AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF FOOD PROCESS ENGINEERING

M.Sc. IN FOOD PROCESSING, PRESERVATION
& BIOTECHNOLOGICAL PROCESSES –
BIO-ECONOMY PRODUCTS DEVELOPMENT

Master's thesis

Valorisation of brewer's spent grain for bacterial nanocellulose production and value added products for the development of biodegradable films as food packaging materials for salmon preservation

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Αξιοποίηση παραπροϊόντων βιομηχανίας ζυθοποιίας για παραγωγή βακτηριακής νανοκυτταρίνης και προϊόντων προστιθέμενης αξίας για την ανάπτυξη βιοαποικοδομήσιμων μεμβρανών ως υλικά συσκευασίας τροφίμων για τη συντήρηση σολομού

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M.Sc. Food Processing, Preservation & Biotechnological, Processes – Bio-economy Products Development
Department of Food Science & Human Nutrition
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Abstract

In this study, the waste from the brewing industry was utilized in order to produce biobased and biodegradable food packaging materials through an innovative biorefinery process. Structural carbohydrates (cellulose 16.6%, hemicellulose 21.8%, and oligosaccharides 21.3%), protein (20.5% ± 0.3 w/w) and lignin (9.6% ± 0.2 w/w) fractions were the most important streams arising from the BSG biorefinery. An innovative and sustainable technology namely dielectric barrier discharged non-thermal plasma was used as a pretreatment method, followed by enzymatic hydrolysis. The produced hydrolysate had a concentration of 34.3 g/L sugars, with glucose be the dominant (74.3%) followed by xylose (14.5%) and arabinose (8.1%). The produced hydrolysate was used as nutrient source for the production of bacterial cellulose by *Komagataeibacter sucrofermentans* DSM 15973 in shake flasks, leading to the production of 3.2 g/L bacterial cellulose, with a yield and productivity of 0.08 g/g and 0.21 g/L/day, respectively. The bacterial cellulose produced was further treated with 50% (w/w) H_2SO_4 in order to produce a bacterial nanocellulose suspension. The residual solids after plasma treatment and enzymatic hydrolysis were rich in protein (24.7%) and lignin (17.0%). This stream was used in order to formulate biobased packaging by different treatments. The selected strategy was to recover a protein-rich fraction (purity 52.8%) by alkaline extraction at 60°C, and subsequently to recover the lignin-rich fraction (148 g from 1000 g initial BSG solids) from the residual solids. The recovered protein fraction was able to form a biobased film by adding 3.5% w/v protein together with glycerol (30%, w/w) and bacterial nanocellulose suspension (10%, w/w). The lignin-rich fraction was used to enrich the antioxidant and light barrier properties by introducing it in the film matrix using three different concentrations (5%, 10%, and 15% w/w) and two different solubilization protocols; lignin solubilization at pH 12.0 and modification of lignin through the antisolvent precipitation method. The produced films had good light barrier (less than 2.3% T_{600nm}) and antioxidant properties (more than 39.8% DPPH inhibition). The produced films named Protein, Pr5L, and Pr5LP were compared to PVC films for its substitution at salmon fillets packaging. For the shelf-life analysis, microbial growth and sensory analysis were conducted. Due to the high starting microbial load, the self-life of salmon

was estimated at 2 days, no matter the film used. The sensory analysis of total appearance resulted not to statistically significant differences between the samples until day 7.

Scientific area: Bioprocess engineering

Keywords: Brewer's spent grain, Biorefinery, Salmon packaging

Αξιοποίηση παραπροϊόντων βιομηχανίας ζυθοποιίας για παραγωγή βακτηριακής νανοκυτταρίνης και προϊόντων προστιθέμενης αξίας για την ανάπτυξη βιοαποικοδομήσιμων μεμβρανών ως υλικά συσκευασίας τροφίμων για τη συντήρηση σολομού

M.Sc. Επεξεργασία, Συντήρηση & Βιοτεχνολογικές Διεργασίες Τροφίμων – Ανάπτυξη Προϊόντων Βιο-οικονομίας
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Περίληψη

Στην παρούσα μελέτη, τα απόβλητα από τη βιομηχανία ζυθοποιίας αξιοποιήθηκαν για την παραγωγή βιοαποικοδομήσιμων υλικών συσκευασίας τροφίμων μέσω μιας καινοτόμου διαδικασίας βιοδιυλιστηρίου. Τα κλάσματα δομικών υδατανθράκων (κυτταρίνη 16.6%, ημικυτταρίνη 21.8% και ολιγοσακχαρίτες 21.3%), πρωτεΐνες (20.5% ± 0.3 w/w) και λιγνίνη (9.6% ± 0.2 w/w) ήταν τα πιο σημαντικά ρεύματα που προέκυψαν από το βιοδιυληστήριο του BSG. Ως μέθοδος προεπεξεργασίας του στερεού χρησιμοποιήθηκε μια καινοτόμος και βιώσιμη τεχνολογία, το μη θερμικό πλάσμα με εκκένωση διηλεκτρικού φραγμού, ακολουθούμενη από ενζυμική υδρόλυση. Το παραγόμενο υδρόλυμα είχε συγκέντρωση σακχάρων 34.3 g/L, με κυρίαρχη τη γλυκόζη (74.3%) ακολουθούμενη από ξυλόζη (14.5%) και αραβινόζη (8.1%). Το παραγόμενο υδρόλυμα χρησιμοποιήθηκε ως πηγή ενέργειας για την παραγωγή βακτηριακής κυτταρίνης από τον μικροοργανισμό *Komagataeibacter sucrofermentans* DSM 15973 σε κωνικές φιάλες, οδηγώντας στην παραγωγή 3.2 g/L βακτηριακής κυτταρίνης, με απόδοση και παραγωγικότητα 0.08 g/g και 0.21 g/L. /ημέρα, αντίστοιχα. Η βακτηριακή κυτταρίνη που παρήχθη υποβλήθηκε σε περαιτέρω επεξεργασία με 50% (w/w) H_2SO_4 προκειμένου να παραχθεί ένα εναιώρημα βακτηριακής νανοκυτταρίνης. Τα υπολειπόμενα στερεά μετά την επεξεργασία πλάσματος και την ενζυμική υδρόλυση ήταν πλούσια σε πρωτεΐνη (24.7%) και λιγνίνη (17.0%). Αυτό το ρεύμα χρησιμοποιήθηκε για τη δημιουργία συσκευασίας με διαφορετικές επεξεργασίες. Η επιλεγμένη στρατηγική ήταν η ανάκτηση ενός πλούσιου σε πρωτεΐνη κλάσματος (καθαρότητα 52.8%) με αλκαλική εκχύλιση στους 60°C, και στη συνέχεια η ανάκτηση του πλούσιου σε λιγνίνη κλάσματος (148 g από 1000 g αρχικών στερεών BSG) από τα υπολειμματικά στερεά. Το ανακτηθέν κλάσμα πρωτεΐνης μπόρεσε να σχηματίσει ένα φιλμ βιολογικής βάσης, προσθέτοντας 3.5% w/v πρωτεΐνη μαζί με γλυκερίνη (30% w/w) και εναιώρημα βακτηριακής νανοκυτταρίνης (10% w/w). Το πλούσιο σε λιγνίνη κλάσμα χρησιμοποιήθηκε για να εμπλουτίσει τις αντιοξειδωτικές ιδιότητες και τις ιδιότητες φραγμού φωτός εισάγοντάς το στη μήτρα χρησιμοποιώντας τρεις διαφορετικές συγκεντρώσεις (5%, 10% και 15% w/w) και δύο διαφορετικά πρωτόκολλα διαλυτοποίησης. Το πρώτο πρωτόκολλο αφορούσε τη διαλυτοποίηση λιγνίνης σε pH 12.0 και το άλλο πρωτόκολλο αφορούσε την

τροποποίηση λιγνίνης μέσω της μεθόδου καταβύθισης με antisolvent. Τα παραγόμενα φιλμ είχαν καλό φραγμό φωτός (λιγότερο από 2.3% T_{600nm}) και αντιοξειδωτικές ιδιότητες (περισσότερο από 39.8% αναστολή του DPPH). Οι παραγόμενες μεμβράνες που ονομάστηκαν Protein, Pr5L και Pr5LP συγκρίθηκαν με μεμβράνες PVC για την αντικατάστασή τους στη συσκευασία φιλέτων σολομού. Για την ανάλυση του χρόνου αποθήκευσης, διεξήχθη μικροβιολογικός έλεγχος και οργανοληπτική αξιολόγηση. Λόγω του υψηλού αρχικού μικροβιακού φορτίου, η διάρκεια ζωής του σολομού υπολογίστηκε σε 2 ημέρες, ανεξάρτητα από το φιλμ που χρησιμοποιήθηκε. Η οργανοληπτική αξιολόγηση της συνολικής εμφάνισης οδήγησε σε μη στατιστικά σημαντικές διαφορές μεταξύ των δειγμάτων μέχρι την $7^{η}$ ημέρα.

Επιστημονική περιοχή: Μηχανική βιοδιεργασιών

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Table of contents

Abstract iii
Περίληψη v
1. Introduction 13
1.1 Plastic packaging 13
1.2 Bioplastics 15
1.3 Biorefinery 20
1.4 BSG 22
1.5 BSG Biorefineries 24
1.6 BSG utilization for packaging development 27
1.7 Plasma pretreatment 28
1.8 Bacterial cellulose 30
1.9 Salmon 32
2. Scope of the study 34
3. Materials and methods 35
3.1 BSG compositional analysis 35
3.1.1 Moisture content 35
3.1.2 Ash 35
3.1.3 Total Kjeldahl Nitrogen 35
3.1.4 Extractives 36
3.1.5 Structural carbohydrates and lignin 37
3.1.6 Free sugars and oligosaccharides 39
3.1.7 Total phenolic content 40
3.2 Biorefinery development 41
3.2.1 BSG pretreatment using atmospheric air pressure plasma 41
3.2.2 Enzymatic hydrolysis 41
3.2.3 Protein extraction 42
3.2.4 Lignin extraction and modification 43
3.3 Bacterial cellulose fermentation 44
3.3.1 Microorganism and inoculum preparation 44
3.3.2 Fermentation conditions 45
3.3.3 BNCs production 45
3.3.4 Free amino acid nitrogen (FAN) 46
3.4 Film formulation 47
3.4.1. Treatment of BSG residual solids after plasma and enzymatic hydrolysis 47
3.4.2. Films using protein extract 48
3.5 Films characterization 49

3.5.1. Antioxidant activity of films 49
3.5.2. Mechanical properties 49
3.5.3. Color and transparency 49
3.6 Salmon packaging 50
3.6.1. Microbiological analysis 51
3.6.2. Sensory analysis 52
3.6.3. Statistical analysis 52
4. Results 53
4.1 Compositional analysis of brewers spent grain 53
4.2 Plasma treatment 53
4.3 Enzymatic hydrolysis 55
4.4 Protein extraction 57
4.4.1 Alkaline extraction 57
4.4.2 Enzymatic extraction 57
4.4.3 Hydrothermal extraction 58
4.4.4 Dilute acid extraction 58
4.6 Bacterial cellulose fermentation 59
4.7 Film production 61
4.7.1 Utilization of BSG residual solid 2 61
4.7.2 Utilization of BSG protein-rich extract 62
4.8 Characterization of protein-based films 63
4.9 Salmon packaging 66
4.10 Microbiological analysis 67
4.11 Sensory analysis 72
5. Conclusions 77
6. Future work 79
References 80

Table of Figures

Figure 1. Plastics use by application at the global level for 2023 (OECD (2023), "Global Plastics Outlook: Plastics use by application - projections", OECD Environment Statistics)..... 14

Figure 2. Plastics use by application at the global level for 2023 (OECD (2023), "Global Plastics Outlook: Plastics use by application - projections", OECD Environment Statistics)..... 15

Figure 3. Material classification system based on their biodegradability and bio-based content (European Bioplastics, 2022). 16

Figure 4. Plastics spectrum (Iles & Martin, 2013)..... 17

Figure 5. Schematic representation of a barley kernel in longitudinal section (Mussatto et al., 2006) 22

Figure 6. Schematic representation of the process to obtain BSG from natural barley (Mussatto et al., 2006)..... 23

Figure 7. Schematic representation of the technological pathways employed in a biorefinery for the valorization of BSG and the potential value-added products resulting from the process (Sganzerla et al., 2021)..... 24

Figure 8. Schematic representation of atmospheric pressure plasma reactor (Ravindran et al., 2019). 30

Figure 9. Glycine standard by the FAN method..... 47

Figure 10. Biorefinery development of BSG..... 56

Figure 11. Kinetic of consumption of sugars (■),bacterial cellulose production (○) and FAN (●) during static fermentation **A.** with commercial sugars and **B.** with hydrolysate. 60

Figure 12 Different films produced by BSG residual solid 2 (1,2 and 3) and protein-rich extract (4). **1.** 1M acetic acid as a solvent with three different initial solids concentrations of A. 5% w/v, B. 7.5% w/v, and C. 10% w/v. **2.** Acidic water as a solvent (final pH 2.0) with three different initial solids concentrations of A. 5% w/v, B. 7.5% w/v, and C. 10% w/v. **3.** 10% w/v BSG residual solid using A. 70% v/v, B. 80% v/v, and C. 90% v/v acetic acid solution. **4.** 3.5% w/v protein A. pH 2, 25% glycerol, B. pH 11, 30% glycerol, and C. pH 11, 30% glycerol, 10% BNC, 1% w/v Tween20. 62

Figure 13. Antioxidant activity of the different films..... 63

Figure 14. Young modulus of the different produced films. 64

Figure 15. Tensile strength of the different produced films. 65

Figure 16. Elongation at break of the different produced films 65

Figure 17. Color measurements for the different produced films..... 65

Figure 18. Transparency of the different produced films 66

Figure 19. Procedure of packaging the salmon fillets under different packaging films. 67

Figure 20. Growth of mesophilic aerobic bacteria at 25℃ on the salmon fillets for the 4 different packaging films. 68

Figure 21. Growth of psychrophilic aerobic bacteria incubated at 4℃ on the salmon fillets for the 4 different packaging films..... 69

Figure 22. Growth of Pseudomonas spp. incubated at 25 ℃ on the salmon fillets for the 4 different packaging films. 70

Figure 23. Growth of Enterobacteriaceae incubated at 37℃ on the salmon fillets for the 4 different packaging films. 71

Figure 24. Sensory analysis of the color in salmon fillets packaged with different packaging films and stored under 4°C. 73

Figure 25. Sensory analysis of the odor in salmon fillets packaged with different packaging films and stored under 4°C. 74

Figure 26. Sensory analysis of the total appearance in salmon fillets packaged with different packaging films and stored under 4°C. 75

Table of Tables

Table 1. Composition of the BSG in this study and comparison with compositions found in literature. 54

Table 2. Protein concentration in each stream after the different conditions of alkaline protein extraction and protein recovery 57

Table 3. Protein concentration and recovery of the protein after enzymatic extraction. 58

Table 4. Protein concentration and recovery of the protein after hydrothermal extraction. 58

Table 5. Protein concentration and recovery of the protein after dilute acid extraction. 59

Table 6. Production, yield and productivity of bacterial cellulose, under fermentation of commercial sugars and hydrolysate. 60

Table 7. Population (CFU/g salmon) of aerobic mesophilic bacteria during incubation of salmon at 25°C. 69

Table 8 Population (CFU/g salmon) of aerobic psychrophilic bacteria during incubation of salmon at 4°C. 70

Table 9. Population (CFU/g salmon) of Pseudomonas spp during incubation of salmon at 25°C. 71

Table 10. Population (CFU/g salmon) of *Enterobacteriaceae* during incubation of salmon at 37 °C. 72

Table 11. Scores of color sensory analysis for the different packaging films of salmon fillets stored under 4°C. 73

Table 12. Scores of odor sensory analysis for the different packaging films of salmon fillets stored under 4°C. 74

Table 13. Scores of total appearance sensory analysis for the different packaging films of salmon fillets stored under 4°C. 75

1. Introduction

1.1 Plastic packaging

The now-defunct Packaging Institute International (Glossary of Packaging Terms, 1988) defined packaging as the enclosure of products, items or packages in a wrapped pouch, bag, box, cup, tray, can, tube, bottle, or other container form to perform one or more of the following functions: containment, protection, preservation, communication, utility and performance. If the device or container performed one or more of these functions, it was considered a package. If the device or container performed one or more of these functions, it was considered a package (Robertson, 2012).

In 2022, the world's population was anticipated to be 8 billion, with this figure expected to rise to 9.7 billion by 2050 (United Nations, 2022). Food waste will rise to over 200 million t annually by 2050 and also an increase of 50% in the global food supply will be needed (Guillard et al., 2018). Europe as a continent produces about 23 million tonnes of plastic packaging each year and it is believed that in 2050 will produce 92 million tonnes of plastic packaging waste (Ncube et al., 2021). Food packaging materials will also have subsequently a higher demand because of the higher food production. Plastics play a significant role in the food industry as a packaging material, finding versatile applications in various forms such as containers and flexible packaging. As a packaging material, they are the first in usage in terms of value with most products being packaged in plastic (Kirwan et al., 2003).

Plastics have a wide range of uses because of their unique properties. They have a wide temperature range in which they can be used, they are very light and very strong for their weight. Apart from these properties, they also have low cost, which is why they are so widely used in the food industry as a packaging material (Andrady & Neal, 2009). In Europe, polyethylene (PE) has the highest consumption of polymers used in the food industry, with the biggest proportion of the market followed by Polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and polyvinyl chloride (PVC). All of the above materials are thermoplastic polymers and each one is based on one or more compounds or monomers, and they are accounting the major plastic market share (Kirwan et al., 2003; Narancic & O'Connor, 2019).

Plastic production requires 10% of the global oil production and moreover, more than one third of the produced plastic is single-use plastic which mainly ends up in landfills after its use (Narancic & O'Connor, 2019; Woldemar D'ambrières, 2019). The global production of

polymers is getting bigger every year and it is expected to reach over 500 million tonnes per year by 2050 with most of these polymers being single-use plastics (Sardon & Dove, 2018).

In most of developed countries, only a small fraction of plastic waste is recycled. For example, USA and Australia have a recycling rate of less than 10% but even countries with significant infrastructure for sorting and processing plastic waste by polymer type can attain recycling rates of 30% (Woldemar D'ambrières, 2019). Only 5% of the single-use plastic packaging material that has been collected for recycling is being successfully recycled into new plastic (Hahladakis & Iacovidou, 2018). The food industry is urged to use waste reduction strategies like reduce, reuse, and recycling rather than the simple direct disposal approach. Methane gas, a greenhouse gas that causes more global warming than carbon dioxide by more than 2000%, is produced when this waste is dumped in landfills (Singh et al., 2017).

Due to the big impact that plastic packaging waste has on the environment, European Union demand that 55% of the plastic packaging waste should be recycled by the year 2030 (EU Directive 2018/852).

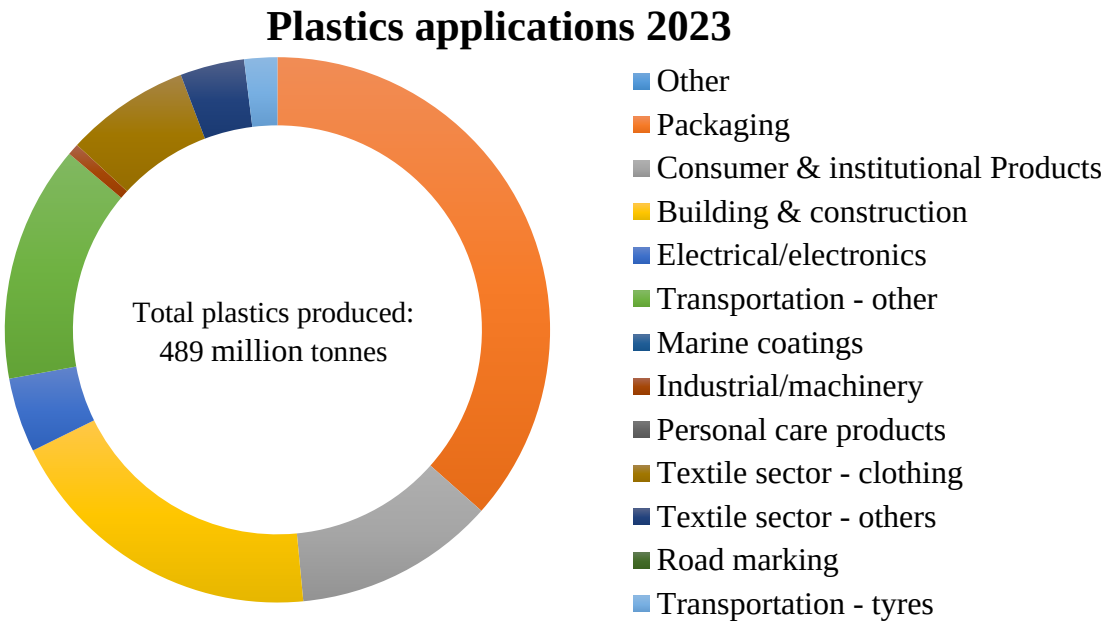


Figure 1. Plastics use by application at the global level for 2023 (OECD (2023), "Global Plastics Outlook: Plastics use by application - projections", OECD Environment Statistics).

Figure 1 illustrates the global utilization of plastics across different sectors and the corresponding quantities employed in each. Notably, the packaging sector constitutes a substantial portion of plastic usage.

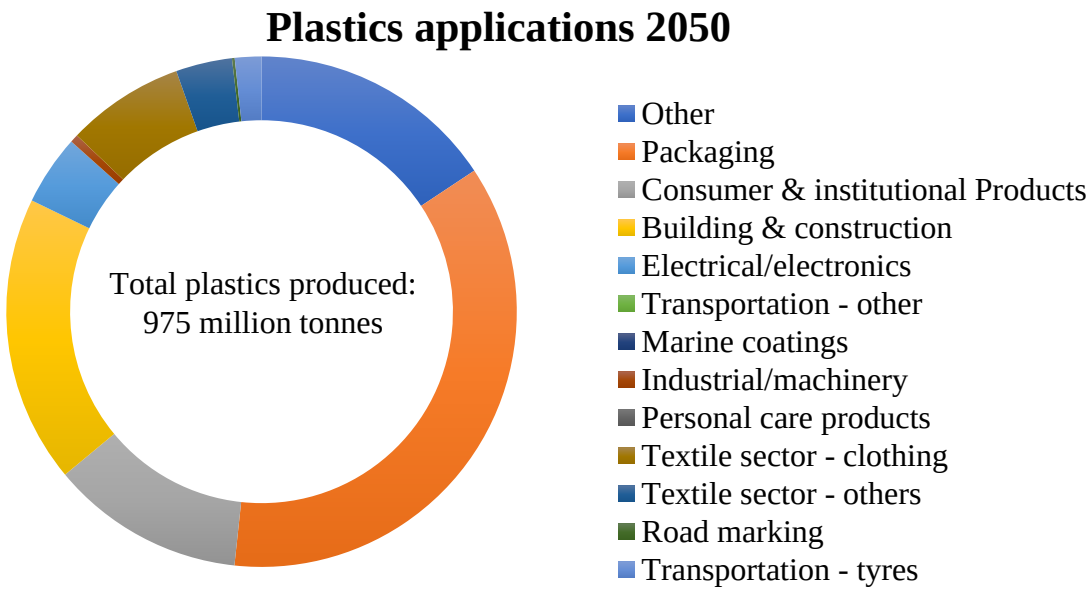


Figure 2. Plastics use by application at the global level for 2023 (OECD (2023), "Global Plastics Outlook: Plastics use by application - projections", OECD Environment Statistics).

In Figure 2, it is evident that plastic production is projected to increase, leading to a subsequent rise in plastic waste generation. Furthermore, the packaging sector is expected to maintain its prominent position as the largest consumer of plastic materials.

1.2 Bioplastics

Bio-based and/or biodegradable are two general categories for bioplastics. It is referred to as being "biobased" when the origin of the carbon building blocks is the primary consideration rather than the destination of the material after it has served its purpose. It's crucial to realize that not all materials described as being biodegradable are also often described as being bio-based, and vice versa. When materials degrade under the influence of microbes and in the proper environments and utilized as a food source, they are called “biodegradable materials”. It is regarded as compostable when the food source is completely assimilated by microorganisms in a compost environment within 180 days. The term 'bio-based' refers to materials or products that are, at least partially, derived from biomass, which primarily comes from plants. Biomass used for bioplastics production includes sources such as corn, sugarcane, or wood, among others (European Bioplastics, 2022).

Bioplastics are divided into three main groups of different materials:

- The first group is composed of bio-based, partially bio-based, non-biodegradable plastics (e.g., bio-PE, bio-PP etc.).

- The second group is composed of plastics that exhibit both bio-based and biodegradable characteristics, like polylactic acid (PLA) or polyhydroxyalkanoate (PHA).
- The third group is composed of plastics that are derived from petroleum-based products and possess a specific molecular weight (MW) and inherent properties that enable them to biodegrade.

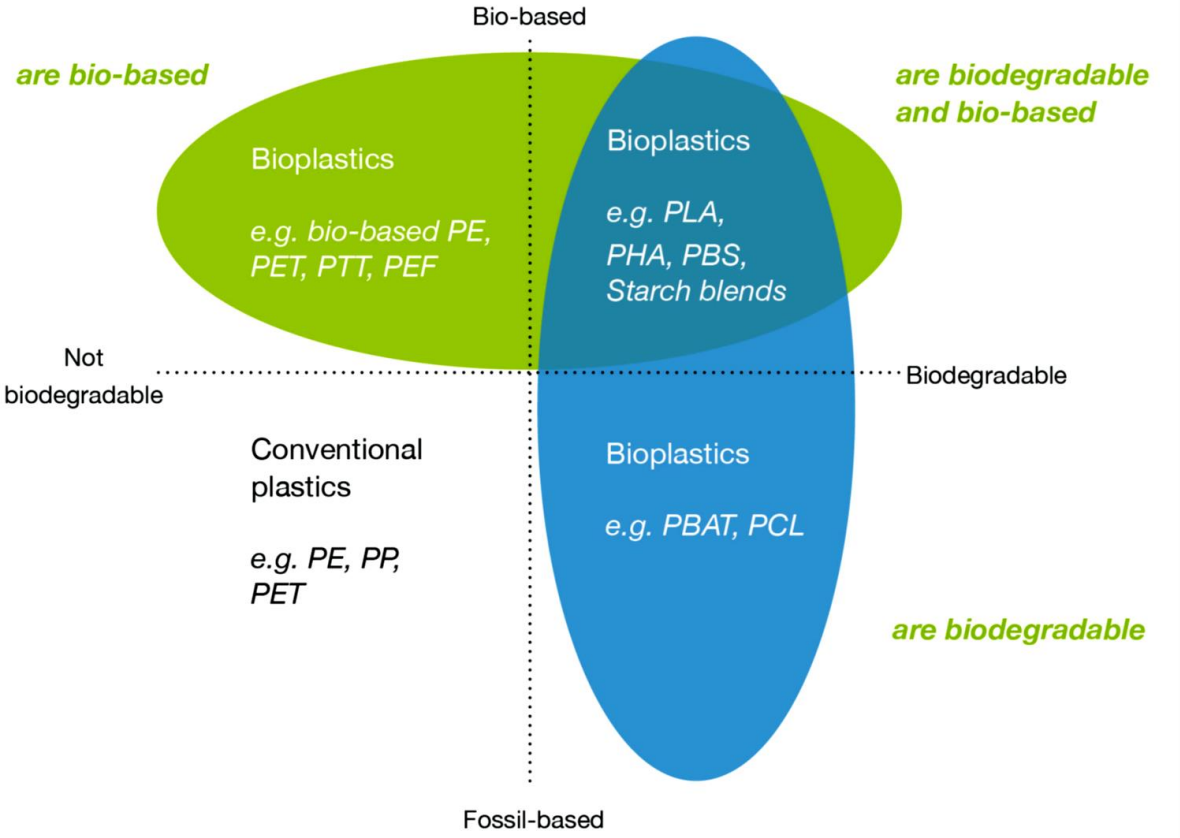


Figure 3. Material classification system based on their biodegradability and bio-based content (European Bioplastics, 2022).

Figure 3 illustrates a material coordinate system used to categorize types based on their bio-based content and biodegradability. The coordinate system is subdivided into four quadrants: bio-based, biodegradable, fossil-based, and non-biodegradable.

It is very important to notice that biodegradability depends on the chemical composition of the product and not on the origin of the material (Piergiovanni & Limbo, 2016).

There are four types of degradable plastics:

1. Photodegradable bioplastics encompass light-sensitive groups that are incorporated directly into the polymer's backbone as additives. Upon extensive exposure to ultraviolet radiation over an extended period, ranging from several weeks to months,

these bioplastics undergo disintegration, leading to their polymeric structure becoming vulnerable to subsequent bacterial degradation (El-Kadi, 2010).

2. The Business-NGO (non-government organization) Working Group for Safer Chemicals and Sustainable Materials provides a definition of bio-based bioplastics as "plastics in which 100% of the carbon originates from renewable agricultural and forestry resources, such as corn starch, soybean protein, and cellulose." (Álvarez-Chávez et al., 2012).
3. Compostable bioplastics undergo biological decomposition during the composting process at a rate comparable to other compostable materials, and they do not leave behind visible toxic remnants. To classify a plastic as bio-compostable, standard tests are conducted to assess its overall biodegradability, degree of disintegration, and the potential eco-toxicity of the degraded material (Sarasa et al., 2009).
4. Biodegradable bioplastics undergo complete degradation facilitated by microorganisms, leaving no visible toxic residues. The term "biodegradable" pertains to materials capable of naturally breaking down into biogases and biomass, primarily carbon dioxide and water, when exposed to a microbial environment and humidity, such as those found in soil. This property contributes to the reduction of plastic waste. In contrast, bio-based sustainable materials refer to those derived from renewable resources, like plants or agricultural products, and are intended to have a reduced environmental impact throughout their lifecycle (Arikan & Ozsoy, 2015).

Figure 4. Plastics spectrum (Iles & Martin, 2013)

The future of biodegradable plastics is very promising because of the need to replace conventional plastics. Bioplastics have the potential to contribute to a much lower carbon footprint compared to conventional plastics. It's essential to consider whether the bioplastic permanently stores the carbon taken from the air during the plant's growth. When a bioplastic is derived from a biological source, it captures and sequesters the CO_2 extracted by the plant during the photosynthesis process. However, if the bioplastic eventually degrades into CO_2 and water, this sequestration is reversed. On the other hand, permanent bioplastics, designed to be

similar to polyethylene or other traditional plastics, store the CO_2 indefinitely. Even through multiple recycling cycles, the initial CO_2 taken from the atmosphere remains sequestered within the permanent bioplastic. This property makes them a potentially sustainable choice for reducing greenhouse gas emissions (Chen, 2014). Another advantage of bioplastics is that bioplastics are derived from renewable resources such as corn, sugarcane, soy, and other plant-based sources. This is in stark contrast to conventional plastics, which predominantly rely on petroleum, a non-renewable and finite resource (Yu & Chen, 2008). Energy efficiency is a notable advantage of bioplastics over conventional plastics (Chen, 2014). The production process of bioplastics typically requires less energy compared to the manufacturing of traditional plastics. This reduced energy demand can lead to lower overall environmental impact and contribute to more sustainable practices in the plastics industry. In contrast, conventional plastics heavily rely on petroleum, accounting for approximately 4% of the world's annual oil consumption. With concerns about oil scarcity and fluctuating oil prices, the production of conventional plastics becomes susceptible to economic uncertainties. Additionally, the extraction and use of petroleum contribute to various environmental issues, including greenhouse gas emissions and habitat degradation (Chen, 2014). Eco-safety is another important advantage of bioplastics. These materials generate fewer greenhouse gases and do not contain toxins, making them environmentally friendly and safer for human health. Researchers (Yu & Chen, 2008) have reported that bioplastics make a significant contribution to the goal of reducing greenhouse gas (GHG) emissions. The production of 1 kg of bioplastic resin emits only 0.49 kg of CO_2, in stark contrast to the 2~3 kg of CO_2 emitted during the production of petrochemical-based plastics. This represents an impressive 80% reduction in global warming potential compared to their petrochemical counterparts.

Nevertheless, the implementation of bioplastics may present certain potential challenges and disadvantages. One significant disadvantage of bioplastics is their higher production costs compared to conventional plastics. At present, it is estimated that bioplastics can cost around two times more to produce. In the future, the large-scale production of bioplastics with a cost reduction of the produced products is expected (Arikan & Ozsoy, 2015). Recycling poses a notable challenge for bioplastics, as their co-mingling with conventional plastics could lead to contamination during the recycling process. For instance, when employing infrared rays in waste separation systems, the differentiation of bioplastics becomes difficult, potentially resulting in contaminated conventional plastic fractions (Arikan & Ozsoy, 2015). The utilization of bioplastics derived from renewable sources could reduce the raw material reserves. To further mitigate energy consumption during bioplastics production and reduce

potential competition with agricultural resources for food, the current trend involves exploring alternative raw material sources, notably the utilization of food by-products (Lagaron & Lopez-Rubio, 2011). Misunderstanding of terminology can lead to confusion, particularly when describing bioplastics as compostable. Not all bioplastics are suitable for home composting, like organic food waste, as many require specialized industrial composting facilities, which may not be accessible in every composting site (Barker & Safford, 2009). Furthermore, certain manufacturers misuse terms associated with bioplastics to enhance the market appeal of their products. Slogans like "environmentally friendly," "non-toxic," and "degradable/totally degradable" are often employed as a ploy to deceive uninformed and overwhelmed consumers. The production of bioplastics is expected to witness significant growth, projected to surpass 6.7 million tons by the year 2018. However, despite this increase, numerous countries have yet to implement any specific laws or legislation concerning the production, usage, or waste management of bioplastics (Arikan & H Ozsoy, 2015).

As per the European Union Plastics Strategy, bioplastics have the potential to play a crucial role in advancing towards a low carbon circular economy, thereby enhancing recycling efficiency. One of the significant advantages of many bioplastics lies in their biodegradability, setting them apart from conventional plastics. The European Union Commission recognizes bioplastics as a key contributor to climate protection and reducing carbon footprint. Considering the significant greenhouse gas emissions associated with fossil fuel dependency, addressing this issue has become crucial in mitigating problems like food supply disruptions, increased wildfires, and extreme weather events (Nandakumar et al., 2021).

Replacing a considerable portion of fossil fuel-based plastics with bio-based alternatives can contribute significantly to achieving the EU Climate Protection targets and align with the UN Sustainable Development Goals. By utilizing bioplastics, we can reduce reliance on fossil fuels and promote a more sustainable and environmentally friendly approach to plastic production and waste management. Emphasizing the adoption of bioplastics can thus be instrumental in reducing the environmental impact of plastics and working towards a more sustainable future (Nandakumar et al., 2021).

Edible packaging represents a rapidly evolving field that utilizes edible compounds sourced from renewable materials, including proteins, polysaccharides, lipids and other edible components. These materials are designed to be an integral part of food products and are intended to be consumed along with the food, making them inherently biodegradable in composting and other biological recycling processes. Edible packaging typically encompasses

various forms such as edible films, sheets, coatings, and pouches. Edible films or sheets are standalone structures that are preformed separately from the food and can be placed on, between, or sealed into edible pouches. On the other hand, edible coatings are thin layers of edible materials directly applied to the surface of food products (Janjarasskul & Krochta, 2010).

1.3 Biorefinery

A biorefinery can be described as a framework or a structure designed to efficiently utilize biomass in a sustainable manner, aiming to produce a diverse range of products. The primary goal of a biorefinery is to maximize the value obtained from biomass resources, ensuring minimal waste and environmental impact (Saral et al., 2022). The biorefinery concept aims to produce environmentally sustainable and economically viable products (Rajesh Banu et al., 2021).

Among the potential large-scale industrial biorefineries, the lignocellulose-feedstock (LCF) biorefinery is likely to achieve the highest success. The LCF biorefinery stands out for two key reasons: firstly, the availability of suitable raw materials is optimal, including straw, reed, grass, wood, paper-waste, and more. Secondly, the conversion products from lignocellulose have favorable prospects in both the traditional petrochemical market and the emerging biobased product markets. These factors make the LCF biorefinery a promising and viable option for sustainable and economically feasible production (Kamm & Kamm, 2004). Approximately 181.5 billion tonnes of lignocellulosic biomass are produced annually, constituting around 90% of the total biomass. However, only a small fraction, approximately 3%, of this lignocellulosic biomass is efficiently utilized and integrated into the circular bioeconomy (Dahmen et al., 2019). This indicates a significant untapped potential for maximizing the sustainable use of lignocellulosic biomass and promoting its incorporation into various bio-based products and processes within a circular economic framework.

Lignocellulosic materials are composed of three main chemical fractions or precursors: (a) hemicellulose/polyoses, which are the combination of polysaccharides that do not have crystallinity, and their degree of polymerisation is low, and it can be further deteriorated into monosaccharides, such as xylose and arabinose, (b) cellulose, which is a linear polymer where β–glucose linked with 1–4 bonds, forming long chains of glucose molecules, and is a crucial structural component in plant cell walls and (c) lignin, which is the polycrystalline polymer composed of phenyl propane linked through C–C or ether bond with irregular repeating unit, providing rigidity and strength to the plant cell walls, and it plays a vital role in supporting the structural integrity of lignocellulosic materials.

Given the estimated annual biosynthesis production of biomass, which amounts to 180×10^9 t, the distribution of its chemical composition is as follows: 75% of the biomass is carbohydrate (cellulose, starch, and saccharose), 20% of the biomass is lignin, and the rest 5% consists of other natural compounds, such as fats (oils), proteins, and various substances (Kamm & Kamm, 2004).

Given this composition, the primary focus should be on efficiently accessing carbohydrates and their subsequent conversion into chemical bulk products and corresponding final products. Glucose, which can be obtained through microbial or chemical methods from starch, sugar, or cellulose, holds a key position as a basic chemical due to its versatility. Glucose serves as a valuable precursor for a wide range of biotechnological or chemical products (Kamm & Kamm, 2004).

The contemporary LCB (lignocellulosic biomass) biorefinery technologies should incorporate two key process steps: (i) the first step is the fractionation of Lignin, Cellulose, and Hemicellulose. This step involves separating lignin, cellulose, and hemicellulose from the lignocellulosic biomass using cost-effective and sustainable technologies (K. Zhang et al., 2016). Fractionation is a crucial initial stage that facilitates the isolation of the individual components of LCB, making them more amenable to subsequent conversion processes. (ii) Integrated biorefining involves the application of various techniques, such as biological conversion, thermochemical processes, and hydrothermal liquefaction, to transform individual fractions of biomass into valuable products. The ultimate aim is to replace fossil-derived products with sustainable alternatives through this comprehensive approach (Rajesh Banu et al., 2021).

The chemical industry is undergoing a significant transformation as it embraces eco-friendly bioprocesses, moving away from traditional fossil fuel dependence. This shift is pivotal in advancing the concept of a circular bioeconomy and reducing environmental impact. Transitioning from a petroleum refinery model to a waste biorefinery model signifies a significant endeavor in carbon management and greenhouse gas (GHG) mitigation. Embracing the waste biorefinery approach entails building a sustainable circular bioeconomy rooted in the principles of recycling, reusing, and remanufacturing, moving away from the traditional linear economy based on the "take, make, and dispose" principle. This transformation represents a powerful step towards managing carbon emissions and effectively mitigating GHGs while promoting a more environmentally responsible and resource-efficient approach to industrial processes (Leong et al., 2021).

A circular economy (CE) is an economic model that focuses on maximizing resource efficiency by minimizing waste, retaining long-term value, reducing the use of primary resources, and establishing closed loops for products, product parts, and materials. This model operates within the framework of environmental protection and socioeconomic benefits. By embracing a circular economy, there is a potential for achieving sustainable development while decoupling economic growth from the adverse effects of resource depletion and environmental degradation (Morseletto, 2020). This shift towards a circular economy presents an opportunity to foster a more sustainable and environmentally responsible approach to economic activities, aiming for positive impacts on both society and the environment.

1.4 BSG

Brewers' spent grain (BSG) is the predominant by-product of the brewing industry, constituting approximately 85% of the total generated by-products. According to (Mussatto & Roberto, 2006), BSG can be classified as a lignocellulosic material, consisting of cellulose, hemicellulose, and lignin. These components together make up nearly 50% (by weight) of the BSG composition. BSG also contains high amount of protein in the range of 18-30%. Despite its abundance, BSG's primary application has been limited to animal feeding, which underutilizes its potential as a valuable resource (Mussatto et al., 2006).

Barley grain, the key raw material used in beer production, is abundant in starch and proteins and is composed of three primary parts: the germ (embryo), the endosperm (comprising the aleurone and starchy endosperm), and the grain coverings. The grain coverings can be further categorized into three fractions: the seed coat, which surrounds the aleurone; the pericarp layers lying above the seed coat, and finally, the husk covering the pericarp layers (Figure 5).

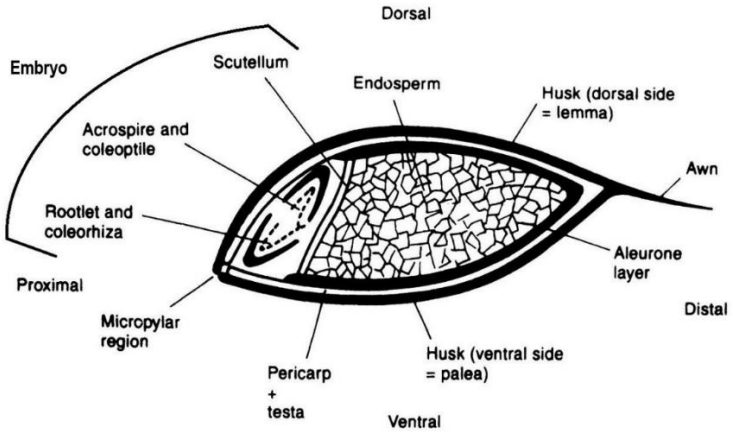


Figure 5. Schematic representation of a barley kernel in longitudinal section (Mussatto et al., 2006)

Figure 6 is showing a schematic representation of the malting and brewing process in order to receive the brewer's spent grain from natural barley after the beer production in a brewing industry.

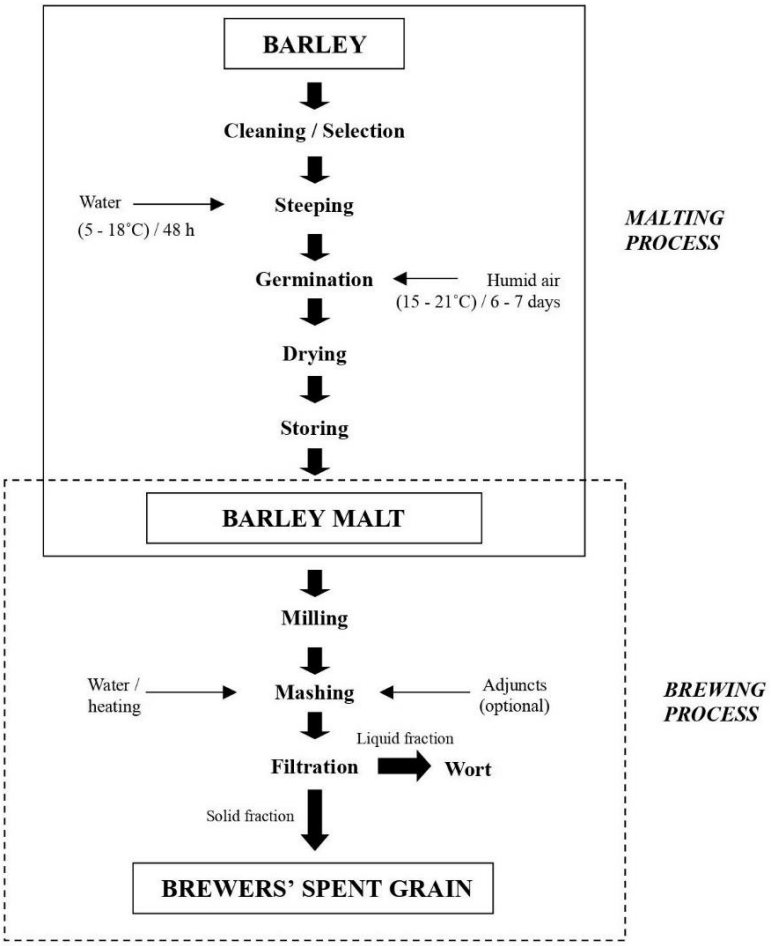


Figure 6. Schematic representation of the process to obtain BSG from natural barley (Mussatto et al., 2006).

The brewing industry is known to produce significant quantities of by-products and wastes, with spent grain, spent hops, and yeast being among the most common. However, the environmentally friendly aspect of the brewing industry lies in its potential for recycling and reusing these by-products. As most of these by-products are derived from agricultural sources, they can be readily repurposed, contributing to a more sustainable and circular economy. By efficiently recycling and reusing these by-products, the brewing industry minimizes waste and optimizes resource utilization, thereby reducing its environmental impact. This environmentally conscious approach sets the brewing industry apart from other sectors, making it relatively more environmentally friendly (Mussatto et al., 2006). BSG and spent yeast are the primary by-products produced in breweries. BSG represents approximately 30% (w/w) of the initial raw materials used in the brewing process and contributes significantly, accounting for 85% of the

total by-product generation in breweries (Tang et al., 2009). On average, the production of 1 m^3 of beer results in approximately 270 kg of solid wastes. Specifically, the breweries in the EU-28 region generated a total of around 10.8 million t of BSG in the year 2016 (Ioannidou et al., 2020).

1.5 BSG Biorefineries

BSG contains valuable structural components including lignin, cellulose, hemicelluloses, and protein which can be recovered through fractionation in a biorefinery process, which involves separating the different fractions of these materials (Outeiriño et al., 2019). However, the comprehensive utilization of lignocellulosic biomass presents a challenge due to its high recalcitrance to chemical and biological degradation (An et al., 2017). Both cellulose and hemicelluloses, exhibit strong stability and adhere firmly to the surrounding polymeric lignin structures. This inherent recalcitrance makes it challenging to efficiently break down and convert lignocellulosic biomass into valuable products (Outeiriño et al., 2019).

Figure 7 illustrates a comprehensive scheme for a biorefinery aimed at valorizing BSG. This scheme outlines the most promising technological pathways for the production of bioenergy, biofuels, and value-added products derived from BSG.

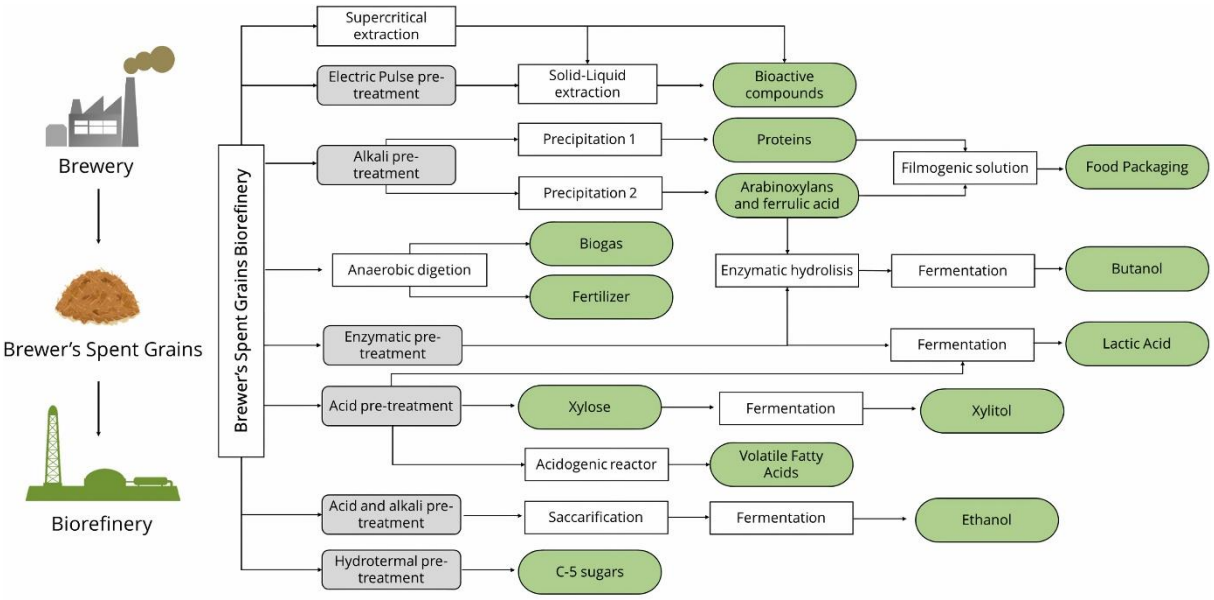


Figure 7. Schematic representation of the technological pathways employed in a biorefinery for the valorization of BSG and the potential value-added products resulting from the process (Sganzerla et al., 2021).

In a study conducted by Bonifácio-Lopes et al. (2020), BSG was utilized as a source of bioactive compounds. The researchers employed a solid-liquid extraction method using a hydroethanolic solution (60% v/v). The results showed the presence of significant bioactive

compounds in the BSG extract, including 4-hydroxybenzoic acid (104.6 ± 0.2 μg per g BSG), vanillin (109.2 ± 0.5 μg per g BSG), catechin (223.6 ± 1.9 μg per g BSG), and vanillic acid (122.0 ± 4.4 μg per g BSG). These extracts demonstrated various beneficial properties, such as antioxidant, antihypertensive, and antibacterial activities.

There are many studies focused on the extraction and valorisation of arabinoxylan fraction of BSG. In a study conducted by (Pérez-Flores et al., 2019), arabinoxylan was the target product extracted from brewers' spent grain (BSG) using an alkaline process with 0.5 mol per L NaOH. The arabinoxylan yield obtained was approximately 5%, and the pure arabinoxylan constituted 71.64±1.18% of the total arabinoxylan in BSG. The composition analysis of BSG-arabinoxylan revealed the presence of glucose (17.94%), arabinose (33.74%), and xylose (37.90%). Furthermore, the researchers found that ferulic acid played a significant role in the antioxidant capacity of arabinoxylan derived from BSG, highlighting its potential as a valuable bioactive compound. Vieira et al. (2014) conducted a study, focusing on the sequential extraction of arabinoxylan and proteins from brewers' spent grain (BSG). The researchers employed a two-step extraction process to recover these valuable components. In the first step, arabinoxylan was extracted and obtained a yield ranging from 66% to 73% of the total arabinoxylan present in BSG. The recovery of arabinoxylan was achieved through ethanol precipitation. In the second step, proteins were extracted, and a yield of 82% to 85% of the total proteins in BSG was obtained. Citric acid was applied to obtain the protein-rich fractions, facilitating the recovery of proteins from the spent grain. Another study refers to the production of butanol from the arabinoxylan fraction of brewers' spent grain (BSG). The researchers employed an enzymatic hydrolysis process using cellulases to break down the arabinoxylan into fermentable sugars. Subsequently, a fermentation using the microorganism *Clostridium beijerinckii* conducted to produce butanol. The results showed a promising yield, with 37 kg of acetone-butanol-ethanol (ABE) per t of BSG and 28 kg of butanol per ton of BSG (López-Linares et al., 2020).

Different kind of fermentation were studied by using BSG as a substrate in order to produce value-added materials. In 2006, Mussatto and Roberto conducted a study to produce xylitol from brewers' spent grain (BSG). They used diluted sulfuric acid to hydrolyze BSG, obtaining a xylose-rich liquor. *Candida guilliermondii* yeast efficiently fermented the xylose to produce xylitol. The process resulted in a xylose consumption rate of 67% to 96.9%, achieving a xylitol yield of 0.70 g/g and a production rate of 0.45g/L/h. The hemicellulose sugars' extraction efficiency was 92.7% without nutrient supplementation during fermentation (Mussatto & Roberto, 2006). The production of lactic acid from BSG hydrolysates have also

been studied by using *Lactobacillus rhamnosus*. The researchers achieved a high lactic acid yield of 91.29% and a volumetric productivity of 1.69 g/L/h, utilizing the 54 g/L initial reducing sugar content in the hydrolysates (Pejin et al., 2017). In 2020, Rojas-Chamorro et al. produced ethanol from BSG using sulfuric acid pretreatment, enzymatic hydrolysis, and fermentation. They achieved an ethanol yield of 22.9 L per 100 kg of dry biomass and recovered 94% of the sugars in raw BSG through optimized pretreatment (Rojas-Chamorro, Romero-García, et al., 2020). In 2021, Castilla-Archilla et al. produced volatile fatty acids (VFAs) from BSG using thermal diluted acid hydrolysis followed by acidogenic batch fermentation. The highest VFAs concentration obtained was 16.89 g COD L−1, mainly composed of acetate and butyrate (99.5–99.8%). The biotransformation of BSG using thermophilic and mesophilic anaerobic methanogenic communities has been studied. Under thermophilic conditions, they achieved a methane yield of up to 58.7 L/kg with 63.5% biodegradation. The optimal BSG concentration for biogas production was found to be 50 and 100 g/L (Malakhova et al., 2015). In 2018, Luft et al. utilized supercritical CO_2 technology to enhance enzymatic hydrolysis of BSG, resulting in a significant increase in sugar yield, up to 3.6 times higher that of conventional enzymatic hydrolysis. The sugars obtained from BSG amounted to 219.39 g/kg BSG and comprised 26.5% glucose, 2.2% cellobiose, 1.7% xylose, and 0.7% arabinose. The optimized conditions were achieved at 40°C, 175 bar, and 80% moisture for 240 minutes (Luft et al., 2018).

Lignin, a byproduct of various agricultural industries, currently has limited applications (Zadeh et al., 2018). Typically regarded as a low-cost raw material and often utilized as a low-value fuel source, holds the potential to be transformed into higher-value lignin-based materials. Its intrinsic hydrophobic nature makes it a suitable reinforcement component for proteins, as it can enhance the moisture resistance of protein-based films. This presents an opportunity to create more valuable materials from lignin. Proteins have found use as packaging films due to their strong oxygen barrier properties and unique structure, which provides excellent mechanical strength. These attributes ascribed to the abundance of polar groups in their structure, facilitating strong intermolecular interactions (Rojas-Lema et al., 2023).

Lignin has demonstrated significant potential across various applications such as biofuels, binders, dispersants, polymeric materials, and packaging. Nevertheless, its utilization as a raw material can be hampered by its intricate and random structure, as well as its limited water solubility. To overcome these challenges, the production of colloidal lignin particles (CLPs) has emerged as a promising approach. CLPs effectively render lignin water-dispersible without necessitating any chemical modifications. Furthermore, these particles exhibit a high surface area-to-volume ratio and beneficial shapes, which enhance lignin's properties. This

includes improved antioxidant and UV protection capabilities, increased antimicrobial activity, and enhanced thermal stability (Colucci et al., 2023).

1.6 BSG utilization for packaging development

Extensive research has been carried out in recent times to explore the potential of utilizing BSG for the development of new packaging materials. The primary goal of these studies is to identify an alternative to conventional plastics that are commonly employed in food packaging, while also leveraging a valuable resource that is a by-product of the brewing industry.

Moreirinha et al. (2020) made a study that focused on the preparation of nanocomposite films using BSG arabinoxylans (AX) as the base material, with the addition of varying amounts of nanofibrillated cellulose (NFC) at different mass fractions (5%, 10%, 25%, 50%, and 75%). The resulting nanocomposite films exhibited homogeneity, excellent thermal stability up to 230°C, and impressive mechanical properties, including a Young's modulus of up to 7.5 GPa. To further enhance the films' functionality, the ones containing 50% NFC were incorporated with either ferulic acid or feruloylated arabinoxylo-oligosaccharides enriched fraction from BSG (at a concentration of 75 mg per g of film). This combination not only improved the films' UV-Vis barrier properties but also endowed them with several additional functionalities: high antioxidant activity, up to 90% in DPPH scavenging activity; antibacterial activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria; and antifungal activity against the polymorphic fungus *Candida albicans*. Overall, these fully biobased nanocomposite films demonstrate significant potential for applications as active food packaging systems.

Another study conducted by Proaño et al. (2020), aimed to develop brewer's spent grain protein concentrate (BSG-PC) films with potential as active packaging materials. Different films were prepared by casting protein dispersions at varying pH levels (2, 8, 11) and using different plasticizers (polyethylene glycol or glycerol) at different levels (0-0.25 g g^{-1}). Mechanical, water-barrier, solubility, optical, antioxidant, and antimicrobial properties of the films were evaluated. Films prepared at pH 2 and plasticized with polyethylene glycol were found to be homogeneous and manipulable, prompting further investigation with different levels of polyethylene glycol at this pH. Increasing polyethylene glycol concentrations led to higher water solubility, water vapor permeability, and elongation at break, while decreasing tensile strength and elastic modulus. The antioxidant activity was dependent on polyethylene glycol concentration, while no antimicrobial properties were observed against specific bacteria

and fungi. The formulations with 0.10 and 0.15 g of polyethylene glycol per gram of BSG-PC showed promise, achieving a balance between mechanical, water-barrier properties, and antioxidant capacity.

In another study conducted by Taner et al. (2022), the researchers investigated a cutting-edge method to coat strawberries by harnessing the abundant protein, phenolic compounds, and cellulose found in BSG. Extracted cellulose, protein, and phenolic compounds from BSG were used to synthesize carboxyl methylcellulose (CMC) films with varying concentrations of BSG protein and phenolic compounds. The CMC films demonstrated significant improvements in physicochemical and biological properties, with increased protein and phenolic compound levels leading to enhanced antioxidant activity and hydrophobicity. Coating fresh strawberries with these CMC films resulted in prolonged freshness, as evidenced by maintained acidity, pH, weight, anthocyanin content, and total dry matter during storage. This innovative approach shows promise in using BSG waste components to develop effective strawberry coatings, potentially extending their shelf life and preserving their quality.

In a study conducted by Ferreira et al. (2019), novel food packaging trays were successfully produced using brewer's spent grains (BSG), a low added-value byproduct of the beer industry, and potato starch. These trays were compared to reference trays made of expanded polystyrene (EPS), a commonly used material in food packaging. The results revealed that all trays with varying proportions of BSG and potato starch exhibited suitable flexural strength, ranging from 1.51 ± 0.32 MPa for 80% BSG content to 2.62 ± 0.46 MPa for 40% BSG content, surpassing the strength of EPS trays (0.64 ± 0.50 MPa). However, the flexural strength and modulus of the trays decreased significantly upon contact with water, primarily due to starch plasticization, resulting in values below those of EPS. Remarkably, trays comprising 60% BSG, and treated with chitosan and glyoxal, demonstrated the highest flexural strength both before and after water contact, measuring 3.75 ± 0.52 MPa and 0.44 ± 0.11 MPa, respectively. The latter value was notably close to the reference EPS tray's strength.

1.7 Plasma pretreatment

Cold plasma represents a cutting-edge non-thermal technology with substantial promise across various sectors, particularly in the food industry. Initially, it found extensive use in the polymer and electronics industries, primarily for surface modification and enhancing the functionality of different polymer materials. However, in recent times, the applications of cold plasma have seen a remarkable expansion into the treatment of biomedical devices and

biological materials, notably within the realm of food processing and preservation. This technology's versatility and ability to safely interact with biological substances make it increasingly relevant and valuable for numerous applications within the food sector (Pankaj & Keener, 2017).

Pretreatment plays a crucial role in biomass processing by degrading the structural network of lignin and enhancing the enzymatic hydrolysis of hemicellulose and cellulose (Chen et al., 2017). Various pretreatment methods are used to improve the digestibility of lignocellulosic biomass, including traditional chemical treatments (alkaline and acid) and innovative processes like non-thermal plasma (Pereira et al., 2021).

According to Merche et. al. (2012) plasma is the fourth state of matter. Non-thermal plasma (NTP) is a partial electrical discharge initiated with sufficient voltages, and it causes the electron's temperature to rise, at a higher temperature than the temperature of the surrounding gas molecules. As a result, the interaction between electrons and gas molecules generates highly energetic species, such as active radicals, reactive species, and ions and they have higher energy levels, which increases the rate of the reactions (Wu et al., 2013). It is a non-toxic and sustainable method that offers an alternative for minimizing lignocellulosic biomass recalcitrance by removing lignin (Ravindran et al., 2019). Operating at low temperatures and atmospheric pressure, NTP technology is relatively simple and cost-effective, making it an a desirable and environmentally friendly alternative for various applications (Cubas et al., 2020).

Ravindran et al., (2019) used a dielectric barrier discharge (DBD) plasma reactor (figure 8) as a pretreatment of BSG. The dielectric barrier discharge (DBD) plasma source utilizes a conventionally coaxial electrode configuration. The high voltage electrode is enclosed within a quartz tube. The DBD tube and the ground electrode are immersed in the treated liquids. The liquid not only serves as an additional dielectric barrier layer but also acts as a coolant for the discharge process. Air was employed as the working gas for the plasma, and its flow rate was regulated using a mass flow controller. The post-discharge afterglow plasma effluent was transferred into the liquid in the form of microbubbles by passing it through four gas diffusers, which are attached at the end of the tube. The pretreatment experiment was devised to identify the optimal voltage and reaction time that effectively reduced recalcitrance in BSG while considering the characteristics of the solvent used.

According to Ravindran et al., (2019), atmospheric plasma in solvents proved to be an effective pretreatment approach for reducing recalcitrance in BSG. When using water as the

solvent, a significant decrease in the total lignin content (36%) of the pretreated BSG was observed. As a result, the subsequent enzymatic hydrolysis yielded 2.14 times more reducing sugars compared to untreated BSG. The atmospheric plasma treatment did not produce any toxic inhibitors, and only trace amounts of organic acids were detected. The successful reduction of recalcitrance enhances the potential for more efficient and sustainable biorefining processes, opening new possibilities for the valorization of biomass in various applications, including bioenergy and bioproducts production.

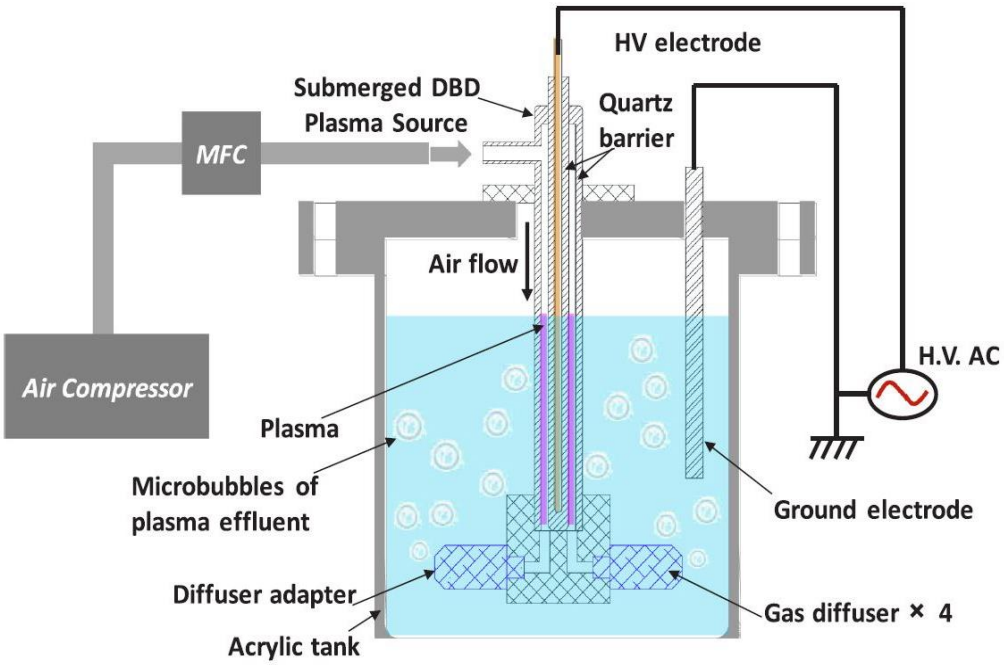


Figure 8. Schematic representation of atmospheric pressure plasma reactor (Ravindran et al., 2019).

1.8 Bacterial cellulose

Bacterial cellulose (BC) has diverse uses, from food additives to biomaterials and nanocomposites, thanks to its special properties. However, its production cost via traditional fermentation methods is high, limiting its widespread use in industries. To make BC more accessible for industrial applications, finding cost-effective production methods is essential. The growth of the BC market relies heavily on the development of biorefinery processes (Filippi et al., 2022). The waste streams of the brewing industry could be used for the fermentation of BC making the production cost-effective. In their study, Donini et al. (2010) compared the productivity of cellulose production from plants and microorganisms to evaluate the advantages of using microorganisms for cellulose production. They found that achieving the same cellulose production from forestry cultivation could be accomplished with bacteria in a hypothetical yield

of 15 g/L in 50 hours of culture in a 500 m^3 bioreactor, taking around 22 days and from forestry cultivation could take up to 7 years.

Cellulose is the most abundant natural polymer globally, with an estimated annual production of 1014 tons of cellulose pulp worldwide (Jozala et al., 2016; Lin et al., 2014). This underscores its immense economic significance. However, to obtain pure cellulose from natural sources, chemical treatments involving highly polluting chemical products such as chlorine gas, caustic soda, carbon disulfide, carbon monoxide, and carbon dioxide are often required due to the presence of other components in the raw materials (Jozala et al., 2016; Klemm et al., 2011). These chemical treatments can have adverse environmental impacts, leading to pollution and the generation of hazardous waste.

Bacterial cellulose (BC) exhibits distinct differences from plant cellulose. It is remarkably thinner, being approximately 100 times thinner than plant cellulose, and forms a unique three-dimensional network. This structural arrangement results in an increased surface area to volume ratio, leading to stronger interactions with surrounding components and moieties. The BC microfibrils are organized in a well-defined 3D web-like structure, composed of monomeric units linked by regular β-1,4-glycosidic bonds. This specific arrangement contributes to BC's exceptional mechanical strength, high degree of polymerization, and a significantly higher crystallinity index (80–90%). Additionally, BC demonstrates superior tensile strength and water holding capacity compared to plant cellulose (Gregory et al., 2021). Various cellulose-producing bacteria have been identified, belonging to genera such as *Acetobacter, Gluconobacter, Komagataeibacter, Rhizobium, Agrobacterium*, and *Sarcina*. Among these, *Komagataeibacter* (formerly known as *Gluconacetobacter*) *xylinus* is the most well-known bacterium for bacterial cellulose production. *Komagataeibacter xylinus* is a Gram-negative obligate aerobic bacterium capable of efficiently metabolizing a diverse range of carbon and nitrogen sources to synthesize BC (Ross et al., 1991) .

BC can be produced under static, agitated, or stirring conditions, leading to various cellulose structures. When cellulose microfibrils are formed under static conditions, they are extruded from the bacterial surface and then aggregate to create a pellicle at the air-liquid interface. This pellicle formation is a result of limited oxygen supply in the bulk, prompting the bacteria to migrate towards the interface. Subsequently, growth and polymer synthesis take place at this location. It has a wide range of applications, including food (as a thickener, water binder, extrusion aid, and film former), paper, packaging, textiles, and bioconcrete. Additionally, it is used in bioremediation, cosmetics, electronics, and sensing applications. Its

versatility and eco-friendliness make it a promising material across various industries (Gregory et al., 2021).

Several techniques are available for obtaining nano-cellulose, including acid hydrolysis, ultrasonic technique, and enzymatic hydrolysis. Among these methods, acid hydrolysis is the most widely used. It is preferred due to its simplicity and efficiency in producing nanocellulose with improved properties. Researchers have reported that nano-cellulose produced through acid hydrolysis exhibits a higher crystallinity index compared to other methods and has a smaller particle size. These advantages make acid hydrolysis the chosen method for obtaining nano-cellulose. Typically, strong acids like H_2SO_4 and HCl are commonly employed to cleave the glycosidic bonds in cellulose. Nanocellulose exhibits distinctive characteristics, including low density, biodegradability, and excellent mechanical properties and is produced in a pure form. Furthermore, it can be easily modified and possesses a high surface area and distinct morphology. In addition, nanocellulose used as reinforcing agent in food packaging in order to increase mechanical and water/gas permeability (Efthymiou et al., 2022; Fu et al., 2013; Wulandari et al., 2016).

1.9 Salmon

The Atlantic salmon (*Salmo salar*) is a ray-finned fish species belonging to the family *Salmonidae*. It is primarily anadromous, starting its life by hatching in streams and rivers, but as it grows, it migrates to the sea where it reaches maturity. Once mature, adult salmon periodically return upstream to spawn. These fish typically measure around 1-1.5 meters in length and weigh approximately 50 kilograms. Atlantic salmon are distributed in the Atlantic Ocean and the Baltic Sea (Schultz, 2003). Salmon, along with other salmonids, ranks as the 9th most cultivated species in aquaculture based on volume. The total global salmon production is approximately 2.5 million tons per year, which translates to a population of around 288 to 674 million fish.

The increasing popularity of salmon consumption is linked to its nutritional advantages, notably its content of omega-3 long-chain polyunsaturated fatty acids (LC-PUFA), in addition to its attractive color, taste, protein richness, vitamins, and antioxidants. However, salmon and other seafood products exhibit high perishability due to characteristics such as their elevated water activity, near-neutral pH, and specific components that encourage biochemical, physical, and microbial deterioration along the production process. This deterioration becomes notably apparent shortly after capture (Romero et al., 2022).

Fresh salmon is susceptible to spoilage due to its limited storage duration, typically maintained within the temperature range of 0 – 4°C. Its shelf life spans around 1–2 weeks, which poses logistical challenges for the distribution and sale of fresh salmon. The deterioration of fresh salmon during refrigerated storage primarily stems from enzymatic and microbial reactions, as well as autolysis degradation, which can result in undesirable odors. Additionally, the oxidation of polyunsaturated fatty acids is a significant contributor to the reduced shelf life of fresh salmon. The formation of volatile compounds during the oxidation process has implications for the sensory characteristics and overall appeal of fresh salmon (Yan et al., 2022).

Fresh or chilled fish is commonly packaged using styrofoam trays that are subsequently wrapped with cling film. The cling film used for this purpose can be made from materials such as polythene or polypropylene. Additionally, the trays themselves can be constructed from materials like polyvinylidene chloride or polystyrene. This packaging method, which combines styrofoam trays and cling film, is a widely adopted and cost-effective solution in the fish market due to its affordability and easy availability (Nowsad, 2007).

2. Scope of the study

In this study, the main byproduct of the brewing industry named BSG was employed in the production of biodegradable packaging films for salmon fillets. A novel biorefinery was developed for this purpose. For the effectiveness of the biorefinery, the utilization of the different fractions were carried out. The initial step involved pretreatment using non-thermal plasma to enhance enzymatic hydrolysis. Enzymatic hydrolysis was carried out using a commercial enzyme preparation, and the resulting hydrolysate was used to produce bacterial cellulose via microbial fermentation using the strain *Komagataeibacter sucrofermentans*. Alkaline extraction was employed to extract protein from the solid residue, and lignin was extracted from the remaining solid material after protein extraction. Following modification of the lignin and cellulose, all biorefinery products, including protein, bacterial nanocellulose, lignin, and lignin particles, were utilized in the production of packaging films. Various concentrations of lignin and lignin particles were tested, and those exhibiting the best properties were selected for shelf-life evaluation. The shelf life of the salmon fillets was assessed over a span of 11 days, during which microbiological and sensory analyses were conducted.

3. Materials and methods

3.1 BSG compositional analysis

The BSG used in this study was kindly provided by Cerveza Mica (Spain) after the malting process of beer named Pilsner. Initially, BSG was subjected to lyophilization using a freeze dryer and subsequently milled using a blender (particle size < 2mm). Milled BSG was stored for further characterization and treatment.

3.1.1 Moisture content

To determine the moisture content of BSG, three crucibles were initially weighed. Subsequently, a quantity of wet BSG was added to each crucible, and their weights were recorded once more. The crucibles were then placed in an oven at 50°C for 24 hours and subsequently transferred to a desiccator to attain room temperature. Once they reached ambient temperature, their weights were measured again. The humidity percentage was computed using Equation (1) for each repetition, and the humidity percentage was obtained as the average of the three repetitions.

$$\%Moisture\ content = \frac{Initial\ weight - Dried\ weight}{Initial\ weight} * 100 \quad (1)$$

3.1.2 Ash

A certain amount of wet BSG was added to incandescent and pre-weighed porcelain crucibles followed by weight recording. The crucibles were then placed in an oven set at 50°C for 24 hours, as described at 3.1.1. The crucibles were transferred to a desiccator to attain room temperature and weighed. Afterward, the crucibles were subjected to a muffle furnace at 575°C for a duration of 7 hours. Subsequent to this step, the crucibles were again placed in a desiccator until they achieved ambient temperature and weighed. The ash percentage was computed using Equation (2), and the final ash percentage was determined as the average of the three measurements.

$$\%\ Ash = \left(\frac{(Weight\ of\ crucible + ash\ (g)) - Weight\ of\ cucible\ (g)}{(Weight\ of\ crucible + dry\ sample\ (g)) - Weight\ of\ cricible\ (g)}\right) \times 100 \quad (2)$$

3.1.3 Total Kjeldahl Nitrogen

To determine the total protein content of the sample, Kjeldahl method was selected by using the Kjeltek TM 8100 distillation unit (Foss, Denmark). Kjeldahl method involves a three-

step process consisting of digestion, distillation, and titration. Dried sample was precisely weighed on rice paper to four decimal places and placed within a digestion tube. Using a bottle-top dispenser, 25 mL of H_2SO_4 was added, along with a Kjeldahl tablet containing Na_2SO_4 (96.5%), $CuSO_4$ (1.5%), and Se (2.0%). The same reagents were incorporated for the blank. Digestion was conducted at 430°C for an hour, and after the tubes returned to room temperature, the distillation process followed. During this step, 30 mL of H_2O and 100 mL of NaOH (40%, w/v) were automatically added in the case of solid samples. In the case of liquid sample, 5 g was added directly within the digestion tube along with 10 mL of H_2SO_4 and a Kjeldahl tablet. During the digestion step, 80 mL of H_2O and 50 mL of NaOH (40%, w/v) were automatically added. The distillate obtained was collected in an Erlenmeyer flask, and to this, 50 mL of a boric acid solution containing 40 g of boric acid, 7 mL of methyl red indicator (0.1%), and 10 mL of bromocresol green indicator (0.1%) in one litre of solution were added. Lastly, the solution was titrated using a 0.1 N HCl standard solution. The volume of HCl standard solution consumed during titration was converted into total nitrogen content according to Equation (3).

$$\%\, N \;=\; \frac{(mL\ sample \;-\; mL\ blank) * 0.1N * 14.007 * 100}{mg\ sample} \quad (3)$$

Where, N is the normality of the HCl solution, mL of sample is the mL of 0.1 N HCl consumed during the titration of the sample, and mL of blank is the mL of 0.1 N HCl consumed during the titration of the blank.

The conversion of %N to % protein was conducted with the Equation (4).

$$\%\, protein \;=\; 6.25 * \%N \quad (4)$$

where 6.25 is the conversion factor of organic nitrogen into proteins because most of the proteins have 16% nitrogen and therefore the conversion factor is 6.25.

3.1.4 Extractives

In order to identify the composition of BSG, Soxhlet extraction was employed to remove the extractable components. The sample is enclosed within a specialized porous pouch known as a thimble and placed within the extraction chamber of the Soxhlet apparatus. A spherical flask containing the selected solvent is positioned at the apparatus's base. Heating was applied to the solvent via a heating mantle, causing vapors to rise through a lateral tube into the extraction chamber. Within the chamber, these vapors condense, moistening the sample. As the

solvent in the extraction chamber reaches a specific level, a siphoning effect was triggered, causing the solvent along with the extracted substance to flow back into the spherical flask. This cyclical process persists until all the extractable components have been absorbed by the solvent (Luque de Castro & Priego-Capote, 2010).

The extraction process adhered to the methodology outlined in Sluiter et al. (2005). The initial step involved weighing the thimble, subsequent addition of dry BSG and reweighing. Cotton wool was employed to seal the thimble, and the entire assembly was reweighed after sealing. The solvent chosen for extraction was tailored to the specific sample component to be extracted. Hexane was utilized for fatty substance extraction, distilled water for water-soluble components, and ethanol for ethanol-soluble constituents. The solvent was placed in the spherical flask, which was then introduced into the Soxhlet apparatus to initiate the extraction process. The heating mantle's temperature was set to yield 4-5 siphon cycles per hour, and the extraction continued for 24 hours. In instances where water was the selected extraction solvent, the resultant extract was transferred to a volumetric flask, with a subsequent dilution to 200 mL using distilled water. Analysis by high-performance liquid chromatography (HPLC) ensued. Alternatively, for the extraction of ethanol-soluble components, the thimble containing the BSG sample remained within the Soxhlet holder. Addition of 97% (v/v) ethanol to the spherical flask facilitated the procedure described above. Concluding the extraction, the thimble was removed, followed by lyophilization and weighing. For the oil and fat determination, hexane was used as solvent. The spherical flask weighted before the extraction, and after the extraction, the spherical flask with the hexane placed to a rotary evaporator (40-50°C). Hexane evaporated from the spherical flask and only oil and fat left inside. The spherical flask weighed again and the % oil – fat composition was calculated. The quantification of extractable matter content in the sample was calculated using the following Equation (5):

$$\%\ extractives = \frac{dry\ sample\ before\ extraction\ (g) - dry\ sample\ after\ extraction\ (g)}{dry\ sample\ before\ extraction\ (g)} * 100 \quad (5)$$

3.1.5 Structural carbohydrates and lignin

The procedure outlined in A. D. Sluiter & Templeton (2012) as provided by the National Renewable Energy Laboratory (NREL) was used to analyze the structural carbohydrate and lignin composition of BSG. The BSG sample used for this method subjected to Soxhlet extraction as described at 2.1.4 to ensure extractable-free samples were used.

Initially, 300 mg of BSG sample was weighed, followed by subjection of a two-step acid hydrolysis process. In the first step, the samples were placed in tared pressure tubes and 3

mL of 72% H_2SO_4 (v/v) added. The mixture was maintained at 30°C with continuous stirring using a magnetic stirrer for 1 hour. Subsequently, the H_2SO_4 solution was diluted to a final concentration of 4% v/v by adding 84 g of distilled water using a balance accurate to 0.01 g to each sample, achieving a total volume of 86.73 mL per sample. The second stage of hydrolysis followed, involving the placement of tared pressure tubes in an autoclave at 121°C for 60 minutes. Simultaneously, the entire procedure was conducted for a sugar solution with a known concentration (Calibration Verification Standard - CVS) to assess the percentage of sugars removed during acid hydrolysis (Sugar Recovery Standards - SRS). Following the 1-hour hydrolysis, the samples were vacuum-filtered using pre-weighed glass fiber filters. The filters, along with the entire solid sample, were transferred to pre-weighed porcelain crucibles and subjected to an oven at 80°C for 24 hours to eliminate moisture. Subsequently, the crucibles were placed in a desiccator until they reached room temperature and were weighed. Finally, the samples were placed in a muffle furnace at 575°C for 6 hours. The difference in weight between 80°C and 575°C was employed to calculate the amount of insoluble lignin (AIL, %).

The liquid fraction obtained through vacuum filtration was quantitatively assessed using volumetric and photometric measurements performed with a UV-Vis spectroscopy device (Shimadzu UV-1900i) at a wavelength of 320nm. The determination of the soluble lignin content (ASL, %) within the sample was executed in accordance with the calculation presented by Equation (6):

$$\mathrm{ASL}(\%) = \left(\alpha\mathrm{bs} * \mathrm{V} * \frac{\mathrm{Dil}}{\varepsilon} * \mathrm{M} * \mathrm{pathlength}\right) * 100 \quad (6)$$

Where,

abs: absorbance of the sample,

V: volume of the filtered sample,

Dil: Dilution = $\frac{Volume_{sample} + Volume_{diluting\ solvent}}{Volume_{sample}}$

ε: absorbance of the sample at the specified wavelength,

M: mass of the solid sample weighed at the initial stage and

Pathlength: thickness of the UV-V cell.

The total amount of lignin (extractives free sample) is calculated by the Equation (7):

$$\%Lignin_{ext.free} = \%AIL + \%ASL \quad (7)$$

To quantitatively and qualitatively analyze the structural carbohydrates of the sample, a High-Performance Liquid Chromatography (HPLC) setup was employed, featuring a Shodex SP0810 column operating at 60°C and a flow rate of 0.8 mL/min, with distilled water serving as the mobile phase. For the analytical process, a 10 mL portion of filtrate was isolated from each sample, and calcium carbonate ($CaCO_3$) was added to neutralize the solution until it reached a pH range of 5.0-6.0. This pH range was maintained to prevent sugar precipitation at higher pH levels. Following neutralization, centrifugation (10 min, 9000 rpm) was performed, and the resulting supernatant was subjected to filtration (0.02 μm). Subsequently, the sugars were quantified through HPLC analysis.

To determine the concentration of sugars, the recovery rate of sugars is initially computed dividing the concentration of the sugars at the SRS after the autoclave treatment by the concentration of the sugar standard solution identified through HPLC prior to autoclave treatment, as outlined in Equation (8):

$$\%\, R_{\text{sugar}} = \frac{concentration\ detected\ by\ HPLC\ (\frac{mg}{L})}{known\ concentration\ of\ sugar\ before\ hydrolysis\ (\frac{mg}{L})} * 100 \quad (8)$$

The previously calculated sugar recovery rate is employed to adjust the observed concentration of each sugar in the analyzed sample, considering potential sample dilution as depicted in Equation (9):

$$sugar\ concentration_{hydrolysed\ sample} = \frac{sugar\ concentration_{HPLC} * dilution}{\frac{\%\, R_{\text{sugar}}}{100}} \quad (9)$$

Next, to determine the concentration of sugar polymers before hydrolysis, an adjustment factor is applied to consider the loss of a water molecule during glycosidic bond formation. This factor stands at 0.88 (132/150) for sugars with 5 carbon atoms (such as xylose and arabinose) and 0.9 (162/180) for sugars with 6 carbon atoms (like glucose, galactose, and mannose). Consequently, the concentration of each polysaccharide in grams per liter (g/L) is obtained, subsequently expressed as a percentage of the initial dry sample. The calculated glucose concentration aids in determining cellulose concentration, while the combined concentrations of other polysaccharides contribute to hemicellulose concentration.

3.1.6 Free sugars and oligosaccharides

To determine the content of free sugars in BSG, a procedure was followed. Initially, 0.5 grams of dry BSG was placed in a 10 mL volumetric flask followed by the addition of distilled water up to the mark. The flask was then positioned on a heated stirrer operating at 40°C for a

duration of 2 hours. After this period, the solid material was separated from the liquid using filter paper. The separated solid was once again suspended in a volumetric flask, followed by filling it up to the mark with distilled water. This flask was placed back on the heated stirrer at 40°C for 2 hours. Following this treatment, the liquid was separated from the solid once again using filter paper. The two separated liquids were then combined and subjected to analysis using HPLC to determine the free sugars present. In order to breakdown the maltodextrins into D-glucose, the enzyme amyloglucosidase (Megazyme, total starch assay kit, 3300 U/mL) was used. At first, 990 μL of the liquid sample and 10 μL of the enzyme were placed into a 2-mL Eppendorf. The Eppendorf placed in a water bath at 50°C and 4 different durations tested. The durations were 3, 4, 6 and 7 hours. After this treatment, the D-glucose of the samples was quantified through HPLC analysis. The glucose that detected by the HPLC converted into oligosaccharides using the factor 1.1 as Equation (10).

$$oligosaccharides\ concentration\ (g/L) = \frac{glucose\ concentration_{HPLC}}{1.1} \quad (10)$$

The concentration of oligosaccharides in the solid was calculated with the equation (11)

$$\%oligosaccharides = \text{oligosaccharides}\ concentration * V_{total\ sample\ liquid} * 100 \quad (11)$$

3.1.7 Total phenolic content

Freeze-dried BSG samples, each weighing 1 g, were accurately weighed and combined with 20 mL of acidified 70% ethanol 0.1M. Ultrasonic extraction was then performed under controlled conditions (70 Hz, 20 minutes, 35°C) to facilitate the extraction of phenolic compounds. The resulting mixture was subsequently filtered, separating the liquid extract from the solid residue. The collected filtrate, enriched with phenolic compounds, underwent evaporation using a rotary evaporator and water bath maintained at 35°C. Post-evaporation, the concentrated extract was weighed, and the container was rinsed with 10 mL methanol. The extraction process was repeated three additional times on the original BSG sample, allowing for the gradual extraction of phenolic components through successive ethanol washes.

The Folin–Ciocalteu method was employed to quantify the Total phenolic content (TPC) in the collected extracts. Crude phenolic extracts obtained from ultrasonic extraction were diluted with distilled water at the ratio of 1:20 v/v. These diluted samples were mixed with Folin solution and Na_2CO_3 solution to induce a reaction. Subsequent to incubation in darkness, spectrophotometric analysis was conducted at 765 nm. TPC concentrations were calculated based on a standard curve constructed using different concentrations of a gallic acid (GA)

solution. Blank solutions were prepared using the same procedure but substituting distilled water for the sample.

3.2 Biorefinery development

3.2.1 BSG pretreatment using atmospheric air pressure plasma

A novel technique for pretreating lignocellulosic biomass called dielectric barrier discharge (DBD) air plasma has emerged as a potential replacement for established pretreatment methods that use high pressure, high temperatures, and acids or alkalis. For this specific procedure, 50 g of BSG was suspended in distilled water to reach a concentration of 50 g/L, utilizing a 1-L Duran bottle. The assembly involved securing a cap containing an electrode, air supply, anode, and a stirring mechanism at the top of the bottle. A controlled flow of air, maintained at 1 vvm, was introduced into the system. The treatment was executed using the Leap100 from Plasmaleap, operating with discharge voltage at 200 V, discharge frequency at 1000 Hz and resonance frequency at 60 kHz. Throughout the treatment, continuous stirring was maintained via a magnetic stirrer. The procedure was carried out at ambient temperature. Following the completion of the treatment process, the solid and liquid components were separated through centrifugation, employing a centrifuge operating at 12,000 rpm for a duration of 15 minutes at 4°C.

3.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreat BSG was conducted utilising a commercial enzyme cocktail (0.05 mL per g BSG). The enzyme cocktail contained endo-β-1,4-glucanase (CMCase) (3.5 U/g), exo-1,4-β-glucanase (Avicelase) (3.9 U/g), β-glucosidase (8.3 U/g), xylanase (2.3 U/g) and total cellulase (4.0 FPU/g). One unit (U) of CMCase, exo-1,4-β-glucanase (Avicelase), β-glucosidase and filter paper unit (FPU) activities were defined as the amount of the enzyme that releases 1 mg glucose in 1 min during hydrolysis of 1% (w/v) CMC, 1% (w/v) microcrystalline cellulose, 0.5% (w/v) cellobiose and 50 mg Whatman No.1 filter paper strip (1.0 ×6.0 cm) in 0.1 M acetate buffer, pH 4.8 and 50ºC within 30, 120, 30, and 60 min, respectively. Additionally, one unit (U) of xylanase activity was defined as the amount of enzyme that releases 1 mg xylose in 1 min during hydrolysis of 0.25% (w/v) xylan from birchwood in 0.1 M acetate buffer, pH 5 and 50ºC within 15 min. Initially, 20 g of dry BSG was placed within a 250-mL Duran bottle followed by 200 mL of plasma liquid addition. The Duran bottle was subjected to sterilization in an autoclave operating at a temperature of 121°C

for a duration of 20 minutes. Following the sterilization procedure, 1 mL /20 g BSG of the enzyme blend was introduced under aseptic conditions. The Durant bottle, containing the enzymatic mixture, was then immersed in a water bath set at 50°C. Subsequent to 24 hours from the initial enzyme addition, an additional 1 mL / 20 g BSG of the enzyme blend was added into the Durant bottle under aseptic conditions. The enzymatic hydrolysis was allowed to proceed for a total duration of 48 hours. The objective of this process was the conversion of a significant portion of cellulose and hemicellulose into monosaccharides and oligosaccharides. After the designated hydrolysis period, the solid component was separated from the liquid, completing the enzymatic hydrolysis process.

Quantification of hydrolysed sugars in the hydrolysate was carried out using HPLC, featuring a Shodex SP0810 column operating at 60°C and a flow rate of 0.8 mL/min, with distilled water serving as the mobile phase.

3.2.3 Protein extraction

Protein recovery was calculated as followed:

$$\%\, Recovery = \frac{g\ of\ protein\ at\ protein\ extract}{g\ protein\ at\ initial\ solids} * 100 \quad (12)$$

A. Alkaline treatment

The extraction of proteins through an alkaline treatment was undertaken, employing diverse conditions to ascertain the most suitable approach. To this end, two distinct temperatures, namely 25°C and 60°C, were investigated. In this method, a portion of BSG was placed within an Erlenmeyer flask, and distilled water added at a ratio of 1/10. The flask was positioned on a heated stirrer. Subsequently, the pH was adjusted to 10.0 by introducing 1 M NaOH, followed by a heating duration of 2 hours. During the heating process, regular monitoring of the pH was conducted, with adjustments made as deemed necessary. Upon completion of the heating phase, the liquid phase was separated from the solid residue using filter paper.

The liquid phase, containing the proteins, adjusted to pH of 3.4 under continuous stirring, (applicable to treatments at both 25°C and 60°C) and 4.5 (exclusive to the 60°C treatment). This pH adjustment facilitated the precipitation of proteins. Following this step, centrifugation was performed at 8000 rpm for a duration of 10 minutes at 4°C. Finally, the solid phase namely protein extract was freeze dried and stored until further characterization.

B. Enzymatic treatment

In this particular treatment by Qin et al. (2018), 5 g of dry BSG suspended in distilled water at a ratio of 1:20 in a Durant bottle. Three different samples made, for the three different durations of enzymatic hydrolysis. The samples were then subjected to sterilization in an autoclave at a temperature of 121°C for a duration of 20 minutes. Following the sterilization process, 2 U per g of BSG added into each Duran bottle. Subsequently, the bottles were immersed in a water bath set at a temperature of 60°C, with continuous stirring at a rate of 250 rpm. At specified time intervals, namely after 2, 8, and 24 hours of incubation, each Duran bottle was exposed to heat for enzyme deactivation. This involved heating the bottle to 95°C for 10 minutes, followed by cooling to room temperature using a water bath.

C. Hydrothermal treatment

The initial step in this procedure involved the precise measurement and placement of 4% w/w of BSG to distilled water into an Erlenmeyer flask according to Qin et al. (2018). The Erlenmeyer flask was then positioned on a heated stirrer, maintaining at 60°C, and stirred at 400 rpm for 24 hours. This conditions for the hydrothermal treatment were the most effective according to Qin et al. (2018). Following this 24-hour treatment period, the liquid phase was separated from the solid residue through centrifugation (8000 rpm, 10 min, 4℃) and analysed in order to determine the protein content of the liquid.

D. Dilute acid treatment

In this procedure according to Mussatto & Roberto (2006), BSG was mixed with an acid solution (120 mg H_2SO_4 solution/g total liquid solution) in a liquid to solid ratio of 10 g/g (1200 mg H_2SO_4/g BSG), and the mixture was autoclaved at 120°C for 27 min. Following the autoclaving process, the bottle was allowed to naturally cool down to room temperature. Once the bottle had sufficiently cooled, the liquid phase was separated from the solid fraction through centrifugation at a speed of 8000 rpm, for 10 minutes, at 4℃. The resulting supernatant, which contained the extracted protein, was collected and subsequently subjected to analysis for its protein content.

3.2.4 Lignin extraction and modification

The lignin extraction process involved utilizing the residual solid material left after protein extraction, which is a byproduct of the BSG biorefinery process and is expected to contain a significant amount of lignin. This procedure, inspired by the method developed by

Rojas-Lema et al. in 2023 with certain modifications, began by suspending the solid residue in a 1.5 M NaOH solution in a Durant bottle with a working volume of 500 mL, using a concentration ratio of 1 part solid to 10 parts NaOH. The bottle was then autoclaved at 140°C for 3 hours to facilitate lignin extraction. Following autoclaving, solid-liquid separation conducted at 12000 rpm, for 15 minutes at 4°C. The resulting supernatant, containing the extracted components, was collected. Sulfuric acid (H_2SO_4) was gradually added to the supernatant under stirring until the pH reached 2.0, causing lignin precipitation. Another round of centrifugation, conducted at 12000 rpm, for 15 minutes at 4°C, was employed to separate the precipitated lignin. The obtained lignin precipitate was then subjected to freeze-drying, resulting in a dry, powdered lignin product ready for various applications.

Lignin solutions were prepared by stirring 30 g/L of lignin in 40 mL of solution for 10 minutes at 500 rpm using a magnetic stirrer. An antisolvent solution, consisting of an aqueous phosphate-citrate buffer with pH 8, was added to the lignin solution at a controlled rate of 14 mL/min using a syringe pump. The amount of antisolvent added was adjusted to achieve a final ethanol concentration of 45%. Subsequently, ethanol was removed, and the resulting LP dispersions were concentrated back to the initial volume of 40 mL using a rotary evaporator at 60°C. These LP dispersions were then stored at room temperature, shielded from light, ensuring that the final lignin concentration matched the initial concentration (Colucci et al., 2023).

3.3 Bacterial cellulose fermentation

3.3.1 Microorganism and inoculum preparation

The bacterial strain employed for the production of bacterial cellulose was *Komagataeibacter sucrofermentans* DSM 15973, sourced from the Leibniz-DSMZ Institute in Germany. The culture initiation involved establishing a preculture in shake flasks, where a single colony was introduced into a liquid medium following the Hestrin and Schramm (HS) medium protocol (Gromet-Elhanan & Hestrin, 1963). The composition of the medium comprised 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L disodium phosphate (Na_2HPO_4), and 1.15 g/L citric acid. To achieve a pH of 6, either 5M HCl or 5M NaOH was used for adjustment. The preculture was then incubated in a rotary shaker incubator at 200 rpm with agitation at 30°C for a duration of 24 hours. For the long-term preservation of bacterial cells, cryovials containing a mixture of 50% (v/v) grown cells in the pre-culture medium and 50% (v/v) pure glycerol were stored at -80°C.

3.3.2 Fermentation conditions

The inoculum preparation involved the transfer of contents from one cryovial into 250 mL Erlenmeyer flasks, each containing 50 mL of working volume. This was followed by incubation, mirroring the conditions of the preculture, and followed by a 24-hour period of static culture at 30°C. The culture medium used in these flasks was based on the adjusted HS medium, where the total sugar concentration was 20 g/L, following the sugar ratio obtained from the BSG hydrolysate. To achieve this, the BSG hydrolysate was suitably adjusted. Additionally, an appropriate amount of yeast extract was added to achieve a carbon-to-free amino nitrogen (FAN) ratio of 28.1. Throughout the fermentation process, the pH was maintained at 5.0, guided by the findings of Efthymiou et al. (2022). The fed-batch fermentations were conducted in shake-flasks with a working volume of 50 mL, using one flask as the inoculum media. These flasks were subjected to static incubation at 30°C for a duration of 17 days.

3.3.3 BNCs production

The modification of bacterial cellulose was carried out through an acid hydrolysis process. Initially, the bacterial cellulose was dried and finely milled to achieve a fine particle size. A precise quantity of 10.023 g of bacterial cellulose powder was weighed and placed into a Duran bottle with a working volume of 250 mL. To this, 169.2 mL of distilled water was added, and the solution was homogenized using an ultraturax device at 12,000 rpm for a duration of 4 minutes. Subsequently, the solution was transferred to a Duran bottle with a working volume of 500 mL. This bottle was then positioned in an ice bath atop a stirrer, and 97.5 mL of H_2SO_4 was gradually added to the solution. Once the solution had stabilized, the bottle was placed on a heated stirrer set to 50°C with agitation at 500 rpm. This step constituted the acid hydrolysis process. To terminate the reaction, 1200 mL of distilled H_2O was added to the solution, followed by centrifugation at 9000 rpm for 10 minutes. The resulting precipitate was mixed with distilled water and subjected to centrifugation again. This procedure was repeated two more times.

The precipitate was then subjected to a bleaching process using 8 mL of H_2O_2 per gram of dry BNC for a duration of 1 hour, while placed in an ice bath. After bleaching, the solution was centrifuged at 9000 rpm for 20 minutes. Following centrifugation, the supernatant was once again centrifuged under the same conditions. The resulting precipitate was collected and ultrasonicated in a small amount of distilled water (approximately 50 mL) for 4 minutes.

The solution was then passed through a reverse osmosis membrane and subsequently placed in a vessel with distilled H_2O. The water in the vessel was regularly changed until the pH approached 6. At this point, the bacterial nano-cellulose was prepared, with a final concentration of 4%.

3.3.4 Free amino acid nitrogen (FAN)

The determination of Free Amino Nitrogen (FAN) concentration in both the hydrolysates and fermentation samples was carried out using the ninhydrin colorimetric method as outlined by the European Brewery Convention (Lie, 1973). This method relies on the oxidative decarboxylation of α-amino acids by ninhydrin when heated under a pH of 6.7. The reaction generates a blue color due to the interaction of reduced ninhydrin with unreduced ninhydrin and the produced ammonia (NH_3). FAN concentration quantifies the nitrogen content present in the free amino groups of amino acids and peptides.

The following reagents were employed for the quantification of FAN:

1. Colour Reagent: This reagent was prepared by mixing 49.71 g of di-sodium hydrogen phosphate dehydrate ($Na_2HPO_4 \bullet 2H_2O$), 5 g of ninhydrin, 3 g of fructose, and approximately 60 g of mono-potassium phosphate (KH_2PO_4) until the pH of the reagent fell within the range of 6.6 – 6.8. The final volume of the mixture was adjusted to 1 L.

2. Dilution Reagent: This reagent was prepared by dissolving 2 g of potassium iodate (KIO_3) in 616 ml of distilled water and then adding 384 mL of absolute ethanol (99% vol/vol Sigma-Aldrich) to reach a final volume of 1 L.

3. Glycine Stock Solution: 0.1072 g of glycine was dissolved in distilled water in a 100 mL volumetric bottle.

4. Glycine Standard Solution: 1 mL of the glycine stock solution was transferred and diluted with distilled water to reach a final volume of 100 mL, resulting in a final FAN concentration of 2 mg/L.

All of these reagents were stored at 4°C and were freshly prepared as needed.

The protocol for the analysis was as follows: After the centrifugation of the samples, the supernatant was collected and appropriately diluted for analysis. 1 mL of the diluted sample was added to glass test tubes, followed by the addition of 0.5 mL of the color reagent. A blank sample was also prepared using water instead of the diluted sample. The tubes were covered, boiled for exactly 16 minutes, and subsequently cooled for 20 minutes. Then, 2.5 mL of the

dilution reagent was added to each tube. The samples were vigorously shaken with a vortex for 20 seconds, and absorbance was measured in a spectrophotometer at 570 nm, against the blank sample. All samples were analyzed in duplicate.

FAN content (mg/L) was quantified based on a calibration curve, which was established by diluting the glycine standard solution to achieve concentrations of FAN at 0.5, 1, 1.5, and 2 mg/L. The equation used to calculate FAN concentration in the samples is presented in the figure 9

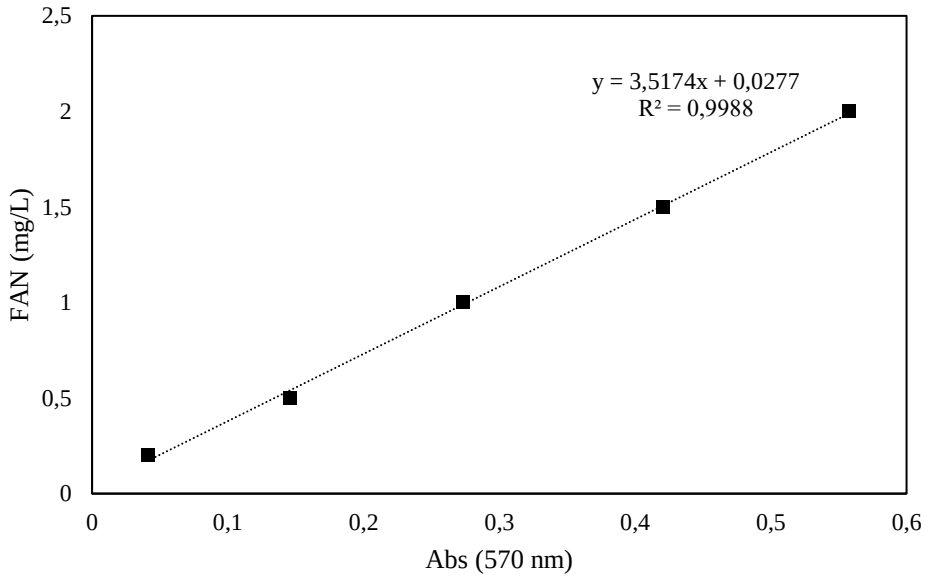


Figure 9. Glycine standard by the FAN method

3.4 Film formulation

Several different methods for producing films were evaluated using BSG residual solids after DBD non-thermal plasma and enzymatic hydrolysis (residual solids 2, figure 10).

3.4.1. Treatment of BSG residual solids after plasma and enzymatic hydrolysis

Dilute acetic acid solution

Three different concentrations of dry BSG to liquid (5%, 7.5%, and 10%) were tested. Dry BSG was weighed and added to 1 M acetic acid in order to achieve the required concentration. This solution was stirred at 400 rpm for 24 h. Then, 30% w/w glycerol was added, heating of the solution at 80°C (400 rpm, 1 h), cooled down, casting into Petri dishes, and allowed to dry at 30°C for 24 h.

Dilute HCl solution

Similar to the above method, three different concentrations of dry BSG (5%, 7.5%, and 10%) were tested. Dry BSG was weighed, distilled water was added in order to achieve the required concentration and the pH was adjusted to 2 with 2 M HCl. This solution was stirred at 400 rpm for 1 hour. Then, 30% w/w glycerol was added, heating of the solution at 80°C (400 rpm, 1 h), cooled down, casting into Petri dishes, and allowed to dry at 30°C for 24 h.

Concentrated acetic acid solution

Dry BSG, milled to be smaller than 1mm, was used. Initially, a ratio of solid BSG to liquid 1:10 placed in a Duran bottle with the addition of 70%, 80%, and 90% v/v acetic aqueous solution. Additionally, 2% v/v of HCl with purity over 99.7% was added. The solution was heated under stirring to 110°C for 1 hour in a bath with silicon oil. This process resulted in a black liquor. To this liquor, 0.3 g of glycerol per gram of BSG was added and stirred for 1 h. The resulting solution was cast into Petri dishes and dried at 30°C for 24 h.

3.4.2. Films using protein extract

Acidic conditions

According to the (Proaño et al., 2020) method, protein mixed with distilled water at a concentration of 7.5%. The pH was adjusted to 2 using 2M HCl, and then 0.25 g of glycerol per gram of protein was added. The solution was stirred for 1 h and casted into Petri dishes, which were placed at 60°C for 5 h.

Alkaline conditions - protein

In this comprehensive method, 3.5% w/v protein was suspended in distilled water, and the pH was adjusted to 11 with 2 M NaOH under heating at 40°C for 20 min, followed by the addition of 30% w/w glycerol. The addition of 10% w/w BNC was tested. Additionally, the incorporation of lignin was also tested by adding lignin (L) and lignin particles (LP) (5%, 10%, and 15% w/w). 1% w/v of tween20 were suspended in the solution in order to achieve better dispersion of the lignin. The different solutions were homogenized with an ultra-turrax at 12000 rpm for 5 minutes and then stirred at 80°C for 30 minutes. Finally, 25 mL of the solution was casted into each Petri dish and dried at room temperature overnight, followed by 50°C for 24 h.

3.5 Films characterization

3.5.1. Antioxidant activity of films

For the DPPH radical scavenging assay, a sample solution of 20 mg of film was mixed with 10 mL distilled water under stirring for 24 h. A DPPH reagent solution at a concentration of 0.2 mM in methnol was made. 2 mL of the sample added into a test tube with the addition of 2 mL DPPH reagent. This mixture mixed for 10 sec and incubated for 30 minutes at room temperature in darkness. After incubation, the absorbance was measured at 517 nm with a spectrophotometer (Zhang et al., 2022).

A control group was prepared by mixing DMSO and the DPPH reagent in equal volumes, and the absorbance of this control group was designated as A0.

The DPPH radical scavenging activity was calculated using the following formula:

$$RSA\% = \left(\frac{A_0 - A_s}{A_0}\right) * 100 \quad (13)$$

In this formula, A0 represents the absorbance of the control (untreated group), and As represents the absorbance of the test sample.

3.5.2. Mechanical properties

The mechanical properties of the film samples were measured under ambient conditions by means of a Simadzu tester equipped (Autograph AGS-X 10kN) with the crosshead speed of 5 mm/ min. The specimens were cut and had a dimension of 20 mm width and 60 mm length.

3.5.3. Color and transparency

The color of the films was quantified using a Minolta CR-400 tri-stimulus colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan). This device measures three key color parameters: L* (luminosity), a* (green-red chromaticity), and b* (blue-yellow chromaticity). A white background was employed as the reference for these measurements. Additionally, ΔE, ΔC and BI (brown index) were calculated according to equations (14), (15), and (16).

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad (14)$$

$$\Delta C = \sqrt{(a - a_0)^2 + (b - b_0)^2} \quad (15)$$

$$BI = \left(\frac{(a^* + 1.75L^*)a^*}{5.645L^* + a^* - 3.012b^*} - 0.31\right) \quad (16)$$

where L_0, a_0, and b_0 are the L, a, and b values on the produced film.

The UV–vis spectrophotometer analysis was conducted within the wavelength range of 400 to 700 nm. Film samples, measuring 1 cm × 4 cm, were directly positioned in the UV-vis spectrophotometer. The absence of a sample served as the reference (blank) for all measurements. Transparency was determined using the following calculation method:

$$T = \frac{A_{600nm}}{t} \quad (17)$$

where the A_{600nm} is the absorbance determined at 600nm, and t is the thickness of each film (nm).

Film thickness was measured at three different points for each film utilizing a calibrated digital micrometer. The final thickness value was determined by averaging the measurements obtained at these three distinct points.

3.6 Salmon packaging

Whole salmon fillets (*Salmo salar*) were supplied from local market and immediately cut into pieces and packaged under aseptic conditions.

The final films that chosen for the experiment were:

- 3.5% w/v protein, 0.3 g glycerol/ g protein, 10% w/w BNC (based on protein) 1% w/v Tween20 and named as **Protein**
- 3.5% w/v protein, 0.3 g glycerol/ g protein, 10% w/w BNC (based on protein) 1% w/v Tween20, 5% w/w lignin based on protein and named as **Pr5L**
- 3.5% w/v protein, 0.3 g glycerol/ g protein, 10% w/w BNC (based on protein) 1% w/v Tween20, 5% w/w linin nanoparticles based on protein and named as **Pr5LP**
- Polyvinyl chloride film (PVC) which named as **Blank**

In the salmon packaging process, strict hygiene and preservation measures were followed. Initially, all cups, lids, and films underwent a 20-minute UV light treatment for sterilization purposes. Under aseptic conditions, the salmon was then meticulously prepared, cut into 3x3x3 cm pieces, with each piece weighing approximately 20 grams. These salmon portions were placed inside individual cups, with volume 50 mL and diameter of the cup 4 cm. A film was carefully positioned on top of each cup to cover the salmon, and lids, which had openings cut into them to secure the films in place, were used to seal the cups. Finally, the assembled salmon packages were stored at a temperature of 4°C to maintain freshness and

safety until further use. This procedure ensured the integrity and quality of the packaged salmon samples.

3.6.1. Microbiological analysis

Microbiological analyses were systematically conducted on samples collected across various days. Sampling was performed immediately upon packaging and after 1, 2, 3, 4, 7, 9 & 11 days of storage. Each analysis commenced with the weighing of 10 grams of the sample, which was then placed in a stomacher bag. Subsequently, 90 mL of Ringer solution (Sigma-Aldrich) was added to the bag. The bag was carefully sealed and introduced into a stomacher machine, where it underwent homogenization for a duration of 1 minute. Following this step, appropriate decimal dilutions were carried out, using the same diluent, in order to prepare the samples for further analysis.

To determine the total colony count, a medium suitable for aerobic plate counts, namely Plate Count Agar (Condalab), was employed. A 0.1 mL inoculum from the diluted sample was evenly spread over the surface of the medium. Incubation was conducted at two different temperatures: 25°C for mesophilic counts and 4°C for psychrophilic counts, with both incubation periods lasting 48 hours. For mesophilic counts, the incubation period lasted 48 hours and for the psychrophilic counts, the incubation period lasted at least 48 hours and till the colonies were visible.

For the enumeration of *Pseudomonas* spp., *Pseudomonas* Agar Base supplemented with CFC (Cetrimide-Fucidin-Cephalosporin) (Condalab) was used. Again, a 0.1 mL inoculum was spread on the surface of the medium, and incubation was carried out at 25°C for a duration of 44 hours.

Selective solid medium, Violet Red Bile Glucose Agar (Condalab), was employed for the enumeration of enterobacteria. Similar to the previous procedures, a 0.1 mL inoculum from the appropriately diluted sample was applied to the medium, and incubation took place at 37°C for 24 hours. Notably, only red-purple colonies displaying biliary precipitate were considered and measured in this context.

3.6.2. Sensory analysis

A sensory analysis panel comprising six experienced individuals was assembled for the evaluation of various attributes. The panelists assessed the odor, color, and overall appearance of the samples, providing ratings on a scale ranging from 1 (indicating the poorest or least favorable) to 9 (representing the highest or most favorable rating). This scale allowed for a comprehensive assessment of the sensory characteristics of the samples, offering insights into their quality and appeal.

3.6.3. Statistical analysis

The statistical analysis of the shelf-life test results and the mechanical properties was carried out using STATGRAPHICS Centurion XVII, Version 17.2.00. This software platform was employed to process and interpret the data, enabling the extraction of meaningful insights and conclusions from the experimental findings.

4. Results

4.1 Compositional analysis of brewers spent grain

The composition of the BSG depends on the type of raw material that used for the production of beer, the production process as well as adjuncts added during mashing processes in breweries.(Robertson et al., 2010). BSG was subjected to detailed chemical analysis to determine its components. The results of this analysis, along with relevant data from previous studies are presented in table 1 in dry weight basis. BSG was rich in structural carbohydrates named glucan and hemicellulose (16.6 and 21.8% db), oligosaccharides (21.3% db), protein (20.5% db) as well as lignin (9.6% db). Regarding hemicellulose composition, BSG consisted of 15.4% db xylan, 1.3% db galactan, 4.8% db arabinan and 0.3% db mannan. BSG was also composed of lipids (6.3% db), ash (2.4% db) and phenolic compounds (0.5% db). The humidity of wet BSG as received by Cerveza Mica (Spain) was around 68.5%. and the ash at 2.4%. According to Pinheiro et al. (2019) and Senthilkumar et al. (2010) and the protein fraction of BSG could be from 17.2% - 24.3% w/w. As for carbohydrates, the biggest fraction was hemicellulose (21.8% w/w) and more specifically, Glucan fraction was 16.6% w/w and Kemppainen et al. (2016) found that the glucan in BSG is 15.2% w/w and Rojas-Chamorro, Romero, et al. (2020) found the glucan fraction 18.1% w/w.

As can been seen, the predominant sugar is glucose delivered from both glucan and oligosaccharides followed by xylose are the predominant sugars in BSG, leading to 41.7 g glucose per 100 g BSG and 24.6 g xylose per 100 g BSG respectively. Conducting a saccharification strategy, those sugars can be recovered in the liquid phase in so called BSG hydrolysate and used as a carbon source in order to produce different chemicals and bioplastics through microbial fermentation.

4.2 Plasma treatment

The pre-treatment of the solid with the use of an innovative technology like plasma conducted. 1000 g of dry BSG were treated with concentration of 50 g/L as described in section 3.2.1 After the pre-treatment the solid and the liquid that occurred analysed both to determine their composition. The composition of the solid and the liquid are shown in figure 10.

Table 1. Composition of the BSG in this study and comparison with compositions found in literature.

Composition (%, dry basis)	This study	Literature	Reference
Moisture	68.5 ± 0.1	65 – 75	Dhiman et al., 2003
Oligosaccharides (glucan equivalents)	21.3 ± 0.1		
Ash	2.4 ± 0.3	1.2 – 4.6	Carvalheiro et al., 2004; Mussatto & Roberto, 2006
Lipids	6.3 ± 0.7	5.2 – 9.2	del Río et al., 2013; Faulds et al., 2008
Protein	20.5 ± 0.3	17.2 – 24.3	Pinheiro et al., 2019; Senthilkumar et al., 2010
Total phenolic content (GA equivalents)	0.5 ± 0.03	0.3 – 1	Fărcaş et al., 2015; Meneses et al., 2013
Glucan	16.6 ± 1.3	15.2 – 18.1	Kemppainen et al., 2016; Rojas-Chamorro, Romero, et al., 2020
Hemicellulose	21.8 ± 0.7	19.2 – 25.1	Meneses et al., 2013; Rojas-Chamorro, Romero, et al., 2020
Xylan	15.4 ± 0.3	7 – 19.9	Mussatto & Roberto, 2006; Pinheiro et al., 2019
Galactan	1.3 ± 0.1	1.1 – 1.3	Kemppainen et al., 2016; Rojas-Chamorro, Romero, et al., 2020
Arabinan	4.8 ± 0.3	2.6 – 5.8	Pinheiro et al., 2019
Manan	0.3 ± 0.02	0.3 – 0.5	Kemppainen et al., 2016; Rojas-Chamorro, Romero, et al., 2020
Lignin	9.6 ± 0.2	8.9 – 14.5	Pinheiro et al., 2019
Soluble	1.4 ± 0.01	1.9 – 8.9	Laine et al., 2015; Meneses et al., 2013
Insoluble	8.3 ± 0.2	7 – 14.8	Laine et al., 2015; Rojas-Chamorro, Romero, et al., 2020

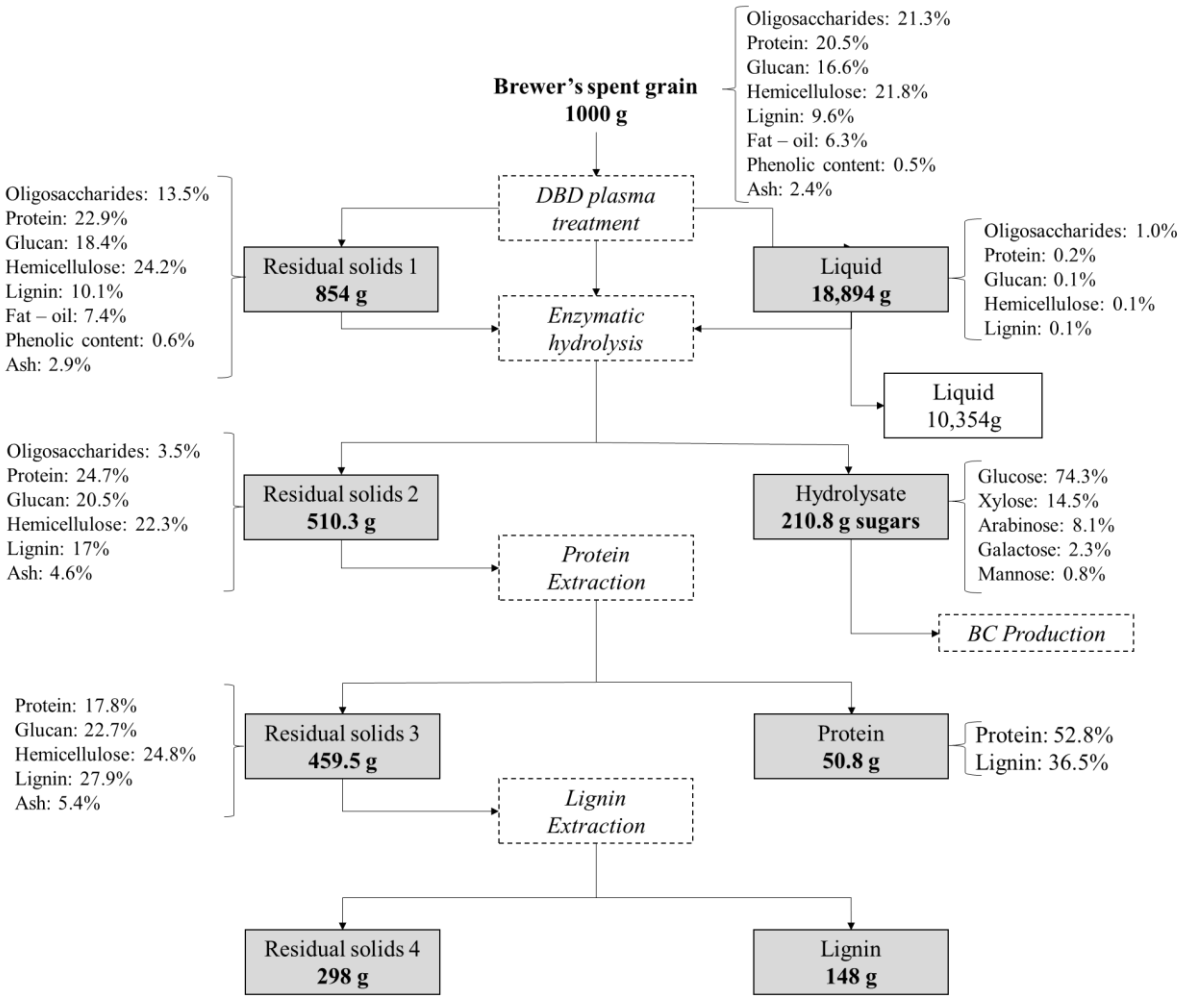
After the pre-treatment, 854 g of solid and 18,894g of liquid occurred (figure 10). It is clearly observed that a small amount of protein, about 16.2%, transferred to the liquid after the treatment. Also, small amounts of glucan (16.9%), hemicellulose (16.8%), mainly xylose, and lignin (21.1%) transferred to the liquid. Miranda et al. (2020) used dielectric barrier discharge plasma reactor as a pretreatment step of lignocellulosic material in order to enhance the enzymatic hydrolysis of biomass. The findings demonstrated that the utilization of plasma had a favorable impact on lignin solubilization. This, in turn, facilitated the enzymatic hydrolysis process and resulted in a substantial yield of carbohydrate solubilization. Notably, this improved yield was achieved with minimal production of inhibitory compounds, ultimately leading to the highest glucose production. Ravindran et al. (2019) used a submerged dielectric barrier discharge plasma reactor system as a pretreatment in order to improve the enzymatic hydrolysis of BSG. According to the results, when 100 g of BSG were treated (10 min, 28 kV) using water as a solvent, 16.29 g of sugars were released from 100 g of BSG. The lignin composition at BSG residual solids was reduced by 36%, whereas both glucan and hemicellulose were increased by 31.7% and 2.2% respectively. Some of the proteins are water soluble and during the pretreatment also migrate to the liquid (Devnani et al., 2023; Perrocheau et al., 2005). Atmospheric pressure plasmas generate highly reactive species like ozone, hydroxyl ions, and hydronium ions. These species can be used to selectively target and break down lignin while leaving cellulose and hemicellulose intact in lignocellulosic biomass (Ravindran et al., 2019).

It is reported that because of the crystalline nature of cellulose and the amorphous nature of hemicellulose, the second one deteriorates more readily in the presence of reactive species. Also, UV light and shock waves produced by plasma can also breakdown the lignocellulose substrate. They were also observed through FTIR profile that the glycosidic linkages, aryl-alkyl ether bonds (C-O-C) and C=C bonds in the aromatic ring of lignin were reduced on the pretreated BSG in contrary to initial BSG (Ravindran et al., 2019).

4.3 Enzymatic hydrolysis

Residual solids of BSG after plasma pretreatment were used for enzymatic hydrolysis by adding plasma liquid as solvent instead of water with an initial solid concentration of 100 g/L. The final sugars concentration of the produced hydrolysate was 34.3 g/L of total sugars composed mainly by glucose (74.3%), followed by xylose (14.5%), arabinose (8.1%), galactose (2.3%) and mannose (0.8%). The glucan and hemicellulose conversion yield were 52.7% and

60.8% respectively. After the enzymatic hydrolysis of BSG, 510.3 g of solid occurred and 6150 g of hydrolysate. It is observed that an amount of protein (4.7%) transferred to the hydrolysate. This may occur due to the relatively high temperature (50°C) and longtime of the treatment (48 h). According to the results of Qin et al. (2018) who used a wide range of temperatures, from 30°C until 135°C, for various times as a hydrothermal treatment for protein extraction, found that protein of BSG can be solubilized and transferred into the liquid phase. More specifically, the conditions of solid to liquid ratio of 4% at 60°C for 24 h had as a result the 66.5% of the protein to solubilize and transfer into the liquid phase. From his results it can be assumed that a fraction of protein is certainly solubilized during the enzymatic hydrolysis and transferred into the hydrolysate.



- DBD air plasma: 50 g/L initial solids concentration (1 vvm, 200 V, 1000 Hz, 60 kHz)
- Enzymatic hydrolysis: 100 g/L initial solids concentration

Figure 10. Biorefinery development of BSG

4.4 Protein extraction

Extracting proteins from BSG presents a challenge due to the strong association between proteins and lignin within this substrate. In pursuit of optimizing protein purity, a range of methods were evaluated to identify the approach yielding the highest protein yield. In the protein extraction process, the residual solid remaining subsequent to enzymatic hydrolysis was utilized.

4.4.1 Alkaline extraction

In this study 4 different methods to extract the protein from the solid that left after the enzymatic hydrolysis were tested. Alkaline extraction was the first method that was tested. The procedure of this method is explained in section 3.2.3 There were performed experiments in different temperatures during the protein solubilisation and different pH values for the precipitation of the protein. The results of this experiments are shown in the tables below.

Table 2. Protein concentration in each stream after the different conditions of alkaline protein extraction and protein recovery

	pH 3.4 T_{Room}	**pH 3.4 60°C**	**pH 4.5 60°C**
Protein (%)	25.4	52.8	28.8
Protein recovery (%)	10.8	21.3	19.9
Solid after protein extraction (%)	26.5	17.8	-
Liquid from protein extraction (%)	0.1	0.1	0.1

It is clearly observed that the temperature in which the protein solubilization is conducted is very important. According to Silva et al. (2023) a temperature higher than the room temperature during the solubilization of the proteins, led to higher protein yield. Also, the pH in which the proteins are precipitate is very important. The pH of 3.4 and temperature 60°C was selected, as higher purity of the protein was obtained.

4.4.2 Enzymatic extraction

This method is based on the solubilisation of the proteins under the influence of enzymes. Table 3 shows the protein extraction obtained after enzymatic extraction at 60°C and also the recovery of the protein.

Table 3. Protein concentration and recovery of the protein after enzymatic extraction.

Time (hours)	Protein (%)	Recovery (%)	FAN (mg/L)
2	0.3	23.5	28.7
17	0.3	20	28.9
24	0.3	21.5	27.1

It is clear that not a big fraction of the protein is recovered and also the liquid had a very small concentration of protein. The longer time also, did not had a positive effect to the extraction of the protein. Further experiments need to be conducted in order to optimize the enzymatic recovery of the protein-rich fraction. Because of these reasons, the enzymatic extraction was not selected as the preferred method.

4.4.3 Hydrothermal extraction

In this method we chose the most efficient method according to Qin et al. (2018). Solid to liquid ratio was 4%, the temperature in which the protein extraction conducted was 60°C for 24 h. In table 4 are shown the protein concentration and the recovery of the protein.

Table 4. Protein concentration and recovery of the protein after hydrothermal extraction.

	Protein (%)	Recovery (%)
Liquid	0.2	16.3

With this method we did not achieve high recovery and also high protein concentration, because a big fraction of the protein did not solubilize during this process.

4.4.4 Dilute acid extraction

The last method that was tested was the dilute acid extraction. With this method, H_2SO_4 in a concentration of 1200 mg of H_2SO_4/g BSG used and autoclaved at 120°C for 20 min. Then, the liquid separated from the solid and both analysed. Table 5 shows the protein concentration and recovery of the protein after the treatment. By using this method for protein separation, the highest recovery of protein fraction was achieved (71.2%). However, the liquid was verry diluted, could not formulate a film, and no further trials were conducted.

Table 5. Protein concentration and recovery of the protein after dilute acid extraction.

	Protein (%)	Recovery (%)
Liquid	1.8	71.2
Solid	10.9	0.9

Finally, the preferred method for our experiment was the alkaline extraction with precipitation pH 3.4 and temperature of the treatment 60℃.

4.6 Bacterial cellulose fermentation

BC fermentation was conducted using commercial sugars as carbon source simulated the ratio of the sugars that were in the hydrolysate. 20 g/L of carbon source according to Hestrin and Schramm fermentation media was used, and FAN concentration was 322 mg/L, leading to a ratio C/N of 28.1. In Figure 11 are shown the sugar consumption and the production of bacterial cellulose for all the days of fermentation (17 days) from the microorganism *K. sucrofermentans.* The higher concentration of BC observed at day 16 of fermentation and reached 1.3 g/L. The same concentration had also the last day. Highest productivity of BC observed at day 6 with 0.14 gBC/L/day and at the end of the fermentation it was 0.08 gBC/L/day. Also, the yield of the fermentation the last day was 0.1 $g_{BC}/g_{consumed\ sugars}$. Finally, the 50.6% of the FAN consumed by the microorganism, and the last day the FAN concentration was 159 mg/L. Tsouko et al. (2023) used commercial sugars for the production of bacterial cellulose using the strain *Komagataeibacter rhaeticus* UNIWA AAK2 and more specifically, when glucose used at a concentration of 20 g/L for the fermentation, it was observed a concentration of BC 0.88 g/L, productivity of 0.088 g/L/day and FAN consumption of 39.7%.

A fermentation using the hydrolysate that produced after the enzymatic hydrolysis was used for this fermentation The concentration of the sugars should be 20 g/L according to Hestrin and Schramm fermentation media that we followed for our fermentations. Because the hydrolysate had higher concentration in sugars, it was firstly diluted with distilled water until its concentration reached the desired value.

The higher concentration of bacterial cellulose observed at day 15 of fermentation and reached 3.2 g/L. The same concentration had also the last day. Highest productivity of BC observed at day 2 with 0.48 gBC/L/day and at the end of the fermentation it was 0.19

gBC/L/day. Also, the yield of the fermentation the last day was 0.1 $g_{BC}/g_{consumed\ sugars}$. Finally, the 74 % of the FAN consumed by the microorganism, and the last day the FAN concentration was 65.4 mg/L.

As in the case of using BSG hydrolysate sugars were consumed from the 3rd day, the batch fermentation converted into fed-batch and two times feeding was conducted as it can be seen in figure 11. The first feeding was conducted on day 3 (20 g/L total sugars), whereas the second one at day 10 (12 g/L total sugars). The productivity of the fermentation using the hydrolysate was higher than that of the one using commercial sugars. This may occur due to vitamins, minerals and protein that exist in the hydrolysate and give better results than the fermentation with commercial sugars (Puligundla & Mok, 2021).

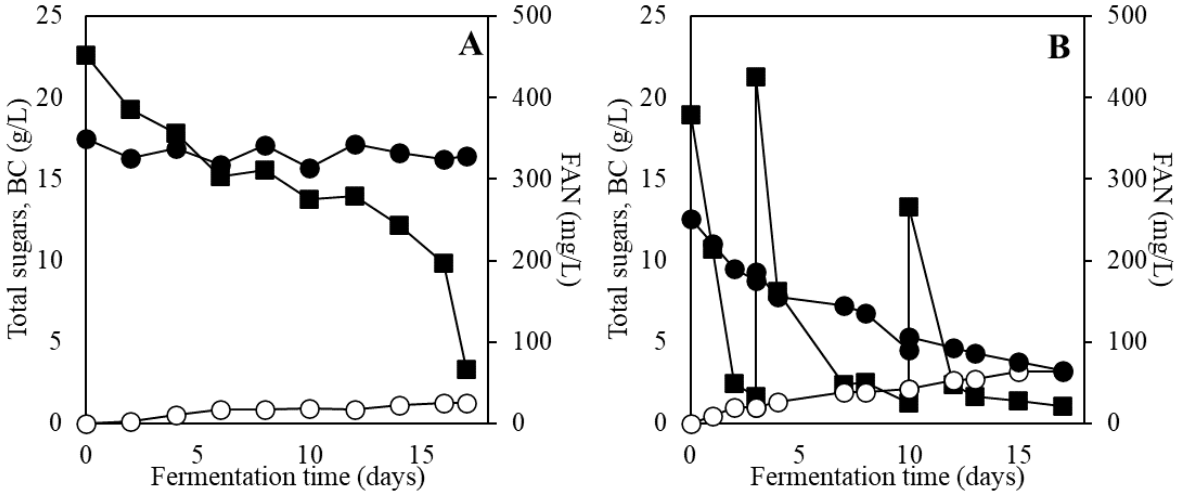


Figure 11. Kinetic of consumption of sugars (■), bacterial cellulose production (○) and FAN (●) during static fermentation **A.** with commercial sugars and **B.** with hydrolysate.

Table 6. Production, yield and productivity of bacterial cellulose, under fermentation of commercial sugars and hydrolysate.

	Simulation	BSG hydrolysate
BC (g/L)	1.3	3.2
Yield (g/g)	0.1	0.08
Productivity (g/L/day)	0.08	0.21

4.7 Film production

4.7.1 Utilization of BSG residual solid 2

In order to utilize the whole residual solid 2 (after DBD air plasma and enzymatic hydrolysis), different methods of solubilization of the solid were tested. Firstly, acid hydrolysis by using 1 M acetic acid solution was tested followed by plasticization, as this method had already used in the literature (Merino et al., 2021). Three different concentrations of BSG solid were tested (5%, 7.5% and 10%). Dried BSG suspended into acetic acid solution 1 M and stirred for 24 h in room temperature. Then, 30% glycerol was added followed by stirring for 10 min, solution casting into Petri dishes and dried for 24 h. The final films can be observed in figure 12 1. A, B and C. The same strategy was followed by using water instead of acetic acid and hydrochloric acid was used in order to fix the pH at 2.0 (Proaño et al., 2020). The final films are represented in figure 12 2. A, B and C. All the samples were rough in touch and their appearance was considered undesirable for use in food packaging. Because of the above-mentioned reasons, all of the films were rejected.

Because of the high lignin concentration in BSG residual solid 2 (17.0%, figure 10), an organosolv process for breakdown the lignin structure was used. Followed the process from de Sousa Nascimento et al. (2021), three different concentrations of acetic aqueous solution were tested, 70%, 80% and 90% v/v. A concentration of 10% w/v BSG was solubilized in the different solvents, 2% v/v HCl was added as catalyst, and the solvent was tired at 110℃ for 1 h. The BSG suspended solution was cooled down and 30% glycerol was added. The resulted black liquor casted into petri dishes and dried at 30℃ for 24 h. Films that resulted from this method were more acceptable even though they were totally dark in color (figure 12 3. A,B,C). However, they were characterized by an unpleasant smell of vinegar, no matter the acetic acid concentration. Because of both smell and color, these films were also rejected for food packaging applications.

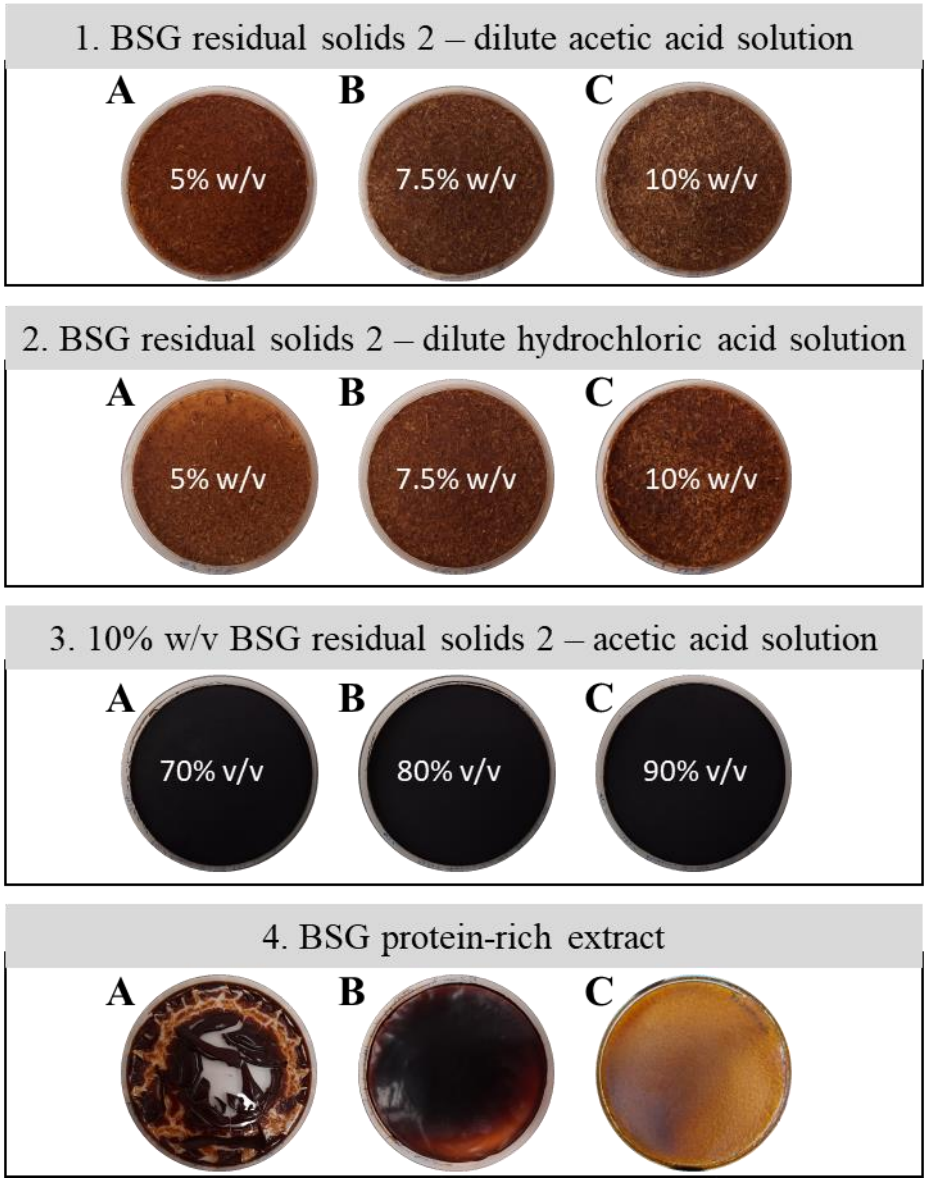


Figure 12 Different films produced by BSG residual solid 2 (1,2 and 3) and protein-rich extract (4). **1.** 1M acetic acid as a solvent with three different initial solids concentrations of A. 5% w/v, B. 7.5% w/v, and C. 10% w/v. **2.** Acidic water as a solvent (final pH 2.0) with three different initial solids concentrations of A. 5% w/v, B. 7.5% w/v, and C. 10% w/v. **3.** 10% w/v BSG residual solid using A. 70% v/v, B. 80% v/v, and C. 90% v/v acetic acid solution. **4.** 3.5% w/v protein A. pH 2, 25% glycerol, B. pH 11, 30% glycerol, and C. pH 11, 30% glycerol, 10% BNC, 1% w/v Tween20.

4.7.2 Utilization of BSG protein-rich extract

After the protein extraction from BSG residual solid 2, the protein rich extract was used as raw material for the production of protein-based films. Firstly, the modified method described by Proaño et al. (2020) was applied. More specifically, 7.5% w/v protein was solubilized at distilled water and pH adjusted to 2.0 in order to be solubilized. After the addition of glycerol as plasticizer and purring of the solvent in Petri dishes, the film was dried at 60°C for 5 h. The resulting film was very brittle with clearly poor cohesion between particles. In

order to improve the properties of the film, less amount of solids and higher pH was used for better protein solubilization. More specifically, a modified protocol described by Efthymiou et al. (2022) was applied. 3.5% w/v protein suspended into distilled water, the pH adjusted to 11 with NaOH 2 M in order the protein to dilute to the solution and 30% glycerol added as plasticizer. Finally, the solution was casted in Petri dishes and dried at 60°C for 5 h. The films that were produced were good dispersed and could be easily removed from the Petri dishes. The produced films were selected for further characterization.

4.8 Characterization of protein-based films

Films that incorporate lignin offer a range of value-added characteristics, including UV-shielding properties, antimicrobial effects, and antioxidant activities. These attributes make them highly versatile and suitable for various packaging materials and applications (Wang et al., 2021). Lignin nanoparticles (LNPs), along with chemical modifications, represent effective strategies for enhancing antioxidant activity by increasing the free content and reducing the bond dissociation enthalpy of phenolic hydroxyl. LNPs find extensive applications in the preparation of films, hydrogels, and conductive materials. This is primarily due to their ability to enhance mechanical strength, thermal stability, resistance to freezing and dehydration, antioxidant properties, and conductivity when incorporated into these materials (Lu et al., 2022).

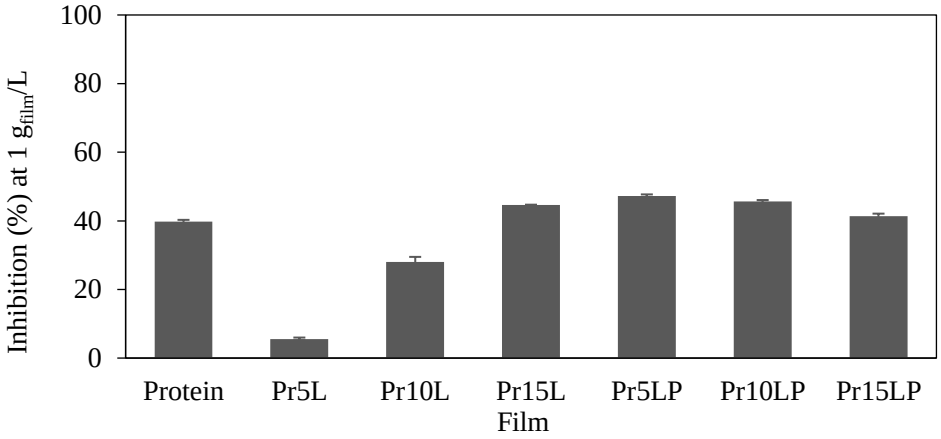


Figure 13. Antioxidant activity of the different films

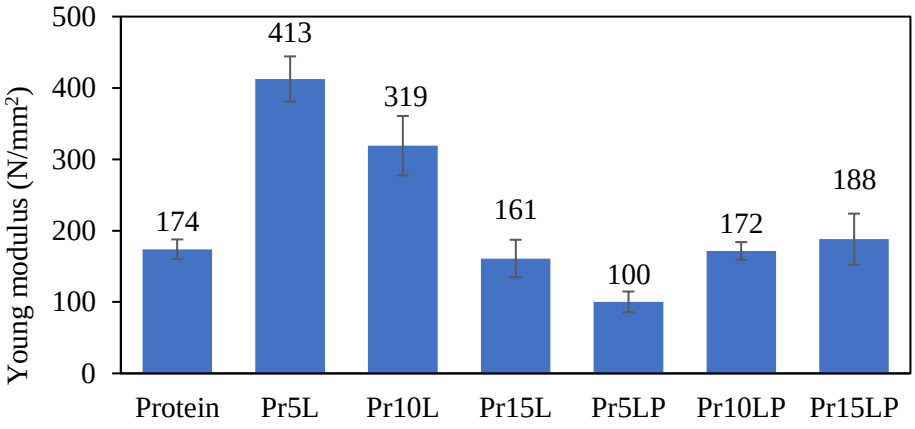


Figure 14. Young modulus of the different produced films.

The film with 5% lignin particles had the higher antioxidant activity (47.2) and the film with 5% lignin had the lowest antioxidant activity (5.6). This may occur due to the lack of homogenous dispersion of lignin in the protein matrix. The produced films showed good antioxidant activity (more than 40%).

From the figure 14 it can be observed that the film with the addition of 5% lignin exhibited a substantial improvement in Young's modulus, increasing it by 137.2% when compared to the film with no added lignin. Notably, there is a significant difference between sample Pr5L and the other samples. Furthermore, sample Pr5LP demonstrated a significantly lower Young's modulus than the films containing lignin particles. From the figure 15 describing tensile strength, it is evident that sample Pr15LP exhibits a significantly lower tensile strength when compared to the other samples. The highest tensile strength is observed in samples Protein and Pr15LP, while the remaining samples do not display significant differences among themselves. In the present work, the incorporation of lignin caused a significant reduction ($p < 0.05$) in tensile strength compared with the control film. Among the samples made with lignin, sample Pr15L exhibits a significantly higher elongation at break. Similarly, among the samples made with lignin particles, sample Pr5LP demonstrates the highest elongation at break when compared to all the other samples.

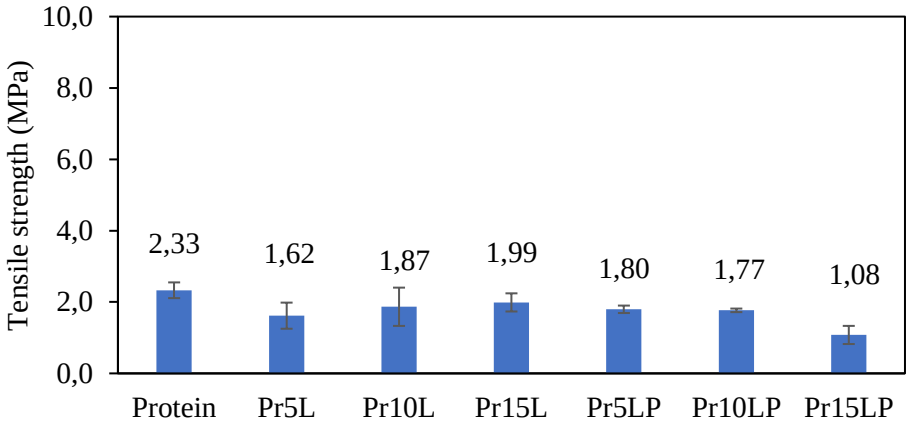


Figure 15. Tensile strength of the different produced films.

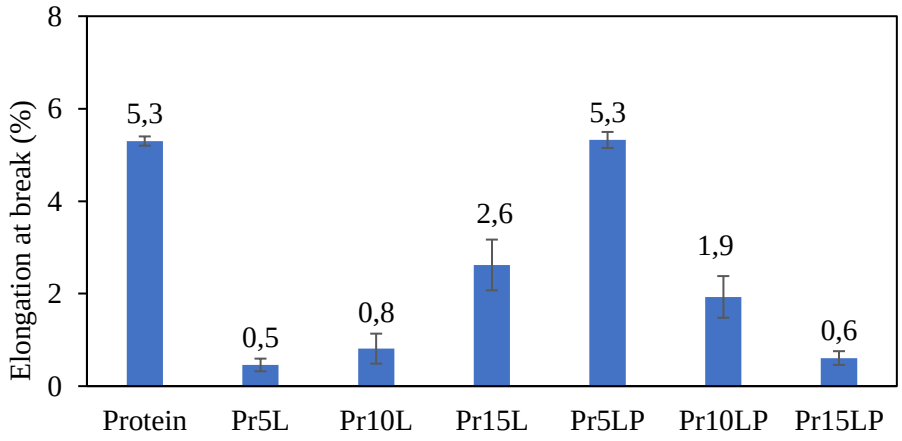


Figure 16. Elongation at break of the different produced films

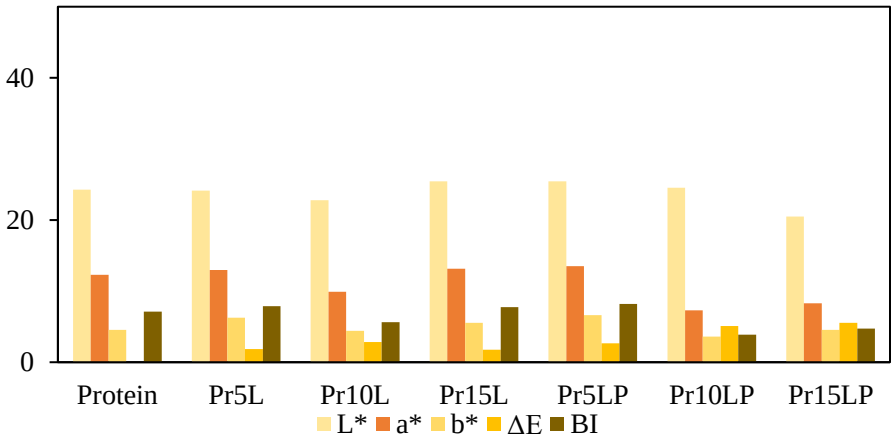


Figure 17. Color measurements for the different produced films.

In figure 17 is showed the color of the produced films. L*, a* and b* of the films were evaluated. The highest L* value observed at the sample Pr15L. There are no significant differences between the samples for each parameter.

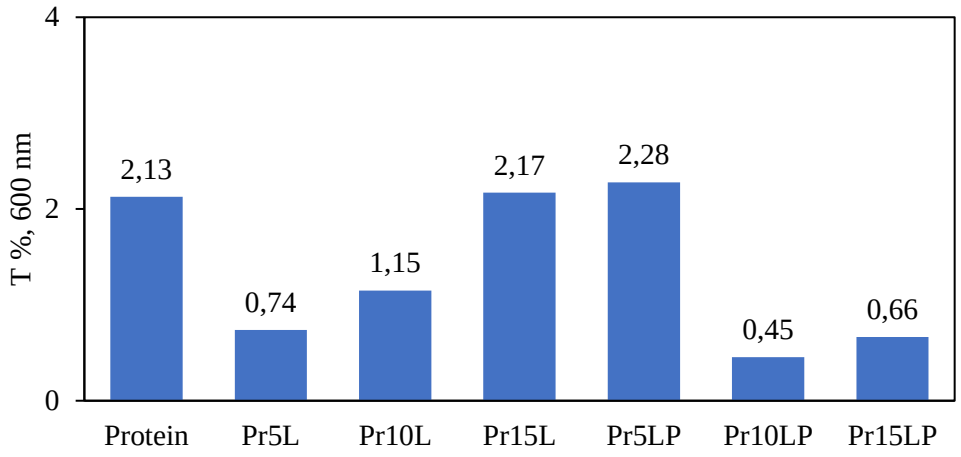


Figure 18. Transparency of the different produced films

All the films that tested, had good light barrier because the transmittance of visible light (600nm) was less than 2.3%.

Three different types of films were selected named Protein, Pr5L and Pr5LP. The resulted films had very good mechanical properties, they had not so big black spots as the other ones and better appearance.

4.9 Salmon packaging

The packaging of the salmon conducted under aseptic condition in order not to contaminate the salmon fillets. Also, the cups, lids and also the films were disinfected under UV radiation. Salmon fillets cut to 3cm x 3cm x 3cm pieces and weighted about 20 grams. The pieces of the fillets placed into the cups and immediately closed with the film. Then, all the cups placed in an incubator at 4℃. In the figure 14 is shown the packaging of the salmon.

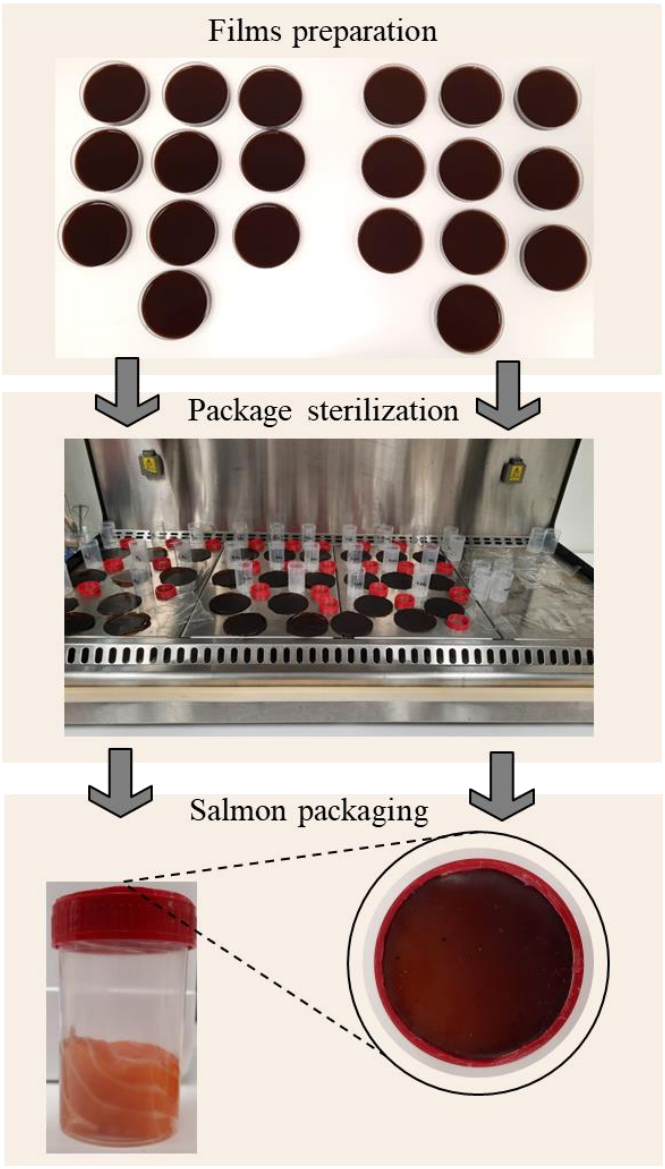


Figure 19. Procedure of packaging the salmon fillets under different packaging films.

4.10 Microbiological analysis

The shelf-life test of the salmon was conducted at 4℃ for 11 days. Three different microbiological analyses were conducted for the evaluation of the shelf-life. Plate count agar was used to determine the population of the aerobic bacteria. Two different incubation temperatures were employed, namely 25℃ and 4℃; the 25℃ is the temperature used for the enumeration of the aerobic mesophilic bacteria and 4℃ is used for the enumeration of the aerobic psychrophilic bacteria. On day 0, the mesophilic aerobic bacteria were 6.13 logCFU/g. In figure 20 the development of the mesophilic bacteria on the salmon fillet under different packaging films is shown.

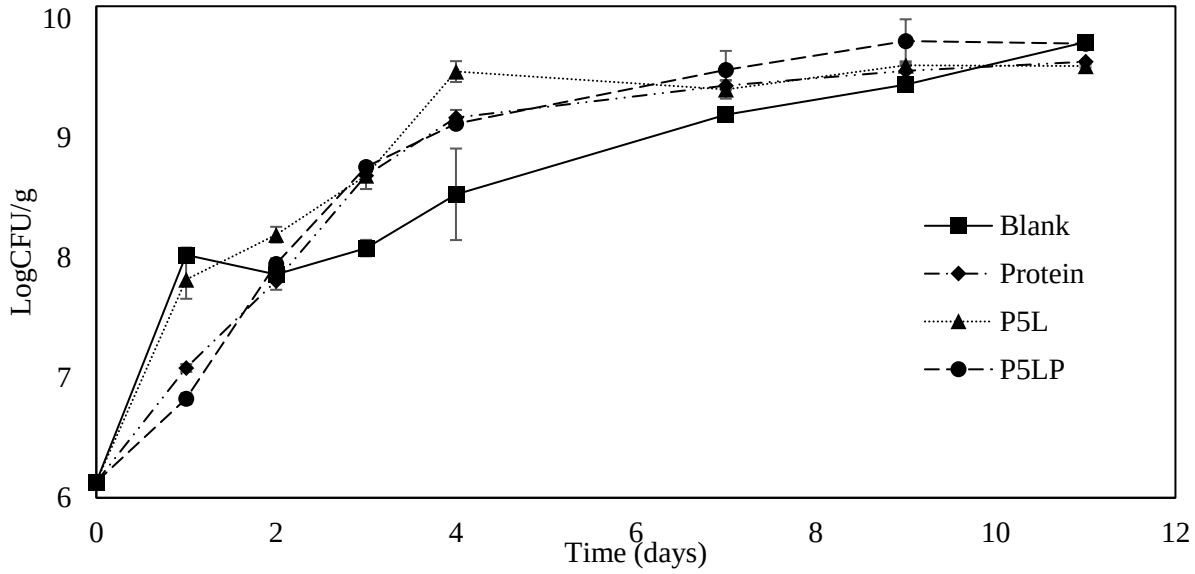


Figure 20. Growth of mesophilic aerobic bacteria at 25℃ on the salmon fillets for the 4 different packaging films.

On day 1 there is a statistically significant difference at the 95.0% confidence level between the Blank – Protein, Blank – P5LP and P5L – P5LP. On day 2 the Blank had no significant difference from the Protein and P5LP but on day 3 the Blank presented significant difference from the 3 other packaging films. On the last day, the P5L and Protein had a significant difference from the other 2 cases. In table 7 the population of the mesophilic aerobic bacteria in each day for the samples under different packaging films is shown. On days 4, 7 and 9 no significant differences between the samples were observed. On day 11, the P5L and Protein had less population than the other 2 samples. The samples Protein and P5L had no significant differences in their population the last 2 days, and the P5LP from day 7 had no significant difference at the population. The last day of the experiment, the sample P5L and Protein had a significant lower population than the other samples, and the other 2 samples had the highest population. In figure 21 the development of the psychrophilic aerobic bacteria on the salmon fillets under different packaging films is shown. At day 0, the psychrophilic aerobic bacteria were 6.15 logCFU/g.

Table 7. Population (CFU/g salmon) of aerobic mesophilic bacteria during incubation of salmon at 25°C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 0	$1.36 \times 10^{6\ a,A}$	$1.36 \times 10^{6\ a,E}$	$1.36 \times 10^{6\ a,I}$	$1.36 \times 10^{6\ a,L}$
Day 1	$1.06 \times 10^{8\ b,A}$	$1.21 \times 10^{7\ c,d,E}$	$6.59 \times 10^{7\ b,c,I}$	$6.70 \times 10^{6\ d,L}$
Day 2	$7.30 \times 10^{7\ e,A}$	$6.43 \times 10^{7\ e,E}$	$1.56 \times 10^{8\ f,I}$	$8.95 \times 10^{7\ e,L}$
Day 3	$1.21 \times 10^{8\ g,A}$	$4.87 \times 10^{8\ h,E}$	$4.87 \times 10^{8\ h,I}$	$5.76 \times 10^{8\ h,L,M}$
Day 4	$3.40 \times 10^{8\ i,A}$	$1.48 \times 10^{9\ i,F}$	$3.59 \times 10^{9\ j,J}$	$1.32 \times 10^{9\ i,L,M}$
Day 7	$1.57 \times 10^{9\ k,B}$	$2.75 \times 10^{9\ k,G}$	$2.55 \times 10^{9\ k,K}$	$3.7 \times 10^{9\ k,M,N}$
Day 9	$2.80 \times 10^{9\ l,C}$	$3.65 \times 10^{9\ l,H}$	$4.05 \times 10^{9\ l,J}$	$6.45 \times 10^{9\ l,N}$
Day 11	$6.32 \times 10^{9\ m,D}$	$5.59 \times 10^{9\ n,H}$	$3.99 \times 10^{9\ n,J}$	$6.12 \times 10^{9\ m,N}$

Statistically significant differences within the same sampling day are indicated with different letters a–n ($p < 0.05$) and within the same sample are indicating with different capital letters A-N ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

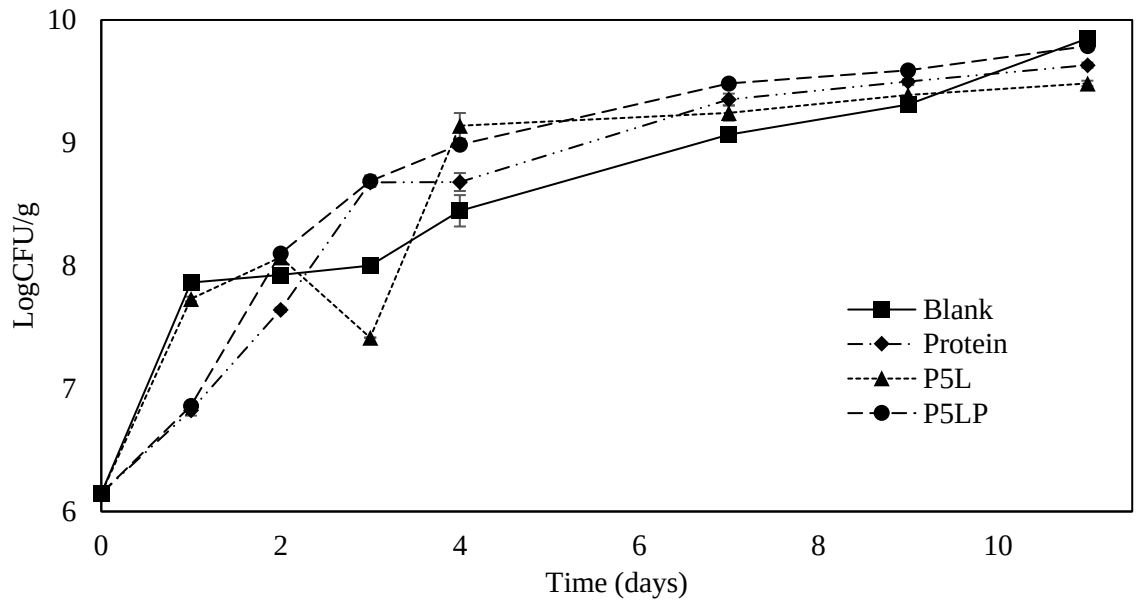


Figure 21. Growth of psychrophilic aerobic bacteria incubated at 4°C on the salmon fillets for the 4 different packaging films.

On day 1 a lower population in the samples under Protein and P5LP films was observed. The last day of the experiment in all samples, a high population of psychrophilic bacteria was reached with Blank having the highest population of the samples. Only the sample P5L has no significant difference in the population from day 9 to day 11, and that means that its higher population is reached. The last day, P5L has a significant lower population than the other samples. At day 1 the samples Blank and P5L exceeded the microbiological limit of 7 logCFU/g but the samples Protein and P5LP exceeded the limit at day 2.

In figure 22 the development of the pseudomonads on the salmon fillets under different packaging films is shown. On day 0, the *Pseudomonas* spp. were 5.33 logCFU/g.

Table 8 Population (CFU/g salmon) of aerobic psychrophilic bacteria during incubation of salmon at 4°C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 0	$1.46 \times 10^{6\ a,A}$	$1.46 \times 10^{6\ a,F}$	$1.46 \times 10^{6\ a,K}$	$1.46 \times 10^{6\ a,O}$
Day 1	$7.29 \times 10^{7\ b,A,B}$	$6.65 \times 10^{6\ c,F}$	$5.36 \times 10^{7\ d,K}$	$7.25 \times 10^{6\ c,O}$
Day 2	$1.94 \times 10^{7\ e,A,B}$	$4.38 \times 10^{7\ f,F}$	$1.17 \times 10^{8\ g,K}$	$1.26 \times 10^{8\ g,O}$
Day 3	$1.01 \times 10^{8\ h,A,B}$	$4.76 \times 10^{8\ i,G}$	$2.30 \times 10^{7\ h,K}$	$4.88 \times 10^{8\ I,P}$
Day 4	$2.80 \times 10^{8\ j,B}$	$4.80 \times 10^{8\ j,G}$	$1.38 \times 10^{9\ k,L}$	$9.65 \times 10^{8\ j,k,Q}$
Day 7	$1.17 \times 10^{9\ l,C}$	$2.25 \times 10^{9\ m,H}$	$1.75 \times 10^{9\ l,m,L,M}$	$3.05 \times 10^{9\ n,R}$
Day 9	$2.05 \times 10^{9\ o,D}$	$3.15 \times 10^{9\ p,q,I}$	$2.45 \times 10^{9\ o,p,M,N}$	$3.90 \times 10^{9\ q,S}$
Day 11	$7.06 \times 10^{9\ r,E}$	$4.29 \times 10^{9\ s,J}$	$3.05 \times 10^{9\ t,N}$	$6.13 \times 10^{9\ u,T}$

Statistically significant differences are indicated with different letters a–u ($p < 0.05$) and within the same sample are indicating with different capital letters A-T ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

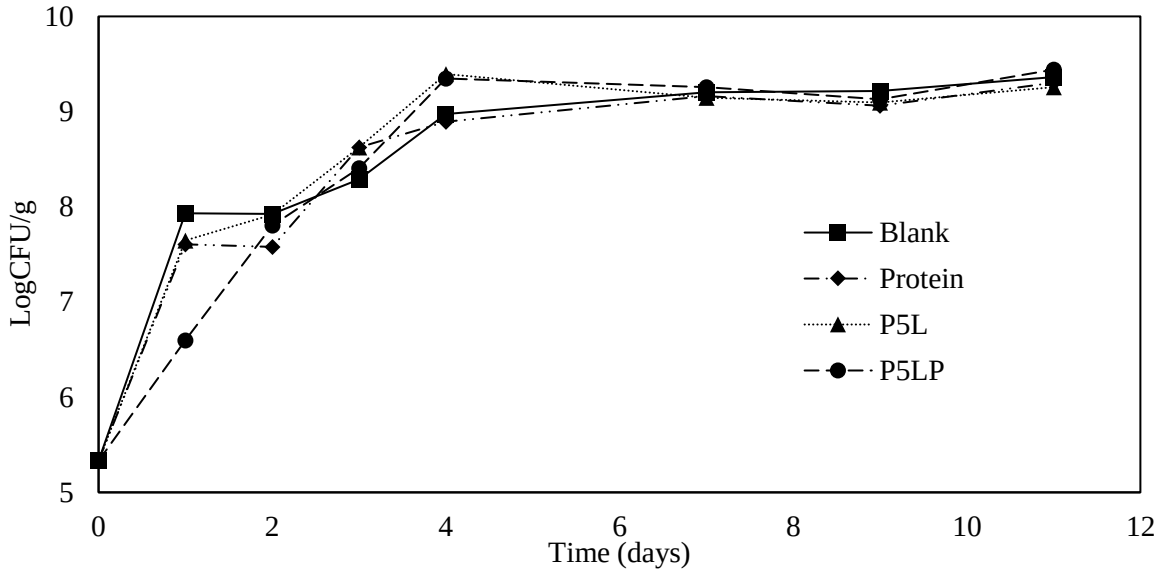


Figure 22. Growth of Pseudomonas spp. incubated at 25 °C on the salmon fillets for the 4 different packaging films.

On day 1 a lower growth of the pseudomonads in the sample P5LP was observed. Also, on day 7 and 9 the samples had no significant differences at the populations between them. The sample P5L from day 7 had no significant difference at its population. That means that it reached the stationary phase of the microorganism. The last day, P5L and Protein had a significant lower population than the other samples.

Table 9. Population (CFU/g salmon) of *Pseudomonas* spp during incubation of salmon at 25°C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 0	$2.15 \times 10^{5\ a,A}$	$2.15 \times 10^{5\ a,E}$	$2.15 \times 10^{5\ a,J}$	$2.15 \times 10^{5\ a,N}$
Day 1	$8.56 \times 10^{7\ b,A}$	$4.03 \times 10^{7\ b,c,E}$	$4.41 \times 10^{7\ b,c,J}$	$3.95 \times 10^{6\ b,c,N}$
Day 2	$8.45 \times 10^{7\ d,A}$	$3.80 \times 10^{7\ e,E}$	$8.30 \times 10^{7\ d,J}$	$6.35 \times 10^{7\ d,e,N}$
Day 3	$1.95 \times 10^{8\ f,A}$	$4.21 \times 10^{8\ g,E,F}$	$4.21 \times 10^{8\ g,J,K}$	$2.55 \times 10^{8\ f,N}$
Day 4	$9{,}40 \times 10^{8\ h,I,B}$	$7{,}85 \times 10^{8\ h,F,G}$	$3{,}19 \times 10^{9\ j,L}$	$2{,}23 \times 10^{9\ i,j,O,P}$
Day 7	$1.6 \times 10^{9\ k,C}$	$1.45 \times 10^{9\ k,H,I}$	$1.40 \times 10^{9\ k,M}$	$1.80 \times 10^{9\ k,O,P}$
Day 9	$1.65 \times 10^{8\ l,C}$	$1.15 \times 10^{9\ l,G,H}$	$1.25 \times 10^{9\ l,K,M}$	$1.35 \times 10^{9\ l,O}$
Day 11	$2.31 \times 10^{9\ m,D}$	$2.01 \times 10^{9\ m,n,I}$	$1.89 \times 10^{9\ n,M}$	$2.76 \times 10^{9\ o,P}$

Statistically significant differences are indicated with different letters a–o ($p < 0.05$) and within the same sample are indicating with different capital letters A-P ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

In figure 23 the development of the *Enterobacteriaceae* on the salmon fillets under different packaging films is shown. The population of *Enterobacteriaceae* at day 0 was 3.6 logCFU/g.

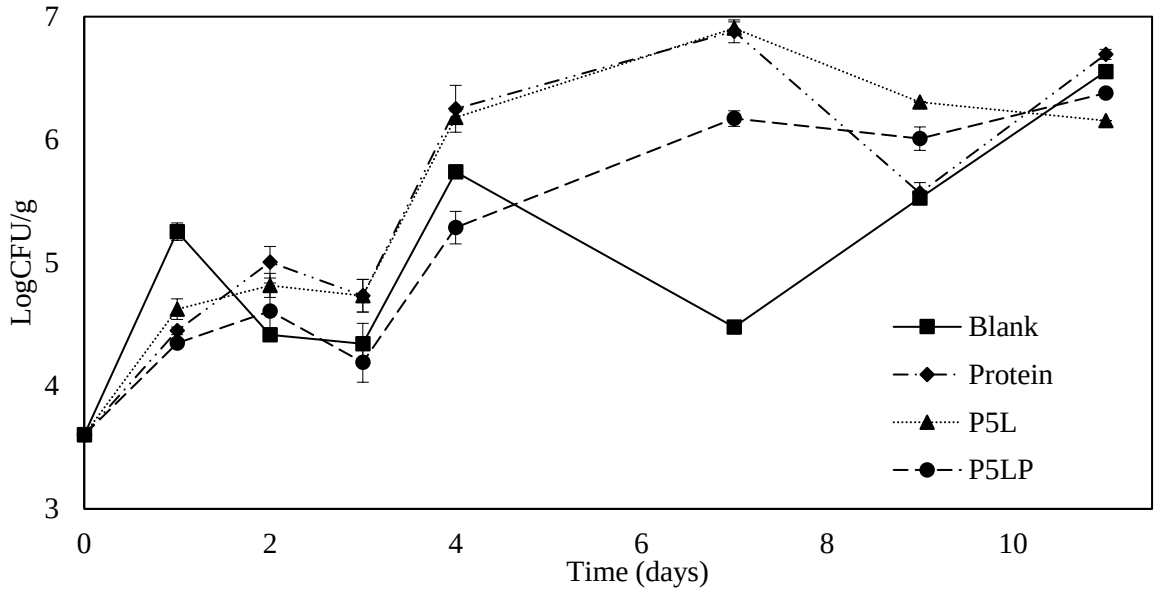


Figure 23. Growth of Enterobacteriaceae incubated at 37°C on the salmon fillets for the 4 different packaging films.

On day 1 the Blank had the highest population while no statistically significant differences were observed between the other samples. The first 7 days the samples Protein and P5L had not significant higher population from day to day. The last day of the experiment, sample Protein had a significant higher population, and P5L and P5LP had a significant lower population. Only the sample P5L had after day 9 not significant difference at the population.

Table 10. Population (CFU/g salmon) of *Enterobacteriaceae* during incubation of salmon at 37 °C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 0	$4.00 \times 10^{3\ a,A}$	$4.00 \times 10^{3\ a,E}$	$4.00 \times 10^{3\ a,H}$	$4.00 \times 10^{3\ a,K}$
Day 1	$1.79 \times 10^{5\ b,A,B}$	$2.80 \times 10^{4\ c,E}$	$4.20 \times 10^{4\ c,H}$	$2.23 \times 10^{4\ c,K}$
Day 2	$2.60 \times 10^{4\ d,A}$	$1.01 \times 10^{5\ e,E}$	$6.55 \times 10^{4\ d,e,H}$	$4.05 \times 10^{4\ d,e,K}$
Day 3	$2.20 \times 10^{4\ f,A}$	$5.40 \times 10^{4\ f,E}$	$5.40 \times 10^{4\ f,H}$	$1.55 \times 10^{4\ f,K}$
Day 4	$5.49 \times 10^{5\ g,h,C}$	$1.78 \times 10^{6\ h,E}$	$1.52 \times 10^{6\ g,h,I}$	$1.93 \times 10^{5\ g,K}$
Day 7	$3.00 \times 10^{4\ i,A}$	$1.49 \times 10^{6\ j,F}$	$8.05 \times 10^{6\ j,J}$	$7.60 \times 10^{6\ i,L}$
Day 9	$3.35 \times 10^{5\ k,B,C}$	$3.70 \times 10^{5\ k,E}$	$2.03 \times 10^{6\ l,I}$	$1.02 \times 10^{6\ k,M}$
Day 11	$3{,}58 \times 10^{6\ m,D}$	$4.94 \times 10^{6\ n,G}$	$1.43 \times 10^{6\ o,I}$	$2.39 \times 10^{6\ m,o,N}$

Statistically significant differences are indicated with different letters a–o ($p < 0.05$) and within the same sample are indicating with different capital letters A-N ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

The microbiological analyses have shown that there are not big differences at the way the microorganisms grow and also at the shelf life with the different packaging films. Only the P5L showed a lower population the last day of the experiment in all the microbiological analyses. From the perspective of microbiological safety and quality, all the samples performed almost the same results. This is very important because these alternative packaging films could be used without changing the shelf life of the products.

4.11 Sensory analysis

The sensory analysis conducted by an experienced panel in which participated 6 people. Color, odor and total appearance evaluated form the panel in a scale 1 – 9 (worst – best). The sensory analysis conducted also in days 0, 1, 2, 3, 4, 7, 9 and 11 as the microbiological analysis. Sensory analysis questionnaire for the evaluation of the color was in a scale for 1-9 (worst to best). At 1 was gray to yellow green color, at 5 orange red to orange brown color and at 9 orange pink to orange red color. Figure 24 shows the development of the color.

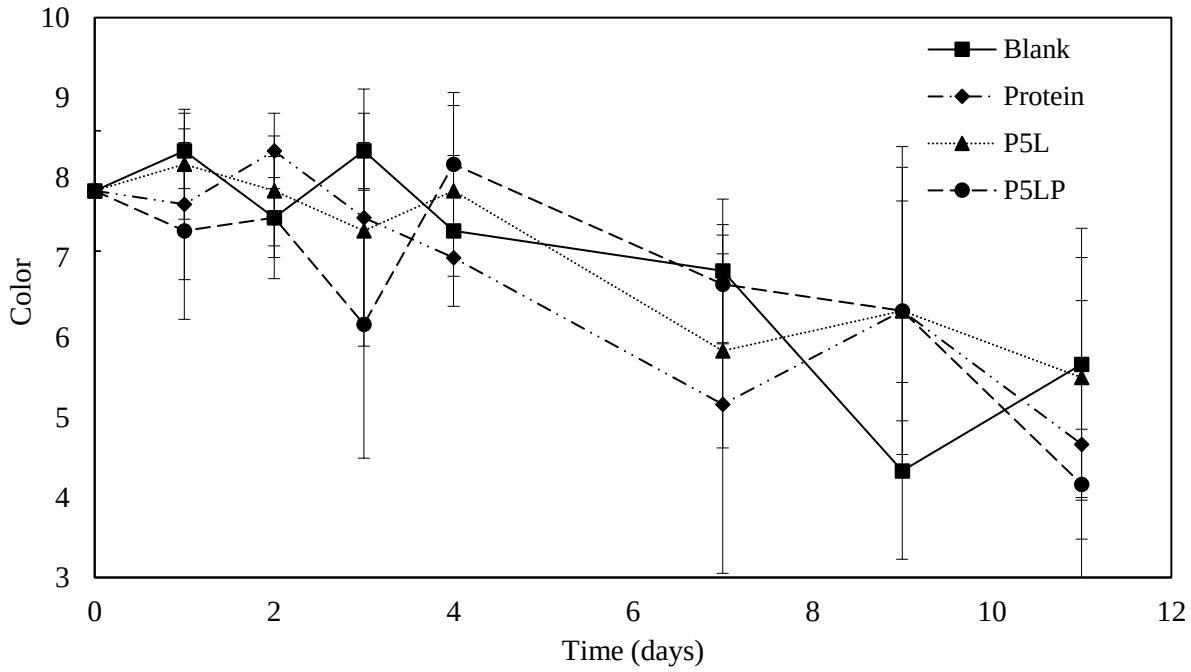


Figure 24. Sensory analysis of the color in salmon fillets packaged with different packaging films and stored under 4°C.

The development of the color is the same the first 4 days, with no big differences between the different packaging films. Day 2 Protein and P5L have a better score on color, on day 3 Blank, Protein and P5L have the better results, but form day 7 all samples have the same score. It can be assumed that the color of the salmon fillets has not big difference when packaged under alternative packaging films.

Table 11. Scores of color sensory analysis for the different packaging films of salmon fillets stored under 4°C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 1	8.3^{a}	7.7^{a}	8.2^{a}	7.3^{a}
Day 2	7.5^{b}	8.3^{c}	$7.8^{b,c}$	7.5^{b}
Day 3	8.3^{d}	$7.5^{d,e}$	$7.3^{d,e}$	6.2^{e}
Day 4	$7.3^{f,g}$	7.0^{f}	$7.8^{f,g}$	8.2^{g}
Day 7	6.8^{h}	5.2^{h}	5.8^{h}	6.7^{h}
Day 9	4.3^{i}	6.3^{i}	6.3^{i}	6.3^{i}
Day 11	5.7^{j}	4.7^{j}	5.5^{j}	4.2^{j}

Statistically significant differences are indicated with different letters a–j ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

The odor of the salmon fillets also evaluated form the sensory panel. The scale that used here was 1 for strong unpleasant rotten odor, 5 for neutral or light acidic odor and 9 for fresh, sea smell. In figure 25 is shown the development of the odor.

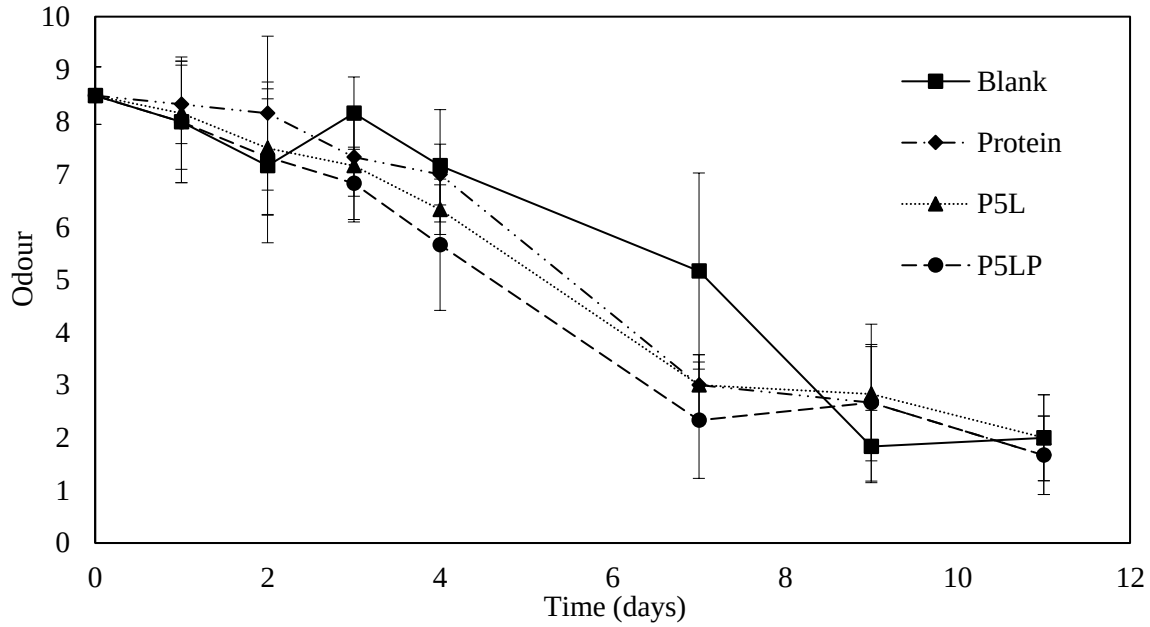


Figure 25. Sensory analysis of the odor in salmon fillets packaged with different packaging films and stored under 4°C.

The development of the odor the first two days was the same for all the samples. The day 3 and 4, Blank, Protein and P5L had better results than the P5LP. Also, in odor there were not big differences in the results between the packaging films.

Table 12. Scores of odor sensory analysis for the different packaging films of salmon fillets stored under 4°C.

	Blank	**Protein**	**P5L**	**P5LP**
Day 1	8.0^{a}	8.3^{a}	8.2^{a}	8.0^{a}
Day 2	7.2^{b}	8.2^{b}	7.5^{b}	7.3^{b}
Day 3	8.2^{c}	$7.3^{c,d}$	$7.2^{c,d}$	6.8^{d}
Day 4	7.2^{e}	7.0^{e}	$6.3^{e,f}$	5.7^{f}
Day 7	5.2^{g}	3.0^{h}	3.0^{h}	2.3^{h}
Day 9	1.8^{i}	2.7^{i}	2.8^{i}	2.7^{i}
Day 11	2.0^{j}	1.7^{j}	2.0^{j}	1.7^{j}

Statistically significant differences are indicated with different letters a–j ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

Lastly, the sensory panel evaluated also the total appearance of the salmon fillets. The scale that used here was 1 for not liked at all, 5 neither liked or disliked and 9 for liked at all. In figure 26 is shown the development of total appearance during the sensory analysis.

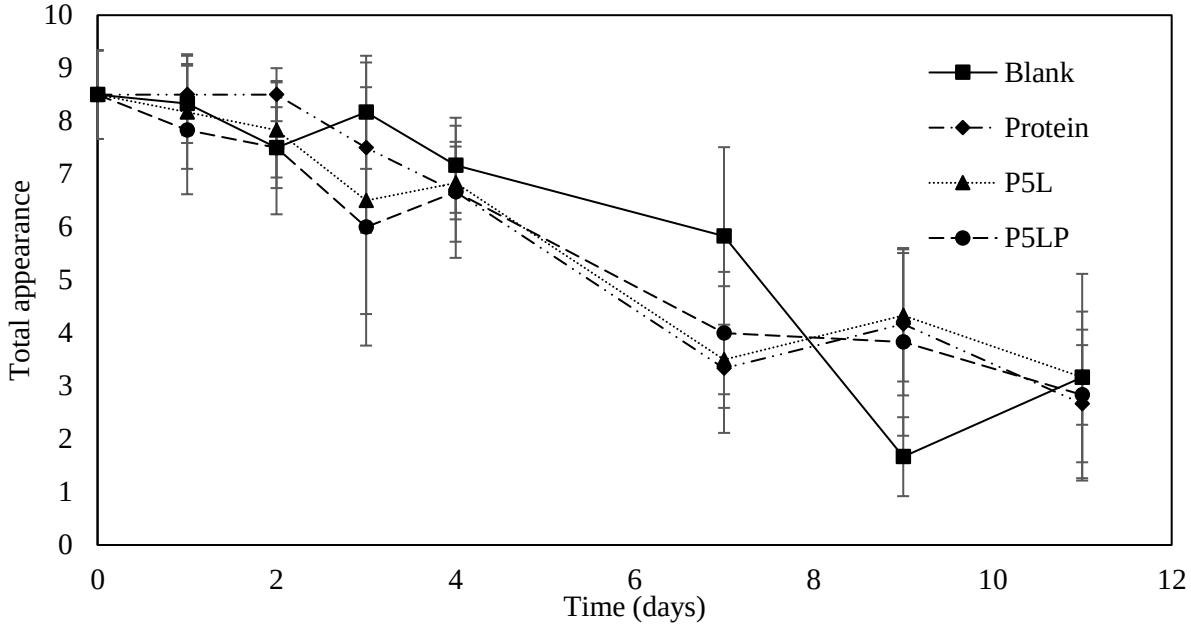


Figure 26. Sensory analysis of the total appearance in salmon fillets packaged with different packaging films and stored under 4℃.

Total appearance of the salmon fillets was the same during the first 7 days for all the samples without significant differences. At day 7, the sample Blank performed better score than the other samples but only for this particular day.

Table 13. Scores of total appearance sensory analysis for the different packaging films of salmon fillets stored under 4℃.

	Blank	**Protein**	**P5L**	**P5LP**
Day 1	8.3^{a}	8.5^{a}	8.2^{a}	7.8^{a}
Day 2	7.5^{b}	8.5^{b}	7.8^{b}	7.5^{b}
Day 3	8.2^{c}	7.5^{c}	6.5^{c}	6.0^{c}
Day 4	7.2^{d}	6.7^{d}	6.8^{d}	6.7^{d}
Day 7	5.8^{e}	3.3^{f}	3.5^{f}	4.0^{f}
Day 9	1.7^{g}	4.2^{h}	4.3^{h}	3.8^{h}
Day 11	3.2^{i}	2.7^{i}	3.2^{i}	2.8^{i}

Statistically significant differences are indicated with different letters a–i ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

As results from the sensory analysis of the samples, not big differences occurred between the different samples. Even though the samples from microbiological perspective

reached the limit the first days of the shelf life evaluation, the sensory analysis showed that all samples are accepted from the panel until day 7 without significant differences between them. The alternative packaging films could be used as packaging film without shortening the shelf life of the product.

5. Conclusions

In this study, was demonstrated the effective utilization of waste generated from the brewing industry to produce biobased and biodegradable food packaging by using all the fractions derived from the process.

More specifically,

- The brewer's spent grain (BSG) emerged as a valuable resource, characterized by its high protein content (20.5%) and significant cellulose (16.6%), hemicellulose (21.8%), and lignin (9.6%) content.
- The enzymatic hydrolysis after non-thermal plasma treatment resulted in a hydrolysate with a sugar concentration of 34.3 g/L, with glucose being the predominant sugar (74.3%).
- The produced BSG hydrolysate was employed as a nutrient source for the production of bacterial cellulose (BC) using *Komagataeibacter sucrofermentans* DSM 15973, achieving a production of 3.2 g/L BC, with a yield and productivity of 0.08g/g and 0.21 g/L/day, respectively.
- The most promising method of protein extraction in order to be formulated protein-based films was alkaline protein extraction (pH 10.0 at 60°C, precipitation at pH 3.4), yielding a protein concentrate with a purity of 52.8%.
- Lignin extraction from BSG yielded 148g of lignin, further enhancing the sustainability of the process.
- The produced protein-based films exhibited excellent light barrier properties (less than 2.3% T_{600nm}) and demonstrated antioxidant capabilities (40% DPPH inhibition).
- The shelf life of salmon fillets was estimated to be two days for all tested films, whereas sensory analysis revealed no significant differences between the film in the overall appearance of the films until day 7.
- The biodegradable films produced from this waste-derived material exhibited promising characteristics for preserving the shelf life of salmon.

In conclusion, this research underscores the potential of repurposing waste from the brewing industry to create environmentally friendly food packaging materials and valuable by-products. These findings contribute to the sustainable utilization of waste resources while

addressing important aspects of food preservation and packaging, aligning with the growing demands for eco-friendly and biodegradable alternatives in the food industry.

6. Future work

For future investigations, this study opens the door to several promising avenues of research. Firstly, an exploration into the films water vapor permeability and oxygen permeability properties is essential, as it could uncover additional opportunities for enhancing its performance and applications. Additionally, investigating the films antimicrobial properties in depth is crucial, potentially leading to the development of novel antimicrobial applications. Furthermore, utilizing FTIR analysis can provide valuable insights into the films molecular structure and bonding, aiding in its refinement. To optimize the enzymatic hydrolysis process, future studies could delve into the modification of enzymatic conditions to maximize efficiency and yield. Lastly, conducting repetition of shelf life tests could offer a comprehensive understanding of the effect of the films on the salmon fillets. These directions for future research promise to extend the practical utility and scientific understanding of our findings.

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