LUNDS UNIVERSITET
Lunds Tekniska Högskola

Master's Programme in Food Technology and Nutrition

MASTER THESIS

Developing microscopy methods for evaluating bio-processed Brewers Spent Grain (BSG)

by

Sera Susan Jacob

Abstract Spent grain (BSG) is the solid residue obtained during brewing containing leftover malt and adjuncts. Though nutrient dense,it is found to negatively impact texture and colour when used its raw form as an ingredient in food. Bio-processing presents an interesting method to alter the structure of BSG, rendering it a possible novel food ingredient to increase the nutrient capacity of products along with improving sustainability in brewing. Three different types of BSG were treated with a xylanase enzyme and separate LAB strains in various combinations. In order to understand the effect of the several treatments, detailed study of the general micro-structure of BSG was conducted. All sample types were studied for micro-structural changes using both CLSM and light microscopy. Micrographs obtained showed that most effective micro-structural degradation was seen at higher temperatures when enzyme treatment was followed by LAB fermentation by the A16 LAB strain.Attempts were also made to analyze EPS slime formation by certain LAB strains on BSG and its characteristics. Light microscopy proved to be most effective in this case. A general comparison of the effectiveness of the microscopy methods was done to conclude the study.
Keywords : micro-structure, CLSM, immuno-labelling, β - glucan, arabinoxylan, cell wall, EPS

KLGM01
Master's Thesis (30 credits ECTS)
July 2019
Supervisors:Dr.Björn Berganstahl (LTH, Lund University) and Dr.Annika Krona (Senior Scientist, RISE)
Examiner: Dr. Lars Nilsson (LTH, Lund University)
Word Count: 12547 words, 73057 characters

Acknowledgements

My thesis was part of the FUNBREW project funded by FORMAS through EU's Horizon 2020 Research & Innovation Programme under grant agreement no. 727473. The opportunity to work as a part of this project was a a true privilege.

I would like to express my sincere gratitude and pure admiration to my supervisor at RISE, Dr. Annika Krona. The enthusiasm and passion she shows towards her work was truly infectious and inspirational. Without her guidance and constant encouragement, my work would not have been as successful.

I would also like to thank my University supervisor , Dr. Björn Berganstahl for his support , help and valuable inputs throughout my thesis period. In addition, I thank my examiner, Dr. Lars Nilsson for taking the time to review my work and give his thoughtful feedback.

I would further like to thank all the employees and other diploma workers at the department of Product Design and Perception, RISE. Your presence and support brought joy to every day I worked at RISE - especially Annika Altskär and Camilla Öhgren for helping me out with the microscopes.

I'd also like to thank my friends, for making my days in Gothenburg happy, light and fun.

Last, but never the least my family,for their constant prayers and words of motivation. Words cannot describe how grateful I am for all of you.Thank you for believing in me relentlessly.

Popular Science Summary

With growing consumer knowledge on health and nutrition, grains such as barley have been gaining consumer acknowledgement and interest as a food ingredient.A main constituent in brewing, barley has a high content of β -glucan and a considerable concentration of starch,protein, vitamins and other components of nutritional importance

Though one of the most popular and heavily consumed beverages today, beer and brewing processes still have a long way to go in terms of environmental consciousness, sustainability and minimal waste generation. Spent grain (BSG) is a collective term that includes barley grain husks and adjuncts obtained as solid residue during brewing. Currently, it accounts for more than 85% of the waste generated by breweries. Several studies have been conducted previously where BSG has been used as an ingredient in products such as bread, cakes and muffins. However, the success of these were limited as BSG was found to significantly alter the final colour and texture. Altering the micro-structure could result in a product that is milder and malleable in terms of utility.

Bio-processing represents the industrial practice of biotechnology,using cell or enzyme systems to produce new substances reactions that would modify natural substances. It presents an interesting approach towards changing the micro-structure of BSG.To test the effectiveness of any particular bio-processing technique in the sample of interest, micro-structure study is crucial.

BSG samples processed in different conditions were frozen in liquid nitrogen and sectioned onto microscope slides.These were then labelled using specific antibodies for confocal laser scanning microscopy(CLSM, a type of fluorescence microscopy). A liquid stain solution mixture was used to visualize starch and proteins in the BSG using light microscopy. The effect of different temperatures on enzyme treatment were also analyzed. Based on comparative study, optimal micro-structural degradation was observed in samples treated with a combination of enzyme treatment and lactic acid bacteria (LAB) fermentation. Different staining and visualization techniques were also attempted to observe slime formation due to some LAB secretions using BSG as a substrate. Light microscopy was understood to be most effective in visualizing the slime.More studies need to be conducted within this space.

Contents

1 Preface **8**

2 Introduction **9**

2.1 Aim and Scope 9

2.2 Background 9

2.2.1 The brewing process 10

2.2.2 Chemistry of spent grain 11

2.2.3 Role of bio-processing and EPS formation 13

2.2.4 Sample preparation 14

2.2.5 Confocal laser scanning microscopy 14

2.2.6 Light Microscopy 15

2.3 Outline of the Thesis 16

3 Methodology **17**

3.1 Sample Preparation 17

3.2 Confocal Laser Scanning Microscopy 18

3.2.1 Immunolabelling 19

3.2.2 Other staining techniques 20

3.3 Light Microscopy 20

3.3.1 Staining techniques 20

3.4 Enzyme treatment 21
3.5 Microbial culturing 21

4 Results **23**

4.1 Spent grain 23
4.2 Dugges 24
4.2.1 CLSM 24
4.2.2 Light Microscopy 25
4.3 Senson 26
4.3.1 CLSM 26
4.3.2 Light Microscopy 29
4.4 Microbial culturing - EPS study 31
4.4.1 CLSM 31
4.4.2 Light Microscopy 32
4.5 Enzyme Treatment 33
4.5.1 CLSM 33
4.5.2 Light Microscopy 34
4.6 Peroni 34
4.6.1 CLSM 35
4.6.2 Light Microscopy 38

5 Discussion **39**

6 Conclusion **40**

A Types of spent grain **41**

B Spectra of stains/Abbreviations **43**

List of Figures

2.1 Different layers of the barley grain (Source:Buglass et al., 2010) 11

2.2 Immuno-labelled outer layers of barley on CLSM (Source: Dornez et al., 2011) . 12

2.3 Schematic diagram of a CLSM setup (Source: Rossetti et al., 2013) 14

2.4 Schematic diagram of a light microscope (Source: A Lavanya et al., 2017) 15

List of Tables

4.1 Micro-structure of spent grain using LM 23

4.2 Effect of enzyme treatment using CLSM 25

4.3 Effect of enzyme treatment using light microscopy (LM) 25

4.4 Bio-processing using enzyme and A16 strain (type 1) - CLSM 27

4.5 Bio-processing using enzyme and 20193 strain (type 1) - CLSM . . . 28

4.6 Bio-processing using enzyme and A16/20193 strain (type 1)- LM . . 29

4.7 Bio-processing using enzyme and A16/20193 strain (type 2) - LM . . 30

4.8 Visualizing EPS slime on spent grain - LM 32

4.9 Influence of temperature during enzyme treatment on spent grain - CLSM 33

4.10 Influence of temperature during enzyme treatment on spent grain - LM 34

4.11 Effect of trial - I on spent grain - CLSM 36

4.12 Effect of trial - II on spent grain - CLSM 37

4.13 Effect of trial I and II on spent grain - LM 38

A.1 Spent Grain samples from Dugges 41

A.2 Spent Grain samples (Trial - I) from Peroni, Italy 41

A.3 Spent Grain samples (Trial - II) from Peroni, Italy 41

A.4 Spent Grain samples from Senson, Finland 42

B.1 Emission spectra of stains tested 43

1

Preface

The Master Thesis was performed at RISE at the Department of Food and Agriculture in the division of Product Design and Perception. It is part of FUNBREW, a SUSFOOD2 ERA-NET project. The project aims at improving the bio-functionality of Brewers Spent Grain (BSG); a unique by-product of brewing with immense potential as a food ingredient due to its unique flavour and nutrient profile. The master thesis contributed towards this by developing microscopy techniques that could effectively visualize micro-structural changes as a result of bio-processing.

Barley has started gaining consumer interest as a food ingredient due to its complex chemistry and nutrient composition. BSG is the solid residue obtained during brewing and mostly contains crushed barley and adjuncts. BSG presents poor technological performance, with detrimental effects seen on texture, flavour and colour in particular even after minimal processing prior to usage (Mussato et al., 2006).Bio-processing is a technology that is currently feasible and possibly capable of altering the matrix.Based on the positive impact of bio-processing on other grains, similar effects are hoped to be seen in the processed BSG. The synergistic action of lactic acid bacteria(LAB) and the enzyme is expected to bring about this change.

Microscopy studies were conducted in an attempt to understand the micro-structure and how it changes with different treatment protocols utilizing the selected xylanase enzyme and strains of LAB. Both confocal laser scanning microscopy (CLSM) and light microscopy were carried out consistently in order to obtain complete and complimentary data. Different techniques to effectively visualize metabolites like exo-polysaccharides (EPS) produced by the bacteria using spent grain as a substrate were also studied in detail. This slime is understood to behave like a hydrocolloid and contribute towards improving the structural and technological properties of food (Caggianiello et al.,2016). It is hoped that these studies will help understand the micro-structure better and in the future, create products that better utilize the processed spent grain as a nutrient-rich and useful ingredient.

Full-time work spanning a total duration of twenty-five weeks at the laboratory facility located in Gothenburg, Sweden was required to fulfill the aims and objectives set for the thesis.

2

Introduction

2.1 Aim and Scope

The Master thesis was conducted with the intention to develop microscopy methods that could help visualize and fully comprehend the effects of different bio-processing techniques on the overall micro-structure of spent grain.

Samples of spent grain sourced from three different breweries and malt producers were processed in Finland and Italy using different combinations of enzyme and bacteria. These were studied for micro-structural changes using CLSM and light microscopy.CLSM allows for thicker section to be studied with minimal background interference. Along with lightmicroscopy, it was hoped that a holistic understanding of microstructural changes could be acheived. The effect of temperature on enzyme treatment and how that would affect the micro-structure was also analyzed. Lastly, attempts to effectively stain the secondary metabolites (exo-polysaccharides) secreted by selected LAB strains were conducted. The effect of such secretions by selected LAB strains due to fermentation on the overall micro-structure of BSG was also attempted to be studied using microscopy studies. In order to obtain a more comprehensive picture, a general comparison of the three different types of spent grain was done to conclude.

2.2 Background

Beer is an alcoholic beverage made with barley malt as a primary ingredient, contributing significantly to its flavour and aroma profile.Utilizing many ingredients, the waste generated by the brewery industry is also quite massive; making it clear that beer and brewing processes still have a long way to go in terms of environmental consciousness, sustainability and minimal waste generation (D. Farías et al., 2017).

2.2.1 The brewing process

Brewing refers to the process of manufacturing beer. Depending on the scale of the industry, it could be either commercial or artisanal.Each ingredient in beer serves a specific function. Barley provides the starch which is converted to maltose and other sugars and subsequently to alcohol and carbon dioxide in the final steps (Kavindra Singh et al., 2016).

The brewing process can be summarized into the following steps - these could vary based on the type of beer and the scale of brewing:

1. **Malting and milling**: Malting is the prerequisite step for barley before it is subjected to brewing. It involves stepping and germination to help develop malt enzymes which would later cause modifications in the structure of the barley endosperm by rupturing the cell walls and the protein matrix (Kavindra Singh et al., 2016).The germinated barley is then dried and milled. Milling is done to improve the mashing process as it allows the endogenous enzymes of the aleurone layer to act on the starchy endosperm and alter the protein matrix(Kavindra Singh et al., 2016).

2. **Mashing and lautering**: Following the process of malting, the barley malt is mixed with adjuncts (rice, corn etc), salts and supplementary enzymes as required with a controlled quantity of liquor (water) at a chosen temperature (E.Briggs et al., 2004). This step is known as mashing.Depending on the region, the type and quality of the ingredients as well as the type of beer, the mashing technique varies to a great extent.In most Central Europe - origin beers, temperature programmed infusion mashing is conducted (E.Briggs et al., 2004).During temperature programmed infusion mashing, a thin liquid mash is heated in a jacketed tun at a temperature range of 45-55°C (James S. Hough, 1991). Based on the end product requirement , the temperature of the mash is then raised using a predetermined program in order to achieve adequate proteolysis and starch conversion. Once this step is completed, the mash mixture is then transferred to the lauter tun at 72°C.

   During lautering, the mash is successfully separated into the sweet wort and the residual spent grain solids. To facilitate this, the lauter tun is required to have a porous bottom to allow effective drainage. In this two-step process, both filtration and extraction is efficiently achieved (Smith & Hui, 2004). Important factors to consider in order to control the quality and final character of the separated products include the permeability of the perforated bed as well as the pressure applied. In more recently adopted brewing practices, mashing and lautering have been combined and conducted in the same vat using specialized stirrers with minimal shear impact (Smith & Hui, 2004). Spent grain is obtained after the lautering step.

3. **Boiling and fermentation**: Hops as pellets or in its further concentrated extract form is added to the sweet wort that has been separated and boiled in order to inactivate all active enzymes among other desire outcomes(E.Briggs et al., 2004). This is done to release the aroma and flavour compounds from the hops.The wort is then cooled and transferred into a fermentation tank. Yeast is added so that the available sugars are converted into ethanol and carbon dioxide (E.Briggs et al., 2004). This gives beer its sparkle and begins ethanol production as well.

4. **Maturation, filtration and pasteurization**: Following fermentation, the young beer is allowed to develop the required body, mouthfeel and flavour profile characteristic to the beer type and region manufactured (E.Briggs et al., 2004). This is done by allowing it to mature in similar tanks, or even specialized barrels based on the final profile required. The time and conditions chosen are also highly variable.The beer is then filtered to remove any suspended yeast cells, leftover hops and malt. Lastly, it is subjected to pasteurization in order to render it microbiologically safe for consumption and packed into bottles, cans or kegs.

2.2.2 Chemistry of spent grain

In recent times, there is a renewed interest seen in offbeat grains such as barley.A whole grain known to contain several micro-nutrients, barley is a rich source of important polysaccharides such as arabinoxylans,β -glucans and dietary fibres.When compared to other grains, barley contains the highest levels of β -glucan (Nikkhah, 2012). A representative diagram indicating the different layers is given in figure 2.1.

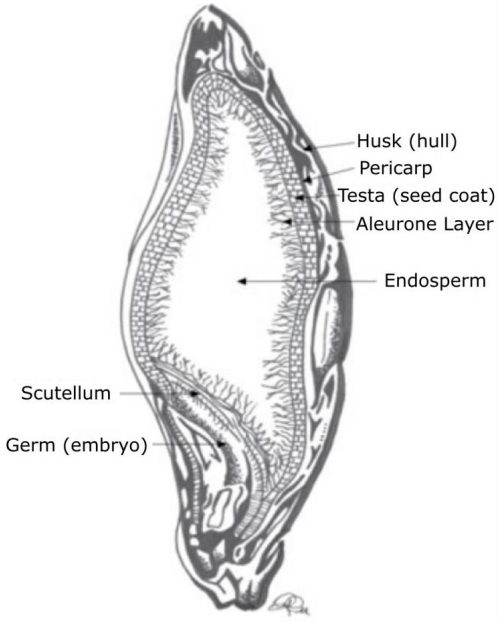
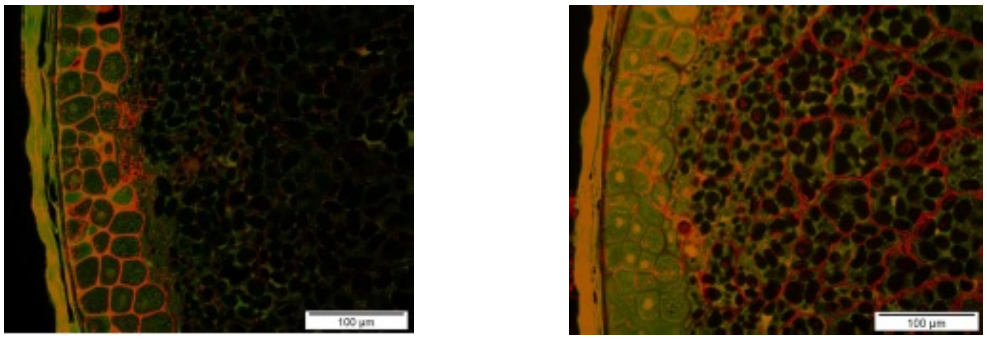


Figure 2.1: Different layers of the barley grain (Source:Buglass et al., 2010)

Based on a recent study conducted on the micro-structure of several grains using different staining techniques, a reference micrograph of selected sections of barley (figure 2.2) was obtained (Dornez et al., 2011).The pericarp, testa , aleurone, sub-aleurone region and endosperm were discernable.In order to achieve localized fluorecence, the grains studied had been immuno-labelled with fluorescent antibodies targeting the important polysaccharides.The polysaccharides of importance in these regions are arabinoxylans and β -glucans.Barley arabinoxylans consist of D-xylanopyranosyl units linked by α -(1-4) bonds forming a molecular backbone and connected to single L-arabinofuranose branches by $\alpha(1\rightarrow2)$ or α -$(1\rightarrow3)$ linkages.The arabinoxylans are mostly found in the aleurone layer (Fig 2.2. (a)).The level and character of the arabinoxylan present in the endosperm tissue differs from the aleurone layer varies average ratio of 1-1.1 to 1.9-2.2 respectively.



(a) Monoclonal arabinoxylan (in red) *(b) Mixed-linkage β -glucan (in green)*

Figure 2.2: Immuno-labelled outer layers of barley on CLSM (Source: Dornez et al., 2011)

Barley β -glucans [(1,3)(1,4)-β -D-glucans] are mostly bound through protein – polysaccharide linkages and phenol-ester linkages formed between the β - glucans, protein and ferulic acid (Jin et al., 2004). The starchy endosperm region of barley is understood to contain more than 70% of the β - glucans (Fig 2.2 (b)).

Barley is subject to malting before being used in brewing .During malting and the subsequent steps involved, the β -glucans are made soluble due to the presence of endogenous enzymes as well as specific processing conditions. The extent of this and type of β -glucan solubilised however, is entirely dependent on the processing and is seen to vary between breweries based on their unique processing parameters (Jin et al., 2004).Spent grain (BSG) is a collective term that majorly consists of barley grain husks and adjuncts obtained as solid residue after the production of wort during brewing. Currently, it accounts for more than 85% of the waste generated by breweries (Xiros et al, 2012).Spent grain from the production of lager beer is used in this project.

As sustainability of manufacturing processes is a growing concern among both industries as well as consumers, studies are now being conducted on effective utilization of the waste BSG. According to recent studies and findings, spent grain is either used as animal feed or subjected to solvent-intensive extraction processes in order to obtain different compounds.The success of these in food for human consumption is limited as the original micro-structure of BSG was found to significantly alter the colour and texture of baked goods(Mussato et al., 2006). Altering the micro-structure would result in a product that is milder and malleable in terms of utility.

Bio-processing presents an interesting approach towards changing the structure of BSG.

2.2.3 Role of bio-processing and EPS formation

Bio-processing represents the industrial practice of biotechnology,integrating the biological, chemical and engineering sciences by using cell or enzyme systems to produce new compounds or catalyze reactions that would modify natural or synthetic substances (Owen P. Ward, 1991). Both fermentation and enzyme treatment are techniques in bio-processing. The two bio-processing treatments used in this study were enzyme treatment alone and in combination with controlled fermentation using selected bacterial strains.Formation of EPS slime as a result of controlled fermentation by capable strains were also utilized.

Enzyme treatment refers to the method by which chosen enzymes are added to a material, or endogenous enzymes are activated. This process facilitates one or multiple chemical reactions that result in changes in selected properties. As spent grain is a rich source of dietary fibres and protein, ongoing studies in partner facilities have concluded that xylanase activity would be most effective in bringing about appreciable changes in the antioxidant profile as well as bio-availability of several nutrients. It is hence expected that treating the spent grain with this enzyme class will yield positive results in terms of micro-structural changes.

Xylanases represents a class of enzymes that are popular as dough improvers in the baking industry. These are also used to help clarify beers in the brewing business (R.Sindhu et al., 2018).They are selective towards certain components that make up hemicellulose called xylans. The enzymatic function of xylanases is due to hydrolysis. In brewing and byproducts (spent grain) generated, it works by facilitating the release of non-starch polysaccharides such as arabinoxylans and lower oligosaccharides, contributing towards improved clarity (R.Sindhu et al., 2018).

Fermentation is a metabolic process that occurs in anaerobic conditions that produces chemical changes in organic substrates ('Fermentation',2019). Lactic Acid Bacteria (LAB) was used to facilitate fermentation based on ongoing studies conducted in partner facilities. Taking into consideration the characteristics of each LAB strain, specific sets were chosen for each type of spent grain that was bioprocessed as part of the project in other facilities.

Fermentation using LAB results in the formation of lactic acid as a by-product due to the breakdown of several complex sugars as well as secondary metabolites such as slime in some cases.This helps to lower the pH and inhibit growth of other microorganisms, improving the shelf-life of the product.Some lactic acid bacteria are capable of producing these extracellular polysaccharides (EPS slime), with desirable technological properties and biological activities. It can be used to alter rheological properties involving viscosity and flocculation among others (Silva, 2019).Based on ongoing studies conducted in this project, the EPS slime produced has been

identified as homo-EPS dextran. Based on information from relevant studies, it is expected that this EPS production would lead to a beneficial change in the micro-structure as well as impart nutritional benefits when the processed spent grain is incorporated into different food products(Silva, 2019).To test the effectiveness of each of the processing techniques in the sample of interest, micro-structure study is crucial.

2.2.4 Sample preparation

Each of the samples studied as part of this thesis project were transferred onto specialized slides using cryo-sectioning techniques.

Preparations of cryo-sections are rapid and relatively easy.Although physically less stable than paraffin- or resin-embedded sections, they are generally superior for the preservation of antigenicity and therefore the detection of antigens by microscopy (H Fischer et al., 2008). Samples are rapidly frozen before sectioning to prevent the formation of large ice crystals that could damage the structural integrity of the tissues of interest. Cryo-sections provide a good method for investigating finer details of the cell. Thin sections are easily stained and visualized using light microscopy while thicker sections can be stained using multiple methods and studied under CLSM

2.2.5 Confocal laser scanning microscopy

CLSM is a specialized microscopy technique. Two pinholes as the focal points and multiple lasers as an energy source make up the crux of the setup of this microscope. The first laser beam passes through one of the pinholes after which the condenser lens focuses it onto the fluorescent section(s) of the sample. The fluorescent light emitted at a selected wavelength is then captured by the objective lens, passed through the second pinhole and transmitted to the detector (Fig 2.3).

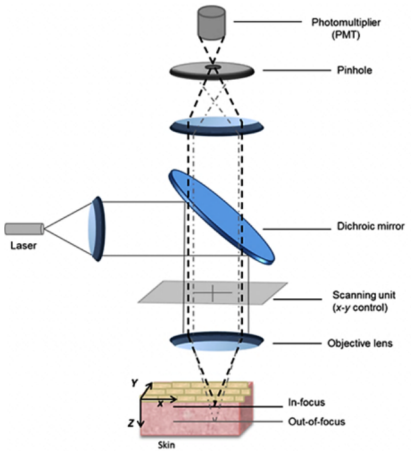


Figure 2.3: Schematic diagram of a CLSM setup (Source: Rossetti et al., 2013)

In order to conduct CLSM, the prepared samples are required to be fluorescent in the regions of interest. In some cases, samples can be auto-fluorescent. These wouldn't require additional staining or labelling. In case of most samples of BSG studied during this thesis, immuno-labelling has been selected as the method of sample preparation for CLSM.

Immuno-labelling

Immuno-labelling or immuno-staining is a quite specific sample preparation technique that labels chosen regions or compounds with fluorescent antibodies , easing the visualization process using CLSM or fluorescence microscopy. One or two antibodies can be used to bind to a single specific antigen - and these are called primary and secondary antibody respectively. This method is called indirect immuno-labelling , while use of only a single antibody is direct immuno-labelling. The quality of the detection is completely reliant on how well the antibody(s) binds to the antigen(s). For better quality and reliability of sample observations, only indirect immuno-labelling has been conducted in this thesis. The detailed protocol for immuno-labelling followed in this project has been described in 3.2.1.

2.2.6 Light Microscopy

Brightfield light microscopy is the second microscopy technique used to study the micro-structure of spent grain in this thesis project. The basic principle of this method is based on the light source and detection objective being placed on opposite sides of the sample (Fig 2.4).The sample is observed by its effect on the light passing through it (D.Kellogg et al., 2015) . As light microscopy requires thin sections, most cells are thin, transparent and absorb enough light to give a clear picture of the sample .

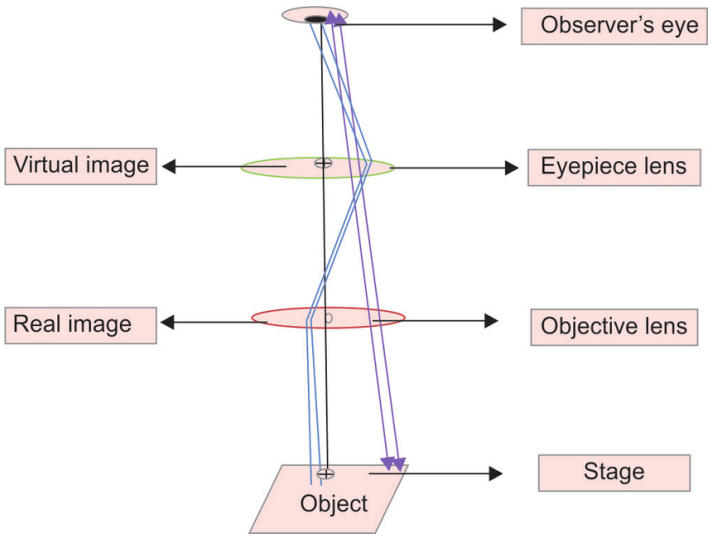


Figure 2.4: Schematic diagram of a light microscope (Source: A Lavanya et al., 2017)

In the case of spent grain, each sample is required to be stained in order to distinguish different layers and components under the microscope. A mixture of Lugol's iodine and Lightgreen has been used to stain samples so as to localize starch and protein respectively when visualized using light microscopy.

Lugol and LightGreen

This particular staining solution mixture was chosen based on the compositional profile of BSG. Protein and starch were the two macronutrients of interest.

Lugol's Iodine is a solution of elemental iodine (5%) and potassium iodide (KI, 10%) together with distilled water (Calissendorff, J. & Falhammar, H.,2017). This solution is used as an indicator test for the presence of starches in organic compounds, with which it reacts by turning a dark-blue/black due to iodine's interaction with the coil structure of the compound(Calissendorff, J. & Falhammar, H.,2017). Starch in any form is observed in a purple - brown hue.

LightGreen is a triphenylmethane dye.It is a component of Papanicolaou's EA series in conjunction with eosin Y and bismarck brown (A. Moawed et al., 2016).It used to selectively stain proteins present in BSG and give an overall view of the general micro-structure.

The combined effect of both staining solutions will provide a holistic image of the micro-structural properties of the different samples of spent grain.

2.3 Outline of the Thesis

During the course of the thesis project, several samples of spent grain bio-processed in Finland and Italy using different techniques were studied using CLSM and light microscopy.

Upon analyzing the micro-structure of the different bio-processed spent grain samples, enzyme treatment was conducted to ensure the effectiveness of the processing steps chosen.Details of each of the different test samples of spent grain (Dugges, Senson, Peroni I and II) studied as part of the thesis project is elaborated in Appendix A.

Since there were samples containing EPS-producing bacteria and their metabolites, these studies were also conducted. Samples were inoculated with three separate strains of bacteria and allowed to ferment in different conditions. Attempts were made to effectively visualize both EPS slime and the degraded BSG along with trying to understand how the EPS slime contributes to the weakening of the cell wall structures.

3

Methodology

3.1 Sample Preparation

Cryo-sectioning was the chosen technique for most samples.The Leica CM1900 was used to section the frozen samples.It offers a stable and well controlled temperature profile settings, which helps to maintain the quality of the sample throughout the sectioning process. Specialized microtome settings help customize the thickness and angle at which the specimens are sliced and transferred onto chosen slide types.

For samples that were later immuno-labelled, the spent grain samples were sectioned onto Polysine^{TM} blue slide (by Thermofisher$^{)}$. These are widely used in immunocytochemistry methods and are specially modified to help the sections adhere to the surface by both electrostatic as well as chemical attractive forces.

For all of the other staining techniques used in this thesis project, Superfrost® microscope slides (sourced from VWR^{TM}) were used. These are of superior quality and offer good support to samples that do not have to be subjected to multiple staining techniques that last long.

i Samples were cut into small blocks using a hand blade such that ice crystals were separated out.These were then in some cases, mixed with glue and mounted or directly placed onto the sample holder with the help of the specialized glue.

ii They were then submerged in liquid nitrogen to facilitate quick freezing. This would result in smaller ice crystals and hence less structural damage than with traditional freezing techniques.

iii They were then placed onto the stand in the Cryostat and allowed to come up to a temperature of -14°C before sectioning was done. The temperature was chosen based on the components of spent grain and their characteristics at very low temperatures.The thickness of the slices were customized based on the intended microscopy use. Thickness was maintained at 10μm for CLSM and 7μm for light microscopy.

For samples that contained EPS(slime),immuno-labelling right after sectioning was not found to be a suitable technique. The EPS was seen to have increased solubility in the solvents and solutions used during immuno-labelling and did not adhere to the slides. Hence, for improved staining, detection and reduced background interference, the samples were first plastic-embedded.

During plastic embedding, small amounts of each spent grain sample containing EPS was fixed using a solution mixture of formaldehyde and a few drops of glutaraldehyde .Following this step,they were then treated with an osmium fixative (A. Keller etal., 1984). Before adding in the 'plastic', a thorough washing using different concentrations of ethanol was achieved to remove water in the sample. Lastly, the samples were placed in moulds along with the polymer (Technovit 1700 by ThermoFisher) for three to four days in order to set.

The entire process of plastic embedding takes atleast seven days. Hence, it was used only sparingly in this project.

3.2 Confocal Laser Scanning Microscopy

Micro-structure studies were primarily conducted on the Leica TCS SP2 confocal microscope. This was used alongside the Leica Confocal Software, both by Cellab Nordia AB, Solletuna, Sweden.

Each of the samples were visualized using objectives at 20X and 63X. This was done to obtain micrographs from different perspectives such that a better understanding of the micr-ostructure could be obtained. Slides were mounted using glycerol for better resolution based on the characteristics of the selcted objectives.

All samples studied using CLSM were immuno-labelled so as to localize arabinoxylan (AX) and β g-glucan (BG) within the cell wall structure of the BSG. Lasers at wavelengths of 647nm and 488nm were used when visualizing. Both lasers were switched on during the whole course of using the CLSM setup.

The laser at a wavelength of 488nm is sensitive to the β -glucan - bound antibody - during which the laser at 647nm causes light to be reflected off the entire sample, providing a better understanding on the localization of the selected β - glucan antibody and the overall micro-structure of the spent grain cross-section. The same system works with the arabinoxylan - bound antibody to which the laser at 647 nm is sensitive and the laser at 488nm provides the reflected view of the overall micro-structure.

When BSG was visualized using other sample preparation methods like lectins (ConA and Lec SB), an additional laser at 547nm was employed based on the properties of the specific stain(s).

3.2.1 Immunolabelling

The process of immuno-labelling is highly specific and time-intensive. Immuno-labelling was conducted in this study in an attempt to individually target and localize the major non-starch polysaccharides present in barley, arabinoxylan and β -glucan so as to understand and follow micro-structural changes better.

Specific primary and secondary antibodies were used during immuno-labelling to ensure that only the desired polysaccharides were most visible.For arabinoxylan detection, **Alexa Fluor™647 goat anti-rat IgG (H+L)** was used as primary antibody; while for β -glucan detection, **Alexa Fluor™488 goat anti-mouse IgG (H+L)** (both by Invitrogen, Thermofisher) was used as secondary antibodies.The antibody provide by Plantprobes diluted to two different ratios was used as the primary antibody. The single immuno-labelling technique used in this study was an adaption of the methods used in similar studies previously (Dornez et al., 2011) (Johansson et al., 2016). Controls were also added to each sample set to ensure reliability of results.

Involving the use of multiple solvents and antibodies that provide specific binding (Müller H. A, 2008), the steps followed can be described as below:

1 Each sample that was sectioned onto Polysine™blue slides was fixated using 4% formaldehyde in 0.01M PBS for thirty minutes

2 The next step involved rinsing each slide three times using 0.1M PBS, with each rinse lasting five minutes

3 Unwanted antigens are blocked in this step using 2.5% BSA diluted in 0.01M PBS. The samples are then allowed to rest for forty minutes

4 The primary antibody is diluted in 0.5% BSA in 0.01M PBS based on a fixed ratio depending on the compound(s) of interest.For arabinoxylan detection,LM11 is diluted in a ratio of 1:50. To ensure detection of β -glucan, a dilution ratio of 1:200 is used.This diluted formula is then coated onto the slides and allowed to rest for around two hours in a dark environment.

5 The next step involved rinsing each slide three times using 0.01M PBS, with each rinse lasting five minutes. The secondary antibody is diluted to 1:500 in a solution of 0.5% BSA in 0.01M PBS.

6 This dilution is then coated onto the slides and allowed to rest for two hours in a dark environment

7 The next step involved rinsing each slide three times using 0.01M PBS, with each rinse lasting five minutes. This is the final rinsing step.

8 Distilled water is then used to clean the sides and rinse off salts.The samples are allowed to rest for approximately five minutes with the layes of distilled water, which is later gently blotted off to remove excess moisture without drying out the sample

9 Prolong™anti-fade diamond mountant (manufactured by Invitrogen, Thermofisher Scientific) is used to mount the coverglass onto the sample so as to delay photo-bleaching. Finally, each slide is individually set using nailpolish to seal samples and prevent them from drying.

3.2.2 Other staining techniques

When studying samples fermented with slime-producing LAB strains, immunolabelling was not feasible due to the high solubility of EPS in the solvents and solutions used. Hence, different staining combinations were used to test for improved visibility and contrast of the spent grain micro-structure in combination with the bacterial cells and EPS using CLSM.

The different staining solutions used were (Appendix B):

1. Con A (EPS in red)

2. Acridine orange - in water (Bacterial cells in green) + Lectin SB (cell wall microstructure and EPS in red)

3.3 Light Microscopy

Complimentary to the micrographs obtained using CLSM, microstructure studies were also done using light microscopy on the Nikon Microphot -FXA microscope which was connected to the DFK33UX264, a 5 megapixel light sensitive camera with short exposure time (by The Imaging Source Europe GmbH, Bremen, Europe).The microscope and camera was linked to the NIS-Elements D software.Samples were investigated using 10X and 20X objectives so as to obtain different perspectives that would lead to a better understanding of the micro-structural profile of the spent grain.

3.3.1 Staining techniques

A mixed staining solution containing both Lugol's Iodine and LightGreen was used on almost all of the samples studied for micro-structural changes using light microscopy.

- Drops of the staining solution was dropped onto the slides with sample sections of interest.
- A coverglass was slid on top, after which all sides were sealed effectively using nailpolish. This prevents the solution from evaporating and drying out the section.

When the samples contain slime (EPS) were studied using CLSM, the same slides were analyzed using light microscopy as well to be able to comprehend the observations fully.

3.4 Enzyme treatment

Enzyme treatment was done separately as part of this thesis project to be able to understand the effects of treatments at different temperature on the micro-structure of spent grain. The protocol followed was provided by the other partners in the project based on analyses conducted previously.

i Milled spent grain (Peroni) was allowed to thaw completely at 4°C for twenty-four hours.

ii The exogenous enzyme E was diluted such that 50 μŁ of distilled water contains 5 μł of the enzyme. This dilution factor has been pre-decided based on the xylanase activity desired. 4,68 μł (100nkat) of the diluted enzyme was then mixed well with 5g distilled water.

iii 5g of untreated milled spent grain was added to this enzyme solution. It was gently folded in so as to reduce the stress on the micro-structure and evenly distribute the enzyme in the sample.

iv The mixture was then weighed and separated into three different beakers to be incubated at 25°C, 35°C and 45°C. Incubation at the selected temperatures was done in three different ovens for twenty-four hours.

Following enzyme treatment, the incubated samples were cryo-sectioned and prepared for microscope studies.

3.5 Microbial culturing

Using spent grain as the substrate, three different strains of LAB were cultured to study EPS formation and its effect on the micro-structure of spent grain.

The selected strains were A16,PT05 and 20193, representative for three different strains capable of producing slime in favourable conditions.They were also known to grow well in MRS and produce a significant quantity of slime (dextran) in a sucrose-enriched substrate. The protocol followed was obtained based on other analyses conducted as part of the FUNBREW project.

i Reviving bacterial cells :

(a) Cells were allowed to thaw after which they were transferred into 10ml of sterile MRS broth.The tube was vortexed for a few minutes and allowed to incubate at 30°C for twenty-four hours.

(b) From the MRS broth culture tubes, 300 μł was transferred into 10ml of sterile MRS broth.The tube was vortexed for a few minutes and allowed to incubate at 30°C for twenty-four hours.

ii The spent grain to be used as a substrate was allowed to thaw completely at 4°C for twenty-four hours. For each bacterial strain, substrates of two different compositions were used.

- Type 1: 10g Spent grain + 15g Water
- Type 2: 9g Spent grain + 1g Sucrose + 15g Water

iii To each of these 28.5 μł of the three selected LAB strains (A16, 20193, PT05)are added.To prepare this bacterial inoculum, the following steps were followed:

(a) 28.5 μł of cells were transferred into Eppendorf tubes and centrifuged at 10000 rpm for 10 minutes.

(b) The MRS broth was discarded and by vortexing, the cells were re-suspended in 12.5 μł from the total quantity of water used to prepare the substrate.

iv After adding the inoculum, the mixture was gently stirred for a few seconds, sealed with aluminum foil and allowed to ferment for twenty-four hours at 25°C.

4

Results

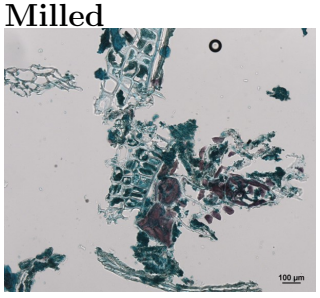
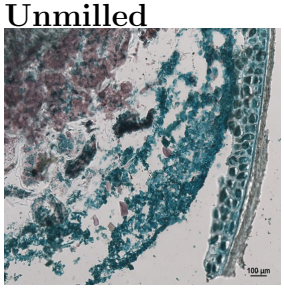
The results have been reported separately based on the unique characteristics and bio-processing methods that were employed for each type of spent grain.Each of them were obtained from different breweries or malt suppliers in three countries; so there is some variation in the composition and percentage of barley contained. All types of BSG used was from the brewing of generic lager beer.

Since sufficient degradation of the cell wall structure is the required outcome for the best bio-processing technique chosen, the main area of focus while studying the samples was the micro-structure and how the different bio-processing methods affected it.Each sample type was analyzed in duplicates to ensure reliability and repeatability of the observations made. Relevant micrographs have been presented and discussed in detail below.

4.1 Spent grain

In order to be able to comprehend the level of degradation in the treated samples, unprocessed spent grain was extensively studied using light microscopy (LM). Both milled and unmilled forms were stained using LightGreen and Lugol's iodine and analyzed. The results are presented in Table 4.1.

Table 4.1: Micro-structure of spent grain using LM

Milled	**Unmilled**
	

As seen in Table 4.1, the micro-structure of the outer layers of the cell walls of the spent grain (barley) is clearly visible and connected. The layers (moving inward-pericarp, aleurone layer, sub-aleurone region and the starchy endosperm) are well connected and easily distinguishable. Protein packages are secure within the cell walls of the aleurone layer and the starch (in purple) is still present in significant quantities even after the mashing step. These structures are visible within the milled sample as well - however, the structure appears ripped and torn in many places owing to the heavy shear involved in milling.

4.2 Dugges

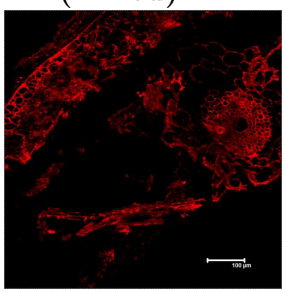
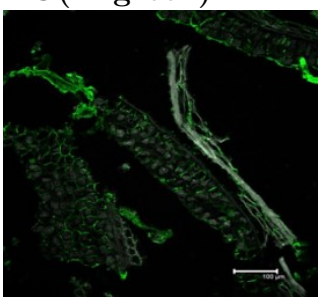
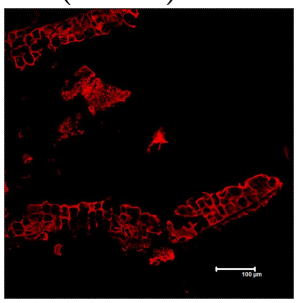
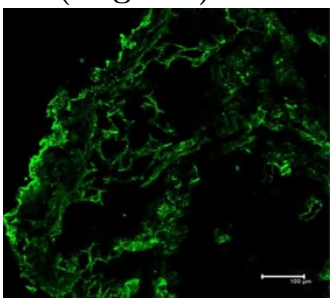
Spent grain was obtained from a local brewery in Göteborg, Sweden.Milled samples had been treated with exogenous enzyme E (with established xylanase activity) and antibiotic mixture for twenty-four hours at 25°C. This was done to ensure no microbiological growth while the enzyme acted on the BSG sample. The sectioned samples were studied for micro-structural changes using both CLSM as well as light microscopy. All observations made were done keeping Fig 2.2 as reference.Immuno-labelled cross-sections of the treated samples were then studied using CLSM (Table 4.2).

4.2.1 CLSM

Milled spent grain had been treated with the xylanase E and an antibiotic mix for twenty- four hours at 25°C.The cryo-sectioned samples were immuno-labelled and observed using a 20X objective (Table 4.2).

Based on the micrographs obtained after CLSM, it was quite clear that some change had occured within the sample. Upon further deliberation, it was seen that there was minimal degradation in the layers of the barley cell structure - most of the cells in the aleurone layer and endosperm region in particular appeared quite stretched and slightly weakened. As discussed earlier, arabinoxylans (AX - in red) are mostly found in the outer parts, especially the aleurone layer while 70% of β -glucan (BG- in green) is localized in the sub-aleurone region and the starchy endosperm. The intensity of these non-starch polysaccharides were also found to remain more or less the same even after exposing the sample to enzyme for twenty-four hours. It was understood that the activity of the xylanase (and possibly the endogenous enzymes present in the barley mash) was not entirely effective, possibly due to low concentrations or insufficient temperature provided for the reactions to occur.

Table 4.2: Effect of enzyme treatment using CLSM

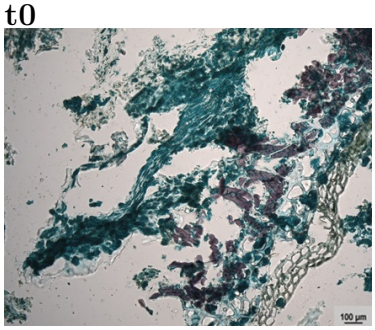
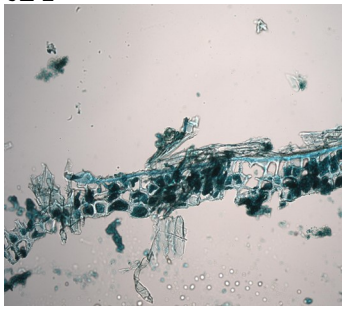
Ab.E.A.t0		
AX(in red)	**BG(in green)**	Aleurone and endosperm are clearly visible and intensities of arabinoxylan and β -glucan are quite high
		
Ab.E.A.t24		
AX(in red)	**BG(in green)**	Except for slight weakening,no significant degradation was seen
		

Hence,degradation was not achieved even after ensuring the solitary effect of enzyme treatment by using the antibiotic mixture. It was understood that the temperature utilized during enzyme treatment was possibly not sufficient.

4.2.2 Light Microscopy

The staining solution mixture containing Lugol's Iodine and LightGreen was used to selectively stain the sample for easier identification of starch and protein/fibres respectively.

Table 4.3: Effect of enzyme treatment using light microscopy (LM)

Ab.E.A	
t0	**t24**
	

Cross-sections of the milled spent grain treated with the xylanase E and antibiotic mixture were visualized using light microscopy. Both the 10X and 20X objectives were used. To ensure consistency with results obtained using CLSM, images at 20X are presented in Table 4.4.

The visible effects of enzymatic treatment studied using light microscopy was seen to be in correlation with the observations drawn from the micro-structural analysis using CLSM. Minimal and erratic breakage was observed - this was not ideal and hence, more trials exploring other bio-processing protocols were advised.

4.3 Senson

These samples of spent grain was sourced from Finland from a malt company - contain only barley remnants. It was supplied in the milled form and treated using a combination of the xylanase E and fermentation by selected LAB strains for a total of twenty-four hours. Samples were collected at four different time points - t0, t10,t16 and t24.

A major motive behind testing this bio-processing technique was the production of EPS slime by LAB and finding a suitable staining technique to effectively visualize it in-situ. Both treatments were done in two different conditions so as to study how it would affect EPS production and micro-structural change of the spent grain.

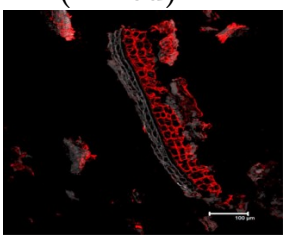
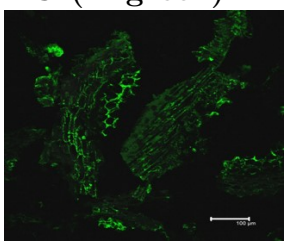
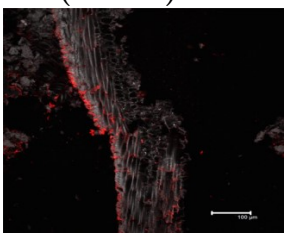
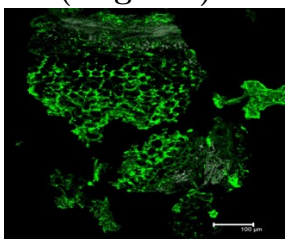
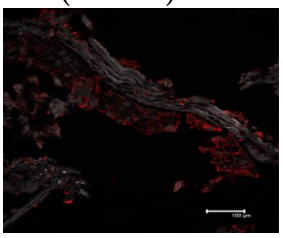
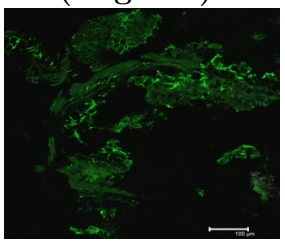
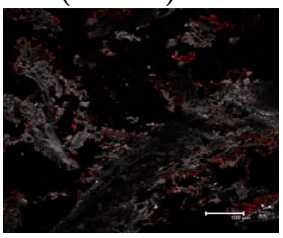
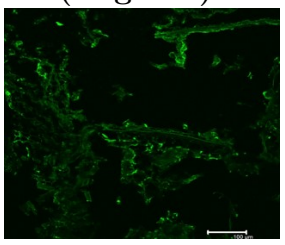
4.3.1 CLSM

The first set of samples (Type - I) involved enzyme (xylanase E) treatment at 25°C for twenty-four hours followed by LAB fermentation at 30°C for an additional twenty-four hours separately by two different strains (A16 and 20193) of milled BSG suspended in sterile water.The results for these using CLSM are discussed in Table 4.4 and 4.5.

Referring to the micrographs obtained from CLSM of the immuno-labelled cross-sections of these samples (Table 4.4/4.5), it can be clearly seen that as time progresses, the level of degradation also increases. At t16, the aleurone layer and endosperm regions can no longer be clearly distinguished.

Since these samples were fermented using LAB strains capable of producing slime, it is speculated that the increased proximity of the structures at t16 and t24 in particular could be attributed to the possible presence of EPS slime. In order to confirm this,more analysis was conducted.

Table 4.4: Bio-processing using enzyme and A16 strain (type 1) - CLSM

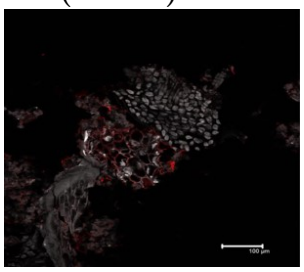
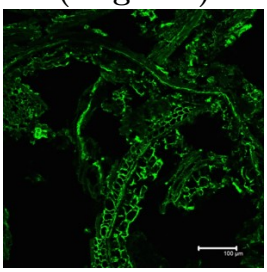
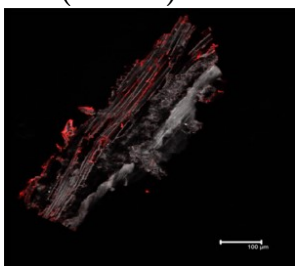
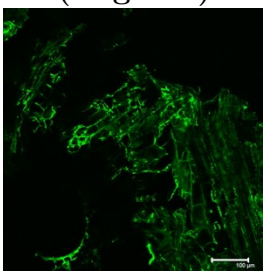
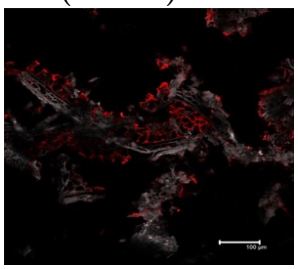
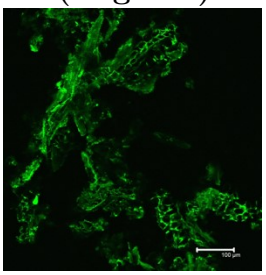
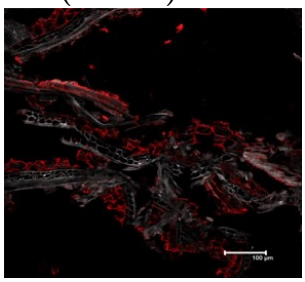
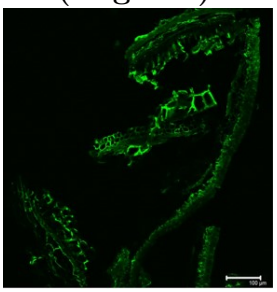
t0		
AX(in red) 	**BG (in green)** 	Arabinoxylan and β -glucan was distinctly visible in the expected regions and good micro-structural integrity was clear
t10		
AX(in red) 	**BG(in green)** 	Slightly reduced intensity in arabinoxylan and β -glucan levels across the sample. Micro-structure shows slight weakening
t16		
AX(in red) 	**BG(in green)** 	Micrograph appeared slightly cloudy from this point. Further reduction in the intensities of arabinoxylan and β -glucan was seen
t24		
AX(in red) 	**BG(in green)** 	Drastic degradation of the overall micro-structure.Arabinoxylan and β -glucan are no longer clearly discernable

In table 4.4, the effects in relation to the A16 strain is presented.Fermentation using this strain is understood to lead to an increased production of EPS slime (could explain the cloudiness of the micrographs obtained) as well as heavy degradation of the cell wall polysaccharides- arabinoxylan (AX-in red) and β - glucan (BG - in green) leading to reduced visibility with time.Significant results were seen when enzyme treatment was combined with fermentation using LAB strain A16.

When the same bio-processing technique was done using a different LAB strain (20193) the resulting micrographs obtained were significantly different from those of the previous set.The results of this trial have been presented in Table 4.5.

When the 20193 LAB strain was used to ferment the enzyme-treated samples, there was a less obvious reduction in the presence of arabinoxylan (AX - in red) while β -glucan (BG - in green) levels seemingly remained the same. However, some degradation is clearly visible from t16 onwards. The extent of this however, is still in question.

Table 4.5: Bio-processing using enzyme and 20193 strain (type 1) - CLSM

t0		
AX(in red)	**BG(in green)**	Cell micro-structure wass intact and clear.More visibility of surrounding structures than arabinoxylan. β -glucan was present in good intensity
		
t10		
AX(in red)	**BG(in green)**	Cell micro-structure was seen to remain more or less the same. Intensities of arabinoxylan and β -glucan did not seem to be affected
		
t16		
AX(in red)	**BG(in green)**	Significant degradation was seen around the aleurone and endosperm regions. Arabinoxylan was sparingly visible; β -glucan had also reduced in intensity
		
t24		
AX(in red)	**BG(in green)**	Severe denaturation was clear with almost no visibility of arabinoxylan and β -glucan
		

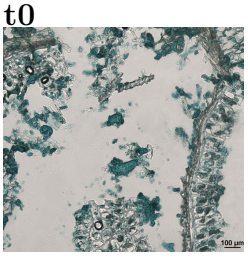
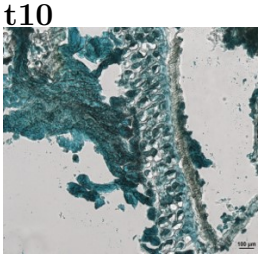
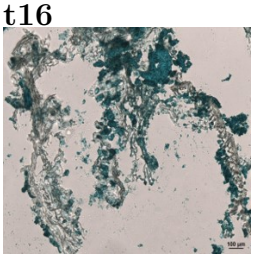
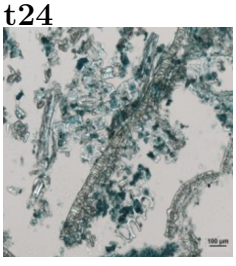
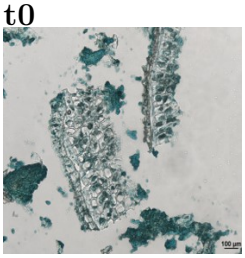
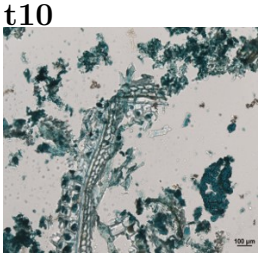
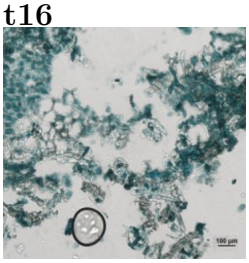
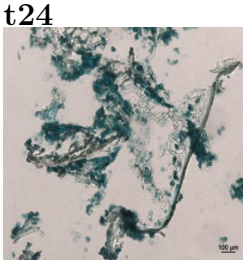
On comparing the degree of micro-structural changes due to degradation , it was observed as a result of using two different strains was apparent. It was hypothesized that there was some characteristic feature of the A16 strain (possibly quantity of EPS slime produced - discussed in section 4.4) that led to this stark difference in results obtained.

The second set of samples from Finland that were studied was bio-processed in the presence of 4% sucrose. Complimentary studies conducted at the partner facilities showed an improved production of EPS slime when sucrose was present in the substrate. Hence tests were conducted on how such processing conditions would affect the micro-structural properties of the spent grain.

However, it was not possible to study the cross-sections of these samples using the standard procedure of immuno-labelling.The several rinsing steps and solvents used according to the protocol washed the samples off the slides- possibly due to the larger quantity of EPS slime present. It is likely that the sizeable presence of EPS in these samples led to increased solubility due to a difference in surface tension causing the sections to only weakly attach onto the Polysine™slides.In this case, samples were plastic embedded prior to immuno-labelling so as to better preserve the structures (prevent it from being rinsed off) and also attempt to capture the qualities of the EPS slime produced.These could not be investigated in time and is expected to be conducted at a later stage.

4.3.2 Light Microscopy

Table 4.6: Bio-processing using enzyme and A16/20193 strain (type 1)- LM

A16			
t0	**t10**	**t16**	**t24**
			
20193			
t0	**t10**	**t16**	**t24**
			

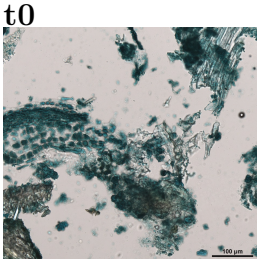
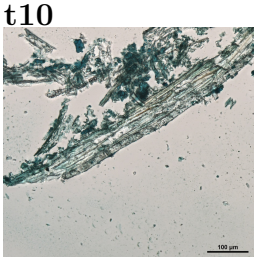
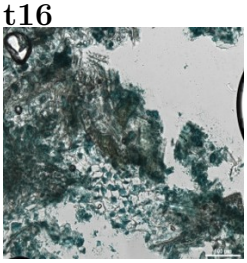
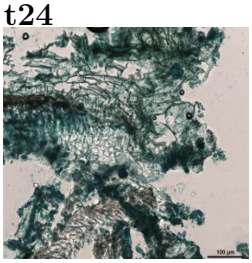
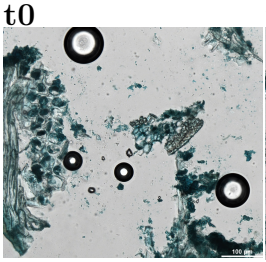
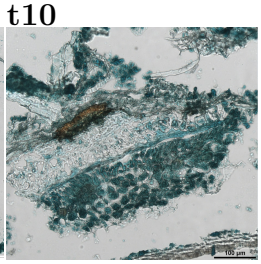
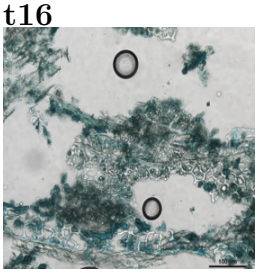
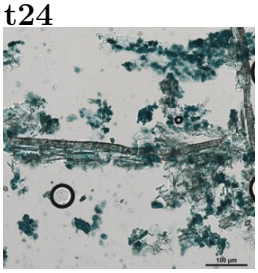
All samples (including those with 4% added sucrose in the spent grain substrate) bio-processed using the above combination of enzyme treatment at 25°C followed by fermentation (A16 and 20193) at 30°C were additionally studied using light microscopy.The staining solution mixture containing Lugol's Iodine and LightGreen was used to selectively stain the sample for easier identification of starch and protein components respectively.

Type - 1 samples (Table 4.6) showed significant degradation in either strain. The cell walls were clearly damaged and protein blocks were observed in clumps outside of the expected regions (aleurone layer in particular). However, there was no visible presence of any structures resembling EPS slime or related components even at t16 and t24. In addition, the different layers seem connected, though substantially degraded in the case of fermentation with the A16 strain.

It is expected that the possible influence of EPS slime (though not effectively visualized here) again could have led to this occurrence. The micro-structure of the cross-sections of samples fermented with the 20193 strain show clear fragmentation, on the contrary. Since these samples are known to produce lesser quantities of EPS slime, this phenomenon could possibly be attributed to the same.

Cross-sections of the sample that were bio-processed using the same techniques as above , but in the presence of 4% added sucrose (type -2) were visualized using light microscopy. Several interesting observations were made. These have been presented in Table 4.7.

Table 4.7: Bio-processing using enzyme and A16/20193 strain (type 2) - LM

A16			
t0	**t10**	**t16**	**t24**
			
20193			
t0	**t10**	**t16**	**t24**
			

Degradation in these aleurone layer and endosperm region appear to be significantly damaged with no evident presence of starch (would be stained purple-brown) any

longer, unlike results obtained from previous bio-processing techniques. The dark blue spots or blocks effectively indicate the protein components in the sample. These were found to be localized in clusters once again, at a distance from the expected region of visualization (aleurone layer and sparsely in the endosperm region)

Furthermore, there was a dark blue haze visible above the micro-structures of the sample fermented using the A16 strain at t16 and t24 (Table 4.7).It was hypothesized that this haze represents the EPS slime produced - more so, because the quantity secreted in the presence of sucrose was expected to be large.

In order to confirm this assumption, some analysis needed to be done on the level of slime produced by microbial culturing.

4.4 Microbial culturing - EPS study

According to the protocol described in section 3.5, samples of milled spent grain (Peroni) was fermented using three different strains of LAB capable of secreting exo-polysaccharides of the same type.Visually, samples containing 4% added sucrose and fermented using strain A16 was seen to produce the most slime. Spent grain appeared to be caught in a large clump of the EPS with the water separated out in the bulk.

In order to effectively understand the characteristics of EPS slime as well as how it affects the micro-structure of the spent grain, microscopy studies were done.

4.4.1 CLSM

The samples were stained using two different stains that were expected to localize selected regions of interest in the sample. The stains used were:

i Con A - Was expected to selectively stain and visualize EPS slime

ii Acridine Orange + Lectin SB - Acridine orange was expected to stain bacterial cells while lecSB was used in order to visualize EPS slime and the cell wall micro-structure.

However, these attempts proved to be unsuccessful as Con A stained the entire structure due to the degree of similarity in the compositional profile of the cell wall and EPS slime.

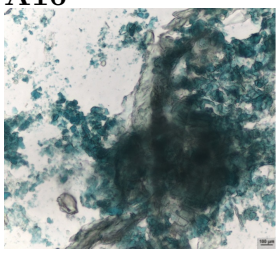
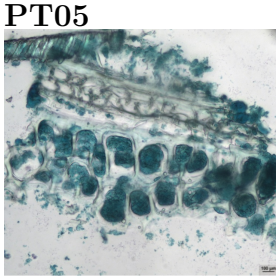
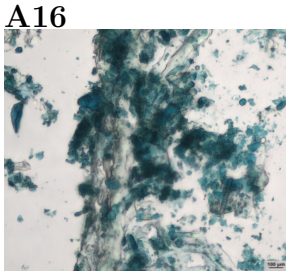
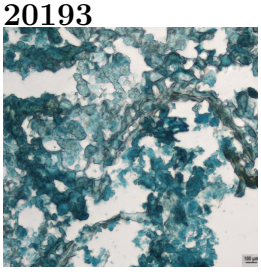
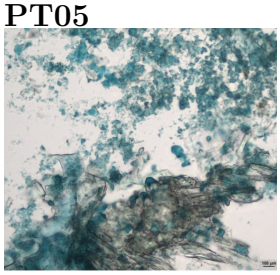
The stain mixture of Acridine Orange and LecSB was also not as effective. It is hypothesized that the quantity if EPS slime in the sample as well as the feature of the dye caused it to be less selective than hoped, making visualization and effective

identification difficult. Since not many studies have been conducted in this area yet, more investigation is needed in identifying suitable stains and methods to visualize the slime and spent grain simultaneously.

4.4.2 Light Microscopy

The cross-sections of the samples that contained EPS slime was studied using light microscopy in an attempt to visualize and understand its characteristic structure and how it affects the micro-structure of fermented spent grain.The micrographs obtained are presented in table 4.8.

Table 4.8: Visualizing EPS slime on spent grain - LM

4% added sucrose		
A16	**20193**	**PT05**
		
0% added sucrose		
A16	**20193**	**PT05**
		

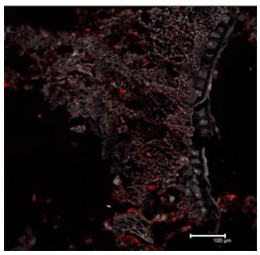
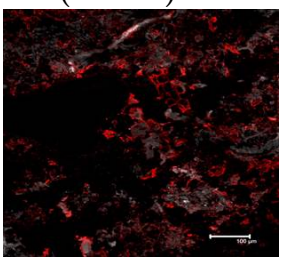
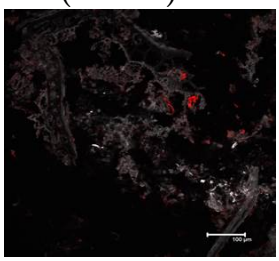
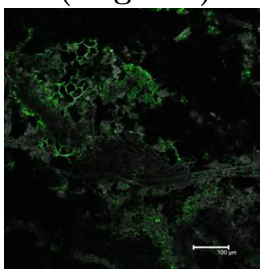
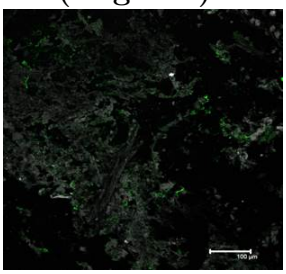
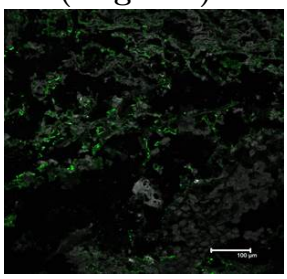
Attempts were made to effectively visualize EPS along woth the spent grain using the standard stain mixture of Lugol's iodine and LightGreen. Similar to micrographs obtained of the Senson samples in Table 4.7, a blue haze was observed in samples fermented using strain A16.Some haze was also visible in the sample fermented woth the same strain in similar conditions with no added sucrose. It is hypothesized that the haze is EPS slime covering the micro-structure of the spent grain. The general blurry nature of the micrographs obtained is understood to be an effect of the presence of EPS.IT can also be observed from these that the micro-structural changes were not drastically different from those obtained using other bio-processing setups done in this project.

4.5 Enzyme Treatment

The protocol described in section 3.4 was followed during the enzyme treatment of milled spent grain (sourced from Peroni, Italy).The effect of temperature was assessed in relation to micro-structural changes due to enzyme treatment.

4.5.1 CLSM

Table 4.9: Influence of temperature during enzyme treatment on spent grain - CLSM

25°C	35°C	45°C	
AX(in red)	**AX(in red)**	**AX(in red)**	Intensity of arabinoxylan showed a drastic decrease along with heavy degradation of the aleurone layer which is no longer recognizable
			
BG(in green)	**BG(in green)**	**BG(in green)**	The levels of β glucan showed a slower decline though micro-structural degradation was as evident as expected.
			

Immuno-labelled cross sections of the enzyme treated spent grain was analyzed using CLSM and the results are tabulated in Table 4.9.As anticipated, the micro-structural degradation is seen to become more evident and drastic with increase in temperature.

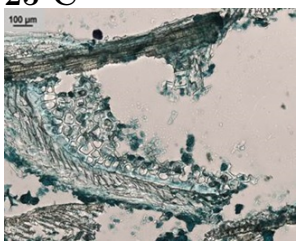
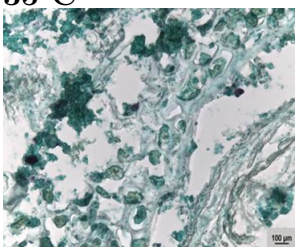
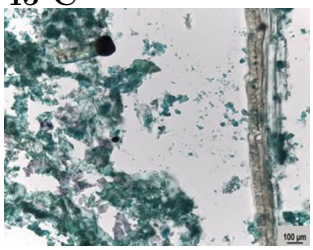
Arabinoxylan (AX- in red) and β glucan (BG - in green) levels are also observed to reduce as the cell walls become more damaged. The micro-structural properties of each layer (in particular, the aleurone layer and the endosperm region) of the barley cell wall micro-structure is no longer identifiable at 45°C.

It was thus concluded from this microscopy technique that 45°C brought about drastic degradation that resulted in almost total loss of both arabinoxylan and β glucan.

4.5.2 Light Microscopy

Cross-sections were stained using Lugol's iodine and LightGreen before being observe using light microscopy (Table 4.10).

Table 4.10: Influence of temperature during enzyme treatment on spent grain - LM

25°C	35°C	45°C
		

At 25°, minimal and erratic degradation was seen. Most structures are still intact and the protein blocks (visualized in blue) are well enclosed within the cell walls of the aleurone layer.On increasing the temperature to 35C, The proteins begin to leach out of the cell walls due to possible perforation and damage.Degradation due to enzymatic activity becomes clear. Finally, at 45°C quite impactful changes are seen in the micro-structure. Protein clumps are seen strewn across the cross-section of the sample.Starch (expected to be seen in purple-brown) was not observed throughout this sample at any temperature point.Further investigation is needed on the maximum possible time temperature combinations that can be utilized for sufficient micro-structural changes.

4.6 Peroni

Spent grain(contain barley and 30% corn) samples sourced from a local brewery in Italy was again bio-processed using combination techniques of both enzyme treatment and fermentation using LAB. The corn cells are expected to have been degraded during the mashing step in brewing.In this trial however, the strains utilized were not capable of producing EPS slime and hence only micro-structural degradation was of interest.Two different bio-processing tests were conducted using the same type of spent grain.

In trial - I, finely milled samples of spent grain were first treated with the exogenous enzyme E for twenty-four hours at 25°C. These were then subjected to fermentation at 30°C for an additional twenty-four hours using three different strains of LAB (PU1, H46 and PRO17).The strains were chosen based on separate studies conducted on their activity and growth parameters. Both CLSM and light microscopy were used to study the micro-structure of all the cross-sections obtained.In trial - II, the effect of higher temperature enzyme treatment on the micro-structure of the same variety of spent grain was analyzed. These samples were treated with the exogenous

enzyme E for twenty-four hours at 50°C, followed by the usual fermentation step for an additional twenty-four hours at 30°C with the same three strains as in trial - I (PU1, H46 and PRO17).

4.6.1 CLSM

In trial - I, samples were first treated with the exogenous enzyme E for twenty-four hours. Fermentation was later conducted using three different strains of LAB. The focus of this trial was observing micro-structural degradation as a result of both enzyme treatment and fermentation on the same sample.Immuno-labelled cross-sections were investigated using CLSM. The results obtained are presented in Table 4.11.

As seen in previous samples subjected solely to enzyme treatment, there are no striking micro-structural changes observed even after twenty-four hours of incubation. However, upon fermentation with LAB at 30°C, significant differences were visualized.The noticeably low intensity of the non-starch polysaccharides (AX arabinoxylan in red and BG β glucan in green) within the sample from the start was duly noted. It is attributed to the possible presence of various constituents of corn-which may have contributed towards significant degradation of the polysaccharides during enzyme treatment itself.

Layers of the cell wall structure as well as individual cells are not recognized in samples that were fermented - especially with the strains H46 and PRO17.

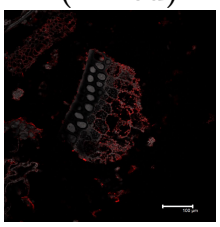
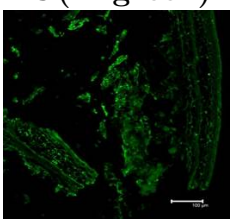
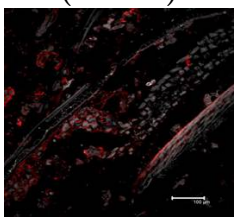
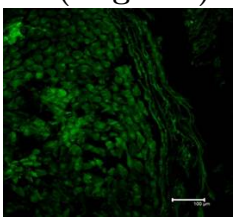
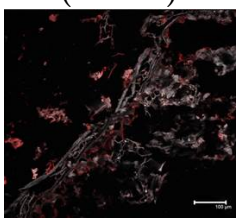
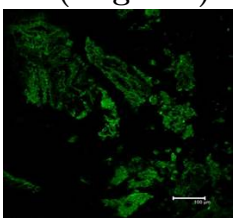
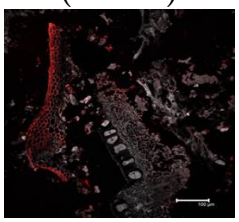
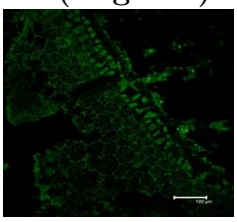
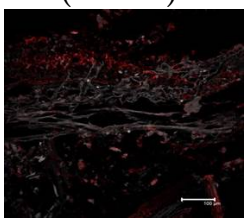
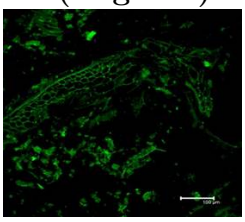
Trial -II using the samples from Peroni, Italy involved enzyme treatment at an increased temperature of 50°C followed by fermentation at 30°C using the same LAB strains as in Trial - I. It was done under the expectation to see drastically degraded structures.

Sections of the samples from this trial were immuno-labelled and effectively visualized using CLSM. The resulting micrographs are presented in Table 4.12.

Unlike previous samples subjected solely to enzyme treatment, there are some micro-structural changes observed even without the addition of any exogenous enzymes. It is speculated that the activated endogenous enzymes are still present in this variety of spent grain from the mashing process and works to some effect towards altering the micro-structure at 50°C. Inner endosperm layers look quite weakened and degraded.

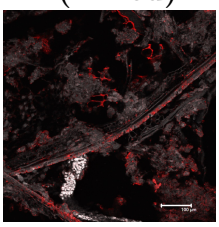
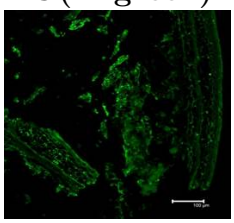
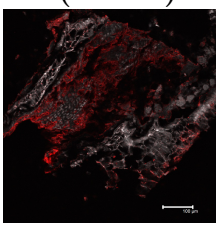
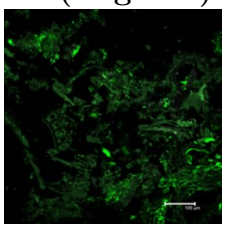
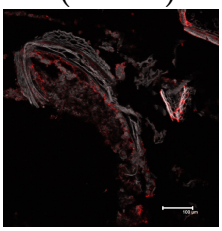
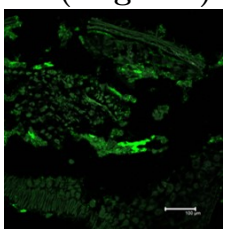
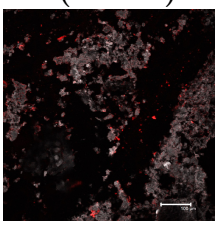
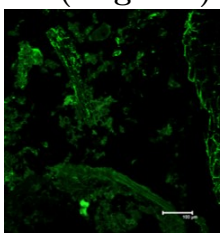
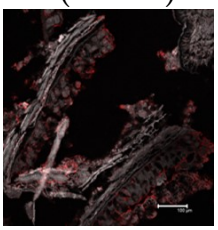
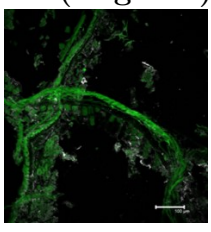
The changes are seen to become more significant upon the addition of the exogenous enzyme E. On fermenting with different LAB strains at 30°C, severely degraded structures were visualized. The noticeably low intensity of the non-starch polysaccharides within the sample from the start was once again duly noted.

Table 4.11: Effect of trial - I on spent grain - CLSM

Ct(t24)		
AX(in red) 	**BG(in green)** 	Good micro-structural integrity of each layer of the cell structure. Sufficient intensity of arabinoxylan and β glucan. No change seen.
Ct.E(t24)		
AX(in red) 	**BG(in green)** 	No evident change was seen in neither the micro-structural properties nor the intensities of arabinoxylan and β glucan
H46 + E		
AX(in red) 	**BG(in green)** 	Drastic degradation was seen within the aleurone layer and weakened cells in the endosperm. Intensity of arabinoxylan was reduced.
PU1 + E		
AX(in red) 	**BG(in green)** 	Extent of degradation was not as severe. Some structures are still visible though intensity of arabinoxylan and β glucan was low
PRO17 + E		
AX(in red) 	**BG(in green)** 	Fragmented cell structures of the aleurone layer and some degradation of the endosperm. Very low intensity of arabinoxylan and β glucan

The aleurone layer and endosperm region which originally contain the highest percentage of localized arabinoxylan (AX - in red)and β glucan (BG - in green) as well as individual cells are not as recognizable in samples that were fermented - especially with the strains H46 and PRO17.

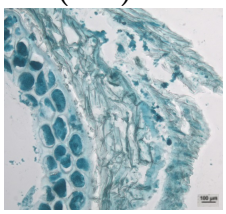
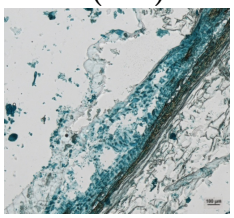
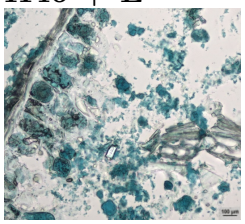
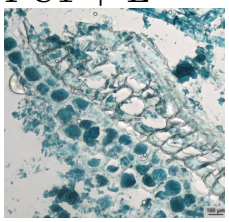
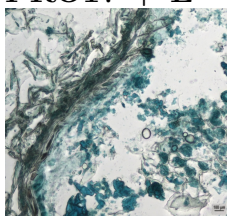
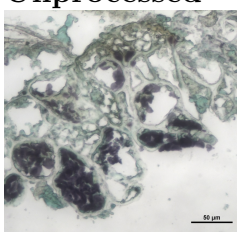
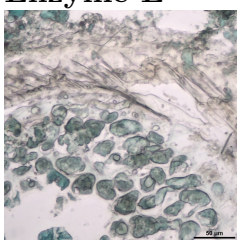
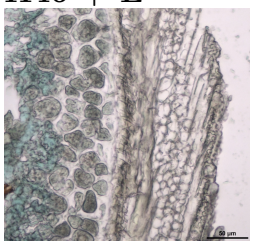
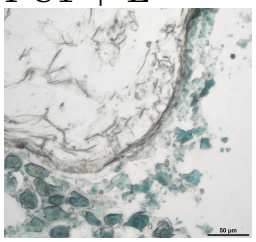
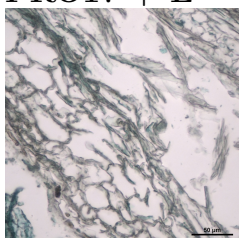
Table 4.12: Effect of trial - II on spent grain - CLSM

Unprocessed		
AX(in red) 	**BG(in green)** 	Clear visibility of the alerone,endosperm and overall micro-structure. Intensities of arabinoxylan and β glucan were weak
Exo. enzyme E		
AX(in red) 	**BG(in green)** 	Erratic stretching of the endosperm.Aleurone layer is almost intact.Intensities of arabinoxylan and β glucan showed no change
H46 + E		
AX(in red) 	**BG(in green)** 	Drastic denaturation was visible. No significant change in the levels of arabinoxylan and β glucan was seen
PU1 + E		
AX(in red) 	**BG(in green)** 	Clear fragmentation of the aleurone layer and endosperm. Structures are no longer distinguishable. Arabinoxylan and β glucan is sparsely visible
PRO17 + E		
AX(in red) 	**BG(in green)** 	Arabinoxylan was no longer clearly visible even though β glucan was recognizable. Some fibres were still seen

The overall micro-structure of each sample set is also quite clearly recognizable and prominent due to the reflected light from the two lasers in both trials.

4.6.2 Light Microscopy

Table 4.13: Effect of trial I and II on spent grain - LM

Trial - I				
Ct (t24)	**Ct.E (t24)**	**H46 + E**	**PU1 + E**	**PRO17 + E**
				
Trial - II				
Unprocessed	**Enzyme E**	**H46 + E**	**PU1 + E**	**PRO17 + E**
				

Sections of samples from both trial-I and II were stained using the stain mixture containing Lugol's iodine and LightGreen. This was expected to stain any starch (in purple-brown) and protein (blue-black) components respectively. Micrographs obtained from each variant is presented in Table 4.13.

Samples from trial- I show only slight degradation after twenty-four hours of enzyme treatment using the exogenous enzyme E at 25°C. However, upon additional fermentation using the three different LAB strains, degradation and separation is significant and quite intense. Most notable micro-structural changes were again observed in samples fermented with strain H46 and PT05.

Sections of samples from trial - II were also studied using light microscopy. The results from these are tabulated in Table 4.13 itself. Several micrographs of the micro-structure of corn was visible in unprocessed samples incubated at 50°C - indicating that not much degradation has occured overall (corn makes up only 30% of the spent grain composition). Due to incubation at a temperature of 50°C, there was some apparent degradation already visible with solitary enzyme treatment using the exogenous enzyme E. Additional fermentation using the three LAB strains was observed to degrade the micro-structure, but not extensively as hoped. It is speculated that following the incubation at 50°C, the efficiency of the bacterial strains is not as high as hoped.

5

Discussion

Each type of BSG and every processing method tested was studied using CLSM and light microscopy.Treating the milled spent grain with the xylanase at 25°C for twenty-four hours was found to be insufficient in bringing about any strong degradation and change in the cell wall micro-structure. The results obtained from both CLSM and light microscopy supported this observation.

Supplementing the enzyme treatment in this setting with fermentation using selected LAB strains brought about quite an intense change in the micro-structure.The strains are understood to secrete EPS slime as a result of fermentation. Most notable differences in micro-structure as well as the general view of the sample was brought about by LAB strain A16 in both trials conducted. Further studies were done to investigate the micro-structure and general appearance of this slime in the presence of the degraded spent grain. These were however not quite successful. Hence, great potential exists in the future that explore possible staining and visualization techniques to effectively observe and understand EPS slime an its impact in changing the micro-structure of spent grain during and after fermentation.Apart from this, other LAB strains that were not capable of producing EPS slime were used to ferment enzyme-treated samples to test for changes following the treatment protocol. To further cement the observations, analyses were done to understand the effect of different temperatures on the effectiveness of enzyme treatment on spent grain. This yielded quite optimistic and positive results that paved the way for developing more treatment setups. Some of these were found quite impactful.

The effect of this setup at a higher incubation temperature of 50°C was thus thoroughly analyzed and found to be drastically strong.Unprocessed milled spent grain was also incubated at this temperature to study the effect of endogenous enzymes, if any.Results were quite interesting to note.In order to identify an appropriate sectioning and staining combination to visualize EPS slime in the presence of milled spent grain , evaluating the effectiveness of the plastic/resin-embedded samples was necessary.However, these did not prove effective when subjected to immuno-labelling. To choose the most optimal bio-processing and microscopy technique with more certainity, the bioprocessed spent grain types must be utilized in products such as pasta, cakes and bread .

6

Conclusion

The thesis work focused on developing methods that would help understand the extent of micro-structural change in spent grain from different breweries bio-processed using different experimental setups. The chosen microscopy methods to observe, identify and assess these changes were CLSM and brightfield light microscopy in all trials.The three variants of spent grain that were studied for micro-structural changes were known to have different compositions of spent grain. Attempts were also made to effectively visualize EPS slime in the presence of spent grain.

Due to the specificity of fluorescence microscopy, CLSM was able to provide more insight into how any selected bio-processing would affect the proportions of the complex polysaccharides of interest (arabinoxylan and β glucan). The possibility of studying thicker sections using this technique helped in understanding each type of BSG. Limitations of this method of microscopy were that there was some level of uncertainity in coming to conclusions based on observing the micrograph - also since the choice of laser(s) and colour chosen greatly affects the visual quality of the micrograph.The compositional profile of the spent grain is also speculated to affect the intensity of the initial visibility of arabinoxylan and β - glucan (especially in the case of Peroni).Another limitation of using CLSM was that the sample preparation methods that could be used were not suitable in identifying EPS slime was not identifiable in the presence of fermented BSG. There is hence potential to conduct more studies and research in this space.Light microscopy is a simpler microscopy method that was used as a compliment to CLSM. Using light microscopy, it was possible to understand and observe the exact location and structure of the proteins and starch as well as the EPS slime (if any). A clear micrograph was captured, which made it possible to correlate images to better understand the effects of each technique.A broader understanding was obtained since these microscopy methods were used on different types of spent grain and is therefore hoped to have provided a more holistic method that could be adapted for treating any type of BSG.

In conclusion, the findings of this thesis project could be a stepping stone towards understanding the micro-structural features and intricate chemistry associated with improving the usability of spent grain, and thus contribute towards the overall sustainability of breweries across the world.

Appendix A

Types of spent grain

Treatment - 25°C for 24 hours					
Sample Code	BSG(g)	Water(g)	Ab1 (5%)	Ab2 (5%)	Enzyme (100 nKat)
Ab_E_A	50	50	200 μL	200 μL	4,68 μL
Ab_E_B	50	50	200 μL	200 μL	4,68 μL
-	-	-	Cyclohexamide 0,01%	Chloramphenicol 0,01%	Exogenous enzyme E

Table A.1: Spent Grain samples from Dugges

Sample [Enzyme(100 nkat/g)(incubate at 25°C for 24 hrs) + Bacteria (ferment at 30°for 24 hrs)]
Ct t0
Ct + E t24
E + H46
E + PU1
E + PRO17

Table A.2: Spent Grain samples (Trial - I) from Peroni, Italy

Sample [Enzyme(100 nkat/g)(incubate at 50°C for 24 hrs) + Bacteria (ferment at 30°for 24 hrs)]
No enzyme (unprocessed)
Only enzyme E
E + H46
E + PU1
E + PRO17

Table A.3: Spent Grain samples (Trial - II) from Peroni, Italy

Treatment - 25°C for 24 hours		
Bacteria	Sample type	% of sucrose
A16	Type 1	0%
	Type 2	4%
20193	Type 1	0%
	Type 2	4%

Table A.4: Spent Grain samples from Senson, Finland

Appendix B

Spectra of stains/Abbreviations

The following stains were used during analyses using CLSM. Only the combination of acridine orange and lec SB was chosen.

Stain	Emission spectra
Con A	488 nm
Acridine Orange	647 nm
Lec SB	597 nm
Congo red	520 nm
Sirius red	550 nm

Table B.1: Emission spectra of stains tested

Abbreviations

BSG: Brewers Spent Grain

CLSM: Confocal Laser Scanning Microscopy , LM: Light Microscopy

EPS: exo-polysaccharide slime, LAB: Lactic Acid Bacteria

AX: Arabinoxylan, BG: β - glucan

BSA: Bovine Serum Albumin, PBS: Phosphate-buffered saline

RISE : Research Institutes of Sweden

Bibliography

Andrade Silva, Liliane & Honorio Pereira Lopes Neto, Jose & Cardarelli, Haíssa (2019) *'Exopolysaccharides produced by Lactobacillus plantarum: technological properties, biological activity, and potential application in the food industry'*, Annals of Microbiology, 10.1007/s13213-019-01456-9.

Briggs, D.E., A. Boulton, Ch Brookes, P.A. & Stevens, R. (2004) *'Brewing: Science and Practice'*, 1-881,10.1201/9780203024195

Buglass, A., McKay, M. Lee, C. G.(2010),*'Handbook of alcoholic beverages: Technical, Analytical and Nutrional Aspects'*,John Wiley Sons, Ltd, pp. 132-210.

Bühler, Thomas M (1996) *'Effects of physical parameters in mashing on lautering performance'*,PhD thesis,Loughborough University, England

Caggianiello, Graziano, Kleerebezem, Michiel & Spano, Giuseppe. (2016) *'Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms'*. Applied Microbiology and Biotechnology. 100. 10.1007/s00253-016-7471-2.

Calissendorff Jan & Falhammar Henrik(2017) *'Rescue preoperative treatment with Lugol's solution in uncontrolled Graves disease'*, Endocrine Connections, 6. 10.1530/EC-17-0025

Dornez, Emmie & Holopainen, Ulla & Cuyvers, Sven & Poutanen, Kaisa & Delcour, Jan & Courtin, Christophe & Nordlund, Emilia (2011). *'Study of grain cell wall structures by microscopic analysis with four different staining techniques'*, Journal of Cereal Science,54,363. 10.1016/j.jcs.2011.07.003.

Farías, R. D., Martínez García, C., Cotes Palomino, T., & Martínez Arellano, M. (2017) *'Effects of Wastes from the Brewing Industry in Lightweight Aggregates Manufactured with Clay for Green Roofs'* Materials , 10(5), 527.

H Fischer, Andrew & A Jacobson, Kenneth & Rose, Jack & Zeller, Rolf(2008) *'Cryosectioning Tissues'*, CSH protocols.

Jin, Y.-L & Speers, Robert & Paulson, A.T. & Stewart, R.J (2004) *'Barley β - glucans and their degradation during malting and brewing'*, Tech. Q. Master Brew. Assoc. Am., 41. 231-240.

J. Scott Smith and Y. H. Hui (2004), *'Food processing : principles and applications'*,1st ed., Blackwell Publishing, Iowa

J. S. Hough (1991),*'The Biotechnology of Malting and Brewing'*, 1st edition, Cambridge University Press,ISBN-10: 0521395534

Kavindra Singh, Atul Anand Mishra & Amit Kumar Yadav (2016) *'Study on beer production from different varieties of barley'*,International Research Journal of Engineering and Technology (IRJET),03,10, e-ISSN: 2395 - 0056

Keller, G. A., Tokuyasu, K. T., Dutton, A. H., & Singer, S. J. (1984), *'An improved procedure for immuno-electron microscopy: ultrathin plastic embedding of immuno-labelled ultrathin frozen sections'*, Proceedings of the National Academy of Sciences of the United States of America, 81(18), 5744–5747.

Meixue Zhou (2010) *'Barley Production and Consumption'*, Genetics and Improvement of Barley Malt Quality, 10.1007/978-3-642-01279-2-1

Moawed, Elhossein. (2012) *'Selective separation of Light green and Safranin O from aqueous solution using Salvadora persica (Miswak) powder as a new biosorbent'*, Journal of Saudi Chemical Society, 10.1016/j.jscs.2012.10.011

Müller, H-Arno.(2008) *'Immuno-labelling of Embryos'*, Methods in molecular biology ,420. 207-18

Mussatto, Solange & Dragone, Giuliano & Roberto, Ines. (2006) *'Brewers' Spent Grain: Generation, Characteristics and Potential Applications'* Journal of Cereal Science. 1-14. 10.1016/j.jcs.2005.06.001.

Nikkhah A. (2012) *'Barley grain for ruminants: A global treasure or tragedy'* Journal of animal science and biotechnology, 3, 22.

Owen P. Ward (1991), *'Bioprocessing'*, USA edition,Van Nostrand Reinhold Inc.,U.S, ISSN - 9780442314392

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A.,Rebello S., Pandey, A. (2018) *'Applications of Microbial Enzymes in Food Industry'*, Food technology and biotechnology, 56(1), 16–30,

Rimsten, Lina (2003) *'Extractable cell-wall polysaccharides in cereals, with emphasis on β -glucan in steeped and germinated barley'*, PhD thesis,Swedish University of Agricultural Sciences, Uppsala.

Thorn K. (2016) *'A quick guide to light microscopy in cell biology'*, Molecular biology of the cell, 27(2), 219–222.

Wikipedia *'Fermentation'* (2019).The Wikipedia Foundation.Available at:https://en.wikipedia.org/wiki/Fermentation (Accessed: 25 June 2019)

Xiros, Charilaos & Christakopoulos, Paul. (2012) *'Biotechnological Potential of Brewers Spent Grain and its Recent Applications'* Waste and Biomass Valorization. 3. 213-232. 10.1007/s12649-012-9108-8.