. In this study, it was seen that *Lactobacillus* strains KS1-4 and CW4 are able to ferment the seaweed *A. esculenta* by utilizing the substrates mannitol and glucose innately present in the seaweed. The effect of heat-treatment on the seaweed and its consequent effect on the fermentation efficiency was analyzed. It was found that irrespective of the method of treatment employed, the novel probiotic strains were able to grow and ferment the seaweed. Thereby highlighting their ability to downregulate the growth of other bacteria in addition to preventing the growth of spoilage micro-organisms like fungi. Utilizing potential probiotic strains for the fermentation of seaweeds not only enhances their nutritional properties and helps in improving their storage faculties, but also helps to enhance the sensory properties of the intended food product. Analyzing the results obtained from this study indicated that the *lactobacillus* strain KS1-4 closely resembled *Lactiplantibacillus plantarum* and CW4 closely resembled *Levilactobacillus brevis.*

OUTLINE

This master's thesis report is divided into six parts. The first part is the 'aim' where the goals outlined for this project are commented upon. This is followed by the second part - 'background' which elaborates on the relevant theory behind the project and reasoning as to why this work was conducted. The background is succeeded by the third part - 'materials and methods' section, where the materials used as well as the methodologies employed for conducting the experiments and analysis of the samples are briefly explained. The fourth part comprises the 'results and discussion', wherein the results obtained from the studies as well as their relevance to the project are discussed in detail with references to literature in cases where previous similar studies have been performed. This part is followed by the fifth part - 'conclusion and future work' section where scope for future work as well as suggestions are provided. The sixth part is the appendix which includes raw data as well as some less important results which could not be included in the main report.

AIM

This master project aims to look at the effect of utilizing two strains of lactic acid bacteria (LAB) in induced fermentation of the brown macroalgae *Alaria esculenta*. In a first step, cultivation of LAB will be performed in serum bottles with MRS media enriched with different sugar sources, to evaluate the substrate preferences of the different strains. In a second step, one or several of the strains will be utilized to induce fermentation of *A. esculenta* as part of the ensiling experiments.

Analyses of the samples will be performed using the: optical density (OD), analysis of single chain fatty acids (SCFA) involving the detection of lactic acid, formic acid, propionic acid, butyric acid, acetic acid, malate, succinate, and ethanol; followed by carbohydrate analysis, water content and total solids and ash determination.

BACKGROUND

What is fermentation?

Fermentation can be explained as an old technique for preserving food and improving its nutritional and sensory values (Bruhn *et.al.,* 2019). The process of fermentation takes advantage of the innate mechanism by which microorganisms convert sugars to various acids such as lactic, acetic and propionic acid (Caplice *et.al.,* 1999). Typically, fermentation can be broadly classified into spontaneous and induced fermentation. Spontaneous fermentation can be referred to as the 'traditional' method of fermentation, where the substance to be fermented is left open to the air/atmosphere. The microorganisms present in the air will then lead to the fermentation of the substance. It is to be noted here that the organism which ferments the substance is not chosen and hence the by- and end- products cannot be controlled. For example, the oldest form of wine/beer making makes use of spontaneous fermentation. Induced fermentation can be considered to be a form of 'selective' fermentation, where the microorganism of interest is induced into the substance which is to be fermented. This method of fermentation therefore yields desired by- and end-products. One common application of induced fermentation is utilizing a specific strain of lactic acid bacteria (LAB) for the fermentation of a particular type of yogurt which will have desired texture and flavor.

Nowadays, food grade lactic acid bacteria are most commonly used for the fermentation of plant-based products and are believed to contribute to positive human health effects due to the increase of probiotic bacteria in the diet combined with the nutritional values of the plant material (Bruhn *et.al.,* 2019).

Lactic acid bacteria (LAB) and fermentation

Lactic acid bacteria have been widely used in the dairy and fermentation industry from the medieval ages. From their usage in production of yoghurt, fermented sourdough, meat or probiotics, they have a broad range of applications in the modern world. Lactobacillus being the largest genus within the LAB group, they belong to the phylum Firmicutes, class Bacilli and family *Lactobacillaceae* (Ibrahim, S.A., 2016). Lactobacillus are classified into three main categories depending on the carbohydrate metabolism (De Angelis *et.al.,* 2016):

Group I: Obligate homofermentative bacteria

> This group of bacilli completely ferments hexoses to lactic acid, whereas pentoses and gluconate are not fermented. *Lactobacillus acidophilus, Lactobacillus helveticus* and *Lactobacillus salivarius* belong to this group.

Group II: Facultative heterofermentative bacteria

This group of bacilli either ferments in its entirety to lactic acid, whereas under glucose limiting conditions, it ferments hexoses to produce lactic acid, acetic acid, ethanol, and formic acid while pentoses being fermented to yield lactic acid and acetic acid. *Lacticaseibacillus casei (L. casei), Lactobacillus curvatus, Lactiplantibacillus plantarum (L. plantarum), Lactobacillus sakei* belong to this group.

Group III: Obligate heterofermentative bacteria

This group of bacilli ferments hexoses to produce lactic acid, CO2, acetic acid and/or ethanol; and the pentoses are fermented to yield lactic acid and acetic acid. *Levilactobacillus brevis (L. brevis), Lactobacillus buchneri, Limosilactobacillus fermentum, Limosilactobacillus reuteri* belong to this group.

These groups of bacteria are utilized in various industries depending on the need and desired properties of the product.

LAB's in focus and their significance

The LAB species discussed in this report have been selected depending on the predictions made based on the results obtained from previously conducted experiments in the lab where this thesis was conducted. It is also to be noted that the LAB's used for this project have not yet been genome sequenced and therefore cannot be commented upon accurately.

L. plantarum

A versatile natural inhabitant of the human gastrointestinal (GI) tract plays an important role in the probiotic industry. One of its applications is in the fermentation of plant products which produces 'plantarin' - an antimicrobial substance. Some commonly sold probiotics containing this gram-positive bacteria are *L. plantarum* 229V, which showcases beneficial adherence properties to the GI tract and *L. plantarum* 01. The adhesive property of *L. plantarum* 229V makes it a valuable probiotic as it is due to this property that the bacteria are able to withstand the acidic pH as well as the bile salts which would otherwise kill the bacteria (Cebeci *et.al.,* 2003). *L. plantarum* has also shown to provide protection against chronic cadmium/aluminium toxicity as well as displayed high resistance to elevated levels of manganese (Tong *et.al.,* 2017). The presence of a large number of surface anchor proteins enables the bacteria to utilize different substrates for growth and proliferation (Kleerebezem *et.al.,* 2003). Some of the food products containing *L. plantarum* are olives, sauerkraut, cassava, cocoa beans, stilton cheese and wine (De Vries, *et.al.,* 2003).

In order for the bacteria to live and thrive in harsh environments, there is an innate mechanism by which *L. plantarum* are able to utilize alternate forms of carbon courses such as mannitol and sorbitol (Yang *et.al.,* 2019). Neves *et.al*, reported that the utilization of mannitol as substrate comes at the cost of NADH burden occurring in the cells due to the formation of excess NADH molecules due to the mannitol-1-phosphate dehydrogenase catalysed reactions. Yang *et.al.,* found that the expression of the genes such as alcohol dehydrogenase -encoding gene (*adhE*), which is responsible for enabling *L. plantarum* to utilize mannitol as substrate was highly upregulated in harsh environments such as the GI tract and stomach of the mice in comparison to when glucose is used as substrate (Marco *et.al.,* 2007). It was also suggested by Yang *et.al.,* that *adhE* is probably upregulated to offset the stress caused due to NADH accumulation in the cells. This was supported by their observation that apart from producing ethanol as a by-product of fermentation, *L. plantarum* N95 converted mannitol into lactic acid and ethanol until 13hr which was followed by the production of acetate which was directly proportional to the consumption of mannitol.

L. brevis

Naturally occurring in the GI tract, it is a gram positive heterofermentative bacteria mostly found in milk, cheese, sauerkraut, sourdough, silage and cow manure. Owing to its long term usage in traditionally fermented food products, it has been recognised as a GRAS organism. Though some research publications suggest that despite being recognised as GRAS, *L. brevis* is not commonly used as probiotics. However, oral administration of *L. brevis subsp. coagulans* was seen to improve the host immunity (in humans) by increased production of IFN-α (Rönkä *et.al.,* 2003). Various strains of *L. brevis* showcasing up to 90% resistance to gastric acids and bile salts making it a valuable novel probiotic (Kim *et.al.,* 2021). The bacterium is known to consume multiple carbon substrates simultaneously for growth and therefore depicts the lack of the traditional control method for carbohydrate metabolism (Ibrahim, S.A., 2016). *L.brevis* has been known to produce mannitol through the 6-phosphogluconate mannitol metabolism pathway.

L. casei

Traditionally extracted from milk, dairy products and the human GI tract, *L.casei* is a gram positive, non-sporulating lactic acid producing bacteria (Caballero *et.al.,* 2015). They have been found to have a wide range of pH and temperature tolerance (Barba *et.al.,* 2018). It is also reported that they are gas producing organisms when used for cheese making. In recent years, due to their advantageous probiotic effects, *L. casei* have been recognised as GRAS by the FDA and are consequently used to enhance the flavour and texture of various foods and beverages (Hill *et.al,* 2018).

Seaweeds and their classification

Owing to the rapid consumption of fossil-based fuels and their greenhouse gas emissions, alternative sources of energy as well as novel food resources have become a necessity for a sustainable future (Hannon *et.al.,* 2010). An approach towards this is to utilize marine biomass such as marine macroalgae (seaweed) which show potential of functioning as resources for sustainable biorefineries and as well as for novel food products (Jönsson *et.al.,* 2020). Given that macroalgae grow in the oceans water and do not consume any land area makes them a viable alternative. In addition to their abundance and easy cultivation, marine macroalgae have many advantages such as high growth rate, high carbohydrate content and other favourable components which are necessary for utilizing them as alternative resources (Allahgholi *et.al.,* 2020). Even though seaweeds have been used as food in the regions of Japan, China and Korea from time immemorial, their commercialization has occurred only in the recent few decades. They are rich in vitamins and minerals, low in calories and are enriched with valuable dietary fibers (Kılınç *et.al.,* 2013). Nevertheless, seaweeds as a food source have not yet been accepted very well in the European consumer market due to their unpleasant sensory properties. Therefore, utilizing fermentation as a method to enhance the taste and texture of the seaweeds to increase their incorporation into the European diet would benefit the health of the consumer. In addition to this, utilizing fermentation coupled with potential probiotic strains will invariably increase their storage time thereby making them an interesting food product to be studies and developed further as a step towards a sustainable future.

Classification of Seaweeds

Seaweeds can be classified into three main categories based on their pigmentation: Chlorophyceae or green algae; Rhodophyceae or red algae; and Phaeophyceae or brown algae.

Chlorophyceae

Chlorophyceae, also called as green algae belong to the family of Chlorophyta. They are known to be found in marine waters as well as in freshwater areas (Sardari *et.al.,* 2018). Green marine macroalgae mainly involve the class Ulvophyceae and display heterogeneity in morphology and cellular diversity (Leliaert *et.al.,* 2012). They have been widely used and consumed in the food industry such as in sushi nori sheets which are used for making sushi. Their cell walls are known to contain monosaccharides such as rhamnose, xylose, glucuronic acid and iduronic acid along with sulfate groups. Despite their traditional use, green algae have not been explored to the same extent as red or brown algae in the industry (Jönsson *et.al.,* 2020).

Rhodophyceae

Rhodophyceae or red algae are made up of 'multicellular photosynthetic eukaryotic organisms'. They are mainly found in marine environments with a small exception of ~ 5 % which can be found in freshwater areas but mainly based in tropical climates. Their prominent red colouration is broadly due to the presence of pigments such as phycocyanin and phycoerythrin which suppress other pigments such as chlorophyll A and carotene. The cell wall of red algae primarily comprises cellulose, carrageenan and agar which have widespread applications in the industrial settings (Viola *et.al.,* 2001).

Phaeophyceae

Phaeophyceae or brown algae require temperate climates for their growth and are consequently found in the northern latitudes in the cold marine waters. They obtain their characteristic brown colour due to the presence of the carotenoid pigment 'fucoxanthin'. Their sizes range from a few millimeters up to 70m. The cell wall of brown algae being rich in polysaccharides are known to majorly contain alginate, fucoidan, laminarin and cellulose where alginate constitutes the majority, whereas fucoidan and laminarin - functioning as a storage polysaccharide (Jönsson *et.al.,* 2020) - occur more prominently only in certain species such as *Laminaria* spp. and *Fucus* spp (Mautner, 1954). However, the percentage of these compounds present in the algae are dependent on the season, species, environment as well as the region they are grown in. It has been observed that monomeric sugars such as glucose, fructose and sucrose are immediately converted into D-mannitol and polysaccharides in brown algae (Graiff *et.al.,* 2016). Taking into account the presence of valuable polysaccharides in brown algae, modern research has focused on utilizing these polysaccharides to produce valuable outputs. It is this algal type which is utilized for conducting our work in this project. The type of algae used for this project and its properties are discussed further in the later parts of the background section.

Detailing the advantages and properties of the main components of the cell wall for brown algae:

Fucoidan - classified as a 'fucose-containing sulfated polysaccharide (FCSP)', it is predominantly composed of fucose but also contains other hexoses such as xylose, galactose, mannose and glucuronic acid (Lim *et.al.,* 2016). It has been reported that the content of fucoidan is highest during the autumn season (Mautner, 1954). Fucoidan plays a vital role in the regulation of water and ion retention in the extracellular matrices of the plants for the prevention of desiccation as well as osmotic stress especially during low tides (Deniaud-Bouët *et.al.,* 2017). Furthermore, fucoidan is also known to have valuable biomedical health benefits thereby finding extensive applications in the biomedical research field (Shiroma *et.al.,* 2008).

Alginate - commonly described as 'salts of alginic acid', are made up of linear unbranched copolymers primarily consisting of β-1,4-d-mannuronic acid (M) and α-1,4-l-

guluronic acid (G). The properties of alginate are influenced by the various ratios and combinations of the M and G subunits (Jönsson *et.al.,* 2020). For instance, the chelating property of the alginate is solely influenced by the number of repetitions of the compound G. Alginate can be considered to be the counterpart to pectin which is present in the terrestrial plants. The alginate present in brown algae when extracted is categorized as 'phycocolloids' i.e., the polysaccharides which have the ability to form colloidal systems when they come in contact with water (Mautner, 1954).

Laminarin - it is defined as a relatively short polymer of β-1,3-glucan consisting of around 20-25 monomer units. It functions as a carbohydrate reserve in many brown seaweeds similar to the function of starch in terrestrial plants. The interchain linkages in laminarin determines its water-soluble properties (Graiff *et.al.,* 2016). Laminarin and its derivatives have been shown to elicit properties which promote and enrich health such as prebiotic, antioxidant and anti-cancerous properties (Jönsson *et.al.,* 2020).

Alaria esculenta

In recent times, cultivation and utilization of brown seaweeds such as *Alaria esculenta* has increased in the European regions. This can be attributed to the ability of *A. esculenta* to reach high biomass yields as well as their advantageous nutritional properties (Stévant *et.al.,* 2017). For this study, the edible brown algae *A. esculenta* also known as 'winged kelp' was considered for performing the reactor experiments. *A.esculenta* belongs to the genus Alaria which are generally olive or yellow-green in colour. They grow on rocks in places exposed to the sea water at low water levels in the shallow subtidal regions. They are the only known type of seaweed kelps in Ireland and Britain making them a valuable resource in those regions (Algae Base. 2021).

Brown seaweed biomass such as *A. esculenta* often contains large quantities of carbohydrate mainly consisting of alginate which are found to be the highest in the summer months. In addition to occurrence of the common structural carbohydrates such as laminarin, cellulose and alginate, kelps are also found to contain mannitol and fucoidan in valuable quantities during the summer and autumn months. In due course, laminarin and mannitol are later utilized by the algae to facilitate fresh tissue growth in the cold winter months (Rosell *et.al.,* 1984). According to Schiener *et.al.,* for *A. esculenta*, the highest levels of moisture and ash contents were reported to be found in the samples obtained during the winter months in comparison to the samples obtained in the autumn months. The average mannitol content was reported to be 12.1 ± 3.5 %. Given that alginate is known to form the majority of the carbohydrates of the brown seaweed, it was reported to be 37.4 ± 4.0 % of dry weight in *A.esculenta* with the lowest values being recorded in the

months of July. The average total carbohydrate yield was reported to be 72.1 ± 6.7 % of the biomass (Schiener *et.al.,* 2015).

Iwamoto *et.al.,* reported that mannitol being produced as one of the major products of photosynthesis has a unique biological significance in brown algae. It has been found to have a very high compatibility with a multitude of organic macromolecules along with being a highly soluble compound and displays the ability to function as an antioxidant. Mannitol in red and brown algae has been found to be responsible for controlling cell turgor (Karsten *et.al.,* 1997) and is found in increased quantities in hypertonic conditions. It was noted that free mannitol comprised up to 30% of the dry weight in brown algal species (Reed *et.al.,* 1985) making them interesting candidates to be used for as substrates.

Based on (unpublished) studies conducted in the lab where this thesis was performed, it has been noted that the total mono-sugars in *A. esculenta* were found to be 20.86 % (w/w) and uronic acids were found to be 14.69 % (w/w) as quantified using ion-exchange chromatography technique.

MATERIALS AND METHODS

MATERIALS

Raw Materials

Brown seaweed (*Alaria esculenta*) cultivated in Frøya farm and harvested in Spring of 2019 (Dated: 2019-05-16) was obtained from 'Seaweed solutions' in Trondheim, Norway. This seaweed was stored at -20˚C prior to being used for experiments. MRS Media chemicals and chemicals used for the preparation of standards were obtained from Sigma life sciences and/or Sigma Aldrich (see Appendix I). Milli-Q water used was purified using a 0.2µm filter. The bacterial strains used for experiments (KS1-4 and CW4) were obtained from Immune-Biotech Company (Sweden).

METHODS

Modified MRS Media Preparation

The De Man, Rogosa and Sharpe (MRS) medium was modified and prepared using peptone from casein - tryptic digest (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), di-potassium hydrogen phosphate (2 g/L), tween 80 (1 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), without the addition of the carbon source (glucose). During the experimentation procedure, various concentrations of the carbon source were used, and their consumption was analyzed.

Pre-culture Preparation

To prepare the pre-culture, 50 mL of the MRS medium containing glucose was measured into a 50 mL falcon tube and inoculated with 1% (v/v) of *lactobacilli* culture. The culture was incubated at 37℃ for at least 12 hours.

Serum Bottle Fermentation

The serum bottles were filled with 80 mL media, sealed, and autoclaved for 20 min. The stock solution of substrate was prepared and autoclaved separately. Prior to starting the cultivation, 20 mL of the sterile substrate stock solution was added to the serum bottles to reach the desired concentration of the carbon source. The serum bottles were then inoculated with 1% (v/v) of prepared inoculum. The inoculated bottles were incubated at 37℃ while samples in duplicates were collected and optical densities measured at 620 nm (OD at 620 nm) at various time points (0,2,4,5,6,7,8,9,10,11,12 h).

Algal Substrate Preparation for Ensiling Experiments

Harvested and frozen *A.esulenta* was weighed and minced using Menuett meat mincer (köttkvarn) (Figure 1). A 20 % (w/v) of seaweed slurry was prepared by mixing 80 g of frozen minced seaweed with Milli-Q water. Care was taken that the algae were weighed when they were still frozen to eliminate the weight which could be added on upon melting of the water present in the harvested algae.

Figure 1. (Left) Menuett meat mincer (köttkvarn); (Right) Minced A.esculenta for ensiling experiments prior to being re-freezed at –20˚C

Ensiling Experiments using Gas Endeavour® Reactor

The ensiling experiments were performed using 500 mL reactors with real-time monitoring of pH and gas production using Bioprocess Control - Gas Endeavour® software. 80 grams of frozen minced algae biomass was measured and mixed with 400 mL of Milli-Q water. The water was filtered through a 0.2 µm filter before adding to the reactor. The reactor was inoculated with 1mL of pre-culture when the OD of pre-culture reached a value of 1.43 (KS1-4 heat-treated), 7.16 (CW4 heat-treated), 2.38 (KS1-4 non-heat treated), and 2.26 (CW4 non-heat treated). The fermentation was performed by placing the reactors in a water-bath maintained at a constant temperature of 37°C. Stirring was performed at 40rpm for 3-5 minutes prior to each sampling to ensure uniform mixing and homogeneity in the sample. The reactor setup is as shown in Figure 2.

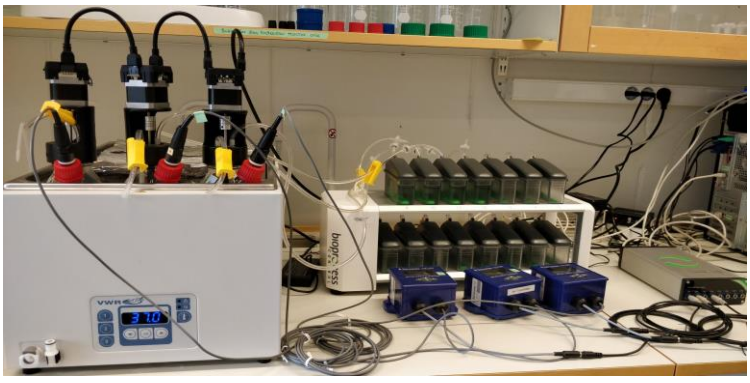


Figure 2. Experimental setup of ensiling experiments. (Left) Reactors equipped with stirring and real-time pH monitoring electrodes kept in a water-bath maintained at 37℃; (Right) The gas measurement and pH display equipment from bioprocess control.

Analysis of Single Chain Fatty Acids (SCFA)

The detection of single chain fatty acids in the samples was accomplished using high-performance liquid chromatography (HPLC) method. For analyzing the single substrate samples, Bio-Rad Aminex 87-HPX column with 0.5mM of sulfuric acid was used as eluent. The flow rate was set to 0.5 mL/min and the column compartment temperature was maintained at 40℃. For analyzing the substrate combination as well as fermentation samples, Aminex HPX-87H column, 5.0 M sulfuric acid was used as eluent with a flow rate of 0.5 mL/min and the column compartment temperature was maintained at 50℃. Samples from the fermentation experiments were centrifuged at 13,000 rpm for 10 mins to remove all cells and debris from the solution. The clear supernatant was used for HPLC sample preparation. The samples obtained from the serum bottle experiments were diluted 20 time and the sample obtained from the bioreactors were diluted 10x with milli-Q water. The resultant diluted solution (with final volume 1 mL) was treated with 20 µL of 20% (v/v) sulfuric acid and incubated for at least 30 mins in the fridge maintained at +4℃. Following the incubation, the samples were again spun at 13,000 rpm for 2 mins to remove any precipitate formation. The centrifuged samples were then filtered into HPLC vials using 0.2 µm filters.

Extraction and Degradation of Carbohydrates

The extraction and degradation of carbohydrates from the algal biomass was based on sample preparation from NREL/TP – 5100-60957 (Van Wychen and Laurens, 2015). It was accomplished by taking 25 ± 2.5 mg of the freeze-dried sample in a 10 mL glass vial and treating it with 250 µL of 72 % sulfuric acid. The samples were vortexed thoroughly to ensure that the solids were immersed in the acid. These samples were then incubated in an incubator-shaker at 30℃ and 300rpm for one hour to ensure carbohydrate degradation. Following which, 7 mL of Milli-Q water was added to the tubes containing the sample to ensure that the concentration of sulfuric acid reduced to 4%. The tubes were vortexed and subsequently, the samples were autoclaved for 15 minutes at 121℃ to extract the carbohydrates. The samples were stored at +4℃ overnight for further analysis the next day. The next day, the samples were neutralized stepwise using 0.1M barium hydroxide ($Ba(OH)_2.H_2O$). The supernatant from the neutralized samples was used for performing analysis using ion exchange chromatography.

Analysis of Total Carbohydrates

The amount of mono-sugars present in the samples was quantified based on NREL/TP – 5100-60957 (Van Wychen and Laurens, 2015) using a High-Performance Ion Exchange Chromatography with Pulsed Amperometric Detection (HPAC-PAD). The analysis protocol followed was as suggested by Allahgholi *et.al.*, 2020. Samples from the fermentation experiments

were centrifuged at 13,000 rpm for 10 mins to remove all cells and debris from the solution. The clear supernatant was used for IC sample preparation. The samples obtained from serum bottle experiments were diluted 500x and the samples from the bioreactor experiments were diluted 50x prior to analysis. The diluted samples were then filtered using a 0.2 µm filter into the analysis vials.

Determination of Total Solids

To determine the amounts of total solids in the freeze-dried samples, a method based on NREL/TP – 5100-60956 (Van Wychen and Laurens, 2015) was employed. The crucibles were pretreated overnight at 575℃ using an incinerator to remove any combustible contaminants present in the crucibles. Following the pretreatment, the empty crucible weights were recorded when the temperature was reached to room temperature. Subsequently, 100 ± 5mg of the freeze-dried samples were weighed into the crucibles and were left for drying in an oven maintained at 105℃ for 24 hrs. The oven dried samples were weighed and their weight after drying was recorded. The total solids were calculated using the following formula:

$$\%\,Total\ solids\ =\ 100 \times \frac{(weight\ of\ dried\ sample\ -\ weight\ of\ empty\ crucible)}{weight\ of\ algal\ sample}$$

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

$$ODW_{Sample} = \frac{(weight\ of\ oven\ dried\ sample\ -\ \%\,Total\ solids)}{100}$$

Determination of Ash Content

Determination of ash was performed based on the method NREL/TP – 5100-60956 (Van Wychen and Laurens, 2015). The oven dried samples were subjected to incineration of the samples at 575℃ for 3 hours. The ash content was determined using following formula:

$$\%\,Ash\ = 100 \times \frac{(weight\ of\ crucible\ with\ ash\ -\ weight\ of\ crucible)}{ODW\ of\ sample}$$

RESULTS

Based on 16S RNA analysis done by Immune-Biotech company (owner of the strains) one strain was thought to be *L. plantarum* and the other *L. casei*. However, based on the results obtained from this project, the identities of the strains under consideration will be discussed in the later parts of this report.

Serum Bottle Fermentation – Single Substrate

To evaluate if the novel probiotics are able to consume glucose and mannitol as substrates, the small-scale fermentation experiments were carried out. The experiments were performed in 100 mL serum bottles which contained modified MRS media and the substrate or carbon source. In addition to validating the ability of the novel strains to consume different carbon sources, experiments were also performed to monitor their growth with varying concentrations of the carbon source (1,5,10,15 and 20 g/L). As a negative control, medium without any carbon source was used i.e. the bottle contained 80 mL modified MRS media and 20 mL Milli-Q water. To eliminate the probability of contamination occurring in the oven, a serum bottle containing media without inoculum was sampled at every time point and its reading was recorded.

The growth curves obtained are as seen in Figure 3 for *Lactobacillus* strain KS1-4 and Figure 5 for *Lactobacillus* strain CW4. The samples were then subjected to analysis using IC to monitor substrate utilization and the results obtained were compared to the growth curves. These results are as shown in Figure 4 for KS1-4 and Figure 6 for CW4.

Serum Bottle Fermentation for *Lactobacillus* strain KS1-4

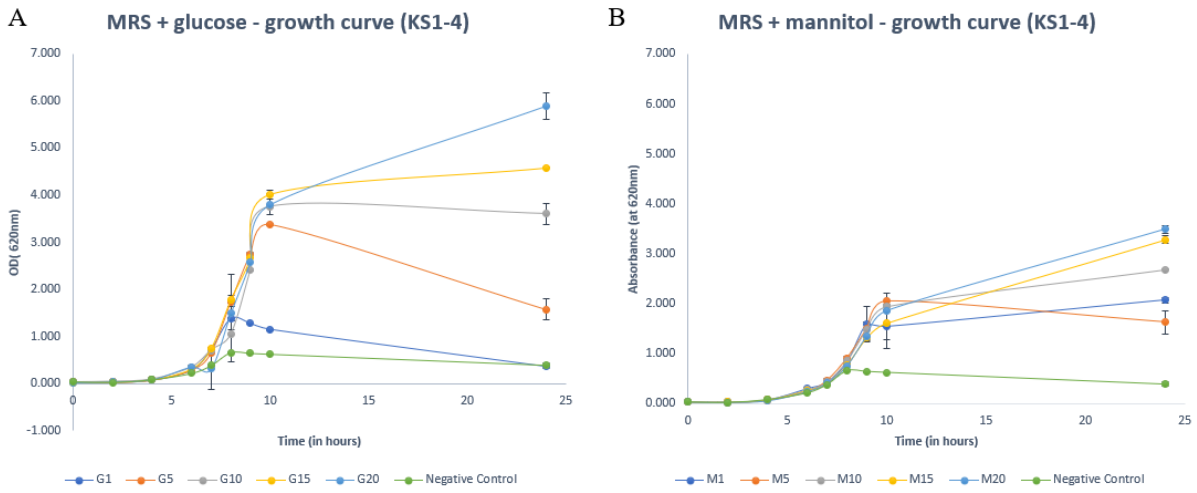


Figure 3. Growth curve trends for Lactobacillus strain KS1-4 utilizing (A) glucose as substrate and (B) mannitol as substrate, with varying concentrations. The notations G1 to G20 indicate the increasing glucose concentrations in g/L and M1 to M20 indicate the increasing mannitol concentration in g/L.

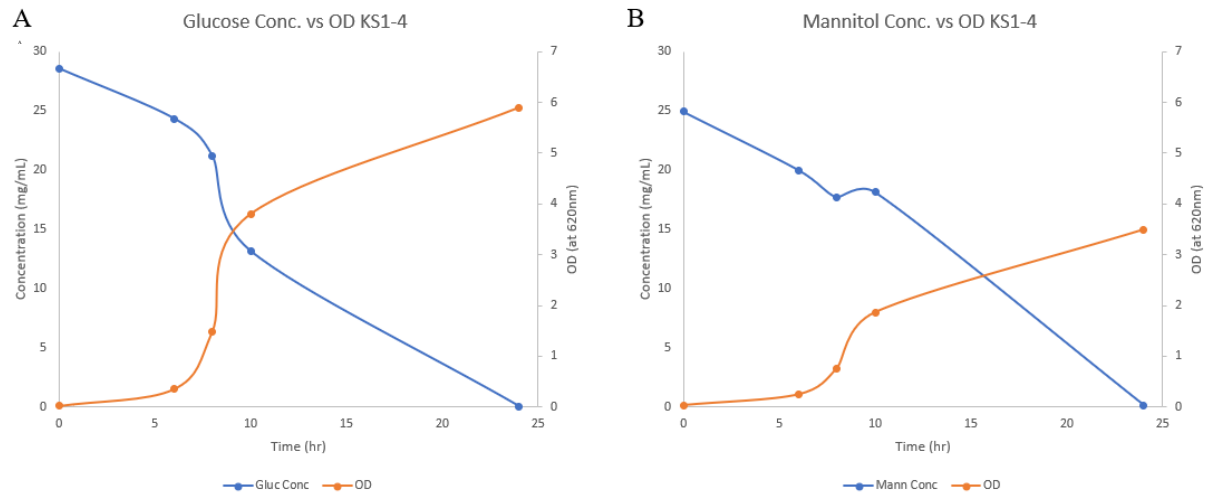


Figure 4. KS1-4 substrate utilization trend for 20g/L substrate concentration based on data obtained from IC analysis compared with the measured OD at 620 nm when (A) glucose was used as substrate and (B) mannitol was used as substrate.

Serum Bottle Fermentation for *Lactobacillus* strain CW4

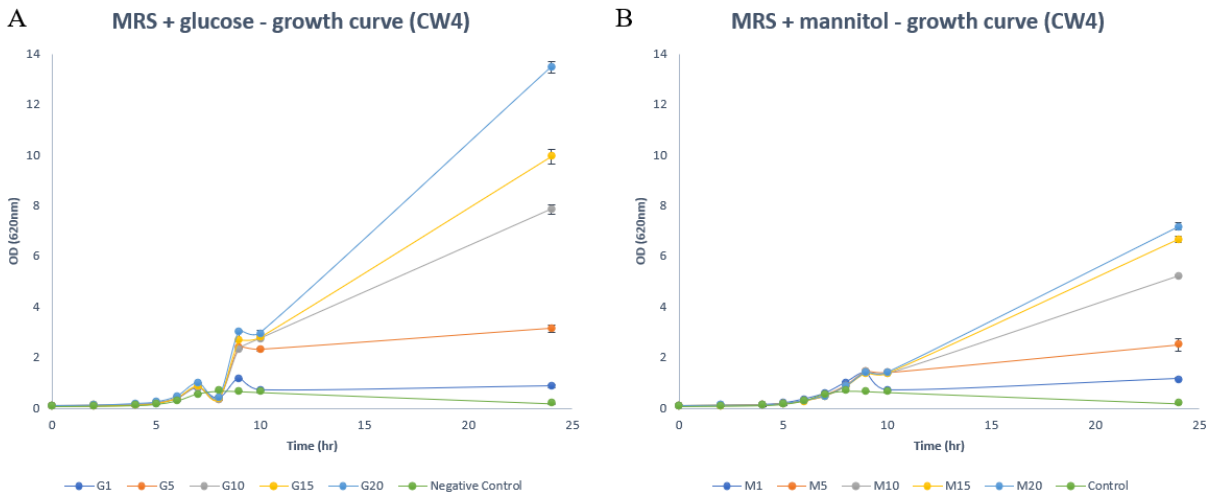


Figure 5. Growth curve trends for CW4 utilizing (A) glucose as substrate and (B) mannitol as substrate, with varying concentrations. concentrations. The notations G1 to G20 indicate the increasing glucose concentrations in g/L and M1 to M20 indicate the increasing mannitol concentration in g/L.

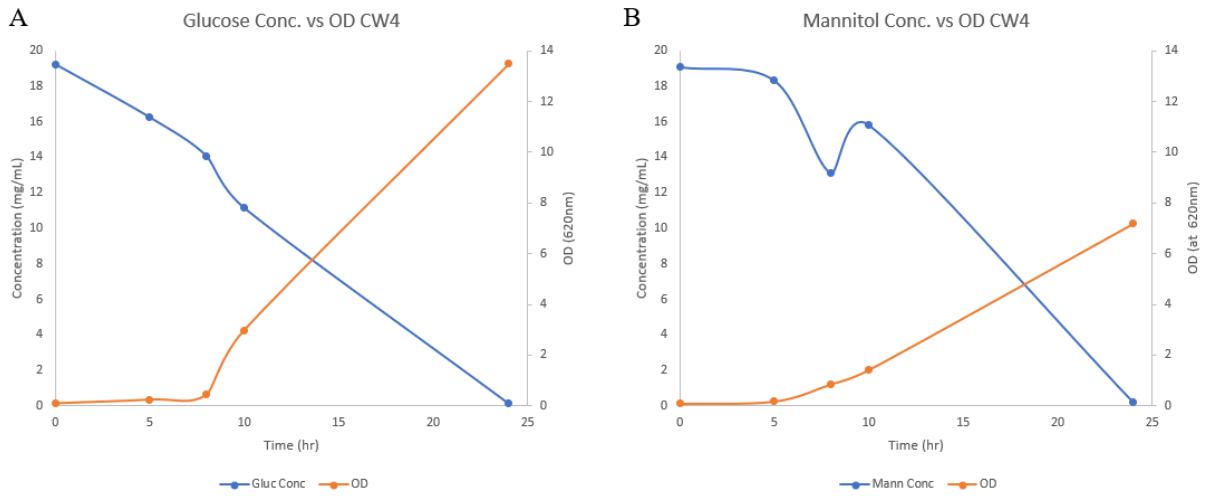


Figure 6. CW4 substrate utilization trend for 20g/L substrate concentration based on data obtained from IC analysis compared with the measured OD at 620 nm when (A) glucose was used as substrate and (B) mannitol was used as substrate.

Serum Bottle Fermentation – Substrate Combination

To study the effect on the growth and substrate consumption preference when the substrates glucose and mannitol are present together like in seaweed, a substrate combination experiment was performed. Here 10 g/L of glucose and 10 g/L of mannitol were fed to the bacteria and their growth curves were plotted as seen in Figure 7. The samples were then subjected to substrate consumption analysis using IC and the results when compared with the OD are as plotted in Figure 8. Comparison of growth curves with different substrates at 10 g/L can be seen in Figure 16 in Appendix II.

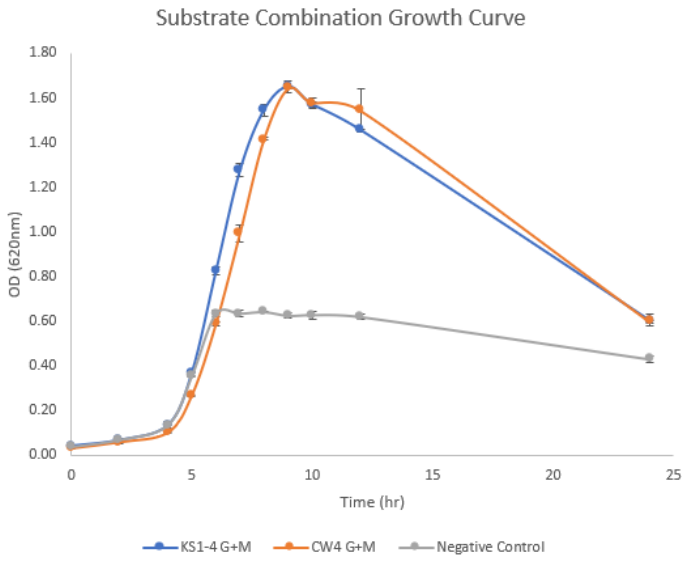


Figure 7. Growth curve results from fermentation experiments for KS1-4 and CW4 as obtained when the substrates glucose (G) and mannitol (M) are combined.

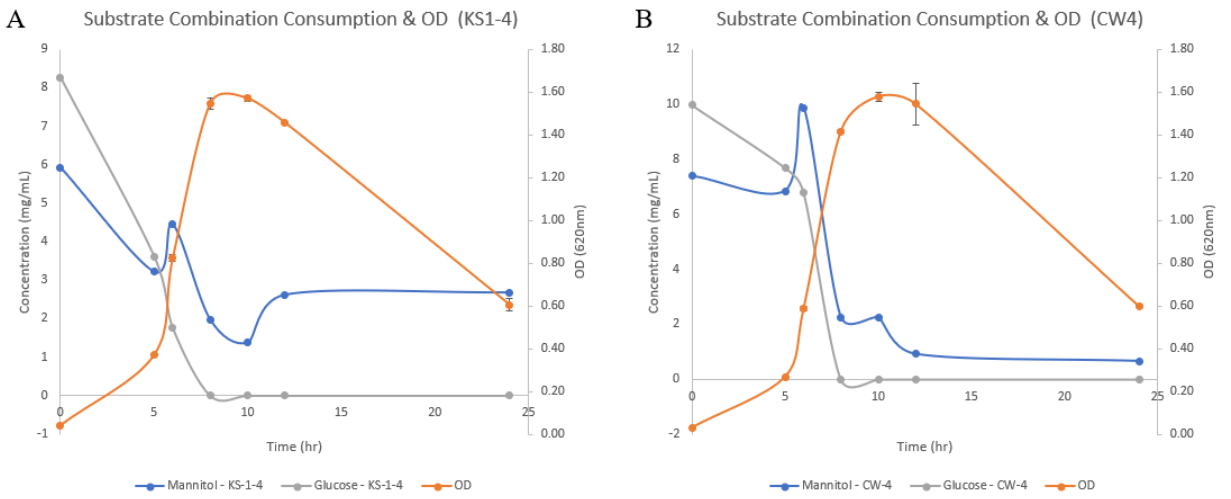


Figure 8. Comparison of glucose and mannitol consumption (with concentration 10 g/L each) analyzed by IC and cell growth (OD) for (A) KS1-4 and (B) CW4.

Ensiling Experiments

To study the ability of *Lactobacillus* strains KS1-4 and CW4 to ferment the seaweed (*A. esculenta*), ensiling experiments with different pre-treatment of the raw material was performed. Samples with pre-treatment had been subjected to autoclaving at 121 ℃ for 15 minutes and the remaining samples were untreated (without pre-treatment). The consolidated growth curve and pH curve results obtained from these experiments are as shown in Figure 9.

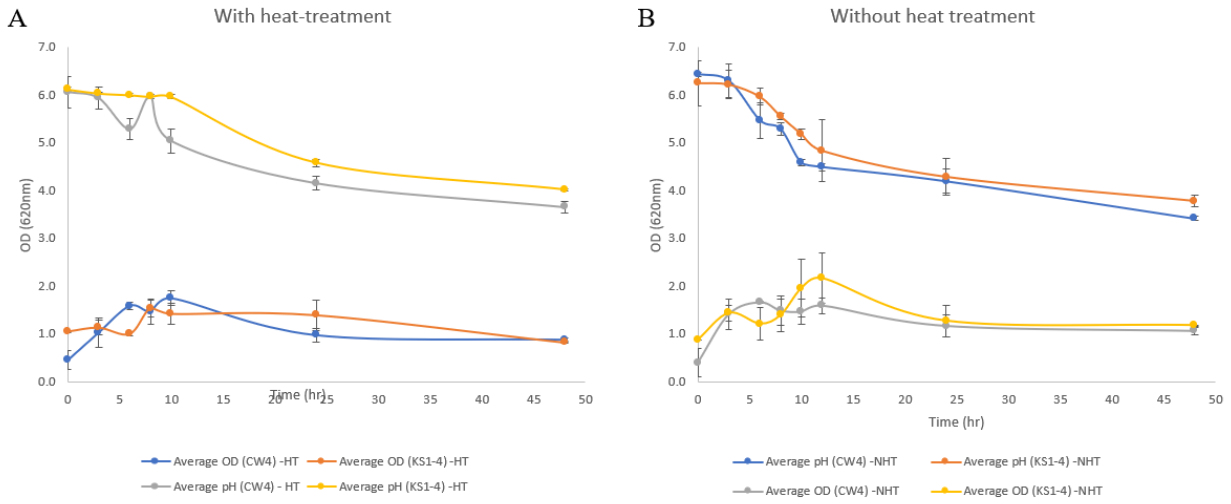


Figure 9. Comparison of growth curves for KS-1-4 and CW-4 with their respective change in pH with the progress of fermentation where (A) Ensiling with heat treatment of algae and (B) Ensiling without heat treatment of algae.

Analysis of Single Chain Fatty Acids (SCFA)

To estimate the and quantify the amount of SCFA produced by the potential probiotic strains, the samples obtained from serum bottle fermentation and reactor experiments were analyzed using HPLC to detect acids such as lactic acid, acetic acid, propionic acid, butyric acid, malate, and ethanol. The acids detected and quantified are as seen in Figure 10, Figure 11and Figure 12 for individual substrate utilization, substrate combination and ensiling experiments with and without heat treatments respectively.

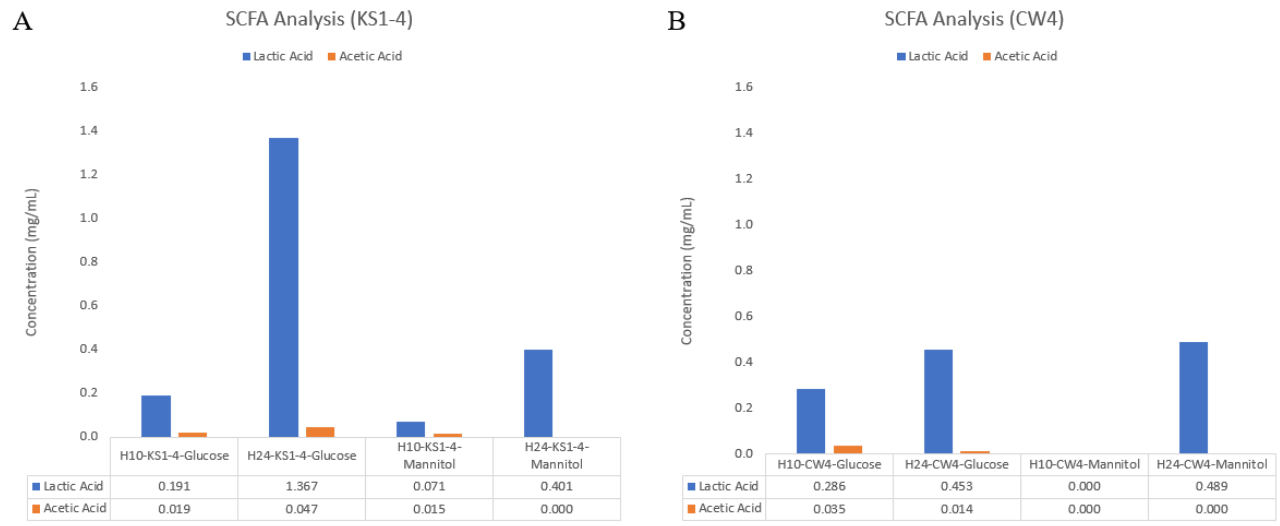
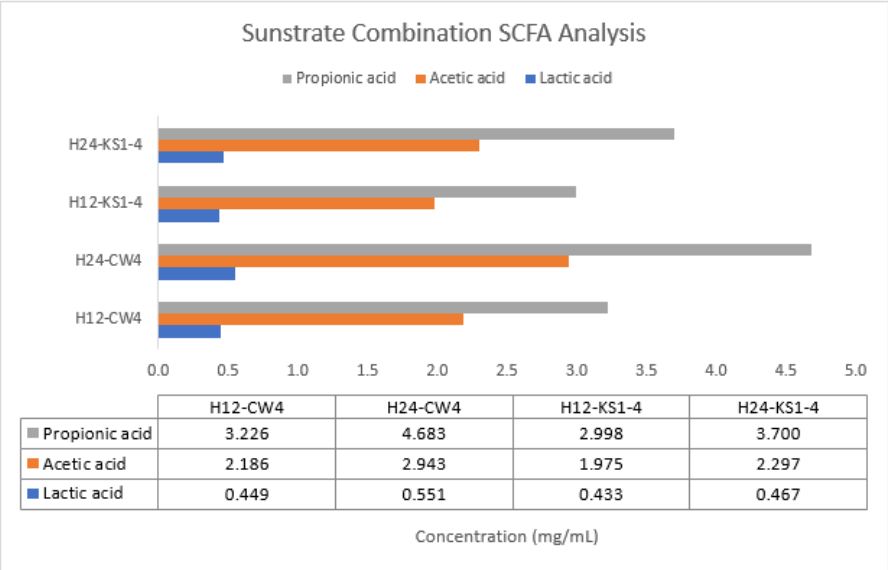


Figure 10. Graphs showing the amounts of lactic acid and acetic acid detected when measured using HPLC when glucose and mannitol were used independently as substrates. No other SCFA were detected in the samples. The samples are named with the Hour (H) & time (10 or 24) and the *Lactobacillus* strain name (KS1-4 or CW-4) followed by the pure substrate used (Glucose or Mannitol).



	H12-CW4	H24-CW4	H12-KS1-4	H24-KS1-4
Propionic acid	3.226	4.683	2.998	3.700
Acetic acid	2.186	2.943	1.975	2.297
Lactic acid	0.449	0.551	0.433	0.467

Figure 11. Production of SCFA - acetic acid, lactic acid and propionic acid as detected by HPLC when glucose and mannitol were used together as substrates. The samples are named with the Hour (H) & time (10 or 24) and the *Lactobacillus* strain name (KS1-4 or CW-4).

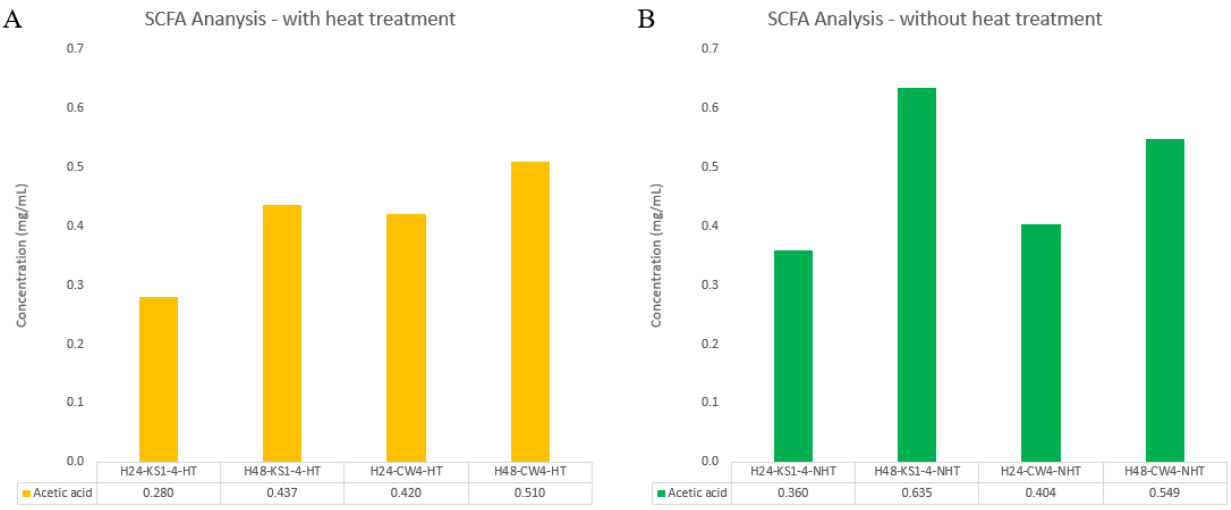
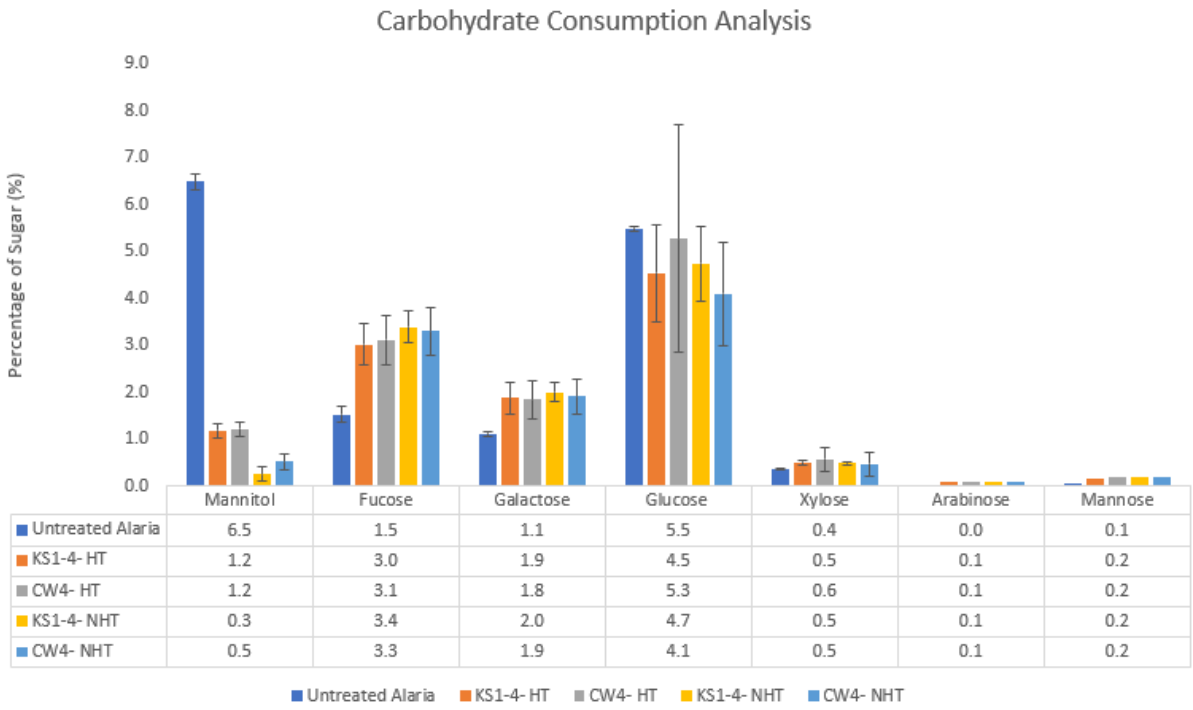


Figure 12. Production of SCFA - acetic acid in heat treated and non-heat-treated samples as detected using HPLC for ensiling experiments. Apart from acetic acid, no other SCFA was detected. The samples are named with the Hour (H) & time (10 or 24) and the *Lactobacillus* strain name (KS1-4 or CW-4) followed by the treatment – heat treated (HT) and non-heat-treated (NHT).

Carbohydrate Consumption Analysis

After 48 hours, the carbohydrate consumption of the bacteria utilizing macroalgae as substrate was analyzed using IC and the results obtained are as shown in Figure 13.



	Mannitol	Fucose	Galactose	Glucose	Xylose	Arabinose	Mannose
Untreated Alaria	6.5	1.5	1.1	5.5	0.4	0.0	0.1
KS1-4- HT	1.2	3.0	1.9	4.5	0.5	0.1	0.2
CW4- HT	1.2	3.1	1.8	5.3	0.6	0.1	0.2
KS1-4- NHT	0.3	3.4	2.0	4.7	0.5	0.1	0.2
CW4- NHT	0.5	3.3	1.9	4.1	0.5	0.1	0.2

Figure 13. Calculated percentage of monomeric sugars per dry weight of algae in different experimented samples as per NREL calculations where HT stands for heat treated and NHT stands for non-heat treated and the *Lactobacillus* strains used for experimentation are indicated as KS1-4 and CW4 accordingly.

Total Solids and Ash Content

The results of total solids and ash content estimation of the fermentation experiments performed using the NREL/TP – 5100-60957 (Van Wychen and Laurens, 2015) protocol are as seen in Table 1.

Table 1. Calculated percentage of Total solids, Moisture and Oven Dry Weight (ODW) of samples along with percentage of Ash as obtained from performing reactor experiments.

Freeze-dried Sample	% Total solids	% Moisture	ODW sample	% Ash
Untreated Algae	90.44 ± 0.31	9.56 ± 0.31	13.20 ± 1.66	38.70 ± 0.06
KS 1-4- HT	94.27 ± 0.22	5.73 ± 0.22	14.14 ± 0.29	13.58 ± 0.12
CW-4 HT	94.01 ± 0.15	5.99 ± 0.15	13.39 ± 1.16	15.92± 0.10
KS 1-4- NHT	94.25 ± 0.22	5.75 ± 0.22	12.91 ± 0.06	12.26 ± 0.04
CW-4 NHT	93.46 ± 0.34	6.54 ± 0.34	13.50 ± 0.53	13.31 ± 1.16

DISCUSSION

It is to be noted that as the novel strains with potential probiotic properties under consideration have not yet been genome sequenced, hence, they will be referred to as KS1-4 and CW4, respectively. Comments upon the possible identities of these strains based on the results obtained from this project will be made in the later parts of this section.

Serum Bottle Fermentation –Single Substrate – KS1-4

Figure 3 shows the growth curves obtained by supplying the bacterial strains with different concentrations of glucose and mannitol. From Figure 3.A. it could be seen that KS1-4 showed a shorter lag phase and began its log/exponential phase between 4-5 hours which lasted until 10 hours after which it was seen to move into the stationary phase or death phase depending upon the concentration of the remaining substrate. Consequently, KS1-4 did not show any significant growth for a concentration of 1 g/L and began approaching the death phase from 10 h for 5g/L due to running out of substrate. Substrate concentrations of 10 and 15 g/L facilitated optimal growth and in the case of 20 g/L of substrate, despite the nearly complete consumption of the substrate (as seen in Figure 4), the bacteria continued to grow even after 24 hours of cultivation. In the case of when mannitol was used as substrate, as seen from Figure 3.B., the 5 g/L sample growth was like that of when glucose was used as substrate. However, due to human error or possible cross contamination, the 1g/L sample showed unusual growth.

The 10,15 and 20 g/L samples showed growth curves similar to that of 20 g/L of glucose, however, they continued to grow even beyond 24 hours suggesting that the rate of consumption of mannitol for growth is slower than that of rate of consumption of glucose for growth. This can be supported by the fact that the final OD for 20 g/L at 24 hours for KS1-4 was 5.90 for glucose as substrate and 3.40 for mannitol as substrate.

To get a better understanding of the substrate consumption behavior of the bacteria, the samples obtained from the highest concentration cultures (20 g/L) were analyzed using IC and were compared with their respective OD as seen from Figure 4.A and Figure 4.B. From this analysis, it was seen that the OD curve for substrate utilization was similar for glucose and mannitol, however the substrate utilization curves varied between the substrates. A more expected curve nature was seen for glucose as in Figure 3.A. i.e., the glucose utilization was slower when the OD was lesser, which is when the bacteria would have been in the lag phase, and then rapidly increases once the bacteria progress into the log phase. Whereas for mannitol, the substrate utilization curve depicted a more rapid and steep curve implying that there is some lag for the bacteria to get adjusted to the new media prior to growth. Irrespective of this, once the bacteria are accustomed, it shows exponential substrate utilization. However, this observation could be biased as it is based on limited time points thereby invariably depicting a linear trend.

An interesting factor to note here is that despite the initial concentration of the substrates being 20 g/L, the measured IC values indicate higher values of 20 ±10 g/L for KS1-4, which is unusually high can be thought to be due to errors caused by pipetting or dilution. Nevertheless, the increased mannitol concentration at the beginning could indicate a new possibility that the LAB strain KS1-4 might be able to produce mannitol in addition to being able to consume mannitol! Based on previous studies conducted in the lab where this thesis was carried out, it is hypothesized that strain KS1-4 could be *L. plantarum*. Assuming that this is true, this could explain the increased mannitol content at the beginning of the cultivation, because, as stated in the background section, Yang *et.al.*, identified an increased expression of the genes *adhE* when *L. plantarum* was subjected to harsh environments and that this could be involved in offsetting the stress caused due to NADH accumulation. Owing to this, there is a possibility that the production of mannitol could occur utilizing fructose – an intermediate in glucose metabolism.

Serum Bottle Fermentation – Single Substrate – CW4

The growth curves for CW4 with glucose and mannitol as substrates can be seen from Figure 5.A and Figure 5.B. It can be inferred from the figures that the growth curve trends for both substrates are very similar to each other. However, it is to be noted that CW4 was seen to display a longer lag phase in comparison to KS1-4. This consequently yielded the late start of the log/exponential phase (between 6-7 h) and lasted up to 10 hours after which it was expected to move into the stationary or death phase depending upon the remaining substrate concentration. On the contrary, it was observed that there was exponential growth after the 10 h time point. This could indicate that the substrate utilization of CW4 is slower compared to that of KS1-4 owing to the longer lag phase of CW4. Yet, this could also have been a result of low concentration of cells in the inoculum. It was noted that the final OD measured at 24 hours for the sample with substrate concentration of 20 g/L of glucose was roughly twice as that of when 20 g/L mannitol was used as substrate. i.e. the final OD with glucose as substrate was found to be 13.50 whereas for that of mannitol as substrate was 7.20 thereby indicating the superior glucose utilization capabilities of CW4 in comparison to mannitol utilization of CW4.

Figure 6.A and 6.B, show the substrate utilization trend data of CW4 as obtained from IC analysis and compared with their respective growth curves. As stated earlier in the discussion, CW-4 shows a longer lag phase. However, from Figure 6.A, it can be seen that the substrate consumption of glucose begins at an earlier stage itself. Therefore, the reason as to why the growth curve does not represent the nearly linear consumption of glucose is a topic to be considered. However, a possible explanation for this could be that the cells are using the substrate for maintenance. As seen in Figure 6.B, as per expectation, mannitol is not immediately consumed and there is a certain delay before which it starts to get utilized for growth. This can be thought to be the time the bacteria take to get used to the new carbon source. But, after this there is a linear

consumption of mannitol. The dip in mannitol concentration at 8 h can be assumed to be due to dilution or pipetting errors and therefore is not important for discussion.

Serum Bottle Fermentation – Substrate Combination

To evaluate the substrate utilization capabilities of KS1-4 and CW4 when both the substrates i.e. glucose and mannitol are present together, a serum bottle experiment was performed where 10 g/L of glucose and 10 g/L of mannitol was mixed with modified MRS media. The growth curves obtained from the experiment can be seen in Figure 7. In comparison to the individual growth curves of KS1-4 and CW4 when mannitol and glucose were separately used as substrates, it was seen that when both the substrates were simultaneously present, the LAB strain KS1-4 utilized glucose wholly and utilized mannitol to a significant amount prior to being saturated with the consumption of mannitol. The LAB strain CW4 was seen to utilize both the substrates completely by the 12 h time point. Despite the IC data indicating the consumption of both the substrates, the OD measurements were very low compared to when the substrates were used independently. This can be attributed to the technical errors faced while using the spectrophotometer for OD measurements. Nevertheless, the exact substrate utilization mechanism in LAB when both glucose and mannitol are present together are not completely understood therefore making them more interesting topics to be studied further.

Ensiling Experiments – *Lactobacillus* strains KS-1-4 and CW-4

Based on assumptions from previous experiments conducted in the lab where this study was conducted – the strains under consideration are hypothesized to belong to the class of heterofermentative bacteria. The suggested mannitol metabolism pathway in heterofermentative lactic acid bacteria by Iwamoto *et.al.,* involves the synthesis of mannitol by the uptake and utilization of fructose with the aid of mannitol 2-dehydrogenase (*3a*) and avoiding the synthesis of mannitol-1-phosphatase. The reaction can be expressed as in Figure 14. In addition to this, mannitol metabolism is also influenced by the environment and regulatory factors which invariably influence the enzymatic activities. It is also interesting to note that the many phaeophycean algae like *Eisenia, Dictyota* and *Spatoglossum* have been able to naturally synthesize mannitol as a product of photosynthesis (Yamaguchi *et.al.,* 1969). Thus, indicating that these brown algae could also be considered for future studies along with *A. esculenta*.

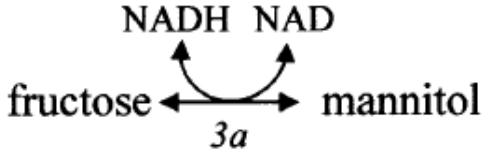


Figure 14. *Mannitol metabolism pathway in heterofermentative LAB. (Iwamoto et.al. 2005)*

Based on studies (unpublished) conducted in the lab where this thesis was performed, it has been noted that the total mono-sugars in *A. esculenta* were found to be 20.86 % (w/w) and uronic acids were found to be 14.69 % (w/w) as quantified using ion-exchange chromatography technique

As mentioned earlier in the background section, based on studies (unpublished) conducted in the lab where this thesis was performed, it has been noted that the A. esculenta comprise 9.16 ± 0.17 % dry weight of mannitol and 5.67 ± 0.07 % dry weight of glucose, thereby making them interesting substrates for fermentation utilizing LAB. It is with line of thought that the brown seaweed *A. esculenta* belonging to the same family was selected to be used for fermentation or ensiling experiments. Ensiling is a process used to facilitate the preservation of moist forage crops by using lactic acid bacteria where the pH drop prevents spoilage. The main products of this process have been reported to be organic acids, the primary one being lactic acid (Weinberg, Z.G. *et.al.*, 2003). In addition to this, to assess the impact of heat treatment upon the raw material and release of substrate into the medium, two conditions were employed – (i) heat treatment for 20 min at 121℃ and, (ii) no heat treatment i.e. the raw material was used without any prior treatment.

The growth curves and the pH drop recorded from the ensiling experiments are as seen in Figure 9. It was seen that as the fermentation process progressed, the growth of lactic acid bacteria caused the pH to drop from the range of 6.30 to 6.10 to the final recorded pH at 48 hours being in the range of 3.60 to 4.00 for heat treated samples and between 3.40 to 3.70 for non-heat-treated samples. In the beginning of the experiments, it was seen that the decline in pH was more rapid when the samples were not subjected to heat treatment as compared to when the samples were heat treated. This behavior could have been due to the growth of bacteria which are innately present along with the seaweed. Even though LAB are known to inhibit the growth of other bacteria, in this case too, it is true; except that the bacteria of interest may take time to compete for substrate with the other bacteria already present and then continue to proliferate.

The fluctuations observed on the growth curves can be assumed to be due to two circumstances. The first being that the minute algal particles which were obtained during sampling might have interfered with the absorbance. To try and correct this interference, the samples were subjected to centrifugation at very low speed for 2 minutes. However, the resultant filtrate still contained algal particles suspended in the solution and did not yield any valuable OD results. This also gave rise to the question if the bacteria are able to utilize the algal biomass as substrate by adhering to their surface or are they utilizing the substrate already present in the solution? To understand this better, larger pore sized filters (like 40 µm) can be used for filtration and consequently evaluated to verify if this question holds value. It would also be of interest to immobilize the algae within a hydrogel and subject it to LAB which can then be continuously monitored using advanced microscopic techniques like confocal microscopy to monitor the substrate consumption behavior of the LAB's in focus.

Mostaert *et.al.*, 1995 observed that the innate mannitol concentration decreased within an hour of exposing the alga to hyposaline conditions. This was reasoned by Karsten *et.al.*, 1997 that the reduction in salt stress disrupted the synthesis of mannitol by deactivating the enzyme mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase opposite to their order of activation. Consequently, mannitol 2-dehydrogenase and hexokinase performed the degradation of mannitol. This factor is important to consider when the pre-treatment of the algae is taken into consideration as this would also determine as to how much of the innately synthesized mannitol is actually available as the raw material for utilization by the LAB for growth.

Algae have been reported to have a defense mechanism upon being harvested where they release their intercellular stored mannitol component in the form of 'sap' into the environment as a brown liquid (Abdullah *et.al.*, 2004). It is in accordance with this fact, as seen in Figure 15, the reactor solution for both the conditions (heat-treated and non-heat-treated) used for ensiling were seen to be dark brown prior to the beginning of ensiling. However, towards the end of the process, the dark brown solution had turned to a nearly transparent light green solution. This brown coloration in the beginning could be assumed to be due to the presence of fucoxanthin which gives the algae its characteristic brown color. However, this cannot be assumed to be the only reasoning based on the color change seen at the end. This phenomenon can also be reasoned that this brown coloration which was observed at the beginning could have been the internal stress storage components of the alga which were released when subjected to hypotonic solution. From this it can be presumed that mannitol being the main product; and glucose, which are of interest were also released into the reaction medium. Therefore, when the LAB strains were introduced to begin the fermentation, they were able to utilize the substrates present in the medium and caused a dip in the pH as the reaction progressed. Owing to which the resultant final difference in the medium coloration can be an indicator of the success of the fermentation process and is valuable to be considered when aiming towards a food product or food formulation.

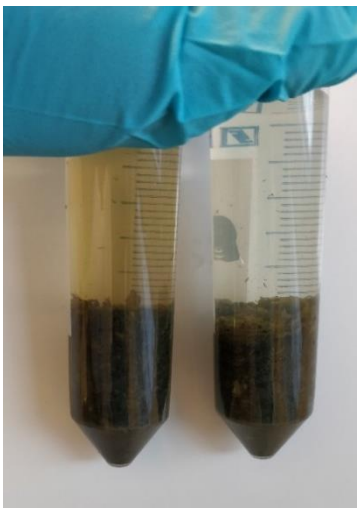


Figure 15. (Left) Brown coloration as obtained from negative control reactor versus (Right) Light green coloration obtained at the end of ensiling experiment from KS1-4/CW4.

Contamination is often one of the largest causes for economic losses in the food industry. Even in this study it was seen that two out of three times, the negative control reactors had fungal contamination. It would be expected that the non-heat-treated samples possess a possibility for fungal growth, but not the pre-heat-treated reactors. But this was not the case. It was seen that one reactor from the heat-treated experiment and one from the non-heat-treated experiment displayed fungal growth after 24 hours of fermentation (as seen in Figure 17 in Appendix II). The fungal growth was accompanied with slimy consistency of the algae and foul smell. It is unclear as to why the heat-treatment was unable to destroy all the fungal spores which might have been present with the algae. It would be an important factor when considering formulation of the food for human consumption and must be investigated further. This also highlighted the important function that LAB may act as spoilage control of micro-organisms when it comes to fungal contamination. Gerez *et.al.,* 2013 reported that *L. plantarum, L. brevis* and *L. casei* isolated from sourdough were shown to inhibit fungal growth with an inhibitory effect >80%. It was also found that the source of isolation for these strains did not alter their antifungal activity thereby explaining why the reactors inoculated with KS1-4 and CW4 during the ensiling experiment did not display any signs to fungal contamination.

It is valuable to note that despite no gas production being detected in the ensiling experiments, there was an internal pressure and 'pop' sound observed when the CW4 experiment was being conducted and concluded by detaching the gas measurement tubes, respectively. A possible explanation is that the gas produced was too little to be quantified or that there was some issue with the filter like clogging or loose connection of the tubes. This could also be an indication that CW4 is *L. brevis* which is well known to be a gas producing micro-organism.

Analyzing the supernatant of the samples obtained from the fermentation experiments, it was seen that final concentration of mannitol in the supernatant was close to half the initial concentration of mannitol thereby confirming the utilization of mannitol by LAB for their growth. It was interesting to note that irrespective of the method of treatment the algae were subjected to, significant quantities of mannitol were found in the medium. This also supports the finding by Abdullah *et.al.,* 2004, about mannitol being one of the components released into the environment when the algae are exposed to harsh conditions. However, it was also seen that glucose in valuable amounts was found in the medium only in heat-treated samples. Thereby indicating that heat-treatment plays an important role to make the innately present glucose more available to the bacteria rather than mannitol. Another interesting finding was that the strains KS1-4 and CW-4 seemed to consume galactose in its entirety in addition to consuming mannitol and glucose. Fucose too was consumed by CW4, however the consumption cannot be said to be of significant amounts.

Single Chain Fatty Acids (SCFA) Analysis

As seen in Figure 10, it was noted that among the acid detected, acetic acid and lactic acid were the major ones identified. After 24 hours of culture, it was found that when 20 g/L glucose was used as the substrate, KS-1-4 showed to produced more lactic acid in comparison to CW-4. However, when 20 g/L mannitol was used as substrate, CW-4 was recorded to produce more lactic acid. This can be assumed to be due to the extended lag phase of CW-4 due to which it begins to grow a bit later but more rapidly. This showed that the probiotic bacteria were able to utilize the substrate and proliferate thereby showing potential to be used for ensiling.

Subsequently, to imitate the substrate availability in algae, mannitol and glucose were fed to the bacteria (10 g/L each) and their growth recorded. From Figure 11, it was clearly seen that despite the low OD measurements, the bacteria were able to grow and produce acetic, propionic, and lactic acid as expected. The levels of acetic acid and lactic acid were seen to be similar in both the strains. However, the level of propionic acid was highly upregulated in CW4 in comparison to KS1-4. It is to be noted that even though lactic acid was detected in the substrate combination samples, the peaks were very small and thus the values had to be extrapolated as they did not fit into the scope of the standard curves. Thus, the quantification of these values may not be accurate.

Proceeding with the ensiling experiments as seen in Figure 12, it was seen that only acetic acid was detected from the analysis. The amount of acetic acid detected was seen to be higher in CW-4 when the algae was subjected to heat treatment than when it was not. Yet, KS-1-4 showcased higher acetic acid production when the algae was not subjected to heat treatment. But this finding does not eliminate the possibility that the increased acetic acid measurement of KS-1-4 could have been contributed by the initial growth of other bacteria present in the medium prior to inoculation. It would also make it interesting to identify the bacteria which might be present along with the algae at various time points – such as at harvest, when frozen, upon defrosting and prior to being used in the lab. The reason as to why lactic acid or propionic or butyric acid were not identified is a topic to be considered for further studies. Regardless, it did showcase the ability of KS-1-4 and CW-4 to utilize the substrate present in algae and bring about fermentation.

Carbohydrate Consumption Analysis

Carbohydrates extracted from algae by acid hydrolysis are reduced to mono-sugars like mannitol, glucose, fucose, galactose, xylose, arabinose, and mannose. Each of these components are stored in the cell walls of algae to withstand and survive harsh changes in climatic conditions. To assess if and how these sugars were utilized by the LAB, the freeze-dried samples obtained from the fermentation experiment as well as untreated *A. esculenta* samples were subjected to a two-step acid hydrolysis using sulfuric acid according to the LAP procedure (based on NREL/TP 5100-60957) where the complex polymeric forms of the carbohydrates were reduced to their monomeric subunits. The percentage of mono-sugars and mannitol quantified after analysis of the samples is as shown in Figure 13 and Table 2.

Table 2. Quantified percentage of sugars in fermentation experiment samples upon carbohydrate analysis. The samples are named with strain names and method of treatment- heat treated (HT) or non-heat treated (NHT) respectively.

Freeze-dried sample	Mannitol	Fucose	Galactose	Glucose	Xylose	Arabinose	Mannose
Untreated Algae	6.5	1.5	1.1	5.5	0.4	0.0	0.1
KS1-4- HT	1.2	3.0	1.9	4.5	0.5	0.1	0.2
CW4- HT	1.2	3.1	1.8	5.3	0.6	0.1	0.2
KS1-4- NHT	0.3	3.4	2.0	4.7	0.5	0.1	0.2
CW4- NHT	0.5	3.3	1.9	4.1	0.5	0.1	0.2

The significant difference in the mannitol content between the fermentation samples and untreated algal samples (as seen in Table 2) undoubtedly proves the utilization of mannitol as substrate by the LAB. Given that LAB was being pre-cultured in medium containing glucose as substrate, it would be presumed that upon introduction into the new medium containing algae, glucose being one of the substrates present would be consumed first. However, this does not seem to be the case as even though the percentage of glucose is lesser than that in untreated sample, it does not seem to be consumed in its entirety. A possible explanation for this could be that glucose is integrated into the cellular structure of algae in the form of the polysaccharide cellulose and therefore is not available for the bacteria for consumption even upon heat treatment. Instead, we see that mannitol is being utilized to the greatest extent supported by the significantly lower sugar percentage in the fermented samples. It could be a possibility that the enzymes responsible for the consumption of mannitol as substrate are preferred over the ones which are used for the utilization of glucose due to lesser energy expenditure or it could also be a method to close the redox balance to compensate the production of NADH. In the future, this would be interesting to correlate and affirm this finding by performing a metabolic profiling of the lactic acid bacteria in focus.

The quantities of xylose (0.5 %) and arabinose (0.1 %) can be seen to be relatively constant throughout the samples. However, the amount of mannose was seen to be two times more in the fermented samples in comparison to the untreated algal samples. Stévant *et.al.*, 2017 found that in

A. esculenta had a greater content of glucose and fucose in comparison to *Saccharina latissima* which showed to possess mannitol in abundance. However, the untreated *A. esculenta* sample in this study shows nearly the same percentage of mannitol and glucose, contrary to the earlier explained finding. Stévant *et.al.,* 2017 also evaluated the effect of seawater storage on the kelps and found that the levels of fucose and polyphenols was significantly reduced when subjected to this method of storage.

Total Solids and Ash Content Analysis

Total solids and moisture content analysis play an important role in the fields of food formulation and innovation. Total solids can be defined as "the dry matter left behind after removal of moisture". Water accounting for a major portion of Seaweeds makes it a valuable factor to be estimated and studied in order to analyze its influence upon the intended food product. The water or moisture content is known to influence the food preservation, stability, and quality. Ash content analysis provides an insight into the mineral profile of the food product under consideration. It is also useful for the nutritional, taxological, and economic evaluation. The ash content for plant foods is found to generally range from 0-12% (Nielsen, 1998).

The results from this study are as presented in *Table 1*. It was observed that the percentage of total solids of 94 % /DW was found to be constant throughout the fermentation samples. The untreated sample was seen to have 90 % /DW of total solids. This difference between the fermented and un-fermented samples could have occurred due to two reasons. The first being that there is a possibility that the samples were not freeze-dried to the same content therefore resulting in the difference. The second reason could be that the total solids in the fermentation samples are inclusive of the bacterial biomass which might have precipitated along with the algal biomass upon centrifugation prior to freeze drying. It is again in this case that it would be interesting to analyze if the bacteria access the substrates by adhering to the algae apart from being able to consume the ones already readily available in the medium.

Given that seaweeds are known to be high sources of vitamins, minerals, and trace elements it makes it useful to analyze the mineral content between the samples. Seaweeds like *A. esculenta* are known to be so rich in minerals that they have been suggested by research to hold potential to be used as a salt-replacing ingredient in food formulation. This replacement is beneficial as the seaweed consumption would reduce the Na/K ratio invariably resulting in a healthier mineral profile in comparison to the usual food substances which have an increased Na/K ratio (Stévant *et.al.,* 2017; López-López *et.al.,* 2009).

It was observed that the percentage of ash was significantly different in the fermented samples (12 to 16 %) in comparison to the untreated algal samples (39%) indicating that the bacteria consume valuable minerals during their growth. However, (unpublished) studies at a

partner institute involved in this project found that a significant amount of ash is solubilized into the soluble fraction of the slurry during the fermentation process thereby resulting in the decrease of the final ash content of the samples. Stévant *et.al.*, 2017 reported that they found the ash content of *A. esculenta* to be approximately 25 % which was in line with the previous findings in literature. However, it was seen in this study that the ash percentage in untreated sample was 39% which is higher than the previously reported percentages. A plausible explanation for this could be the difference in the place of origin of the seaweed as well as the time of harvest.

Implications for the Food Industry

- Mannitol being 50-70 % sweet as sucrose makes it a valuable substitute as an artificial sweetener for human consumption.
- Even though the percentage of amino acids in brown algae is reported to be lesser than that in red algae, brown algae are still hold the possibility to fulfill all the essential amino acid requirements for humans (Mæhre *et.al.*, 2014).
- *Alaria esculenta* comprising 40% of its DW in the form of alginates (Stévant *et.al.*, 2017) makes its an invaluable substitute for increasing the dietary fiber intake thereby enhancing the colonic and cardiovascular health (Brownlee *et.al.*, 2005).
- Utilization of probiotics like *L. plantarum, L. brevis and/or L. casei* for fermentation makes the intended food product potentially more beneficial to the human health and wellbeing when consumed. A good gut equals to a good mind!
- LAB like KS-1-4 and CW-4 can be used as spoilage control micro-organisms to control fungal contamination as well as an inhibitor or undesired bacteria.

CONCLUSION

From this study conducted to evaluate the effect of probiotic bacteria on the use of macroalgal substrate, it was seen that the probiotic bacteria under consideration in this project – KS-1-4 and CW-4 belonging to the class of lactic acid bacteria can indeed utilize the substrates glucose and mannitol which are innately present in the seaweed *A. esculenta* to ferment the seaweed. This highlighted the advantage of utilizing probiotics to ferment seaweed along with displaying how probiotics can be utilized to enhance the nutritional content of food products intended for human consumption. Having seen that both the bacterial strains displayed their ability to utilize mannitol and glucose as substrates accompanied with the production of gas, and the unique trend of increased mannitol content by KS-1-4 suggested that the identity of the strain KS-1-4 closely resembled *L. plantarum* and that of the strain CW-4 closely resembled *L. brevis.*

FUTURE WORK

Future work related to this project would involve the confirmation of the identities of the strains used for experimentation by genome sequencing. Upon confirmation of the identities, the strain KS-1-4 could be studied further to confirm its ability to synthesize mannitol during fermentation. Another important aspect would involve the study of how the LAB are utilizing the substrate present in algae i.e., if they access the substrates through adhesion to the algal surface or utilize the substate readily available in the medium. Later part of the project could involve genetic modification of the LAB to synthesize mannitol in addition to consuming it. In this approach, half of the quantity of LAB intended for inoculation can be genetically engineered to produce mannitol and the other half can utilize this additional substrate for growth and therefore bring about greater fermentation in a shorter duration. Upon characterizing the strains, it would be worthwhile to investigate the taste and texture of the fermented food product obtained using seaweed.

REFERENCES

Scientific Articles

1. Allahgholi, L., Sardari, R.R., Hakvåg, S., Ara, K.Z., 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.
2. Abdullah, Mohammed I., and Stein Fredriksen. "Production, respiration and exudation of dissolved organic matter by the kelp Laminaria hyperborea along the west coast of Norway." *Marine Biological Association of the United Kingdom. Journal of the Marine Biological Association of the United Kingdom* 84, no. 5 (2004): 887.
3. Brownlee, I.A., Allen, A., Pearson, J.P., Dettmar, P.W., Havler, M.E., Atherton, M.R. and Onsøyen, E., 2005. Alginate as a source of dietary fiber. *Critical reviews in food science and nutrition*, 45(6), pp.497-510.
4. Bruhn, A., Brynning, G., Johansen, A., Lindegaard, M.S., Sveigaard, H.H., Aarup, B., Fonager, L., Andersen, L.L., Rasmussen, M.B., Larsen, M.M. and Elsser-Gravesen, D., 2019. Fermentation of sugar kelp (Saccharina latissima)—effects on sensory properties, and content of minerals and metals. *Journal of Applied Phycology*, *31*(5), pp.3175-3187.
5. Caplice, E. and Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. *International journal of food microbiology*, *50*(1-2), pp.131-149.
6. Cebeci, A. and Gürakan, C., 2003. Properties of potential probiotic Lactobacillus plantarum strains. *Food Microbiology*, *20*(5), pp.511-518.
7. De Angelis, M. and Gobbetti, M., 2016. Lactobacillus spp.: general characteristics.
8. Deniaud-Bouët, E., Hardouin, K., Potin, P., Kloareg, B. and Hervé, C., 2017. A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges. *Carbohydrate polymers*, 175, pp.395-408.
9. 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. *International Dairy Journal*, *16*(9), pp.1018-1028.
10. Gerez, C.L., Torres, M.J., De Valdez, G.F. and Rollán, G., 2013. Control of spoilage fungi by lactic acid bacteria. *Biological Control*, 64(3), pp.231-237.
11. Graiff, A., Ruth, W., Kragl, U. and Karsten, U., 2016. Chemical characterization and quantification of the brown algal storage compound laminarin—A new methodological approach. *Journal of applied phycology*, 28(1), pp.533-543.
12. Hannon, M., Gimpel, J., Tran, M., Rasala, B. and Mayfield, S., 2010. Biofuels from algae: challenges and potential. *Biofuels*, 1(5), pp.763-784.

13. Hill, D., Sugrue, I., Tobin, C., Hill, C., Stanton, C. and Ross, R.P., 2018. The Lactobacillus casei group: history and health related applications. *Frontiers in microbiology*, 9, p.2107.
14. Ibrahim, S.A., 2016. Lactic Acid Bacteria: Lactobacillus spp.: Other Species.
15. Iwamoto, K. and Shiraiwa, Y., 2005. Salt-regulated mannitol metabolism in algae. *Marine Biotechnology*, 7(5), pp.407-415.
16. Jönsson, M., Allahgholi, L., Sardari, R.R., Hreggviðsson, G.O. and Nordberg Karlsson, E., 2020. Extraction and modification of macroalgal polysaccharides for current and next-generation applications. *Molecules*, 25(4), p.930.
17. Karsten, U., Barrow, K.D., West, J.A. and King, R.J., 1997. Mannitol metabolism in the intertidal mangrove red alga Caloglossa leprieurii: salinity effects on enzymatic activity. *Phycologia*, 36(2), pp.150-156.
18. Kim, K.T., Yang, S.J. and Paik, H.D., 2021. Probiotic properties of novel probiotic Levilactobacillus brevis KU15147 isolated from radish kimchi and its antioxidant and immune-enhancing activities. *Food Science and Biotechnology*, pp.1-9.
19. Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W. and Stiekema, W., 2003. Complete genome sequence of Lactobacillus plantarum WCFS1. *Proceedings of the National Academy of Sciences*, *100*(4), pp.1990-1995.
20. Leliaert, F., Smith, D.R., Moreau, H., Herron, M.D., Verbruggen, H., Delwiche, C.F. and De Clerck, O., 2012. Phylogeny and molecular evolution of the green algae. *Critical reviews in plant sciences*, 31(1), pp.1-46.
21. Lim, S.J., Mustapha, W.A.W., Maskat, M.Y., Latip, J., Badri, K.H. and Hassan, O., 2016. Chemical properties and toxicology studies of fucoidan extracted from Malaysian Sargassum binderi. *Food science and biotechnology*, 25(1), pp.23-29.
22. López-López, I., Bastida, S., Ruiz-Capillas, C., Bravo, L., Larrea, M.T., Sánchez-Muniz, F., Cofrades, S. and Jiménez-Colmenero, F., 2009. Composition and antioxidant capacity of low-salt meat emulsion model systems containing edible seaweeds. *Meat Science*, 83(3), pp.492-498.
23. Mæhre, H.K., Malde, M.K., Eilertsen, K.E. and Elvevoll, E.O., 2014. Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. *Journal of the Science of Food and Agriculture*, 94(15), pp.3281-3290.
24. Marco, M.L., Bongers, R.S., De Vos, W.M. and Kleerebezem, M., 2007. Spatial and temporal expression of Lactobacillus plantarum genes in the gastrointestinal tracts of mice. *Applied and environmental microbiology*, 73(1), pp.124-132.
25. Mautner, H.G., 1954. The chemistry of brown algae. Economic Botany, 8(2), pp.174-192.
26. Mostaert, A.S., Karsten, U. and King, R.J., 1995. Inorganic ions and mannitol in the red alga Caloglossa leprieurii (Ceramiales, Rhodophyta): response to salinity change. *Phycologia*, 34(6), pp.501-507.

27. Neves, A.R., Ramos, A., Shearman, C., Gasson, M.J. and Santos, H., 2002. Catabolism of mannitol in Lactococcus lactis MG1363 and a mutant defective in lactate dehydrogenase. *Microbiology*, 148(11), pp.3467-3476.
28. Rönkä, E., Malinen, E., Saarela, M., Rinta-Koski, M., Aarnikunnas, J. and Palva, A., 2003. Probiotic and milk technological properties of Lactobacillus brevis. *International journal of food microbiology*, *83*(1), pp.63-74.
29. Rosell, K.G. and Srivastava, L.M., 1984. Seasonal variation in the chemical constituents of the brown algae Macrocystis integrifolia and Nereocystis luetkeana. *Canadian journal of botany*, 62(11), pp.2229-2236.
30. Reed, R.H., Davison, I.R., Chudek, J.A. and Foster, R., 1985. The osmotic role of mannitol in the Phaeophyta: an appraisal. *Phycologia*, 24(1), pp.35-47.
31. Sardari, R.R. and Nordberg Karlsson, E., 2018. Marine poly-and oligosaccharides as prebiotics. *Journal of agricultural and food chemistry*, 66(44), pp.11544-11549.
32. Schiener, P., Black, K.D., Stanley, M.S. and Green, D.H., 2015. The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta. *Journal of Applied Phycology*, 27(1), pp.363-373.
33. Shiroma, R., KoniShi, T., Uechi, S. and TaKo, M., 2008. Structural study of fucoidan from the brown seaweed Hizikia fusiformis. *Food science and technology research*, 14(2), pp.176-182.
34. 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.
35. Tong, Y., Zhai, Q., Lu, W., Tian, F., Zhao, J., Zhang, H. and Chen, W., 2017. New insights in integrated response mechanism of Lactobacillus plantarum under excessive manganese stress. *Food Research International*, *102*, pp.323-332.
36. Viola, R., Nyvall, P. and Pedersen, M., 2001. The unique features of starch metabolism in red algae. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 268(1474), pp.1417-1422.
37. Weinberg, Z.G. and Ashbell, G., 2003. Engineering aspects of ensiling. *Biochemical Engineering Journal*, 13(2-3), pp.181-188.
38. Yang, X., Teng, K., Su, R., Li, L., Zhang, T., Fan, K., Zhang, J. and Zhong, J., 2019. AcrR and Rex control mannitol and sorbitol utilization through their cross-regulation of aldehyde-alcohol dehydrogenase (AdhE) in Lactobacillus plantarum. *Applied and environmental microbiology*, 85(4).
39. Yamaguchi, T., Ikawa, T. and NISIZAWA, K., 1969. Pathway of mannitol formation during photosynthesis in brown algae. *Plant and cell physiology*, 10(2), pp.425-440.

Website References

1. Algae Base. 2021. Alaria esculenta (*Linnaeus*) Greville 1830. [online] Available at: <https://www.algaebase.org/search/species/detail/?tc=accept&species_id=82> [Accessed 24 March 2021].

Books

1. Caballero, B., Finglas, P. and Toldrá, F., 2015. *Encyclopedia of food and health*. Academic Press.
2. Barba, F.J., Ahrné, L., Xanthakis, E., Landerslev, M.G. and Orlien, V., 2018. *Innovative technologies for food preservation*. In Innovative Technologies for Food Preservation (pp. 25-51). Academic Press.
3. Kılınç, B., Cirik, S., Turan, G., Tekogul, H. and Koru, E., 2013. Seaweeds for food and industrial applications. *In Food industry*. IntechOpen.
4. Nielsen, S.S. ed., 1998. *Food analysis* (p. 630). Gaithersburg: Aspen Publishers.

Documents

1. NREL/TP-5100-60956 – https://www.nrel.gov/docs/fy16osti/60956.pdf
2. NREL/TP-5100-60957 – https://www.nrel.gov/docs/fy16osti/60957.pdf

APPENDIX I

Chemical Name	Molecular Formula	Supplier	CAS	Purity (%)
Ammonium citrate dibasic	C6H14N2O	Sigma Aldrich	3012-65-5	98
Sodium acetate	C2H3NaO2	Sigma Life Science	127-09-3	≥99.0
Magnesium sulfate heptahydrate	MgO4S.7H2O	Sigma Aldrich	10034-99-8	-
Manganese sulfate monohydrate	MnO4S.H2O	Sigma Aldrich	10034-96-5	-
Potassium phosphate dibasic	K2HPO4	Sigma Aldrich	7758-11 4	-
Tween 80	-	Sigma Life Science	9005-65-6	-
Yeast Extract	-	Sigma Aldrich	8013-01 2	-
Meat Extract	-	Sigma Aldrich	70164-500G	-
Peptone from casein, tryptic digest	-	Sigma Aldrich	91079-40-2	-
D(+) Glucose	C6H12O6	Sigma	50-99-7	≥99.5
D-Mannitol	C6H14O6	Sigma		≥99.0

Chemical Name	Molecular Formula	Supplier	CAS	Purity (%)
D-Mannitol	C6H14O6	Sigma	69-65-8	≥99.0
D-(+)- Glucose	C6H12O6	Sigma	50-99-7	≥99.5
D-(+)- Galactose	C6H12O6	Sigma Aldrich	59-23-4	≥99.0
D-(+)-Mannose	C6H12O6	Fluka Analytical	3458-28-4	≥99.0
L-(-)- Fucose	C6H12O5	Sigma Life Science	2438-80-4	≥99.0
D-(+)-Xylose	C5H10O5	Sigma	58-86-8	≥99.0
L-(+)- Arabinose	C5H10O5	Sigma	5328-37-0	≥99.5

APPENDIX II

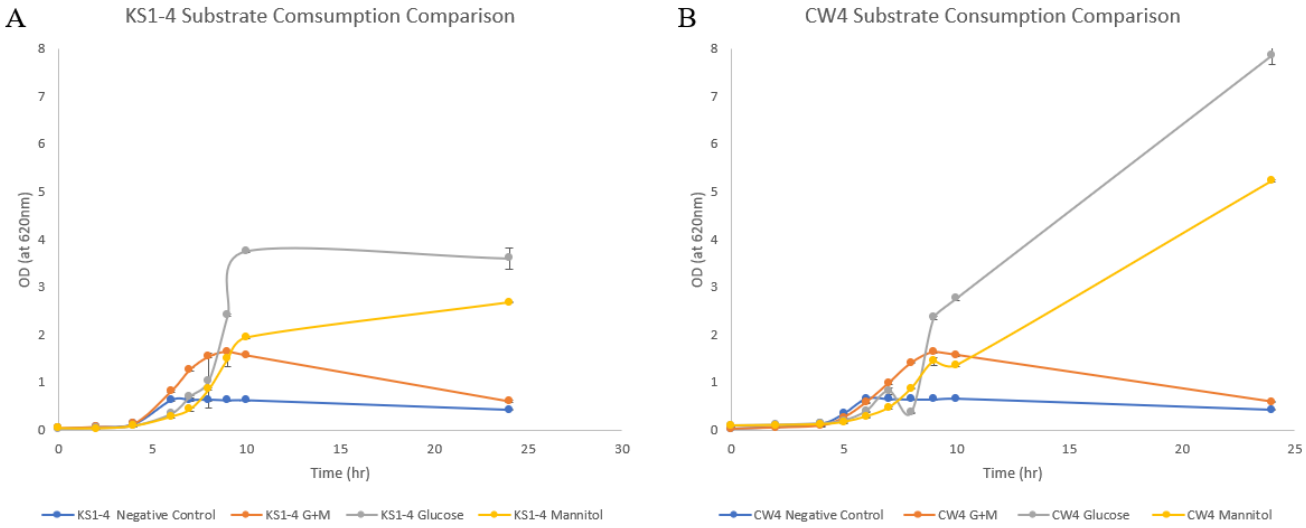


Figure 16. Growth curves of KS-1-4 and CW-4 when various combinations of the substrates glucose and mannitol were used (10 g/L for all).

Figure 17. Image showing Fungal contamination in the negative control reactors with thier dates.