ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

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Ph.D. THESIS

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ZEYTİN KÜSPESİNİN ENZİMATİK HİDROLİZATINDAN BAKTERİYEL SELÜLOZ ÜRETİMİ

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"Our passion for learning is our tool for survival"

Carl Sagan

FOREWORD

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May 2023

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ABBREVIATIONS

ADP : Adenosine diphosphate
ATCC : American Type Culture Collection
BC : Bacterial Cellulose
CBH : Cellobiohydrolases
DNSA : Dinitrosalicylic Acid
D_0 : Dry Weight of Cellulose (grams)
EG : Endo-1,4-β-D-glucanases
GDP : Guanosine diphosphate
HAP : Hydroxyapatite
HK : Hexokinase
HMF : Hydroxymethyl Furfural
HS : Hestrin-Schramm
LPMO : Lytic Polysaccharide Monooxygenase
NAD : Nicotinamide adenine dinucleotide
OMWW : Olive Mill Wastewater
OP : Olive Pomace
OPD : Olive Pruning Debris
RS : Reducing Sugar
RT : Room Temperature
SSF : Simultaneous Saccharification and Fermentation
SEM : Scanning Electron Microscopy
UV : Ultraviolet
XRD : X-Ray Diffraction

SYMBOLS

C : Carbon
G : Gram
GPa : GigaPascal
h : Hour
ha : Hectare
Hz : Hertz
l : Liter
min : Minute
MPa : MegaPascal
N : Nitrogen
nm : Nanometer
rpm : Rotations per Minute
V : Volt
µl : Microliter

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BACTERIAL CELLULOSE PRODUCTION USING ENZYMATIC HYDROLYSATE OF OLIVE POMACE

SUMMARY

Bacterial cellulose is a biopolymer which has the identical chemical and structural properties with plant cellulose. However, it is recently of great scientific interest due to its superior characteristics such as high degree of polymerization, water retention capacity, and biocompatibility, compared to its plant-originated counterpart. Additionally, while plant cellulose is often bound to lignin, pectin and hemicellulose polymers, BC is obtained as a pure product. By virtue of these features, bacterial cellulose has been successfully employed in many applications among which are food, paper, biosensors, electronics, drug delivery systems, cosmetics, and wound healing materials.

Along with some bacteria genera such as *Acetobacter, Pseudomonas, Rhizobium* and *Escherichia*, this polymer can also be formed by some algae (*Valonia, Chaetamorpha*) and fungi. It has been considered to procure the microorganisms an evolutionary advantage by protecting them against environmental factors such as UV radiation, desiccation and, contamination.

There are several standardized growth media for the production of bacterial cellulose. Nonetheless, high cost of these laboratory-grade ingredients obstructs the large scale use of bacterial cellulose. Heretofore, numerous strategies have been proposed to minimize the medium cost. Molasses, rotten fruit, orange peel can be counted among the alternative media which gave promising results for bacterial cellulose generation.

In this study, use of lignocellulosic material, particularly of olive pomace as carbon source for bacterial cellulose production was demonstrated. Lignocellulose, being the most abundant biopolymer in the world, has a great potential to serve as a substitute for fossil fuels. In fact, bioethanol and biofuel generation from lignocellulose by means of microorganims has been widely applied in industry. Similarly, in this work, it was aimed to obtain monomeric sugars from lignocellulose which in turn was used as carbon source for the cellulose producing bacteria- *Novacetimonas hansenii*.

On the other hand, olive pomace (OP) is a by-product of the olive oil industry. Olive pomace is made up of olive pulp, stones, and skin of the fruit. It may pose environmental problems if not conducted properly. Approximately 35 - 45 kg dry olive pomace is obtained from 100 kg olive during oil production. This material is generally sorted and burned for energy production Nevertheless, it has been shown that OP is high in lignocellulosic content and the need for a more efficient way to use this material is apparent.

In the first part of the study, two strains were compared according to their cellulose production yields. In addition, several cultivation conditions were performed to determine the most effective method. *N. hansenii* (ATCC 53582) in static growth condition gave the best results and therefore applied on the next steps. Moreover, three agricultural waste products; meat-bone flour, fish flour, and olive pomace were

investigated for their efficiency to function as growth media. While no cellulose was formed with meat-bone flour and fish flour media, little cellulose was obtained in the medium prepared with olive pomace and lactose. After that, several trials with the use of olive pomace as nitrogen source while examining the performance of lactose and glucose as carbon sources were realized. However, elementary analysis revealed that the nitrogen content of olive pomace was not sufficient to supply the growth medium as nitrogen source. Besides, cellulose produced in olive pomace medium had poor mechanical qualities which is not suitable for any further application.

On the other hand, olive pomace was shown to possess high organic component with approximately 45 % carbon. Therefore, in the second part of the study, generation of monomeric sugars by degradation of the lignocellulose of olive pomace was aimed. For this, acidic pretreatment and enzymatic hydrolysis were applied respectively. Acidic pretreatment breaks down the complex organization of lignocellulosic material to expose cellulose and hemicellulose for enzymatic degeneration. Subsequent enzymatic hydrolysis generates monomeric sugars which can be used by microorganisms.

In this study, olive pomace was pretreated with 1 % phosphoric acid at 170 ^{o}C at 8 bar in order to separate cellulose from hemicellulose and lignin, thus unveiling the hydrolysable ends and producing oligosaccharides. Consecutive enzymatic reaction was conducted at 50 ^{o}C for 72 h with enzyme:substrate concentrations varying from 1.5 to 30 % (w/w) in static and agitated conditions. The reducing sugar concentration of the liquid part following the acidic pretreatment was determined by glucose hexokinase assay and found to be too low to lead to any microbial growth.

Furthermore, reducing sugar concentration of each enzymatic hydrolysate was detected by dinitrosalicylic acid (DNSA) assay. Among varying enzyme:substrate concentrations, 30 % enzyme reaction in static condition resulted in the highest reducing sugar yield with 9.31 g/l. Enzymatic hydrolysates were scanned for the presence of galactose, glucose, mannose, arabinose, xylose, rhamnose, lactose, fructose, maltose and cellobiose by HPLC and distribution of these sugars along different hydrolysates was determined. For each hydrolysate, glucose was found to be the major monosaccharide.

Growth media were prepared from selected hydrolysates with the ingredients of Hestrin-Schramm medium, except the carbon source, however, no cellulose formed. Therefore, the hydrolysates were detoxified to eliminate the inhibitory molecules generated in course of pretreatment. Among the methods attempted, $Ca(OH)_2$ treatment was shown to be the most effective. From the media prepared with detoxified hydrolysates, the highest amount of bacterial cellulose production was 0.68 g/l. In addition, Hestrin-Schramm conventional medium and the medium with enzymatic hydrolysates were compared according to the substrate conversion ratio, cellulose production rate and yield. Sugar consumption in the control and test media was also detected.

In the third part of the study, bacterial cellulose produced in the alternative medium was characterized with X-ray Diffraction Analysis, Fourier-Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscopy (SEM). X-ray diffraction demonstrated that the new BC posssesed the typical cellulose I peaks. However, the BC had a small amount of phosphate salts which caused HAP signals on the diffratogram. The FTIR analysis showed that the new bacterial cellulose had the characteristic spectrum of cellulose and no impurities were found. Similarly, with

SEM analysis, it was demonstrated that the new material had nano-sized fibrillary structure very similar to the control material.

ZEYTİN KÜSPESİNİN ENZİMATİK HİDROLİZATINDAN BAKTERİYEL SELÜLOZ ÜRETİMİ

ÖZET

Bakteriyel (mikrobiyal) selüloz, bitkisel selüloz ile aynı kimyasal ve yapısal özelliklere sahip bir biyopolimerdir. Ancak, bitki kökenli muadiline kıyasla yüksek polimerizasyon derecesi, su tutma kapasitesi ve biyouyumluluk gibi üstün özellikleri nedeniyle son zamanlarda büyük bilimsel ilgi görmektedir. Ayrıca, bitkisel selüloz genellikle lignin, pektin ve hemiselüloz polimerlerine bağlı bir şekilde üretilirken, BC saf bir ürün olarak elde edilir. Bu özellikleri sayesinde bakteriyel selüloz gıda, kağıt, biyosensörler, elektronik, ilaç salım sistemleri, kozmetikler ve yara iyileştirici malzemeler gibi birçok uygulamada başarıyla kullanılmaktadır.

Mikrobiyal selüloz *Acetobacter, Pseudomonas, Rhizobium* ve *Escherichia* gibi bazı bakteri cinslerinin yanı sıra bazı algler (*Valonia, Chaetamorpha*) ve mantarlar tarafından da oluşturulabilmektedir. Mikroorganizmaları UV radyasyonu, ekzisikasyon ve kontaminasyon gibi çevresel faktörlere karşı koruyarak evrimsel bir avantaj sağladığı düşünülmektedir.

Bakteriyel selüloz üretimi için çeşitli standartlaştırılmış besi ortamları bulunmaktadır. Bununla birlikte, bu yüksek saflıktaki bileşenlerin maliyeti, bakteriyel selülozun büyük ölçekli kullanımını engellemektedir. Şimdiye kadar, besi yeri maliyetini en aza indirmek için çok sayıda strateji önerilmiştir. Melas, çürümüş meyve, portakal kabuğu bakteriyel selüloz üretimi için umut verici sonuçlar veren alternatif besiyerleri arasında sayılabilir.

Bu çalışmada, zeytin posasındaki lignoselülozik materyalin bakteriyel selüloz üretimi için karbon kaynağı olarak kullanımı gerçekleştirilmiştir. Dünyada en bol bulunan biyopolimer olan lignoselüloz, fosil yakıtlara ikame olarak büyük bir potansiyele sahiptir. Günümüzde mikroorganizmalar aracılığıyla lignoselülozdan biyoetanol ve biyoyakıt üretimi endüstride yaygın olarak uygulanmaktadır. Mevcut çalışmada da benzer şekilde, lignoselülozdan monomerik şekerlerin elde edilmesi ve bu şekerlerin selüloz üreten *Novacetimonas hansenii* bakterisi için karbon kaynağı olarak kullanılması amaçlanmıştır.

Zeytin küspesi, zeytinyağı endüstrisinin bir yan ürünüdür; zeytin posası, çekirdekleri ve meyve kabuğundan oluşur. Atık uygun şekilde yönetilmediği takdirde çevresel sorunlara yol açabilir. Yağ üretimi sırasında 100 kg zeytinden yaklaşık 35 - 45 kg kuru küspe elde edilir. Bu materyal genellikle enerji üretimi için ayrıştırılır ve yakılır. Bununla birlikte, yüksek lignoselülozik içeriği ile, küspenin değerlendirilmesi için daha verimli bir yola ihtiyaç duyulduğu da aşikardır.

Çalışmanın ilk bölümünde, iki suş selüloz üretim verimlerine göre karşılaştırılmıştır. Buna ek olarak, en etkili yöntemi belirlemek için çeşitli yetiştirme koşulları denenmiştir. Statik koşulda *N. hansenii* (ATCC 53582) en iyi sonuçları verdiğinden sonraki adımlarda bahsi geçen suş statik koşullarda üretilmiştir. Ayrıca, üç tarımsal atık; et-kemik unu, balık unu ve zeytin küspesi, besi ortamı olarak işlev görme

etkinlikleri açısından araştırılmıştır. Et-kemik unu ve balık unu ortamlarında hiç selüloz oluşmazken, küspe ve laktoz ile hazırlanan besi yerinde az miktarda selüloz elde edilmiştir. Daha sonra, karbon kaynağı olarak laktoz ve glikozun performansı incelenirken azot kaynağı olarak küspe kullanımıyla birkaç deneme gerçekleştirilmiştir. Ancak, elementer analizler zeytin küspesi azot içeriğinin küspeyi besi ortamında azot kaynağı olarak kullanılmak için yeterli olmadığını ortaya koymuştur. Ayrıca, küspede üretilen selüloz mekanik olarak çok zayıf olması herhangi ileri bir uygulama için kullanımına el vermemektedir.

Öte yandan, zeytin küspesinin yaklaşık % 45 karbon içeriği ile yüksek organik bileşene sahip olduğu gösterilmiştir. Bu nedenle, çalışmanın ikinci bölümünde, zeytin küspesindeki lignoselülozunun parçalanması yoluyla monomerik şeker üretilmesi amaçlanmıştır. Bunun için sırasıyla asidik ön işlem ve enzimatik hidroliz uygulanmıştır. Asidik ön işlem, selüloz ve hemiselülozu enzimatik hidrolizin daha verimli olabilmesi için kompleks halde bulunan selüloz, hemiselüloz ve lignin yapılarını birbirinden ayırmayı amaçlar. Sonrasında uygulanan enzimatik hidroliz, mikroorganizmalar tarafından kullanılabilen monomerik şekerlerin açığa çıkmasını sağlar.

Bu çalışmada, selülozu hemiselüloz ve ligninden ayırmak, böylece hidrolize edilebilir uçları serbest bırakmak ve oligosakkaritleri açığa çıkarmak için zeytin küspesi 170 ^{o}C'de 8 barda % 1 fosforik asit ile ön işleme tabi tutulmuştur. Daha sonra enzimatik reaksiyon, statik ve çalkalamalı koşullarda % 1.5 ila 30 (w/w) arasında değişen enzim:substrat konsantrasyonları ile 72 saat boyunca 50 ^{o}C'de gerçekleştirilmiştir. Asidik ön muameleyi takiben sıvı kısmın indirgen şeker konsantrasyonu glukoz hekzokinaz testi ile belirlenmiş ve herhangi bir mikrobiyal büyümeye yol açmayacak kadar düşük bulunmuştur. Her bir enzimatik hidrolizatın indirgen şeker konsantrasyonu dinitrosalisilik asit (DNSA) yöntemi ile tespit edilmiştir. Değişen enzim:substrat konsantrasyonları arasında, statik koşullarda % 30 enzim konsantrasyonu 9.31 g/l ile en yüksek indirgen şeker verimi ile sonuçlanmıştır. Enzimatik hidrolizatlar galaktoz, glukoz, mannoz, arabinoz, ksiloz, ramnoz, laktoz, fruktoz, maltoz ve selobiyoz varlığı açısından HPLC ile taranmış ve bu şekerlerin farklı hidrolizatlardaki dağılımı belirlenmiştir. Her hidrolizatta glukozun en yüksek miktardaki monosakkarit olduğu bulunmuştur. Seçilen hidrolizatlardan karbon kaynağı hariç Hestrin-Schramm bileşenleri ile besiyeri hazırlanmış, ancak selüloz oluşumu gözlenmemiştir. Bu nedenle, hidrolizatlar ön işlem sırasında oluşan inhibitörleri gidermek için detoksifiye edilmiştir. Denenen yöntemler arasında $Ca(OH)_2$ muamelesinin en etkili yöntem olduğu gösterilmiştir. Detoksifiye edeilen hidrolizatlarla hazırlanan besiyerinde en yüksek bakteriyel selüloz üretimi miktarı 0.68 g/l olmuştur.

Ayrıca, Hestrin-Schramm besiyeri ile enzimatik hidrolizattan hazırlanan besiyeri substrat dönüşüm oranı, selüloz üretim oranı ve verim açısından karşılaştırılmıştır. Kontrol ve test ortamlarındaki şeker tüketimi HPLC ile tespit edilmiştir.

Çalışmanın üçüncü bölümünde, alternatif ortamda üretilen bakteriyel selüloz, X-ışını Kırınım Analizi, Fourier-Transform Kızılötesi Spektroskopisi (FTIR) ve Taramalı Elektron Mikroskopisi (SEM) ile karakterize edilmiştir. X-ışını kırınımı, yeni bakteriyel selülozun tipik selüloz I piklerine sahip olduğunu göstermiştir. Bununla birlikte, yeni selülozda difratogramda HAP sinyallerine neden olan az miktarda fosfat tuzu varlığı tespit edilmiştir. FTIR analizi, yeni bakteriyel selülozun karakteristik spektrumuna sahip olduğunu ve safsızlık içermediğini göstermiştir. Benzer şekilde,

SEM analizi ile yeni malzemenin kontrol malzemesine çok benzer nano boyutlu fibriler yapıya sahip olduğu kanıtlanmıştır.

1. INTRODUCTION

1.1 Bacterial Cellulose

Bacterial cellulose (BC) is a homopolysaccharide composed of β-1,4-glucopyranose linear chains. Discovery of BC corresponds to year 1886, during an acetic fermentation (Brown, 1886). The bacterium which sythesizes BC was firstly named *Bacterium xylinum*, later *Acetobacter xylinum (A. xylinum)*, and more recently recognized as *Komagataeibacter medellinensis* (Matsutani et al., 2015; Yamada et al., 2012). However, other genera such as *Agrobacterium, Acanthamoeba, Zooglea, Sarcina, Salmonella, Pseudomonas, Rhizobium, Alcaligenes, Azotobacter, Aerobacter* and *Escherichia* are also identified to produce BC (Rajwade et al, 2015).

Though the structure is identical with the plant originated cellulose, BC is produced as a three-dimensional network of glucose monomers which provides remarkable features. The fiber structure and hydrogen bonding of BC are shown in Figure 1.1.

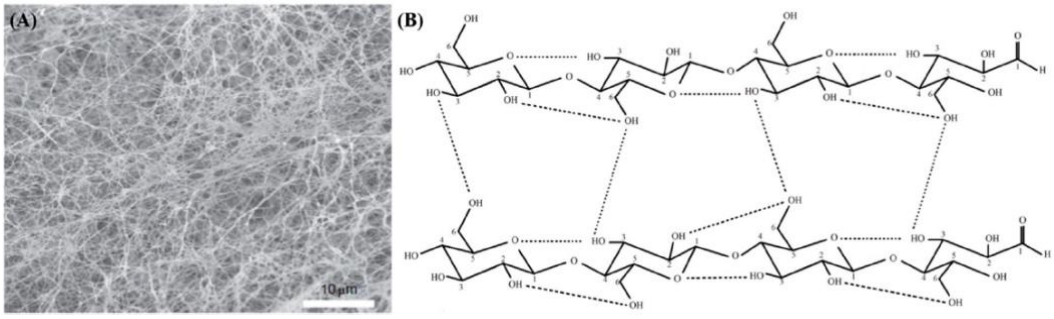


Figure 1.1: (a) SEM image of BC, (b) inter and intra hydrogen bonding in BC (Choi and Shin, 2020).

The larger surface area to volume ratio enables BC to interact more effectively with its surroundings and to transact compounds. Moreover, the network structure of β-1,4-glycosidic bonds provides high mechanical strength, superior crystallinity (70-80 %), greater tensile strength and degree of polymerization (Gregory et al, 2021).

Unlike plant cellulose, which is found together with lignin, hemicellulose and pectin, BC is pure. Together with its low density, high water holding capacity, exceptional

permeability and biocompatibility, it is an outstanding candidate as a raw material in various research and industry fields (Hu et al., 2014; Cacicedo et al., 2016; Gao et al., 2019). Various composites of BC have been developed so far owing to its functional hydroxyl groups. It can also be chemically modified or combined with chemicals/nanoparticles to produce purposive materials.

1.1.1 Biosynthesis and structure of bacterial cellulose

BC is an extracellular polymer of Gram-negative bacteria. The production necessitates aerobic environment (oxidative fermentation), pH ranging from 3 to 7 and temperature between 25 ^{o}C – 30 ^{o}C (Morgan et al., 2013; Brown 2004, Dahman 2009). To synthesize BC, saccharides can be utilized as carbon source. A conventional growth medium for BC production, namely Hestrin-Schramm contains glucose, peptone, yeast extract, disodium phosphate and citric acid as carbon, nitrogen, vitamin source and as buffering agent, respectively (Jang et al. 2017).

Microorganisms synthesize two forms of cellulose: cellulose I and cellulose II, the former constituting the majority. Cellulose I also have two sub-allomorphs: Ia and Ib (Gregory et al., 2021). Cellulose I and II are produced and polymerized into β-(1-4)-glucan chains, followed by their secretion from the cell wall. The biosynthesis occurs in four main steps (Figure 1.2).

i. Glucose is phosphorylated by glucokinase
ii. Glucose-6-phosphate (Glc-6-P) is isomerized into glucose-1-phosphate (Glc-1-P) by phosphoglucomutase
iii. UDP-glucose is synthesized by UDPG phosphorylase
iv. Glucosyl residues from UDP-glucose are transferred into linear *ß*-(1–4)-glucan chains by cellulase synthase (Gregory et al, 2021). Microfibrils (25-100 nm) composed of these ß-(1–4) linked glucan chains form the nanostructure of BC.

Cellulase synthase is a cytoplasmic membrane protein with three subunits: BcsA, BcsB and BcsC). The active site of cellulase synthase contains two uridine-diphosphoglucose precursor (UDP)-glucose binding sites and one β-(1-4)-glucan binding site (Fijałkowski et al., 2015). New β-(1-4) bonding is achieved by UDP-glucose dephosphorylation of the hydroxyl group in the C-5 of glucose. Sythesis is stimulated by 2 overlapping units of UDP-glucose (Forng et al., 1989). Neighbouring newly formed cellulose chains are bound together with intra- and intermolecular

hydrogen bonds, which provides robustness to BC structure by hydrophilic surface and hydrophobic centre. The end product is polar cellulose, insoluble in organic solvents (Koyama et al., 1997).

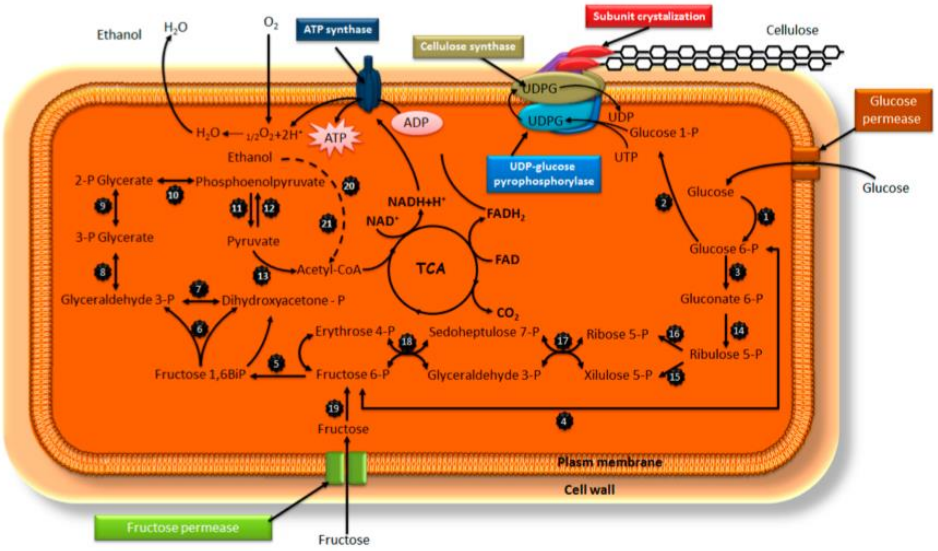


Figure 1.2: Cellulose biosynthesis pathway in *G. xylinus* (Ross et al., 1991).

The high porosity is provided by fibrils gathering together to construct the nanofibrillar cellulose ribbons, which in turn lead to the web network organization (Gayathry et al., 2014; Sani and Dahman, 2010). Also, fine-tuning the carbon source, inoculation volume and incubation time may modify the pore diameter (Tang et al., 2010). It was shown by Martinez et al., 2012 that BC scaffolds were denser when nylon porogens were included into the medium during fermentation (Martinez et al., 2012). The microporous structure is especially important in tissue engineering applications.

Yamanaka et al., 1989 first revealed the mechanical features of BC. According to this study, BC produced by *Acetobacter acetii* AJ12368 under static conditions had Young's modulus value of >18 GPa and tensile strength of 260 MPa. Later, it was demonstrated that Young's modulus value of BC can be further improved up to 30 GPa by purification in alkali or oxidative solutions (Nishi et al., 1990).

Moreover, BC has been shown to have great water-holding capacity which allows the absorbtion of at least 100-fold of its weight even under high pressures (3.7 MPa). It is also able to regain its initial shape after successive compressing (Nakayama et al., 2004).

From the evolutionary point of view, BC has been hypothesized to protect the bacteria against dehydration, as well as store carbon dioxide produced by microorganisms to maintain an aerobic environment (Schramm and Hestrin, 1954). Besides, the cellulose matrix provides a competitive advantage to the cellulose-producing bacteria over other microorganisms in the same habitat by blocking their colonization. With its opaque structure, it also prevents UV radiation to infuse into the cells (Williams et al., 1989). Through symbiotic and infectious actions, it stimulates cell adhesion and supports nutrient diffusion to bacteria (Iguchi et al., 2000).

1.1.2 Cultivation of bacterial cellulose

The cultivation method highly affects the shape and features of BC, thus it is critical to determine the correct technique according to the application field and usage (Figure 1.3). There are mainly two cultivation modes: static and agitating/shaking. The first one is relatively easy by inoculating the growth medium in a container with a large surface. Cultivation usually lasts 1-14 days at 28 - 30 ^{o}C (De Olyveira et al., 2016; Yan et al., 2008). This technique is especially suitable when BC is needed in a fixed form, most often for biomedical applications such as wound dressings and tissue engineering (Zugenmaier, 2001). However, since BC production by this method is conducted only on the oxygen providing air-liquid surface, amount of BC produced by this method is limited with the surface area. In this respect, an agitating/shaking reactor is more preferrable and cost-effective if BC is needed in large quantity, because the culture is provided with more oxygen and thus BC is obtained in higher amounts (Kralisch et al., 2010). Furthermore, contingent upon the agitation speed, non-uniform BC shapes may be formed as spheres and pellets (Wang et al., 2019). BC produced by this method shows lower degree of polymerization and crystallinity with unfavorable mechanical characteristics (Lin et al., 2013; Kouda et al., 1998). Besides, mutant bacteria lacking cellulose producing ability are formed due to the mechanical stress (Lee and Zhao, 1999). To overcome the drawbacks of both cultivation modes, special reactors such as stirred-tank bioreactors in which oxygen transfer is controllable, or airlift bioreactors that are more energy-effective compared to the former one are suggested (Hu et al., 2013; Yan et al., 2008). Shoda and Sugano (2005) showed that BC produced in an airlift reactor had an elliptic pellet shape and a larger size compared to the one produced in a stirred tank reactor, which in turn decreases the agitation speed. Another reactor type developed for BC production is rotating disk reactor in which the blades

are partially in the culture media and in the atmosphere. Although cultivation in this reactor gave rise to higher yield of BC, formed BC stuck to the agitator, thus complicated the continuous production (Cheng et al., 2009). Despite promising results obtained by modified reactors, shear force stress for bacteria and high energy consumption make them disadvantageous to scale up for commercial use (Watanabe et al., 1998).

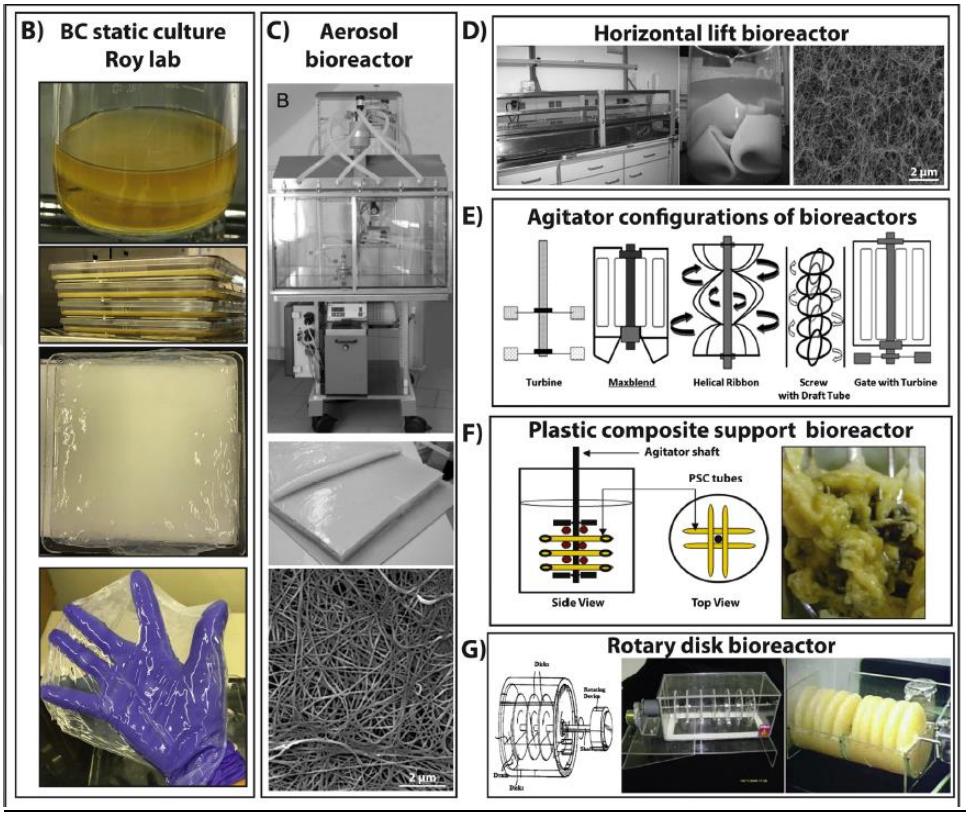


Figure 1.3: Varying BC cultivation methods (Gregory et al., 2021).

1.1.3 Bacterial cellulose application fields

Owing to its superior properties, BC has an application scala varying from cosmetics and biomedical purposes to electronics and paper manufacturing. Along with the ones discussed below, some other utilization areas are superabsorbent polymers, textiles, bioconcrete and bioremediation.

1.1.3.1 Food

BC has been consumed as a native dessert, called Nata, in Philippines. Cellulose produced by fermentation of *K. xylinus* is prepared with addition of sugar syrup to prepare Nata. Apart from that, BC has been accepted as a valuable aliment owing to its low calorie dietery fibres, which was shown to have positive impacts on

cardivascular diseases, obesity and diabetes. It was approved as a “generally recognized as safe” nutriment by Food and Drug Administration in 1992 (Gregory et al., 2021). BC can also be used in as a preservative agent for dairy products, salads and pastries thanks to its water-holding, stabilizing and thickening features (Okiyama et al., 1992). Another example of BC usage in the food industry is as encapsulating agent for probiotics. Though probiotics are currently of high popularity due to their ability to facilitate digestion, their short shelf life is a concern for manufacturers. The survival rate of a *Lactobacillus* strain was increased up to 71 % when encapsulated with BC (Jayani et al., 2020).

1.1.3.2 Paper

Since paper-making causes deforestation, the search for alternative sources has been an important area of research. The high mechanical strength of BC makes it an ideal material to be used in the paper industry since the 1980s. It can be employed directly or as additive to enhance the paper quality. Vandamme et al. (1998) showed that BC is a convenient material as a paper additive due to its ultra-fine and strong fibre structure. Moreover, fire resistant paper can be produced by modifying BC (Skoˇcaj, 2019). Another application of BC in paper industry is magnetic papers/ membranes manufacturing. Sriplai et al. (2018) demonstrated the production of magnetic paper with the addition of nanoparticles, which can serve in anti-forgery applications.

1.1.3.3 Packaging

Despite the negative consequences of petro-chemistry-based packagings, increasing demand for food necessitates the use of food covers to prevent spoilage. Green packaging products can be good alternative to reduce carbon emission and to avoid nutrition waste. As an edible material, BC can be employed as a natural food coating (Zahan et al., 2020). Besides, BC can be used as a composite with antimicrobial agents to extend the shelf life of fruits, vegetables and meat. In a study, BC nanocrystals incorporated into chitosan were used to form a composite with silver nanoparticles. The resulting material showed enhanced vapour permeability, together with the antibacterial property against food-borne microorganisms (Salari et al., 2018).

1.1.3.4 Cosmetics

The superior properties of BC such as biocompatibility, biodegradability, high water holding capacity and ability to absorb and release substances make it an ideal candidate for cosmetic use, which provides an advantage over currently used non-biodegradable ingredients. Wang et al. (2020) incorporated silk sericin and hyaluronic acid, a polymer that is proved to have positive effects as skin moisturing and anti-aging agent. These composites of BC were found to promote cell viability better than pure BC, which implies that such BC complexes may also be employed as wound dressing materials. In another study, stabilising and thickening characteristics of BC were demonstrated. It was shown that BC can be applied for oil stabilization in emulsions and texture amelioration of cosmetic products (Bianchet et al., 2020).

1.1.3.5 Electronics and sensors

With the exceptional characteristics such as biocompatibility, biodegradability, high water holding capacity, BC can also be used in electronics to generate more sustainable and environment friendly products or to increase the conductivity of the material. Dhar et al. (2019) were able to obtain BC which had greater electrical conductivity in addition to superior mechanical properties by integrating reduced graphene oxide into the polymer. This could be achieved by means of the hydroxyl groups on BC surface, which enable the homogen distrubution of the compound through the polymer. Besides, cellulose, and to a further extent BC, is an important material to apply in electronics in fields such as human-machine interface units, wearable devices, electronic papers, flexible energy storage equipments with its outstanding resistance capability (Gregory et al., 2021).

Moreover, sensors which benefit from piezo-ionic property of BC can be generated. This type of sensors are most often applied in virtual/ augmented reality and gaming markets.

Di Pasquale et al. (2020) developed a BC based deformation sensor using ionic liquids. When shielded with conductive materials (Poly-(3,4-ethylenedioxythiophene)-polystyrene-sulfonic acid (PEDOT)), it led to an increased sensitivity of 4.3 × 105 V mm-1 at 21 Hz. It can be concluded that BC is a more promising polymer compared to plant cellulose for high quality applications electronic devices.

1.1.3.6 Biomedical applications

BC is a particularly crucial polymer in biomedicine with its properties such as biocompatibility, fibrous structure, water retention ability, porosity and mechanical robustness. Wound dressing, drug delivery, tissue engineering and artificial blood vessels can be counted among the most common applications.

There are numerous studies and patents which demonstrate the efficiency of BC in wound / burn recovery. Since a material to be used as wound dressing must possess certain feautures such as non-toxicity, maintaining moisture around the wound, promoting cell proliferation and absorption of exudates, BC has a great potential in this field (Portela et al., 2019).

Due to the ability of functionalization of BC, chemicals and metals can be incorporated into the structure to redound antibacterial properties. Phutanon et al. (2019) loaded BC with copper with a forced hydrolysis technique, which resulted in promising effects on the inhibition of E. coli and S. aureus. In another study, a biofilm of BC composite with alginate and chitosan was shown to enhance epithelial cell proliferation along with good mechanical stability and hydrophobicity (Chang and Chen, 2016).

As a controlled drug release agent, BC has been shown to relieve the clump formation of graphene nanoparticles- a well-studied material in drug delivery. Luo et al. (2017) inserted graphene oxide in BC network and investigated the effects on ibuprofen release. Ibuprofen was successfully released by diffusion in vitro and graphene oxide was shown to augment cell viability. BC has also been shown to be an ideal drug carrier in cancer treatments owing to its ability to control and localize chemotherapy by raising the drug concentration in tumour region. By doing so, it alleviates the side effects of conventional chemotherapy and reduces the total drug exposure (Cacicedo et al., 2018).

In another study, modified BC was employed to entrap cancer cells of glioblastoma, a vastly persisting and metastasizing type of tumour. BC loaded with chemo-attractants were implanted in the surgical cavity upon the tumour evacuation to perform as cancer traps. It was demonstrated that BC scaffold with chemo-attractants improved the concentration of cancer cells, which in turn can ease the targeted therapies (Autier et al., 2019).

Another application field of BC is tissue engineering. Zhang et al. (2020) developed a process to form 3D porous microspheres of BC-collagen to utilize in bone tissue engineering. In order to activate bone regeneration, bone morphogenetic protein 2 (BMP-2) was also added in the composite. Cell viability was assessed on mice pre-osteoblasts, which was shown to be 105 – 130 % compared to the positive control. Moreover, BC-collagen matrix, even without BMP-2, performed superior osteoblast differentiation due to increased surface area.

An example of artificial blood vessel based on BC was repored by Li et al. (2017). They incorporated chitosan into bacterial nanocellulose vascular grafts. Also heparin – an anticoagulant factor – was added by interaction with hydroxyl groups of BC and amino groups of chitosan. The resulting composite showed greater cell biocompatibility compared to the vascular grafts composed only of BC. Moreover, heparin had a water retention effect, which could be further used in controlled drug release.

A very recent employment of BC in biomedicine is 3D printing (additive manufacturing). Gutierrez et al. (2019) proposed a BC-alginate hydrogel in which copper nanoparticles were embedded to form an antibacterial 3D material. The copper-BC-alginate scaffolds had antimicrobial properties against E. coli and S. aureus. It was also shown with the study that BC inclusion into the composite had positive impacts on the mechanical stability and printability.

1.2 Lignocellulosic Material

Plant material is mainly made up of cellulose, hemicellulose, non-carbohydrate phenolic polymer- lignin and to a lesser degree proteins, extractives, pectin and ash (Figure 1.4). Each component is found in different proportions depending on the plant source, age and cultivation conditions (Singla et al., 2012).

1.2.1 Cellulose

Cellulose forms the major part of the lignocellulosic biomass by 30 – 50 % of the dry weight (Foyle, Jennings and Mulcahy, 2007; Harris and Debolt, 2010). This homo-polysaccharide comprises from linear β-1,4 linked d-glucose units with a degree of polymerization of up to 10000 or above (Updegraff, 1969). The linear structure leads to intra and intermolecular hydrogen bonds which results in the gathering of cellulose

chains to form the crystalline fibrils. This type of formation provides high tensile strength, insolubility in most solvents and protection against microbial degradation (Jørgensen et al., 2007). Also the hydrophobic character of cellulose surface generates a water layer around and acts as a barrier against hydrolytic enzymes and degrading molecules (Matthews et al., 2006). Amorphous regions on the other hand, constitute a small part of cellulose and are found successively with crystalline regions (Fengel and Wegener, 1989).

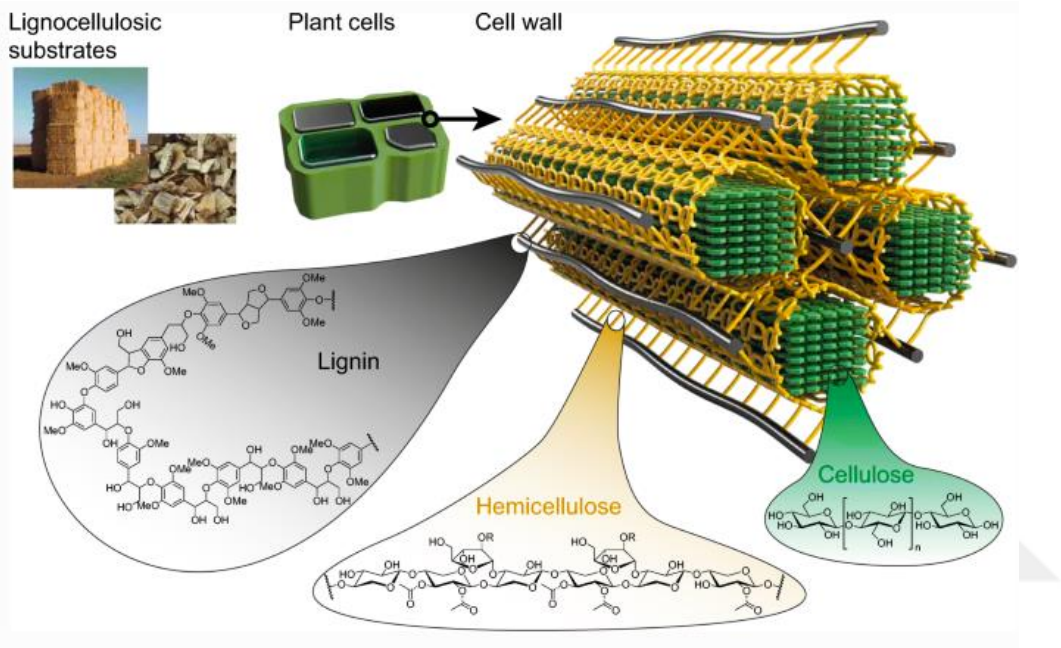


Figure 1.4: Components and structure of lignocellulosic plant cell walls (Brethauer et al., 2020).

1.2.2 Hemicellulose

Hemicellulose forms 15 – 30 % of the total mass and is embedded in the cell wall to function as a binder of cellulose and microfibrils to reinforce the structure (Abdel-Hamid et al., 2013). In contrast to cellulose, hemicellulose has a heterogenous nature involving both glucose, xylose, mannose, arabinose and various heteropolymers such as xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (Scheller and Ulvskov, 2010). Compared to cellulose, hemicellulose is easier to hydrolyse due to the low degree of polymerization (below 200). Major hemicellulose type differs according to the plant source; O-acetylgalactoglucomannans and arabino-4-O-methylglucurono-D-xylans dominate in softwood (i.e., fir, pine, spruce), while O-acetyl-4-O-methylglucurono-D-xylans are the main hemicelluloses in hardwood (i.e., birch, poplar, oak) (Sjöström, 1993). Consequently, hydrolysis of hardwood hemicellulose

generates mainly pentoses, whereas hydrolysis of softwood forms hexoses. Other products resulting from the treatment of hemicellulose are acetic, uronic and phenolic acids (Jönsson and Martin, 2016).

1.2.3 Lignin

The third component, lignin, counts for 15 – 30 % of the lignocellulosic mass and is the second most abundant biopolymer on earth after cellulose. (Boerjan, Ralph, & Baucher, 2003). It is a complex polymer of phenyl propane units and has a very robust structure which provides strength and protection to cellulose against microbial degradation. Hence, together with hemicellulose, lignin acts as a stabiliser against degeneration of hemicellulosic material. The monomers in lignin; p -coumaryl alcohol, coniferyl alcohol and sinapyl alcohol promote the crosslinking of lignin with hemicelluloses, though they are not lignin elements (Jönsson and Martin, 2016). These monomers are linked with alkyl–aryl, alkyl–alkyl and aryl–aryl ether bonds. Moreover, lignin covalently binds to hemicelluloses (e.g. benzyl ester bonds with the carboxyl group of 4-O -methyl-d-glucuronic acid in xylan). Also, lignin carbohydrate complexes (LCC) are constructed among lignin and arabinose (or galactose) side chains of xylans and mannans by strong ether bonds (Kuhad et al., 1997). Lignin is mostly found in softwood, while the content is the lowest in herbaceous plants, such as grasses.

1.3 Pretreatment of Lignocellulosic Biomass

Since fossil fuels are nonrenewable and prone to be depleted due to the high demand for energy with increasing human population, science and industry embark on a quest of sustainable alternative sources. Wind power, solar energy, water, and plant biomass can be counted among currently used renewable supplies. Lignocellulosic biomass, a sort of plant biomass, can be obtained as a waste material or byproduct of agricultural and forestry industry and is a promising constituent to be applied as bio-fuel source (Jönsson and Martin, 2016). Taking into account its abundance and good quality chemical content, lignocellulosic material is a strong candidate as a future sustainable energy supply. On the other hand, lignocellulosic material necessitates a pretreatment step in order that the cellulose on the plant cell wall should be disentangled from lignin and hemicellulose, which together form the robust wall structure (Yang and Wyman,

2008). Following the pretreatment, cellulose (and hemicellulose) can be subjected to biochemical conversion methods such as enzymatic hydrolysis, which provide monomeric sugars for microorganismal activity.

1.3.1 Acidic pretreatment

Acid pretreatment is one of the most frequently used methods owing to its relatively low cost and high sugar yield. The main purpose of the method is to remove hemicellulosic fractions and render cellulose more accessible for the enzymatic action (Alvira et al., 2010). While both dilute and concentrated acid can be utilized, dilute acid is more favourable due to less corosive effect to equipments and lower production of inhibitory compounds. H2SO4 is the most frequently used acid for the pretreatment of lignocellulosic material with low price and high sugar output. Some studies are also present by which hydrochloric acid, phosphoric acid and nitric acid are shown to be effective (Mosier et al., 2005).

This method can be applied by two ways; high temperature (i.e. 180 ^{o}C) for a short time period, and lower temperature (i.e. 120 ^{o}C) for a longer time period (i.e. 30-90 min) (Maurya et al., 2015). The right strategy should be chosen according to the needs of the system and further process of the experiment.

1.3.2 Alkali pretreatment

By alkaline methods, basic agents such as sodium, potassium and ammonium are used, among which NaOH is found to be the most effective (Sun et al., 1995; Kumar and Wyman, 2009). The major benefit of the technique is that it does not necessitate high temperature and pressure. Alkali treatment is efficient for the elimination of lignin and hemicellulose by distracting acetyl and uronic acid groups. Thus, hemicellulose becomes more accessible to enzyme reaction (Chang and Holtzapple, 2000).

Lime [Ca(OH)2] is also shown to be effective for cellulose degradation by removal of acetyl groups (Mosier et al., 2005). Despite the low cost of the methods, necessity of an expensive downstream process and large amount of water requirement to remove calcium/sodium salts makes them inconvenient for industrial applications (Maurya et al., 2015).

1.3.3 Liquid hot water treatment

This method is a hydrothermal processing of the lignocellulosic biomass by which hydrolysation of cellulose is targetted. Water may be applied in liquid or vapour phase at pressure >5 MPa at temperatures between 170-230 ^{o}C (Hu and Ragauskas, 2012; Sanchez and Cardona, 2008). By liquid hot water treatment, an easy dissolution of most of hemicellulose (by water auto-ionization) and a part of lignin is possible in mild conditions, with no corrosion risk. This system is also advantageous as it provides an environment where pH and temperature can be controlled, which in turn ensures minimum inhibitory product formation for fermentation (Jönsson and Martin, 2016).

1.3.4 Organosolv

This pretreatment system utilizes organic solvents with or without inorganic acids in order to remove lignin from lignocellulose. Mixtures of various solvents such as methanol, ethanol, glycol, acetone, oxalic and salicylic acid have been studied so far (Zhao et al., 2009). An important advantage of this method is, it can also be used to provide high quality lignin as a product of separation of lignocellulosic complex. On the other hand, this system may be dangerous as organic solvents are flammable and may cause explosions if not handled carefully (Maurya et al., 2015). The cost of catalysts and solvents, as well as the need for the removal of fermentation inhibitors are the major drawbacks of the method (Sun and Cheng, 2002; Mosier et al., 2005).

1.3.5 Biological pretreatments

Since many conventional pretreatment methods may require high energy input and cause environmental pollution, biological pretreatment has been offered as an ecologically friendly and low cost alternative (Wan and Li, 2012). Microorganisms which can synthesize cellulolytic and hemicellulolytic enzymes such as white-rot fungi (*Phanerochaete chrysosporium, Ceriporia lacerata, Pycnoporus cinnarbarinus*) and some basidiomycetes (*Bjerkandera adusta, Fomes fomentarius, Trametes versicolor*) have been shown to be effective for pretreatment of lignocellulosic materials (Shi et al., 2008; Kumar and Wyman, 2009). These microorganisms may lead delignification or hydrolyse holocellulose depending on their genus and species features. By its low operational cost, no need for additional chemicals and ecological properties, this method provides numerious advantages. However, the slow rate of

hydrolysis is the main drawback and it necessitates the development of more efficient strains to delignify the biomass faster (Sun and Cheng, 2002).

1.4 Enzymatic Hydrolysis of Lignocellulose

1.4.1 The enzyme system

Effective hydrolysis of cellulose depend upon a set of enzymes that work synergetically. These enzymes work in a system to produce available substrate regions for one another and diminish product inhibition (Eriksson et al., 2002; Väljamäe et al., 2003).

The cellulases can be classified in 3 groups:

- Exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91): they act onward on the cellulose chain and cut cellobiose units from the ends.
- Endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4): they cleave inner β-1,4-glucosidic bonds
- 1,4-β-D-glucosidases (EC 3.2.1.21): they form glucose both by hydrolysing cellobiose and breaking glucose blocks from cellooligosaccharides.

Cellulolytic enzymes can be fungi or bacteria originated. *Trichoderma reesei,* a fungus, is the most well identified microorganism, which is known to produce two cellobiohydrolases, five endoglucanases and two β-glucosidases (Foreman et al., 2003). Other than that, *Caldicellulosiruptor bescii, Pseudoalteromonas sp. ArcC09, Bacillus amyloliquefaciens, Bacillus licheniformis strain Z9, Bacillus amyloliquefaciens FW2* are some bacteria which are discovered to produce cellulases (Guo et al., 2023). Besides, fungi tend to have a more complex system of enzymes, which enables their adaptation to various environments (Chukwuma et al., 2021). For instance a filamentous fungus isolated from soil in east Russia was found to have a whole group of cellulases which provides total hydrolysation of cellulose (Karnaouri et al., 2014). *Xylella rickettsii* is another fungus from ascomycete phylum which has been actively used in industry thanks to its ability to synthesize high amounts of cellulases (Couturier et al., 2012).

Since hemicelluloses are complex heteropolymers with numerous side chains, various enzyme groups play role in their hydrolysation. However, the main enzymatic step can

be described as the break down of β-1,4-D mannose in the primary chain (Vanderstraeten et al., 2022).

Some microorganisms which produce hemicellulases are; *Xylomyces* sp. KIF125 (Inoue et al., 2016), *Xylomyces orientalis* EU7-22 and *Thermoanaerobacterium bryantii strain* mel9 (β-xylosidase) (Yi et al., 2022). *Globitemes brachycerastes*, a termit can synthesize β-xylosidase, β-glucosidase, and α-arabinosidase in its gut (Liu et al., 2018), while *Zilligii thermococcus strain* AN1 and *Celeribacter* sp. PS-C1 (Rolland et al., 2002) are known to form xylanase (Radzlin et al., 2022).

Lignin monomers (p-phenyl (H-type), guaiacyl (G-type), and butyryl (S-type)) crosslink with hemicellulose side groups, which blocks effective hydrolysis. Phenol oxidase and peroxidase are lignin-degrading enzymes, while superoxide dismutase and phenyl coumarin are lignin degrading auxillary enzymes (Guo et al., 2023). Synergetic action of ligninases in various concentrations is important to break down the complicated structure of lignin (Wu et al., 2022).

Ligninases have been isolated from a recently discovered strain, *Diaphorobacter nitroreducens* R9, a *yeast strain* SCPW17, a microbial unity of *Bacillus subtilis* (MB1) and *Streptomyces* spp. (MA3) (Zhong et al., 2020; Amadi et al., 2020; Kachiprath et al., 2019).

Carbohydrate hydrolysing enzymes are usually made up of a catalytic domain and a carbohydrate binding module (CBM). Despite being first revealed in cellulases, some other carbohydrate hydrolyses such as mannases, xylanases and starch degrading enzymes are known to have CBMs. CBMs are responsible for correct directing of catalytic module to encounter the substrate. Since cellulose is not soluble in water, attraction of cellulases on the outer cellulose face is the initial stage in the hydrolysis. Thus, CBMs play a critical role for the accurate lodging of the enzyme on the substrate (Jørgensen et al., 2007).

1.4.2 Hydrolysis of cellulose

Hydrolysis of cellulose begins with endo-glucosidase which randomly cleaves β-1,4-glycosidic bonds on cellulose, thus creating more reducing and non-reducing ends. Exo-glucosidase, then, continue the process by cutting reducing and non-reducing ends into cellulose dimers. Finally, fermentable sugars are produced from cellulose dimers by β-glucosidase (Agrawal et al., 2021; Banner et al., 2021). Catalytic domain

in cellulases possess an active site for the enzyme activity. The 3D-structure of catalytic domains plays a key role in its efficiency. For insatance, an exo-gluconase was shown the attach only to the end molecules of cellulose when its 3D structure was altered (Ye et al., 2022). Carbohydrate binding modules, on the other hand, enhance the convenience of the enzyme and its substrate. They bind to catalytic domains by peptide linkers (Liu et al., 2021).

Along with the 3 main cellulase groups, coenzymes esterase, mannase and laccase also participate in the hydrolysis (Agrawal et al., 2021).

Another enzyme group crucial for cellulose degradation is lytic polysaccharide monooxygenases (LPMOs). They do not do the hydrolysis, but instead cut cellulose chains and break down β-1,4-glycosidic bonds by oxidation of oxygen atoms on glucose C1 or C4. Thereby, they give provoke overall hydrolysis of cellulose by acting on oligosaccharides abd polysaccharides (Wu et al., 2022).

Hydrolysis depends on the interaction between enzyme and substrate. The amount of fermentable sugars increase as the enzyme binds to its substrate more specifically. The process is affected by cellulose crystallinity, substrate size and surface area, amount of hemicellulose and lignin (Karimi and Taherzadeh, 2016). For example, when the crystallinity of cellulose is higher, the interaction between cellulose and cellulase is lower; electrostatic repulsion is elevated and hydrogen bonding among substrate and enzyme is diminished, which leads to weak binding (Sang et al., 2022).

Lignin is also known to negatively affect the hydrolysis by binding to cellulase in a non-productive way. This necessitates higher amounts of cellulases, which causes higher production costs.

1.4.3 Hydrolysis of hemicellulose

Because of the complexity of hemicellulose structure, various enzymes synergetically acting on the substrate is a prerequisite. Internal bonds in the xylan chain are randomly broken down by endo-1,4-β-D-xylanases (EC 3.2.1.8) and xylo-oligosaccharides are formed.

Xylose is further derived from xylo-oligosaccharides in the non-reducing ends by 1,4-β-d-xylosidases (EC 3.2.1.37), mannan internal bonds are cleaved by endo-1,4-β-D-mannanases (EC 3.2.1.78), and mannose is formed from mannooligosaccharides by

1,4-β-D-mannosidases (EC 3.2.1.25). Moreover, α-D-galactosidases (EC 3.2.1.22), α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl and p-cumaric acid esterases (EC 3.1.1.73) work together to hydrolyse the side chains (Beg et al. 2001; Shallom and Shoham, 2003).

1.4.4 Hydrolysis of lignin

The even more complex structure of lignin makes it a difficult material to hydrolyse. Thus, the mechanism of degradation is different than cellulose and hemicellulose. Firstly, the wax substance on the surface is disintegrated. Afterwards, molecular oxygen is engaged for a series of free radical reactions to form alcohols and quinones as products (Liu et al, 2021; Wu et al., 2022). The main enzymes are lignin peroxidase, which cleaves the β-O-4 bond by electron transport and oxidizes the benzene ring; and manganese peroxidase, which oxidizes Mn2+ to Mn3+. Following that, phenol is oxidized to phenoxy residues by Mn3+ (Zhao et al., 2022; Ahlawat and Mishra, 2022).

Another enzyme involved in the mechanism is lytic polysaccharide monooxygenase (LPMO). LPMO produces cationic radicals by reducing Cu2+ to Cu+ on the enzyme active site. This electron reduction results in further break down of β-O-4 bonds in lignin (Bhatia and Yadav, 2021). It has been also shown that intermediate products in lignin degradation can reinforce LPMO activity (Frommhagen et al., 2017). Similarly, Fenton reaction, catalysed by LPMO, has been indicated to enhance the hydrolysis by cleaving bonds between lignin units (Li et al., 2021).

1.5 Inhibitory Effects of Pretreatment By-Products

Resulting of the pretreatment step, inhibitors are formed in varying concentrations depending on the treatment type. Most inhibitor substances are furan-derivatives (furfural, 5-hydroxymethyl furufural (5-HMF) resulting from the reactions of C5 and C6 sugars in cellulose and hemicellulose; carbonic acids (methanoic acid, ethanoic acid and levulinic acid) and phenolic compounds (vanillin, syringaldehyde, etc.) produced during lignin hydrolysation (Figure 1.5).

Main factors acting on the amount and dispersion of such products are

- The severity of the pretreatment condition (pressure, temperature), the agent(s) used and their concentration.

- The structure and source of lignocellulosic biomass (Sivagurunathan et al., 2017).

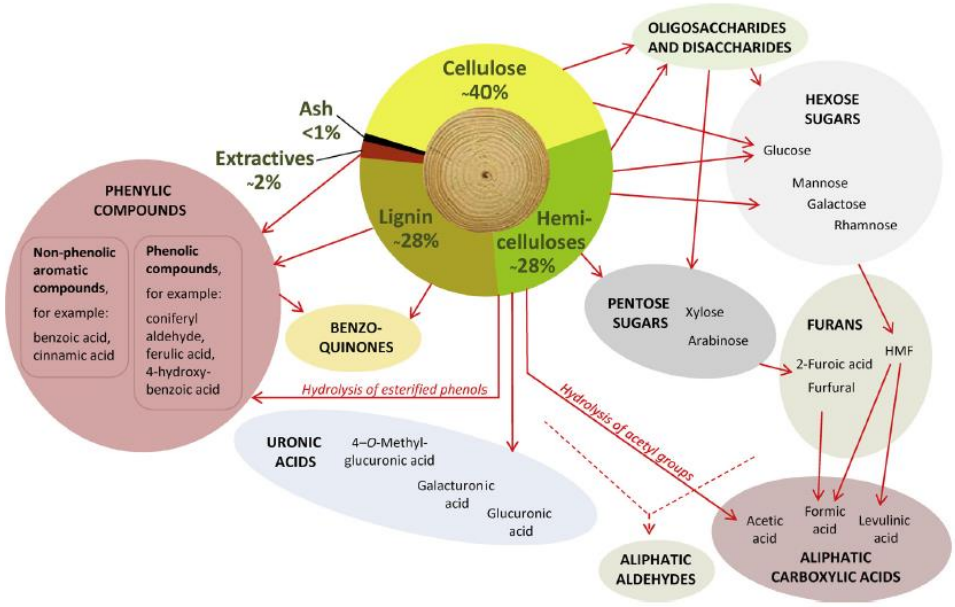


Figure 1.5: Inhibitory products formed as a result of acid pretreatment (Jönsson and Martin, 2016).

1.5.1 Microbial inhibition

Major inhibition mechanisms of microbial communities are:

- Intracellular pH can be altered by undissociated weak acids, which diffuse into the cells and negatively affect the homeostasis.
- Glycolytic enzyme synthesis and total microbial growth can be damaged by furanic substances.
- Intercellular communication and cell membrane integrity can be demolished by phenolic compounds (Monlau et al., 2014; Quéméneur et al., 2012)

While common aliphatic carboxylic acids such as acetic acid, formic acid and levulinic acid and furan aldehydes such as furfural and hydroxymethylfurfural (HMF) are found to be less toxic compared to aromatic byproducts, their high concentration may play a strong inhibitory role (Jönsson and Martin, 2016).

Aromatic carboxylic acids, on the other hand, are produced in lower amounts in hydrolysates, but have more devastating inhibition effect. Phenolic aromatic carboxylic acids such as ferulic acid and 4-hydroxybenzoic acid and non-phenolic aromatic carboxylic acids such as cinnamic acid can be counted under this group.

These compounds are formed by hydrolysis of esterified phenols or degradation of lignin. It was found that *S. cerevisiae* is inhibited by 0.2 g/l ferulic acid, while this concentration is almost 2-fold higher in case of aliphatic carboxylic acids (Larsson et al., 2000).

1.5.2 Inhibition of enzyme activity

Inhibition may also be formed by the competitive binding of solid part elements, such as lignins and hemicelluloses to cellulolytic enzymes. Lignin non-productively binds to cellulase and this results in improper degradation of the substrate, which leads to higher costs in lignocellulose biorefinery (Han et al., 2022). Though with few studies, this non-productive adsorption is shown to be dependent on the hydrogen bonding, hydrophobic and electrostatic interferences (Yuan et al., 2021a).

Cellulases can also incur product inhibition by mono-(glucose) and disaccharides (cellobiose) (Berlin et al., 2007; Teugjas and Väljamäe, 2013). Moreover, it has been shown with various studies that aromatic compounds interfere with the cellulolytic enzyme activity by hydrophobic interactions (Alriksson et al., 2011; Cavka et al., 2011). A study indicated that when aromatic compounds are treated with sulfur oxyanions, they get negatively charged and hydrophilic, which in turn makes them less reactive. In the same study, it was also shown that when treated with sodium borohydride, they get less reactive in a similar way, but without a dramatic change in the hydrophilicity (Cavka and Jönnson, 2013).

1.5.3 Strategies to overcome inhibition

The generation of inhibitors depends on the type of lignocellulosic material along with the pretreatment process. Therefore, employing low recalcitrant value feedstock can be beneficial to avoid inhibition (Jönsson et al., 2013). Application of mild conditions at pretreatment step could also be a possibility. Studies showed that hydrothermal pretreatment of Miscanthus grass and wheat straw resulted in lower concentrations of furan aldehydes and phenols, when no acid was used during the process (Chiaramonti et al., 2012; Larsen et al., 2012).

Detoxification (conditioning) is one of the most efficient strategies. Addition of alkali or reducing agents as well as various polymers, liquid-liquid or liquid-solid extraction, enzymatic treatment and vaporization can be counted as detoxification techniques

However, the necesssity of an additional step is a disadvantage of the method (Jönnsson and Martin, 2016). Overliming (addition of calcium hydroxide) has been shown to be a very powerful, yet cost effective process. By this way, the concentration of soluble salts remains low, which enhances the fermentation activity (Alriksson et al., 2005; Martinez et al., 2001).

As a biological method, simultaneous saccharification and fermentation (SSF) can be applied. In this process, enzymatic hydrolysis of lignocellulose and fermentation take place at the same time in fed-batch or continuous cultivation (Olofsson et al., 2008). Similarly, in consolidated bioprocessing (CBP), fermentation microorganisms also act as enzyme suppliers (Olson et al., 2012). Both techniques aim to prevent product inhibition. Moreover, microorganisms can be screened to select the strains with inhibitors resistance. For example, some strains of *S. cerevisiae* isolated from a wine yard were found to possess high resistance to aliphatic carboxylic acids, furan aldehydes, and a sugarcane bagasse hydrolysate (Favaro et al., 2013).

Genetic engineering is another tool to produce recombinant microorganisms which are able to survive in lignocellulosic hydrolysates. Wang et al. (2013) succesfully developed an *Escherichia coli* strain with furfural resistance in sugarcane bagasse hydrolysate.

Despite resistance for a number of inhibitors has been demonstrated by various studies, biological methods remain less effective compared to the well-established chemical methods, since resistance do not provide efficient fermentation capabilities in industrial scale (Jönsson and Martin, 2016).

1.6 Olive Oil Industry

Olivefruit is obtained from *Olea europaea* L., subsp. *europaea* plant and have been cultivated for thousands of years, particularly in Mediterranean region. Besides fruit, it is mainly grown for its oil, which has been the shown as the basis of healty Mediterranean diet. Although the cultivation prevails in countries such as Spain, Portugal, Italy, Greece, Turkey, Tunisia, Morocco, etc., current cultivation has been dispersed worldwide (USA, Australia, South America, China, etc.) owing to the beneficial effects of olive oil (Martin et al., 2020; Cuevas et al., 2010) (Figure 1.6).

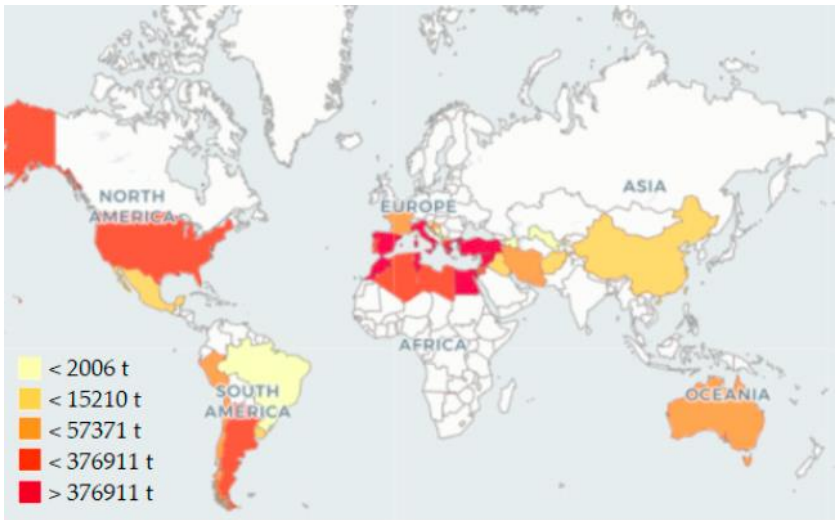


Figure 1.6: Average olive production of countries between 1994 and 2017 (Martin et al., 2020).

Presently, 11 x 10^6 ha land is in use for olive tree plantation, from which approximately 20 x 10^6 t olives are harvested each year (Martin et al., 2020).

Besides olive fruit and olive oil, olive tree cultivation leads to the production of numerous by products namely, olive tree prunning, stones, leaves, pomace, and olive mill wastewater. A mass balance of a 1 ha land of cultivation is depicted in Figure 1.7.

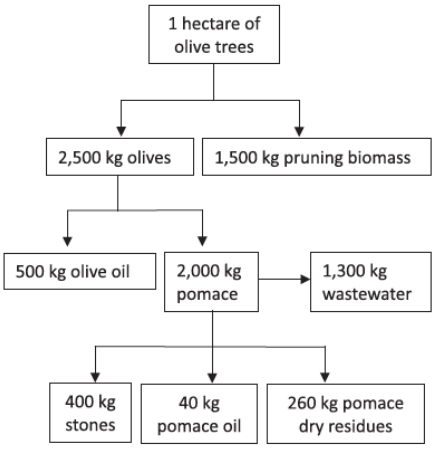


Figure 1.7: Average mass balance derived from 1 hectare of olive trees corresponding to typical cultivation method (Romero-Garcia et al., 2014).

1.6.1 Olive oil production process and byproducts

After olive fruit is collected and transferred to olive mill, leaves still remaining (5 % of olives by weight) are removed, which in turn used as animal feed or thrown away. Milling, mixing (malaxation) and separation in a centrifuge or decanter are the next

steps of the process. There are mainly two methods to isolate olive oil from the byproducts such as olive stones and olive pomace; two phase separation, in which formation of two components (oil and byproducts) in the vessel is followed by removal of olive stones and pomace to obtain olive oil, and three phase separation, through which water is added into the vessel to produce three fractions: oil, olive pomace and olive mill waste water. This water generated by washing of the olive oil contains high amounts of sugars, phenols and other water soluble compounds which pose significant evironmental problems if not handled properly (Ruiz et al., 2017).

1.6.2 Olive derived biomass

1.6.2.1 Olive pruning debris (OPD)

Olive trees are trimmed biennially by farmers in order that the tree can get rid of the old branches and regenerate. As well as the distribution depends on the culture region, methods of pruning activity, maturity of trees and cultivation type, a classical olive pruning debris comprises thin branches (< 5 cm diameter and app. 50 % by weight), thick branches and wood (app. 25 % by weight) and leaves (25 % by weight) (Romero-Garcia et al., 2014).

According to Sánchez et al., 2002, the amount of olive pruning debris obtained from 1 ha olive tree plantation is app. 3 t, which leads to about 3.3×10^7 t biomass. Chemical characterization of OPD shows that it is mainly composed of lignocellulosic material; cellulose, hemicellulose and lignin, proportions of which vary due to the factors such as climate, soil composition and maturity of the plant. The other components of OPD are soluble compounds and ash. Some minimal and maximal values stated by various studies are 25.0 % and 39.1 % (Cara et al., 2008; Garcia-Martin et al., 2008) for cellulose; 17.9 % and 25.7 % (Cuevas et al., 2010; Garcia-Martin et al., 2008) for hemicellulose; 14.3 % and 24.1 % for lignin (Garcia-Martin et al., 2008; Cuevas et al., 2010). Despite its high potential for biofuel production, OPD is usually crushed and left on the land to be combusted. The ton price of the material is stated to be 30 – 40 euro in Andalusia region (Cuevas et al., 2019), which makes it a significant candidate for use in green chemistry applications.

1.6.2.2 Olive stones

Olive stones constitutes 10 – 20 % of the olive fruit by weight and are composed of the seeds and endocarps, the wooden part which cover and protect the seed (Romero-Garcia et al., 2014; Garcia Martin et al., 2020). Each year app. 30,000 tonnes olive stone is formed from olive oil industry (Mata-Sánchez et al., 2013). They are generated either by olive oil extraction or olive pomace extraction facilities and generally broken down to smaller pieces whose size differ between 1.2 mm and 6.2 mm (Saleh at al., 2014; Fernández-Bolaños et al., 1999). Similar to OPD, it is mainly made up of lignocellulosic material with percentage of cellulose varying between 27.1 and 36.4 % (Martín-Lara et al., 2009; Fernández-Bolaños et al., 2001); hemicellulose 24.5 and 32.2; and lignin 23.1 and 40.4 % (Rodríguez et al., 2008; Martín-Lara et al., 2009). Owing to its remarkable lignin amount, olive stones stand as an attractive material to be used as solid biofuel and for thermal combustion. With various methods, the higher heating value of olive stones have been found out to be between 18.8 MJ/kg and 20.9 MJ/kg (Cuevas et al., 2019; Mata-Sánchez et al., 2013). Correspondingly, the vast majority of olive stones are employed today in thermal power production.

1.6.2.3 Olive mill wastewater (OMWW)

OMWW is a blend of liquids generated at different stages of olive oil extraction: olive fruit washing waters (~ 5 % of the weight of the total olives refined), fruit pulp water (~ 45 % of the initial weight of fruit), water supplemented in the centrifugation step, and water acquired from the extraction appliances (5 – 10 % of the weight of refined olives) (Cassano et al., 2016). It is mainly composed of organic substances; along with carbohydrates, proteins and fatty acids, it has a high concentration of carotenoids, tocopherols and phenols. Since these organic compounds are well dissolved in water, OMWW may contaminate water sources, thus must be disposed very carefully. On the other hand, the high phenol concentration (up to 53 % of total phenolics in olive fruit) may act as a source of antioxidants (La Scalia et al., 2017). For example, solar distillation method was proposed in order to isolate the antioxidants in OMWW (Sklavos et al., 2015). In another study, OMWW was treated with *Aspergillus niger* enzymes by membrane filtration and ultrafiltration, which led to 7.2 g/l hydroxytyrosol (Hamza and Sayadi, 2015).

1.6.2.4 Olive pomace

Olive pomace (OP) constitutes the largest solid moiety upon olive oil extraction which counts up to 40 % of the total olive weight (Chanioti and Tzia, 2018). OP is composed of crushed olive stones, olive skin and pulp, along with water. The water amount depends on the extraction method the OP is obtained. When generated by two phase method, the moisture content of OP is 50 – 70 %, while it is 40 – 54 % with three phase method. OP can be further treated to separate the residual oil it contains. As a result, olive cake (dry pomace) is obtained, most of which is used as biofuels (Gullon et al., 2020; Ruiz et al., 2017).

Moreover, OP is rich in phenolic compounds, proteins and carbohydrates, which makes it an ideal candidate for further value added applications. As lignocellulosic biomass, it comprises 30.0 – 41.6 % lignin, 35.3 – 49.0 % cell wall polysaccharides such as cellulose, pectin and hemicellulose, 7.5 – 14 % oil and 4.4 – 6 % minerals (Miranda et al., 2019; Rodrigues et al., 2015).

Owing to its valuable contents, it has been suggested as an antioxidant and antiproliferative agent (Bermúdez-Oria et al., 2019). In another study, OP generated by two phase method was used to extract polyphenols namely tyrosol, apigenin and oleuropein in order to employ against oxidative stress (Vergani et al., 2016).

OP can also be used as a carbon source for microorganisms to supply biosurfactants as demonstrated by Moya Ramírez et al. (2015). In a study conducted by Salgado et al. (2015), lignocellulolytic enzymes were produced by *Aspergillus uvarum* cultivated in a growth medium prepared with OP. In a different application, OP was shown to provide superior characteristics when integrated into ceramic. The resulting material, including 3 – 12 % wt OP, had lower density and lower thermal conductivity, which may be an effective use of the substance regarding the environmental concerns (De La Casa et al., 2012).

Olive derived biomass parts are shown in Figure 1.8.

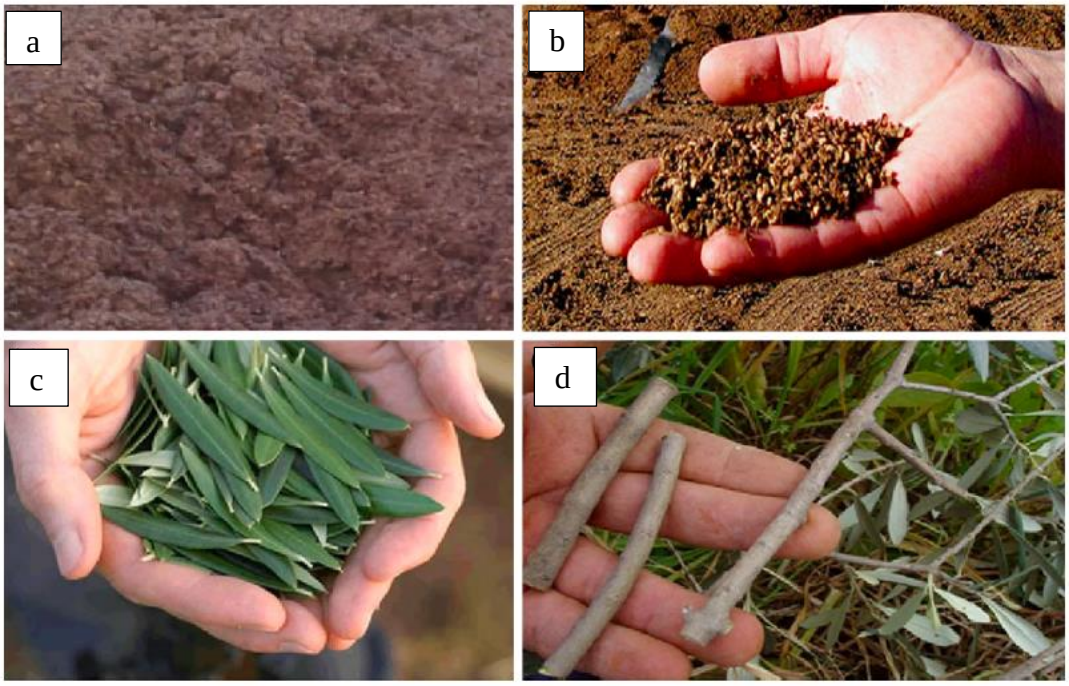


Figure 1.8: Olive biomass (a) olive pomace from two-phase separation system, (b) olive pomace from three-phase separation system, (c) olive leaves, (d) olive prunning debris (Maragkaki et al., 2016).

1.7 Aim of the Thesis

This study aims to produce a functional biopolymer, bacterial cellulose, making use of olive pomace, an industrial byproduct obtained abundantly in the Mediterranean region. It is focused on three main steps: firstly to acquire reducing sugars by acid pretreatment and enzymatic hydrolysis of olive pomace. Secondly, the cultivation of *Novacetimonas hansenii* in a growth medium prepared with enzymatic hydrolysates. Finally, characterization of the bacterial cellulose produced in the alternative media.

By elementary analysis, high content of carbon of olive pomace was revealed. Therefore, techniques targeting lignocellulose hydrolysis into reducing sugars, which can be subsequently utilized for growth medium preparation were applied. Dilute acid pretreatment was employed to partially hydrolyse cellulose and hemicellulose. By proceeding with enzymatic hydrolysation, it was aimed to achieve monomeric sugars that could be metabolized by cellulose producing bacteria. Monosaccharide types and distributions in varying hydrolysates were tracked with HPLC.

Since pretreatment process leads to inhibitor formation both for cellulases and microorganisms, detoxification of the hydrolysates was also a critical step in this study.

Once the hydrolysates were detoxified, the cultivation of bacterial cellulose was realized in the alternative media. Within the scope of the study, cellulose production rates and yields were also calculated in standard and alternative media. Cellulose form and structure were analyzed and compared with the control material by Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy and X-Ray Diffraction.

With this study, the production of bacterial cellulose using olive pomace as carbon source was achieved and described.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Medium

Novacetimonas hansenii (formerly *Gluconacetobacter hansenii)* ATCC 23767 and ATCC 53582 strains were purchased from American Type Culture Collection. Freze-dried samples were dissolved in sterile Hestrin-Schramm medium and placed in -80 ^{o}C for further use.

Hestrin-Schramm (HS) medium was prepared as mentioned in Table 2.1. In case of HS-agar preparation, medium was supplied with 1.5% (w/v) agar. Growth medium was sterilized in autoclave at 120 ^{o}C for 20 min before use. All chemicals were purchased from Merck KGaA.

Table 2.1: Ingredients of Hestrin-Schramm medium.

Reagents	% (w/v)
Glucose	2
Peptone	0.5
Yeast Extract	0.5
Na_2HPO_4	0.27
Citric Acid	0.115

2.1.1 Preliminary efficiency analysis of strains

ATCC 23767 and ATCC 53582 were compared according to their yield in static and agitated conditions. For this, selected single colonies of ATCC 23767 and ATCC 53582 were inoculated in HS medium and incubated at 30 ^{o}C for 3, 6, 10 and 14 days in separate Erlenmayer flasks either statically or at 150 rpm agitation speed. At the end of each incubation period, the culture medium was discarded and bacterial cellulose samples were collected. BCs were washed firstly in distilled water until pH 7 and then in 0.1 M NaOH solution for 1 hour at 100 ^{o}C. Then, they were vacuumed in a vacuum-filter system and dried in oven at 85 ^{o}C until a constant weight is obtained. All conditions were applied as duplicates. BC production is schematically shown in Figure 2.1.

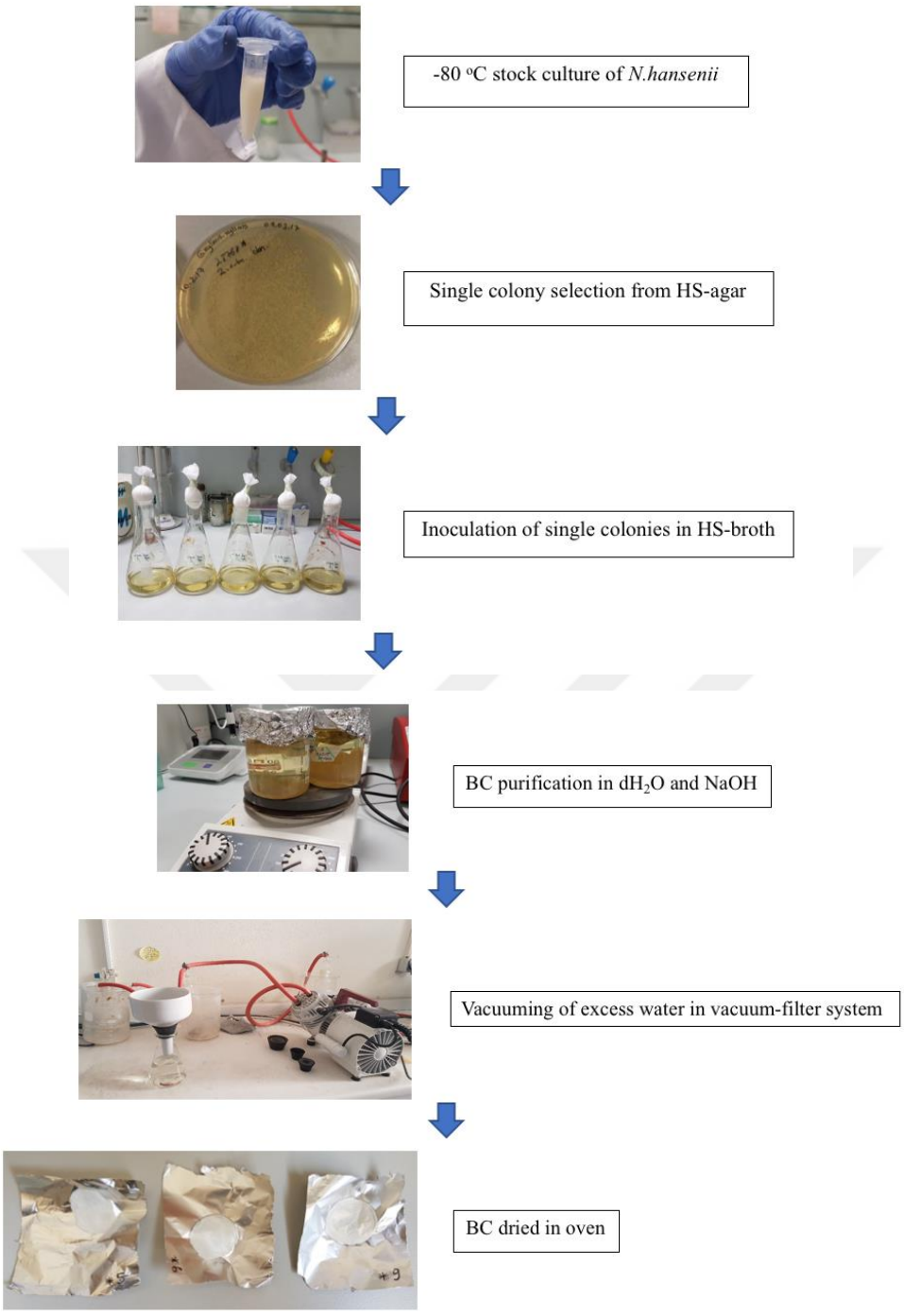


Figure 2.1: BC preparation steps.

2.1.1.1 Effect of low agitation speeds in BC production

To investigate the effect of varying agitation speeds, 20 rpm, 40 rpm and 80 rpm were applied in 500 ml Erlenmeyer flasks and 50 mL centrifuge tubes. The broth volumes were 150 ml and 15 ml for Erlenmeyer flasks and centrifuge tubes, respectively. 50 µL of starter culture prepared in 2.1.1 were inoculated in each flask and tube. Cultures

were incubated for 14 days and purified as mentioned before. All conditions were applied as duplicates.

2.1.1.2 Surface area / volume ratio effect in bacterial cellulose production

Surface areas of erlenmeyer flask and centrifuge tube were calculated according to the equations 2.1, 2.2, and 2.3.

$$S = \prod r^2 \quad (2.1)$$

The surface area of the erlenmeyer flask was 88.25 cm^2 and the surface area of the centrifuge tube was found to be 8.04 cm^2. According to the surface areas found, the surface area (S) /volume (V) values were calculated as follows:

For erlenmeyer flask:

r = 5.3 cm , S ($\prod r^2$)= 88.25 cm^2 , V= 150 cm^3

$$S / V = 88.25\ cm^2 / 150\ cm^3 = 0.5883\ cm^{-1} \quad (2.2)$$

For centrifuge tube:

r = 1.6 cm, S ($\prod r^2$)= 8.04 cm^2 , V= 15 cm^3

$$8.04\ cm^2 / 15 cm^3 = 0.5360\ cm^{-1} \quad (2.3)$$

2.1.2 Calculation of nitrogen and carbon conversion rates during bacterial cellulose production

Conversion rates of carbon source (glucose) and nitrogen source (peptone + yeast extract) in HS medium to bacterial cellulose were calculated with Equations 2.4 and 2.5. D_0 represents the dry weight of cellulose in grams, C indicates the amount of carbon in grams, and N represents the nitrogen source in grams (Yim et al., 2017; Jalili Tabaii and Emtiazi, 2016). The calculated conversion rates the bacterial cellulose production efficiency according to the consumption of carbon and nitrogen sources.

$$(D_0/C)\times 100\ \% \quad (2.4)$$

$$(D_0/N)\times 100\ \% \quad (2.5)$$

The weights of bacterial cellulose samples produced in flasks and falcons were converted into g/L units using Equations 2.6 and Equation 2.7, respectively, and yield was obtained according to bacterial cellulose dry weight. mD refers to the dry weight of bacterial cellulose.

$m_D \times (1000\ mL/150\ mL)$ (2.6)

$m_D \times (1000\ mL/15\ mL)$ (2.7)

2.2 Bacterial Cellulose Production in Alternative Media

2.2.1 Media preparation

Industrial and agrowastes namely fish flour, meat-bone flour and olive pomace were tried as potent growth media for *N. hansenii* on static culture. All alternative media were prepared as follows: 50 g meat-bone flour/ fish flour/ dry olive mill and 12.5 g lactose (obtained from whey) were dissolved in dH_2O and the total volume was completed to 250 ml. as shown in Figure 2.2. pHs were adjusted to 6 and the media were autoclaved for sterilization. 150 µl of -80 ^oC stock culture was inoculated in 150 ml HS Broth to obtain a starter culture. After 5 days, 1 mL of this starter culture was inoculated in each flask. After 21 days following the inoculation, cultures were discarded and BC samples were purified and dried as mentioned before.

Figure 2.2: Medium prepared with olive pomace.

2.2.2 Lactose concentration effect in olive pomace medium

To determine lactose concentration effect on BC production; 5, 10, 15, 20 and 25 g lactose amounts were tested on both conditions. The experiments were conducted as duplicates. Before inoculation, a starter culture was prepared as mentioned before. 5 days later, 1 ml of this starter culture was inoculated in each flask. After 21 days, BC samples were collected, purified and dried.

2.2.3 Lactase application on standard medium with lactose

To investigate the lactase affect on BC production, Dairyzym Y50L (Cat. No: 0109039) provided from SternEnzyme company. HS Media were prepared with lactose instead of glucose to demonstrate the effect clearly. 0, 7.5, 15, 30, 60, 90 µl lactase were added to 6 flasks, each containing 2 g lactose and 100 mL dH_2O and incubated for 16 hours in room temperature. When the incubation process is over, flasks were controlled against a probable pH change that may have been caused by the enzymatic reaction and all flasks were arranged at pH 6. Other ingredients of the HS media were added into the flasks and they were sterilised in autoclave. As positive control, 100 mL HS medium containing glucose instead of lactose was used. 100 µl *N. hansenii* culture from -80 ^oC stock solution was inoculated to each flask. The flasks were statically incubated at 30 ^oC for 15 days. At the end of the incubation time, BC from all flasks were isolated and washed with 0.1 M NaOH with stirring. BCs were washed until pH 7 and dried in vacuum oven at 60 ^oC.

2.2.4 Elementary analysis of olive pomace and BC produced in OP medium

The elemental composition was determined on a VARIO EL III CHNS analyzer (Elementaranalysensysteme GmbH). Carbon, hydrogen, nitrogen and sulphur content of a variety of materials can be determined by CHNS elemental analyzers. The samples may be in solid or liquid form as well as volatile and viscous. The device has the capability to be adjusted to very little (mg) and large amount (g) of samples. The working principle depends on the combustion at high temperature in an oxygen-rich environment. During the combustion (at ca. 1000 ^oC), carbon, hydrogen and nitrogen are transformed into carbon dioxide, water, and oxides of nitrogen, respectively. An inert gas, generally helium functions to remove the combustion products. Next, the gases are stored in absorbent traps in a way that only carbon dioxide, sulphur oxide, water and nitrogen are reserved. A schematic figure of an elemental analyzer is shown in Figure 2.3.

For the elemental analysis of samples, BC samples were shivered into tiny pieces and OP was powderized for the analysis of carbon, hydrogen, nitrogen and sulfur molecules.

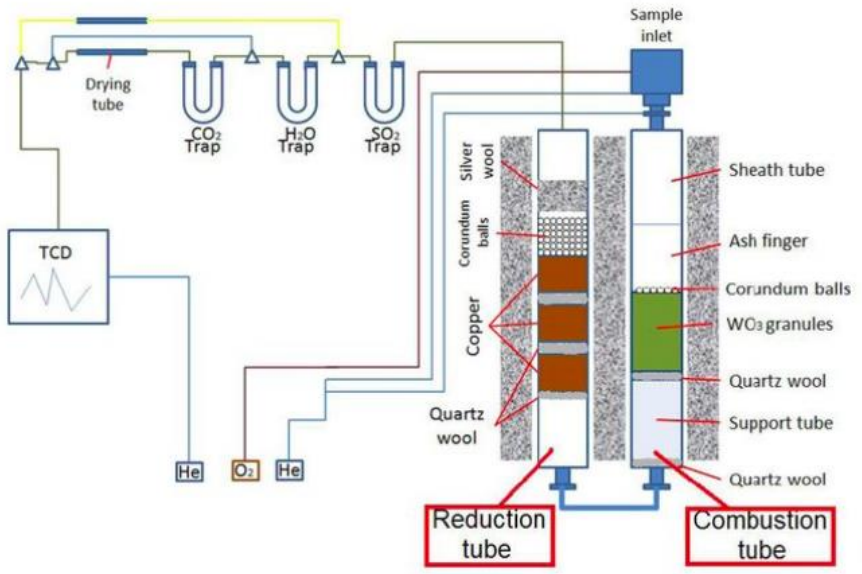


Figure 2.3: Representative view of an elemental anayzer (Horvat, 2016).

2.2.5 Removal of protein contamination

In order to remove the N contamination in BC-OP determined by elemental analysis, test and control samples were subjected to longer washing periods. Following their production and collection, they were subjected to stirring washing in 0.1M NaOH. At the days 4, 7 and 11; small pieces of each BC were collected in 1.5 ml vials and stored in -20 ^{o}C. Before elementary analysis samples were lyophilized and vacuum-dried.

2.3 Degradation of Olive Pomace

2.3.1 Acidic pretreatment

OP was treated with diluted weak acid in order to degrade lignocellulosic material into monomeric sugars. 150 g OP was added into 500 ml 1 % phosphoric acid. 170 ^{o}C temperature at 8 bar and 200 rpm shaking condition was applied to the mixture in a 1 liter reactor for 45 min. Parts of the reactor and schematic view are shown in Figure 2.4 and Figure 2.5. At the end of the process, liquid and solid phases were collected in separate tubes. Water-soluble carbohydrates are found in the liquid phase and could be directly measured after the acid pretreatment. On the other hand, water-insoluble carbohydrates found in the solid phase have to be enzymatically broken to be served as growth media. Thus, glucose concentration results of the liquid phase indicates the

direct measurement after acid pretreatment, while results of the solid phase results demonstrate the measurements after enzymatic process.

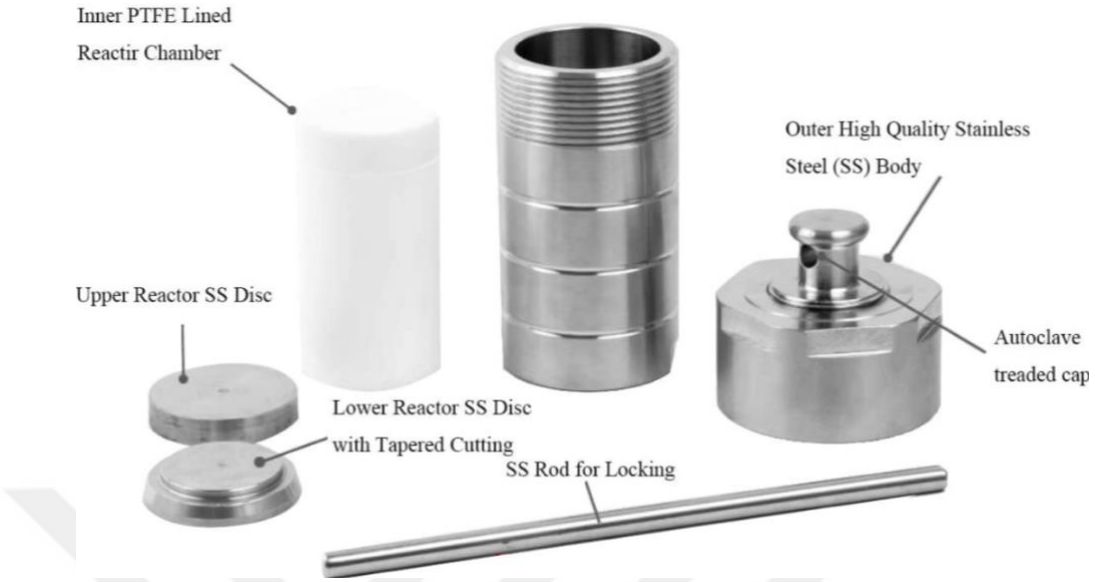


Figure 2.4: Parts of a hydrothermal autoclave (URL-1).

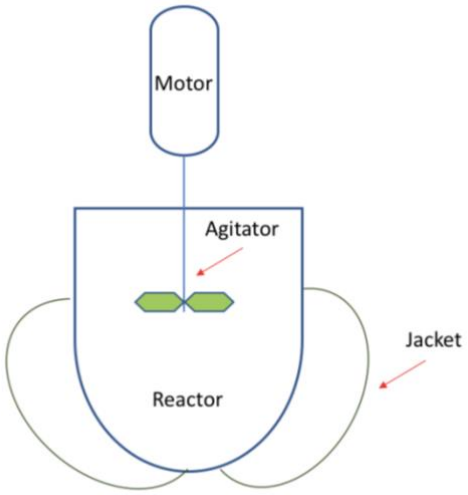


Figure 2.5: Schematic inner view of a hydrothermal reactor.

2.3.2 Glucose concentration analysis of the liquid phase by glucose hexokinase assay

For glucose concentration indication Sigma-Aldrich Glucose (HK) Assay Kit (Cat. No. GAHK20) was purchased. Determination of glucose concentration by this kit relies on the spectrophotometric measurement (at 340 nm) of the NADH produced by the chemical reaction below:

Glucose + ATP → Glucose-6-phosphate + ADP (in presence of hexokinase)

GDP+NAD → 6- Phosphogluconate + NADH (in presence of G6PDH)

0.2 mL from the liquid phase was deproteinized and decolorized according to the kit manual by 1 mL 0.3 N $Ba(OH)_2$ and 1 mL 0.3 N $ZnSO_4$ (deproteinization). Then, a small amount of activated carbon was added into the solution and decolorized by filtering through 0.45 μL pore-sized filter. The absorbance of this sample along with reagent blank and sample blank was measured at 340 nm. Glucose concentration was calculated with the Equations 2.8, 2.9, 2.10, and 2.11.

$$\mathbf{A_{Total\ Blank} = A_{Sample\ Blank} + A_{Reagent\ Blank}} \quad (2.8)$$

$$\mathbf{mg\ glucose\ /\ ml = \frac{(\Delta A)(TV)(Glucose\ molecular\ weight)(F)}{(\mathcal{E})(d)(SV)(Conversion\ factor\ for\ \mu g\ to\ mg)}} \quad (2.9)$$

$$\mathbf{mg\ glucose\ /\ ml = \frac{(\Delta A)(TV)(180.2)(F)}{(6.22)(1)(SV)(1000)}} \quad (2.10)$$

$$\mathbf{mg\ glucose\ /\ ml = \frac{(\Delta A)(TV)(F)(0.029)}{(SV)}} \quad (2.11)$$

$\Delta A = A_{Test} - A_{Total\ Blank}$

TV = Total Assay Volume (ml)

SV = Sample Volume (ml)

Glucose MW = 180.2 g/mole or equivalently 180.2 μg/μmoles

F = Dilution factor from sample preparation

$\mathcal{E}$ = Milimolar extinction coefficient for NADH at 340 nm, Milimolar $^{-1}$ cm $^{-1}$ or equivalently (ml/ /μmoles) (1/cm)

d = Light path (cm) = 1 cm

1000 = Conversion factor for μg to mg

2.3.3 Enzymatic treatment of the solid part of olive pomace

The solid phase was washed with excess dH_2O to clean the acidic residues. It was dried in vacuum-drier at ~3 mbar. A multi enzyme blend, Cellic® CTec2 (Novozymes) was used for the enzymatic hydrolysation of the solid material. Cellic Ctec2 is a new generation mixture of cellulases, β-glucosidases and hemicellulases. It is used to generate monomeric sugars from lignocellulosic materials. Cleavage sites of the enzyme are shown in Figure 2.6.

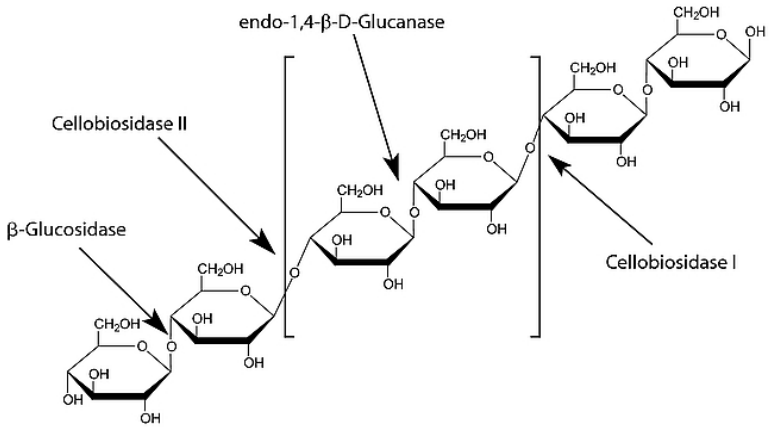


Figure 2.6: Activity sites of Cellic® CTec2 on cellulose polymer (URL-2).

Since lignin serves as a protective barrier for cellulose and hemicellulose, it should be removed by a pretreatment method i.e. acidic pretreatment. Following this process, enzymatic degradation is employed to generate monomeric sugars from cellulose and hemicellulose which contain the sugars in polymeric form. Afterwards, these monomers can be used for fermentation/ microbial activity. Some superior properties of Cellic® CTec2 compared to standard cellulase is shown in Table 2.2.

Table 2.2: Qualifications of the enzyme Cellic® CTec2 (URL-3).

Product	**Cellic® CTec2**
Technical description	a mixture of: - aggressive cellulasses - high level of B-glucosidases - hemicellulase
Properties	- high conversion yield - effective at high solids loading - inhibitor tolerant - compatible with multiple feedstocks and pretreatments - high concentration and stability
Benefits	- up to 50 % lower enzyme dosage - lower operating and capital costs from optimization of process - increased process flexibility

The optimal temperature and pH for the enzyme were tested on corn stover lignocellulosic biomass. the material was primarily pretreated with dilute acid. The enzyme hydrolysation was applied at 5 % solids loading. For pH profile, the

temperature was conducted constantly at 50 ºC and for the temperature profile, the pH was conducted constant at 5. For both assays, the reactions were maintained for 72 h. The relative performances are shown in Figure 2.7. Based on the data, it was concluded that the optimal temperature and pH for Cellic® CTec2 are 50 ºC and 5, respectively (URL-3).

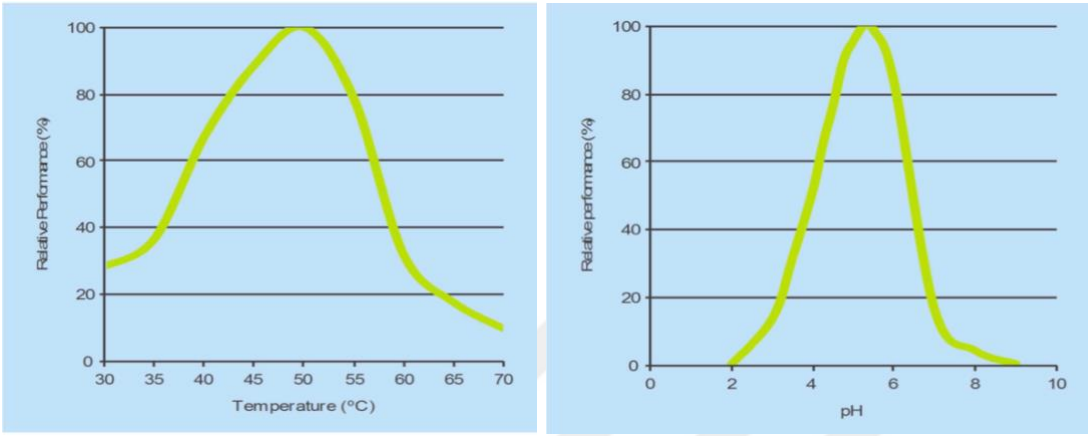


Figure 2.7: The temperature (left) and pH (right) profiles for the relative performance of the enzyme Cellic® CTec2 (URL-3).

Enzyme trial concentrations were calculated as suggested in the user manual of Cellic Ctec2 enzyme: 1.5 %, 3 %, 6 % and 30 % (w/w g enzyme/g cellulose). Keeping the substrate amount constant (1.25 g solid material), 100 ml reaction flasks with 25 ml 0.05 M sodium citrate buffer (pH 4.8) were prepared with varying amounts of enzyme. Solid phase was dried in vacuum-oven at 60 ºC and 1.25 g of the sample were transferred to 4 flasks containing 25 mL citrate buffer and 5.5 µl (1.5 % enzyme), 110 µl (3 % enzyme), 22 µl (6 % enzyme), 110 µl (30 % enzyme) of Cellic cTec2 to test the enzyme concentration effect on hydrolysation. Flasks were incubated at 50 ºC water bath for 72 h either at static or agitated condition (150 rpm agitation speed) as shown in Figure 2.8. At the end of the incubation period, samples were taken into 90 ºC to stop enzymatic reaction. Then, samples were centrifuged at 4000 rpm for 5 min and supernatants were collected.

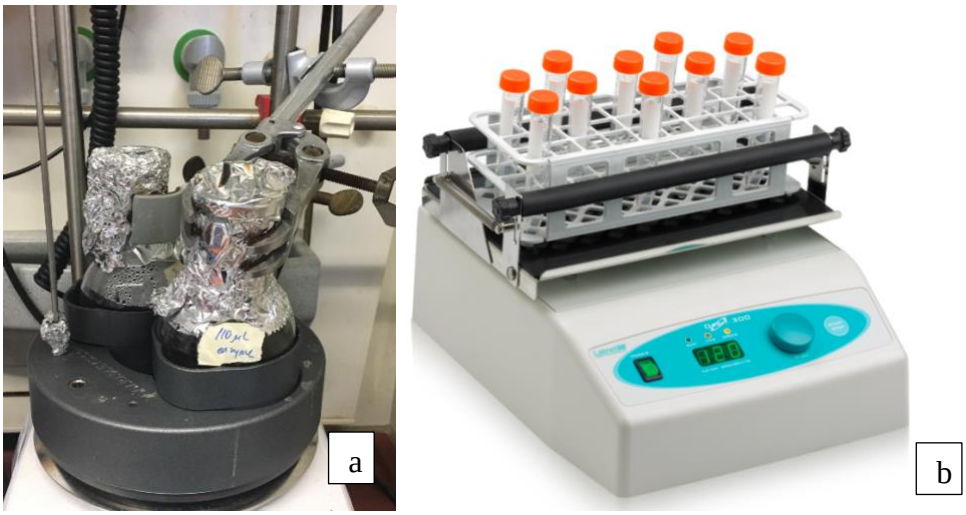


Figure 2.8: Enzymatic reaction of OP solid phase following the phosphoric acid pretreatment, (a) in static (b) in agitated condition.

2.3.4 Reducing sugar determination of the solid phase by 3,5-dinitrosalicylic acid method

Hydrolysates were treated with activated carbon to prevent blur which may in turn cause spectrophotometric interference.

3,5-dinitrosalicylic acid method tests for the presence of free carbonyl group (C=O), namely reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

$$\text{aldehyde group} \xrightarrow{\text{oxidation}} \text{carboxyl group}$$

$$\text{3,5-dinitrosalicylic acid} \xrightarrow{\text{reduction}} \text{3-amino,5-nitrosalicylic acid}$$

The NREL (National Renewable Energy Laboratory) protocol (URL-1) has been modified according to the needed amount of chemicals and use of the experiment. DNS Acid Reagent Solution (1%) was prepared as follows:

-DNS 10 g

-Sodium sulfite 0.5 g

-Sodium hydroxide 10 g

-dH_2O added up to 1 l.

-Potassium sodium tartrate solution (40 %) was prepared separately.

To obtain a glucose calibration curve, glucose standart solutions in citrate buffer on 1-10 g/l range were prepared.

1 ml DNS reagent was added to 1 ml sample. The mixture was heated to 90 ^{o}C for 15 min to develop red-brown colour. 0.33 ml Potassium sodium tartrate solution was added to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance at 575 nm was recorded with a 96-well-plate reader spectrophotometer. All samples were measured as triplicates.

2.4 HPLC Analysis of the Reducing Sugars After Enzymatic Reaction

In order to estimate the amounts of various reducing sugars at the enzymatic hydrolysates, HPLC analysis was applied to selected samples. The presence of galactose, glucose, mannose, arabinose, xylose, rhamnose, lactose, fructose, maltose and cellobiose was investigated with the analysis.

JASCO device (eluent: 0.005 M H_2SO_4, flow rate: 0.5 ml/min) with a refractive index detector (RI-930), an intelligent pump (PU-980) and an Aminex® HPX-87H column from Bio-Rad Laboratories (length: 300 mm, inner diameter: 7.8 mm). For HPLC analysis, the polymer (ca. 100 mg) was treated with 70% (v/v) $HClO_4$ (2 ml) within 10 minutes at room temperature. After dilution with water (18 ml), the mixture was shaken at 100 °C for 16 hours. The samples were neutralized using a 2 M KOH. Afterwards, the samples were kept at 4 °C for 1 hour for complete precipitation of $KClO_4$. In a further step, the samples were concentrated to an amount of about 4 ml.

2.5 Growth Media Preparation with Enzymatic Hydrolysates

2.5.1 Role of citric acid

Selected enzymatic hydrolysates were supported with HS growth medium ingredients except for glucose. When hydrolysate was used directly (with no pretreatment), no BC production was observed. Since sodium citrate buffer is used as enzymatic reaction buffer, effect of citric acid on BC production was investigated. In order to test this, 24-well plates were prepared as shown in Figure 2.9.

All media were prepared accordingly and autoclaved at 120 ^{o}C for 30 min for sterilization. All wells were designed as duplicates. For each inoculation, 2 ml growth media was used and each inoculated with 200 µl of the primary culture of *N. hansenii.*

After 21 days incubation period, all BC samples were collected and washed as described before. Afterwards, they were freeze-dried and dry weights were recorded.

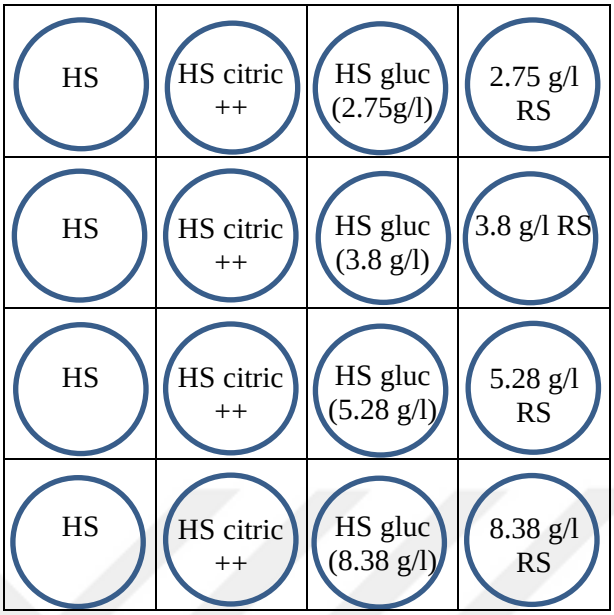


Figure 2.9: Schematic design of a 24-well plate with media prepared for citric acid effect determination. HS: Hestrin-Schramm medium; HS citric ++: HS media including the same concentration of citric acid found in the sodium citrate buffer (4.5 g/l); HS gluc: HS media containing the same concentration of glucose as the reducing sugar concentration of the related enzymatic solution.

2.5.2 Comparison of methods for the detoxification of the enzymatic hydrolysates

An assay was conducted to investigate the most appropriate detoxification procedure which cause minimal sugar loss in this assay. $Ca(OH)_2$, NaOH and activated charcoal were used as detoxifying agents. The method suggested by Guo et al. (2013) was modified as follows:

20g/L glucose solution was prepared for each detoxification flask and for control flask.

- Activated charcoal: Charcoal was added to obtain a solution of 2% (w/v). Solution was shaken well and incubated at RT for 5 min. It was centrifuged at 8300 g. for 5 min and supernatant was collected in a clean tube. pH was adjusted to 5.
- $Ca(OH)_2$: $Ca(OH)_2$ was added to pH 11. Solution was incubated at 30 ºC for 3h. It was centrifuged at 8300 g. for 5 min and supernatant was collected in a clean tube. pH was adjusted to 5 with 4M H_2SO_4.

- NaOH: NaOH (powder) was added to pH 9. Solution was incubated at 80 ^{o}C for 3 h. It was centrifuged at 8300 g. for 5 min and supernatant was collected in a clean tube. pH was adjusted to 5 with 4 M H_2SO_4.

1 ml of sample from each flask was collected and DNSA method was applied as mentioned before. Absorbance at 575 nm was recorded for duplicated wells.

2.5.3 Overliming of the enzymatic solutions for bacterial cellulose production

Enzymatic solution of 9.64 g/l RS concentration was used for activated charcoal and $Ca(OH)_2$ detoxifications. Growth media ingredients except glucose were added into the enzymatic solution according to the volume. Detoxification steps were applied as stated at the previous title. 24-well plate was prepared as shown in Figure 2.10.

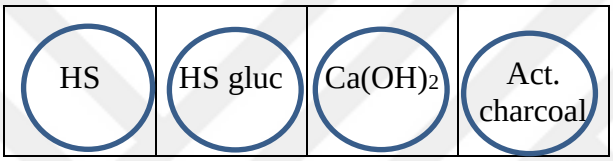


Figure 2.10: Schematic design of a 24-well plate with media prepared for overliming effect determination. HS: Hestrin-Schramm medium; HS gluc: HS media containing the same concentration of glucose as the reducing sugar concentration of the related enzymatic solution.(9.64g/L); $Ca(OH)_2$: enzymatic solution treated with $Ca(OH)_2$; Act. charcoal: enzymatic solution treated with activated charcoal.

4 wells were used for each condition. For each inoculation, 2 ml growth medium was inoculated with 200 µl of the primary culture of *N. hansenii.* After 21 days of incubation period, all BC were collected and washed twice with dH_2O at room temperature and with 0.1 M NaOH at 80 ^{o}C for 1 hour, respectively. Then, they were freeze-dried and dry weights were recorded.

2.5.4 Detoxification and growth media preparation from enzymatic hydrolysates

For detoxification of the hydrolysates, $Ca(OH)_2$ and activated charcoal treatments were applied as stated by Guo et al. (2013). According to that;

a) $Ca(OH)_2$ was added to the hydrolysate until pH 11 and incubated for 3 h at 30 ^{o}C.
b) Activated charcoal was added to form a 2% (w/v) suspension, then mixed vigorously and incubated at room temperature (RT) for 5 min.

Both mixtures were centrifuged at 8300 g for 5 min. Supernatants were collected and pH adjusted to 6 after adding Hestrin-Schramm ingredients except for glucose. These media were named ENZ media depending on the enzyme amount they comprise.

2.5.5 Preparation of control growth media

HS medium was prepared as mentioned before. Another media, which is here named HG (enzyme amount in μl), were designed to test the efficiency of glucose compared to a mix of reducing sugars in terms of BC production. HG growth media were prepared with the same concentration of glucose as the reducing sugar in each enzymatic hydrolysate. Therefore, each HG medium is the HS medium including exactly the same glucose concentration that corresponds to the reducing sugar in each enzymatic medium (e.g. HG 110 is a HS medium with 9.3 g/l glucose, HG 55 is a HS medium with 7.5 g/l glucose).

2.6 Calculations of BC production efficiencies in Hestrin-Schramm and enzymatic media

To compare the substrate conversion ratio, BC production rate and yield of both media, Equations 2.12, 2.13, and 2.14 were modified from Gomes et al. (2013).

Substrate conversion ratio (α) (2.12)

$$\alpha = \frac{S_i - S_f}{S_i} \cdot 100\ \%$$

BC production rate r_{BC} (g l^{-1} day^{-1}) (2.13)

$$r_{BC} = \frac{m_{BC}}{V \cdot t}$$

BC production yield $Y_{BC/S}$ (2.14)

$$Y_{BC} = \frac{\frac{m_{BC}}{V}}{S_i - S_f} \cdot 100\ \%$$

Where Si is the initial concentration (mg ml^{-1}), Sf is the final concentration (mg ml^{-1}) of the substrate, mBC is the amount of BC produced (mg), V is the reaction volume (ml) and t is the time of cultivation (day). The HPLC column (Aminex® HPX-87H) has the same retention time for xylose and fructose. Therefore, only glucose concentration was taken into account for Si and Sf values. Since glucose in the ENZ medium was depleted at the end of day 5 of incubation, Sf concentrations were calculated with the data at the end of day 5, for both media.

2.7 Characterization of bacterial cellulose

2.7.1 Scanning electron microscopy

The scanning electron microscopy (SEM) depends on the targeted high energy electron beam to produce diverse signals at the surface of solid materials. Information about morphology, chemical structure and crystallinity can be obtained by the signals generated upon electron-specimen reciprocal action. Generally, data are assembled along a determined part of the material and by this way, a 2 dimensional figure which exhibit spatial divergence is created. A SEM device is depicted in Figure 2.11 and 2.12. With standard SEM techniques, sections from 5 μm to 1 cm could be analyzed with magnification range between 20 X and ca. 30,000 X (URL-4).

In this study, scanning electron microscopy (SEM) imaging was performed with a Sigma VP Field Emission Scanning Electron Microscope (Carl-Zeiss AG, Germany) using the InLens detector with an accelerating voltage of 6 kV. The samples were coated with a thin layer of platinum (10 nm) via sputter coating (CCU-010 HV, Safematic, Switzerland) before the measurement.

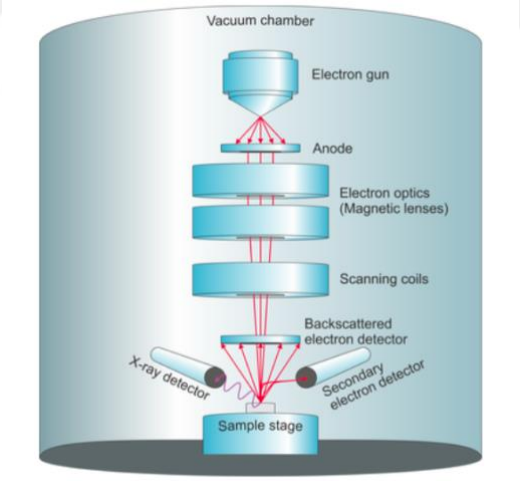


Figure 2.11: Schematic demonstration of a SEM device (URL-5).

2.7.2 FTIR analysis

FTIR represents Fourier Transform Infrared Spectroscopy. This technique evaluates the electromagnetic radiation scale in the infrared region. When a sample is subjected to infrared radiation (IR), the radiation spectrum can be determined by using the difference of light absoption frequencies of different bonds. For the analysis, IR is

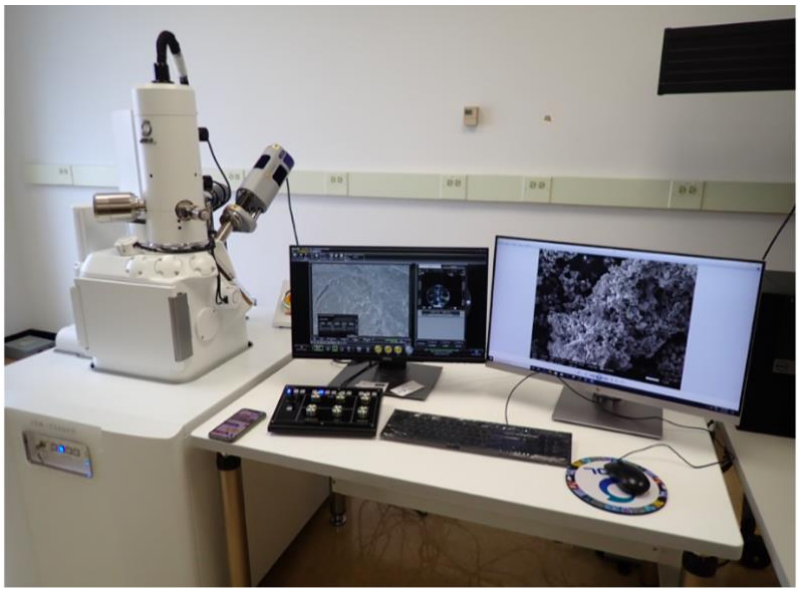


Figure 2.12: SEM instrument with electron column, sample chamber, detectors and visual display monitors (URL-6).

applied to the material and its typical behaviour to absorb the light at various wavelengths is detected. By this way, the molecular arrangement and composition of the sample can be revealed. A schematic view of a FTIR spectrometer is demonstrated in Figure 2.13.

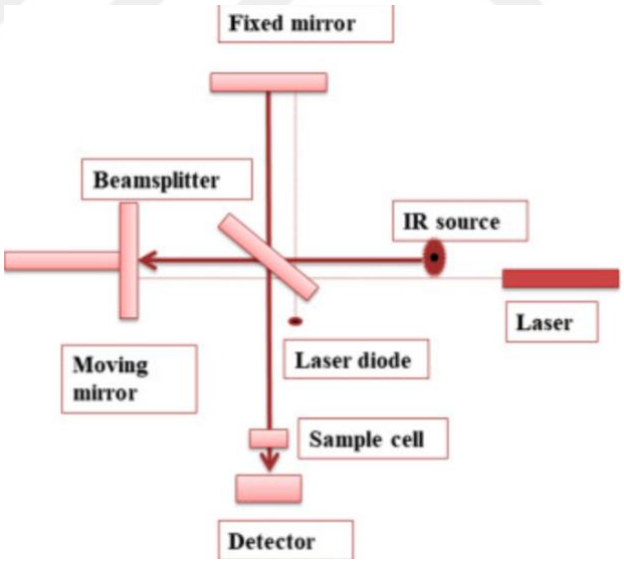


Figure 2.13: A schematic view of a FTIR spectrometer (URL-7).

Cellulose features of the samples were investigated with Fourier Transform Infrared Spectroscopy (FTIR). Spectra were recorded on a Nicolet iS5 spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) using translucent KBr pellets containing

the solid PS samples. The resolution was 4 cm^{-1} and 32 scans were collected in the range from 4000 to 600 cm^{-1}.

2.7.3 X-ray diffraction (XRD)

X-ray diffraction is an analytical method principally applied for crystalline substance verification and characterization. The working principle depends on the interference between a crystalline material and monochromatic X-rays. The X-rays are produced by a cathode ray tube and transformed into monochromatic radiation. The beams are then assembled and conducted towards the sample. The interaction of the X-rays and the sample lead to an interference and diffraction when the arrangement fits Bragg's Law ($n\lambda=2d \sin\theta$). Bragg's Law is used to associate electromagnetic radiation wavelength to the diffraction angle and web-like spacing of the sample. Afterwards, the diffracted beams are identified, processed and evaluated. Analyzed diffraction peaks are converted to d-spacings, which are exceptional to each compound. Finally, standard reference patterns are used to match the material with the correct material (URL-8). A schematic diagram of an X-ray diffractometer is shown in Figure 2.14.

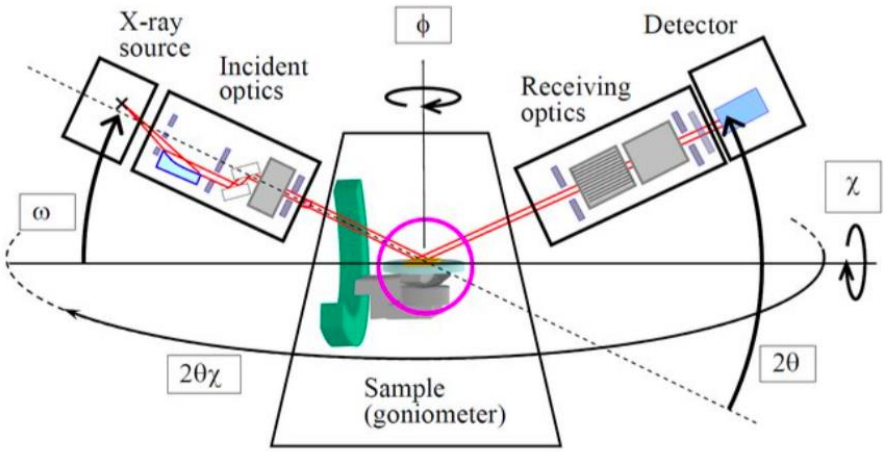


Figure 2.14: Schematic diagram of an X-ray diffractometer (Inaba et al., 2013).

X-ray diffractograms of all samples were measured with D8 Advance (Manufacturer: Bruker AXS GmbH) diffractometer using Cu Kα-Dublett, λ = 1,54184 Å radiation. The raw data were smoothed by means of SAVITZKY-GOLAY-filtration, whereupon the scattering background was modelled by fitting spline-functions onto significant base marks of the scan and subtracted afterwards. The measurement and evaluation were implemented as per European Standard EN 13925-13 "Non-destructive testing –

X-ray diffraction of polycrystalline and amorphous materials (2003)". Instrument settings are shown in Table 2.3.

Table 2.3: Instrument settings of X-ray diffractometer.

Type of equipment	D8 Advance (Manufacturer : Bruker AXS GmbH)
Operation mode	θ/2θ–coupled, continuous scan
X-Ray tube	K FL CU 2 K
X-Ray generator	Kristalloflex K760-A21
Radiation	• CuKα-Dublett, λ = 1,54184 Å (average) • no monochromatization • β-portion suppressed by Ni-Filter
Tube voltage	40 kV
Anode current	40 mA
Goniometer-diameter	600 mm (Measurement circle)
Sample translation	closed Eulerian cradle, Model 511.4
Mode of radiography	Transmission
Primary optics	• 1-mm-Divergence slit (fixed) • Soller slit 2°
Secondary optics	• 8-mm plug–in slit • Ni-Kβ-Filter • Soller slit 2,5° • No anti-scattering slit installed
Detector	LynxEye® (PSD)-Detector • Opening angle: 3° • Resolution: 0,0143°/channel • Zero-Offset– 1,488°
Step size	0,05° 8° ≤ 2θ ≤ 50°
Time per step	0,5 sec
Number of steps	843

3. RESULTS AND DISCUSSION

3.1 Comparison of Strains and Optimization of Bacterical Cellulose Production Conditions

3.1.1 Comparison of bacterial cellulose production efficiencies of strain ATCC 23767 and 53582

Two strains used in this study: ATCC 23767 and 53582, were inoculated in HS medium and incubated at 30 ^{o}C for varying times either under static or agitated conditions. BC morphology differs according to the incubation method as shown in Figure 3.1. Average BC dry weights after washing and drying processes are given in Table 3.1. At the end of 14 days, since the BC yield obtained by ATCC 53582 was the highest with 51.2 mg, it was selected as the strain for further experiments.

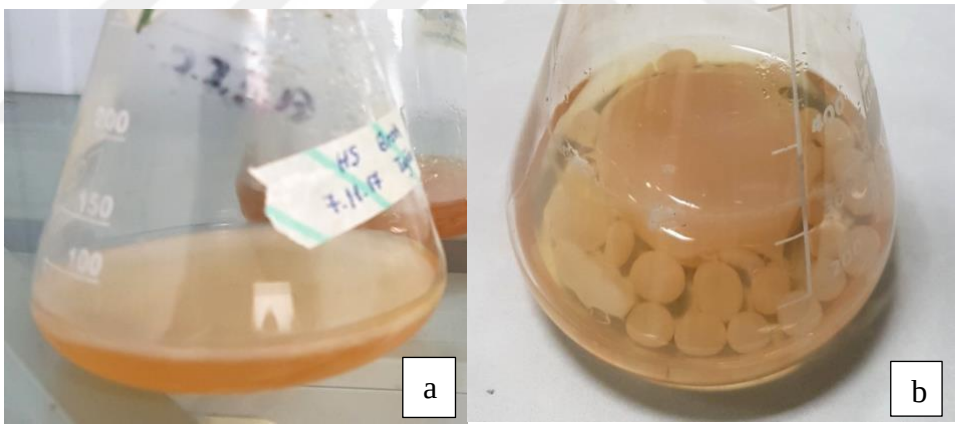


Figure 3.1: BC produced in (a) static culture, (b) agitated culture.

3.1.2 Optimization of the culture method for bacterical cellulose production

With the strain ATCC 53582, it was shown that statical culture led to much higher production yields. On the other hand, it has been reported that agitation could provide an increase in BC generation due to more effective aeration on the surface of the culture (Son et al., 2003; Mohite et al., 2013). This is particularly important since BC-producing bacteria are strongly oxygen-dependent microorganisms. To test this hypothesis, lower agitation speeds: 20, 40 and 80 rpm were investigated with different surface area/volume ratios in terms of BC production. To investigate the effect of the

S/V ratio on BC production, 500 ml Erlenmeyer flasks and 50 ml centrifuge tubes were used as shown in Figure 3.2. To provide a respectively larger surface area, 150 ml HS was prepared in 500 ml Erlenmeyer flasks (Figure 3.2a), while the volume for centrifuge tubes was 15 ml (Figure 3.2b).

Table 3.1: BC production by different strains.

		BC dry weight (mg)	
		ATCC 23767	**ATCC 53582**
Day 3	Static	-	3.9
	agitated	-	0.6
Day 6	Static	0.1	13.6
	Agitated	0.8	9.7
Day 10	Static	1.5	23.8
	Agitated	2.6	20.2
Day 14	Static	3.4	51.2
	agitated	3.8	25.7

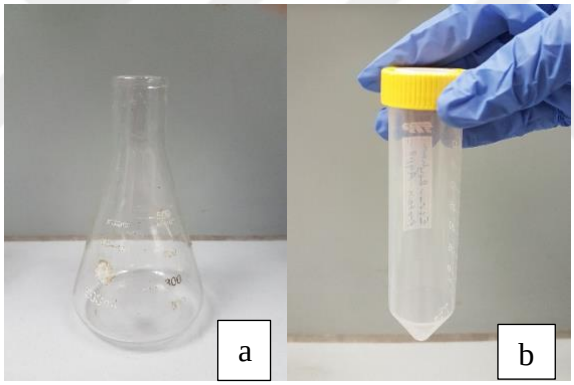


Figure 3.2 : (a) 500 ml Erlenmeyer flask, (b) 50 ml centrifuge tube.

Bacterial cellulose production efficiencies by dry weight (g/l) were compared for static conditions and at agitation speeds of 20 rpm, 40 rpm, and 80 rpm. BC production both in Erlenmeyer flasks and centrifuge tubes reached their highest values at 20 rpm with 4.53 g/l and 2.77 g/l, respectively as shown in Table 3.2. Moreover, regardless of the culture vessel, the BC amount decreased gradually from 20 rpm to 80 rpm agitation speed.

Singhsa et al. (2018) stated that agitated conditions enhanced the oxygen diffusion in the culture, resulting in higher bacterial cellulose production. It has been concluded that agitated cultures provide improved aeration by increasing the air-liquid surface

area and positively affect the microbial activity and thus bacterial cellulose production (Mohite et al., 2013).

Table 3.2: BC production according to the varying culture conditions.

	BC dry weight				
Culture condition	**Erlenmeyer (500 ml)**		**Centrifuge tube (50 ml)**		
	g/150 ml	**g/l**	**g/15 ml**	**g/150 ml**	**g/l**
Static	0.641	4.27	0.026	0.26	1.72
20 rpm	0.679	4.53	0.042	0.42	2.77
40 rpm	0.382	2.55	0.041	0.41	2.74
80 rpm	0.203	1.35	0.017	0.17	1.14

Therefore, the increase in bacterial cellulose production when shifting from static condition to 20 rpm agitation speed can be explained by enhanced oxygen diffusion. On the other hand, it has been shown by several studies that agitation can increase mutant cell formation which leads to decreased BC production, as observed in this study (Singhsa et al., 2018; Aydın Yaşar and Deveci Aksoy, 2014). The gradual decrease in bacterial cellulose production from 20 rpm to 80 rpm can be explained by the increase in mutant cell formation may be associated with the raise in mutant cell formation.

Previous studies dealing with the relation between agitation speed and BC production efficiencies are mainly conducted at speeds of 100 rpm and above. However, Krystynowicz et al. (2002) performed BC production at 4, 6, 8, 10, 12 and 16 rpm and showed that BC production efficiency decreased gradually below 4 rpm and increased above 12 rpm. These findings support our study with higher efficiency at 20 rpm than at static conditions.

3.1.3 Surface area / volume relation in bacterical cellulose production

The surface areas of Erlenmeyer flask and centrifuge tube were calculated according to the equations 3.1 and 3.2.

The surface area of the Erlenmeyer was 88.25 cm^2 and the surface area of the centrifuge tube was found to be 8.04 cm^2. According to the surface areas found, the surface area (S) /volume (V) values were calculated with the following equations:

For Erlenmeyer flask:

r = 5.3 cm , S ($\prod r^2$)= 88.25 cm^2 , V= 150 cm^3

$S / V = 88.25\ cm^2 / 150\ cm^3 = 0.5883\ cm^{-1}$ (3.1)

For centrifuge tube:

$r = 1.6$ cm, $S\ (\prod r^2) = 8.04\ cm^2$, $V = 15\ cm^3$

$8.04\ cm^2 / 15 cm^3 = 0.5360\ cm^{-1}$ (3.2)

It was observed that a higher S/V ratio augmented BC production at static conditions (1.72 g/l to 4.27 g/l) and at 20 rpm agitation speed (2.7 g/l to 4.53 g/l) as shown in Figure 3.3. The surface area/volume ratio did not seem to considerably affect the production at 40 and 80 rpm agitation speeds.

Similarly, Çakar et al. (2014) demonstrated that an increase in the surface area/volume ratio increased BC production in static culture. This rise has been related to the enhanced oxygen transfer in larger surface areas, which enable higher microbial activity on the top layer of the culture (Cakar et al., 2014; Krystynowicz et al., 2002).

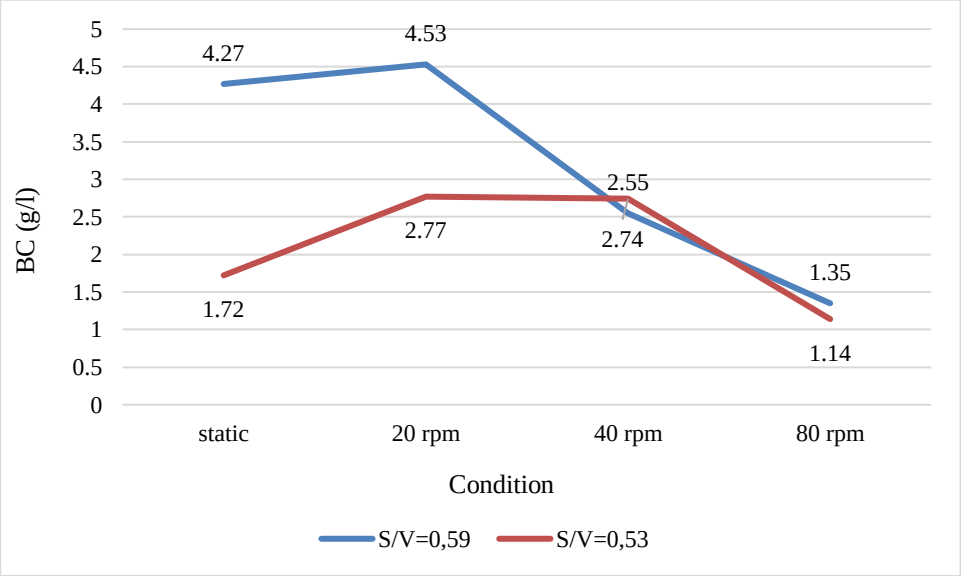


Figure 3.3: BC production according to S/V ratio.

3.1.4 Nitrogen and carbon conversion rates in different conditions

Conversion rates of carbon source (glucose) and nitrogen source (peptone + yeast extract) in HS medium to bacterial cellulose were calculated with Equations 2.4 and 2.5. The calculated conversion rates refer to the bacterial cellulose production efficiency according to the consumption of carbon and nitrogen source. Carbon and nitrogen conversion rates in Erlenmeyer flasks and 50 ml centrifuge tubes in static conditions and at various agitation speeds are given in Table 3.3.

Table 3.3: C and N conversion rates in different cultivation types and S/V ratios.

S/V ratio	Cultivation type	Conversion rate of C (%)	Conversion rate of N (%)
0.59	Static	21.36	42.71
	20 rpm	22.63	45.26
	40 rpm	12.73	25.46
	80 rpm	6.77	13.53
0.53	Static	8.58	17.16
	20 rpm	13.83	27.66
	40 rpm	13.72	27.43
	80 rpm	5.7	11.4

Using the results given in Table 3.3, the conversion rates of carbon and nitrogen sources in the HS medium are shown in Figures 3.4 and 3.5.

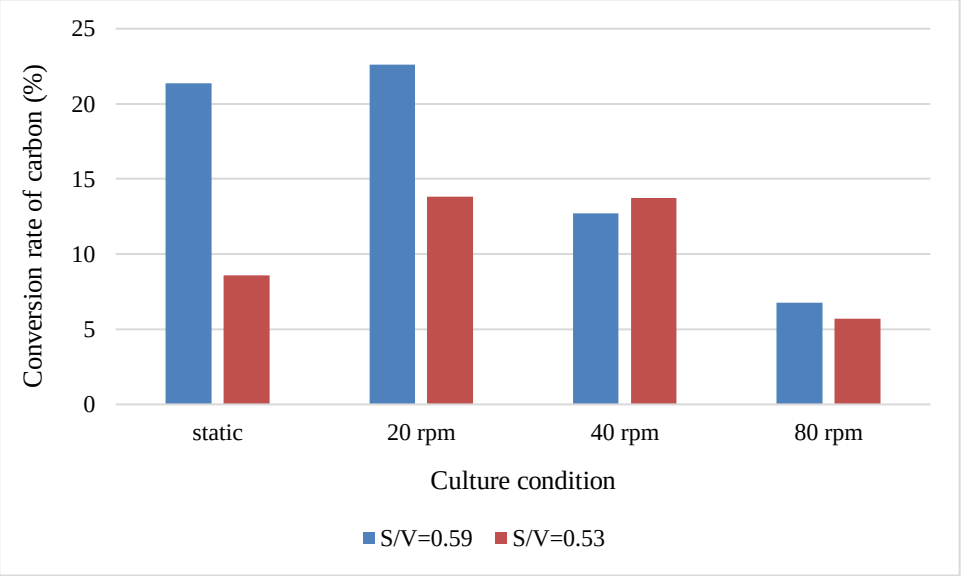


Figure 3.4: Conversion rates of carbon sources in different culture conditions and S/V ratios.

Krystynowicz et al. (2002) reported that the total glucose in the HS medium was consumed by *Gluconacetobacter xylinus* in 7 days. Therefore, for the calculation of conversion rates, the glucose was assumed to have depleted completely. As seen in Figure 3.4, the carbon source conversion rate is highest for 20 rpm agitation speed in both S/V 0.59 and S/V 0.53: 22.63 % and 13.83 %, respectively.

To calculate the conversion rates of nitrogen sources in the HS medium, the total amount of yeast extract and peptone in the medium was considered as nitrogen sources (Puglia et al., 2016). As shown in Figure 3.5, nitrogen source conversion was the highest at 20 rpm agitation speed. Conversion rates were found to be 45.26 % and 27.66 % for S/V 0.59 and S/V 0.53, respectively.

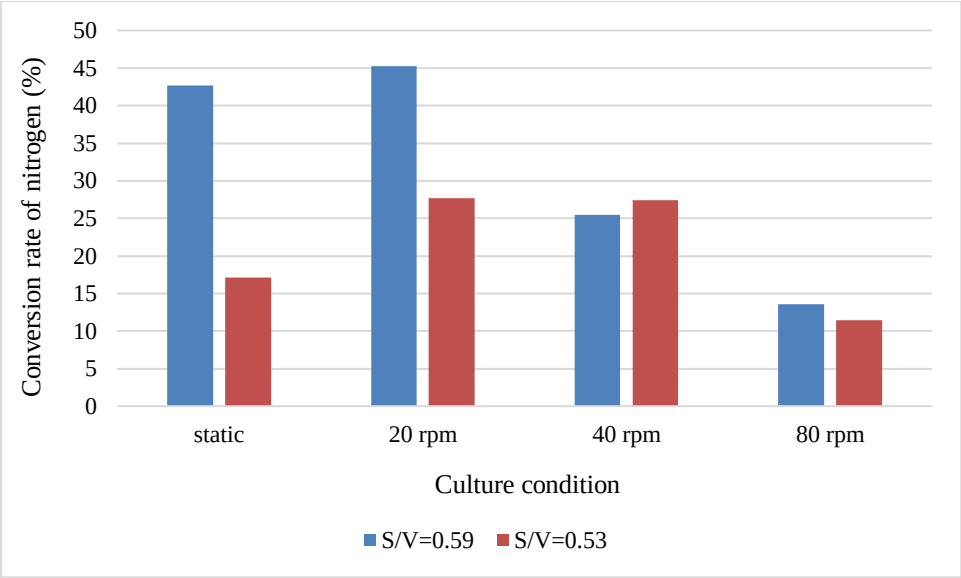


Figure 3.5: Conversion rates of nitrogen sources in different culture conditions and S/V ratios.

3.2 Bacterical Cellulose Production in Alternative Media

3.2.1 Bacterical cellulose yield comparison in different sources

Meat-bone flour, fish flour and olive pomace were tested as nitrogen sources for BC production. As carbon source, lactose obtained from whey was utilized. At the end of 14 days of incubation time, no BC was observed in the meat-bone flour medium, while a very little amount was obtained in fish flour. The dry olive mill had the highest BC production value with 0.37 mg as shown in Table 3.4.

Table 3.4: BC production in different alternative media.

Medium	BC (mg)
Meat-bone flour	-
Fish flour	0.008
Olive pomace	0.368

3.2.2 Lactose concentration effect on static and 20 rpm-stirred culture in olive pomace

As starting amount of lactose was 12.5 g (50 g/l), an optimization setup to investigate the lactose concentration effect on BC production was conducted with lactose amounts varying from 5 g (20 g/l) to 25 g (100 g/l).

Results indicate that while there is an increasing BC production trend up to 80 g/l lactose and BC amount decreases dramatically with 100 g/l lactose in static conditions. However, lactose concentration does not seem to affect BC production in vessels with a proper trend at 20 rpm agitation speed as shown in Figure 3.6.

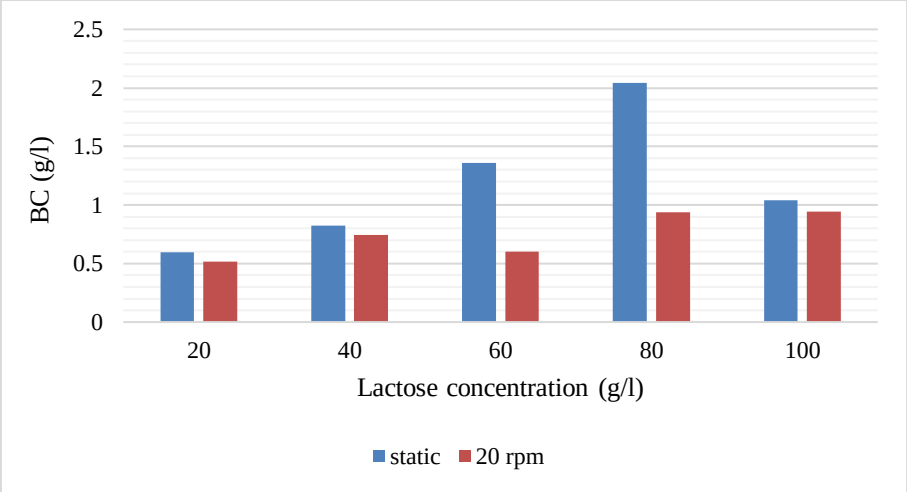


Figure 3.6: BC production in varying lactose concentrations at static and 20 rpm-stirred conditions.

This experiment aimed to minimize the carbon source cost by using lactose as it was obtained from whey. The cost is much lower compared to laboratory-grade glucose in standard HS medium. However, while 4.27 g/l BC was generated in static conditions with HS medium, of which the carbon source (glucose) concentration is 20 g/l; the BC amount is only 0.59 g/l for the medium prepared with olive pomace and 20 g/l lactose. In static conditions, it is possible to obtain approximately 2 g/l BC when the lactose amount is increased to 80 g/l, which is still much lower than the amount achieved with HS medium, despite the 4-fold higher lactose amount.

3.2.3 Investigation of lactase application on standard medium with lactose

While there is little BC production in the medium prepared with olive pomace (OP) and lactose, no BC was formed in the HS medium with lactose instead of glucose (Figure 3.7). Indeed, in many studies, lactose was found to generate much lower BC production compared to glucose. Keshk and Sameshima (2005) reported that BC yield obtained with lactose as the carbon source was only 22 % of the amount produced with glucose. Similarly, Masaoka et al. (1993) stated the same yield as 16 % of the reference. Accordingly, in this set of experiments, the effect of lactase application was investigated to test whether the glucose obtained by lactose hydrolysis would lead to higher BC production. For this, different lactase enzyme amounts were tested varying from 7.5 to 90 µl. Results are shown in Figure 3.8.

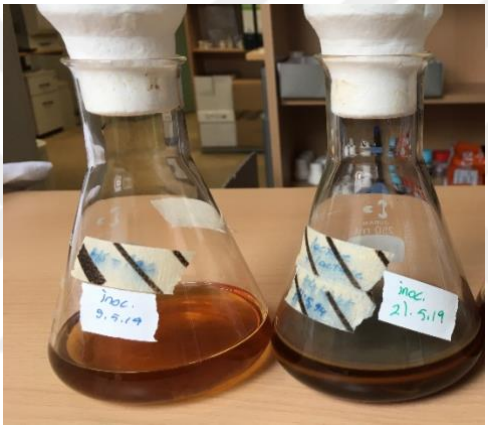


Figure 3.7: BC production in HS medium with lactose (left) and OP with lactose (right).

Results show that there is no direct correlation between the lactase amount and BC production. Although the lactase amount increased 12-fold from 7.5 µl to 90 µl, the BC amount increased only 0.3 g/l. On the other hand, 30 µl lactase application resulted in a lower amount of BC compared to 7.5 µl lactase. Therefore, it can be said that lactase addition to obtaining glucose is not an effective method to generate higher yields of BC. Moreover, since the use of any enzyme would increase the production cost in this step, it was concluded not to proceed with lactose in the growth medium prepared with olive pomace.

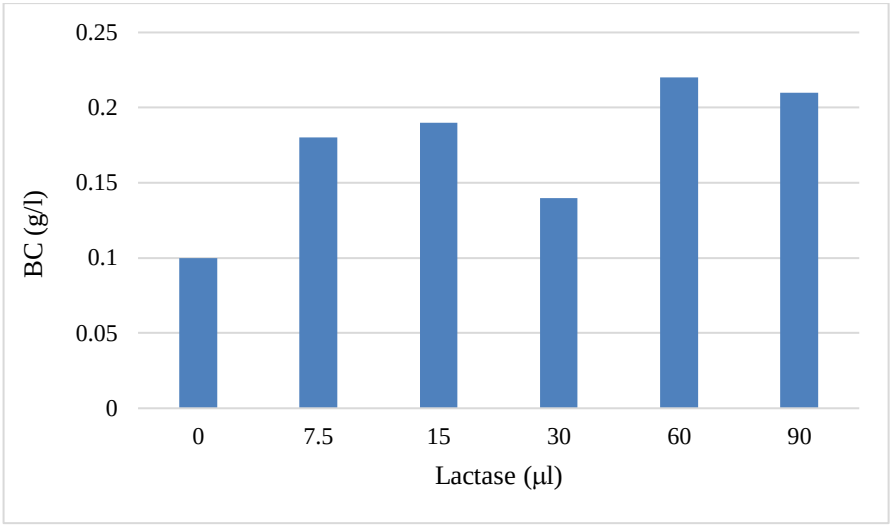


Figure 3.8: BC dry weights obtained according to lactase amounts in media.

Results show that there is no direct correlation between the lactase amount and BC production. Although the lactase amount increased 12-fold from 7.5 µl to 90 µl, the BC amount increased only 0.3 g/l. On the other hand, 30 µl lactase application resulted in a lower amount of BC compared to 7.5 µl lactase. Therefore, it can be said that lactase addition to obtaining glucose is not an effective method to generate higher yields of BC. Moreover, since the use of any enzyme would increase the production cost in this step, it was concluded not to proceed with lactose in the growth medium prepared with olive pomace.

3.2.4 Elementary analysis of bacterical cellulose produced in olive pomace medium

Elementary analysis was applied to investigate carbon, hydrogen, nitrogen and sulfur concentration of BC produced in OP medium. The content of these elements is shown in Table 3.5. The results indicate that the BC produced in OP (BC-OP) is mainly composed of C and H, as expected. On the other hand, it contains about 2.5 % N. According to Jung et al. (2003) and Simonne et al. (1997), N percentage can be converted into total protein percentage by a conversion factor of 6.25. As a result it is concluded that the sample had 14.25 % protein contamination.

Table 3.5: C, H, N and S contents of BC-OP indicated by Elementary Analysis.

Elements	% in total (w/w)
N	2.28
H	5.98
C	45.88

3.2.5 Removal of protein contamination

To compare the protein contamination removal efficiencies of BC-HS and BC-OP, the samples were subjected to stirring washing in 0.1M NaOH for 11 days. At the end of days 4, 7 and 11; small pieces of BC were collected from each sample. The N percentage values of samples are shown in Figure 3.9. It can be concluded that increasing the washing period positively affects the removal of protein contamination from both BC. On the other hand, while all protein contamination in BC-HS was eliminated at the end of the test time, BC-OP still contained 2.75 % protein. This amount of contamination might also be removed by extending the washing time, however, this is undesirable since it would lead to more energy consumption and elongate the production process.

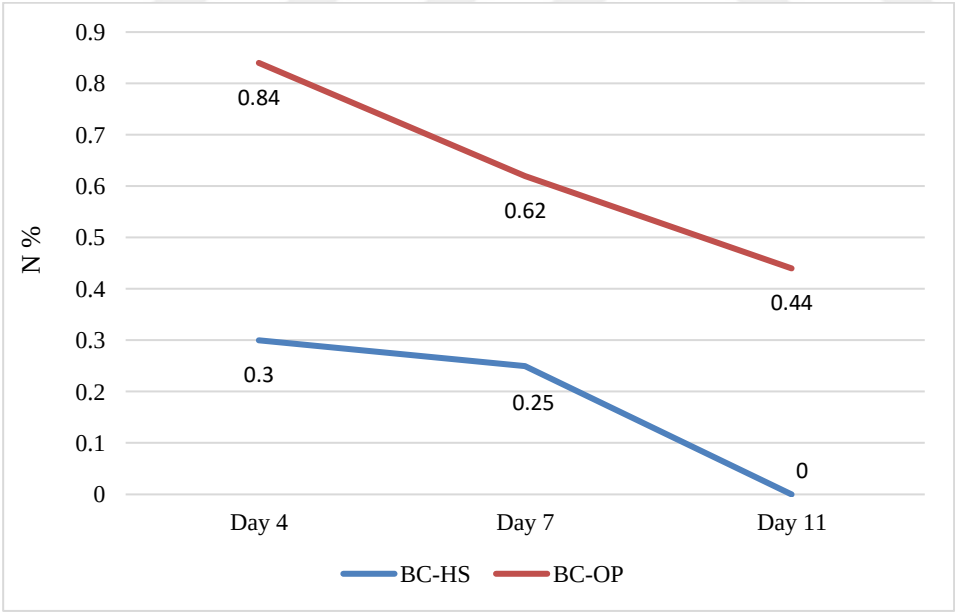


Figure 3.9: N percentage of BC-HS and BC-OP according to days, during NaOH washing.

3.2.6 Elementary analysis of olive pomace

Since the BC produced in OP medium was very low in amount, mechanically unstable and had protein contamination, another elementary analysis was applied to investigate

if OP was an ideal nitrogen source for microbial activity. N, H and C content of OP is shown in Table 3.6. According to the results, it was concluded that OP had a very low amount of nitrogen (2.31 %), which is not sufficient to provide a good quality BC.

Table 3.6: N, H and C contents of OP indicated by Elementary Analysis.

Elements	% in total (w/w)
N	2.31
H	6.16
C	51.02

With the experiments conducted so far, it was presumed that OP was not a suitable material to be used as a growth medium for BC production. Although some BC fleece could be obtained from olive pomace and lactose mixture medium, the amount of BC is too low as mentioned in Table 3.4. Moreover, unlike the BC produced in HS, it takes much longer to get non-contaminated BC from OP to be used for medical applications, as demonstrated in Figure 3.9.

On the other hand, OP is composed of up to 50 % cellulose and hemicellulose. Therefore, in further steps, lignocellulosic hydrolysis of OP was realized to obtain reducing sugars - mainly glucose, which can be used as a carbon source for BC production.

3.3 Hydrolysis of Olive Pomace

3.3.1 Pretreatment of olive pomace with phosphoric acid

For effective enzymatic hydrolysis of lignocellulosic biomass, a pretreatment step is required to disrupt the complex structure and disembody hemicellulose and lignin from cellulose. Moreover, cellulose becomes more accessible to enzymatic degradation with decreased crystallinity and enhanced porosity upon pretreatment. Dilute acid pretreatment is one of the most widely used method which can be applied either at high temperatures (> 160 ^{o}C) with short retention times or at lower temperatures with longer retention times. It has been reported by various studies that higher temperatures with shorter retention times lead to higher sugar yields (de Vasconcelos et al., 2013). By dilute acid pretreatment, 80 – 95 % of hemicellulose-originated sugar recovery can be obtained (Balat et al., 2008).

Since the hydrogen ion of H_3PO_4 is small and can easily penetrate cellulose, it has been shown as one of the most competent dissolving agents. Phosphoric acid also provides an advantage by its ability to perform at lower temperatures (Zhang et al., 2006). Besides, after H_3PO_4 treatment, cellulose was found to remain in amorphous form, which is preferable for degradation, and the remaining phosphorous acid had no inhibitory impacts for further fermentation applications (Zhang et al., 2007). De Vasconcelos et al. (2013) demonstrated that the pretreatment of sugarcane bagasse with dilute phosphoric acid was more efficient to eliminate hemicellulose compared to hydrothermal treatment. In comparison with another study conducted by Rocha et al. (2011), phosphoric acid was found to be more powerful in hemicellulose solubilization than sulphuric and acetic acid mixtures.

Therefore, in this study, pretreatment of olive pomace was conducted with 1 % H_3PO_4 at 170 ^oC. By this, minimal generation of enzymatic and microbial inhibitors was aimed at maximal cellulose degradation.

3.3.1.1 Glucose concentration analysis of the liquid phase by glucose hexokinase assay

The absorbance of the OP liquid phase after pretreatment with 1 % phoshoric acid is shown in Figures 3.10 and 3.11.

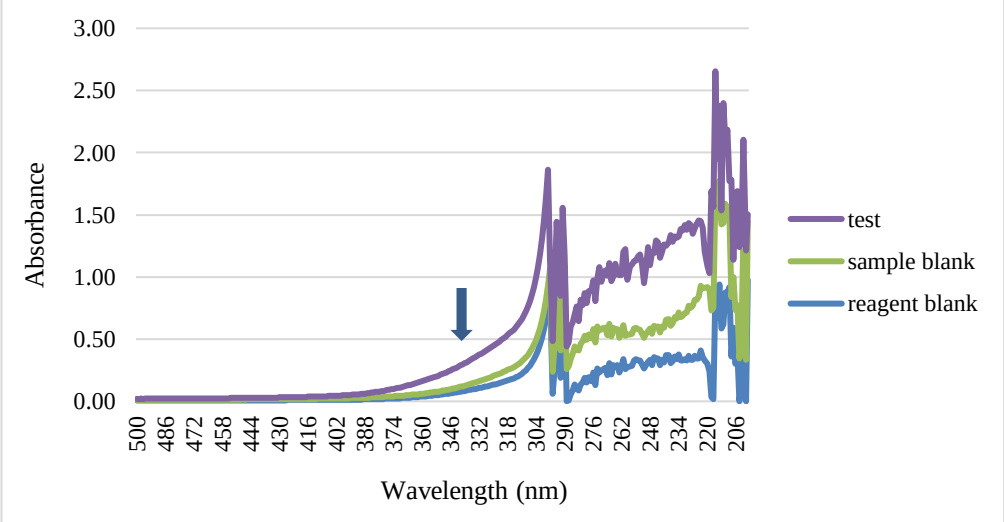


Figure 3.10: Absorbance range of the liquid phase after acid treatment between 200-500 nm.

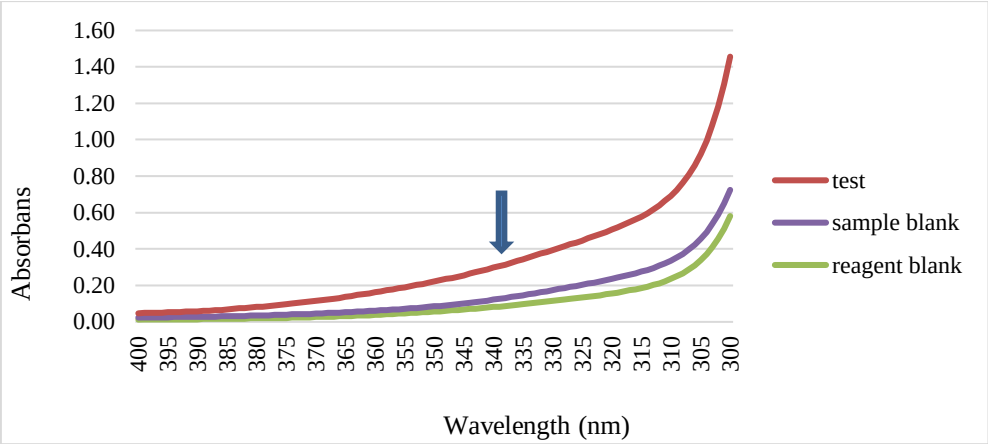


Figure 3.11: Absorbance range of the liquid phase after acid treatment between 300 – 400 nm.

Calculation of glucose concentration according to the absorbance values was done according to the conversion factors and Equations 2.8 - 2.11. The glucose concentration of the OP sample that was treated with 1 % phosphoric acid was found to be 0.185 g/l. It is stated in the kit manual that ΔA value must be between 0.03 and 1.6. Thus, it can be concluded that the result is in the confidence interval.

On the other hand, the glucose concentration was too low to lead to microbial activity that result in bacterial cellulose production.

Moreover, acid pretreatment of lignocellulosic material is known to generate inhibitors, which decreases microbial and enzymatic activity significantly. In this case, another downstream process is highly needed to remove these chemicals. However, since the glucose concentration was too low, an additional detoxification step would not be economically feasible. Therefore, it was concluded not to proceed with the sugars obtained from the acidic pretreatment.

3.3.2 Enzymatic hydrolysis of olive pomace solid phase

3.3.2.1 Reducing sugar determination of the solid phase by 3,5-dinitrosalicylic acid method

To determine the efficiency of reducing sugar yield in terms of enzyme amount, various enzyme/substrate ratios were applied in static and agitated conditions as stated in Figure 3.12. It was observed that, regardless of the condition (static or agitated), the concentration of reducing sugar increased as the enzyme amount increased. However, though enzyme amount increased by around 2 folds each time from 5 µl to 110 µl,

reducing sugar concentration did not differ dramatically. This could be due to the supersaturation of substrate by the enzyme, thus no product can be formed after a certain concentration.

Moreover, correlative to previous findings, the static condition led to a slightly higher reducing sugar yield compared to the agitated condition in each case, possibly due to the enzyme deactivation caused by agitation (Gunjikar et al., 2001; Ye et al., 2012). This result was particularly convenient in terms of reducing energy consumption. Since the static condition led to slightly higher reducing sugar concentrations, it was determined as the principal method for further growth media preparation.

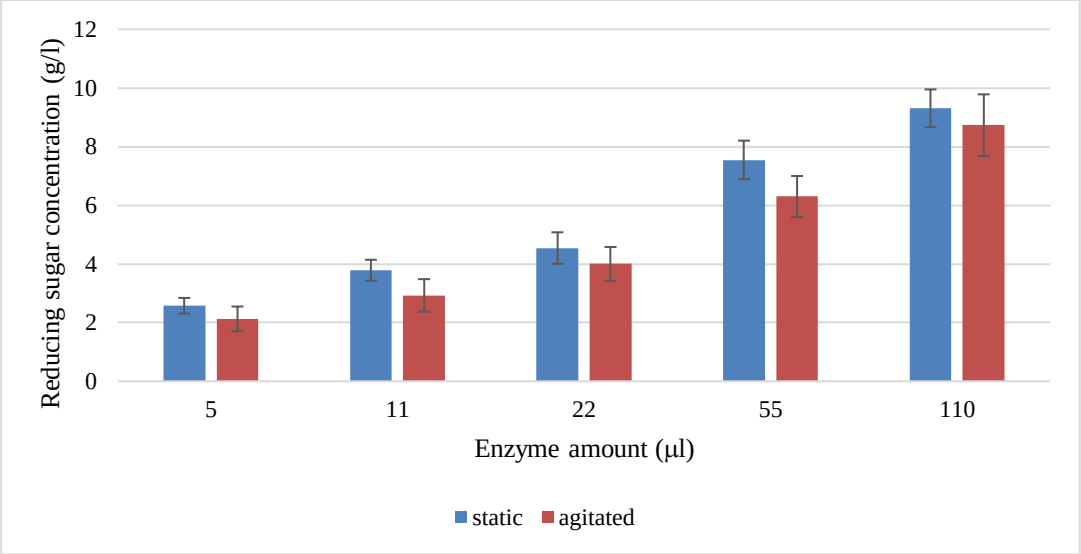


Figure 3.12: Reducing sugar concentration obtained due to enzyme amount in OP hydrolysates.

Inhibition of cellulolytic enzymes is a well-known and widely studied phenomenon, though not fully understood. Currently, enzymes obtained from *Trichoderma reesei*, which are commonly used in cellulose degradation, are known to be dramatically inhibited by products glucose and cellobiose. This product inhibition leads to decreased total transformation rate of cellulose to glucose (Gan et al., 2002; Katz and Reese, 1968). Cellobiose, formed by the action of cellobiohydrolase and to a lesser degree of endoglucanase, is a direct inhibitor of these two enzymes (Gruno et al., 2004).

Glucose, on the other hand, is an inhibitor of β-glucosidases, cellobiohydrolases and endoglucanases (Dekker, 1986; Holtzapple et al., 1990). Nevertheless, the primary mechanism for glucose inhibition is indirect, by inhibiting β-glucosidase and thus leading to cellobiose accumulation. Interestingly, it was also shown by a few studies

that cellulose and cellobiose, as substrates, can cause cellobiohydrolase, endoglucanase and β-glucosidase inhibition (Gong et al., 1977; Huang and Penner, 1991; Väljamäe et al., 2001; Oh et al., 2000).

The current data indicate that the reaction rate can be reduced by 10-100 % by glucose inhibition, depending on the substrate, enzyme, inhibitor concentrations and also on the enzyme complex utilized (Andrić et al., 2010).

Besides, the glucose formation rate from lignocellulose is slower in comparison with the other enzymatic reactions, mostly as a result of the insoluble and crystalline structure of the cellulose (Himmel et al., 2007; Zhang and Lynd, 2004). More in detail, cellulose degradation occurs in a complex and heterogeneous process during which endoglucanases and exoglucanases (cellobiohydrolases) act synergetically to disintegrate cellulose at the solid-liquid interface. Then, intermediate products such as cellulooligosaccharides and cellobiose are broken down to glucose via liquid-phase hydrolysis (Kubicek, 1992; Zhang and Lynd, 2004). When lignocellulose is pretreated physically or chemically, both crystalline and amorphous forms of cellulose are generated (Lynd et al., 2008; Mosier et al., 2005). As a result, the enzymatic reaction takes place at different rates for each form of substrate. Moreover, catalytic activity differs throughout the reaction depending on the substrate type; amorphous cellulose tends to degrade at the initial phase while crystalline is broken later at a slower rate (Lynd et al., 2002). Thus, due to the heterogeneity of reactions, conventional Michaelis-Menten equations do not correctly fit to define these reactions and their inhibition schemes. Several inhibition patterns have been suggested for cellulases such as hyperbolic inhibition -a type of partial inhibition- and a special scheme in which enzyme-inhibitor complex (EI) is formed with the inhibitor or the substrate (Bezerra and Dias, 2004). In this case, the excess amount of substrate may act as an inbititor of the catalysis.

Moreover, the enzyme to substrate ratio (E/S) is another important factor in terms of cellulase inhibition. When E/S is comparatively high, reactive sites on cellulose can be fully saturated by cellulase. This may lead to competitive or pseudo-competitive inhibition in which cellulases negatively affect the reaction because of the insufficient substrate amount (Gusakov and Sinitsyn, 1992). The competitive enzyme-substrate interaction may also cause unproductive binding which decreases the reaction rate (Medve et al., 1998; Ryu et al., 1984). In this case, enzymes bind to the substrate non-

productively and thus render it inactive. These inactive enzymes also hinder the binding of active enzymes to substrate (Andrić et al., 2010).

However, the strongest inhibition of cellulases is accepted to be caused by lignin. During hydrothermal pretreatments including dilute acid catalyzed hydrolysis, hemicellulose becomes more accessible and thus soluble. In this way, hemicellulose and hemicellulose-originated inhibitors can be washed and separated from the slurry. However, most of the lignin remains in the solid fragment, which in turn causes strong inhibition of cellulases (Ko et al., 2015a). The macropores in the lignocellulosic substrate are opened up when pretreated, which also increases the amount of lignin that would bind to cellulases non-productively and generate physical blockages for cellulase activity (Kim et al., 2015; Chang and Holtzapple 2000). Therefore, lignin not only prevents the enzyme access to cellulose but also diminishes the active enzyme amount to bind cellulose (Nakagame et al. 2011). Moreover, the inhibition of lignin renders more dramatical as the harshness of the pretreatment increases due to the higher amount of lignin formed. High lignin content induces alterations in lignin structure by de- and re-polymerization (Ko et al., 2015a). Ko et al. (2015b) presented that liquid hot water pretreated lignin transforms into a denser structure which affects cellulase activity more severely than unpretreated one. This condensed and hydrophobic structure of lignin also adsorbs the enzymes more strongly due to increased hydrophobic interactions (Nakagame et al., 2011). Related to this thesis research and the enzyme used in the work, Ko et al. (2015b) also demonstrated that 50 – 60 % of cellulase was inactivated when 10 mg cellulase was treated with lignin obtained from the pretreatment of hardwoods. In the same study, the Cellic CTec2 enzyme was shown to lose more than 90 % of the β-glucosidase activity through lignin inhibition.

Furthermore, lignin derived phenolics (syringyl aldehyde, syringic acid, vanillin, vanillic acid, ferulic acid, p-hydroxybenzoic acid, trans-cinnamic acid, coumaric acid) are also reported to interfere with cellulolytic enzyme activity even at micromolar to millimolar concentrations (Ximenes et al., 2010). Indeed, phenolics, among other soluble inhibitory compounds such as sugars, weak acids, furan aldehydes, were found to have a more toxic effect on cellulase (Kim et al., 2011). In a study, syringyl aldehyde at 10 mM concentration was shown to lower cellulose degradation by around 70 %. Oligomeric phenolics, on the other hand, were reported to have a higher inhibition effect on cellulases compared to simple phenolics. 1mM of tannic acid decreased the

cellulase activity by 70 – 80 % (Tejirian and Xu, 2011). In addition, phenolics inhibition of cellulase activity leads to the constant loss of the enzyme since phenolics in the hydrolysate aggregate with the enzyme (Kim et al., 2011). The degree to which the enzyme is deactivated depends on the microorganism source and type of the enzyme as well as the phenolic compound type and concentration in the hydrolysate. For example, β-glucosidase isolated from Trichoderma reesei was found to be more susceptible to gallic acid and p-coumaric acid inactivation compared to the one obtained from Aspergillus niger (Ximenes et al., 2011).

Reducing sugar amount obtained upon enzymatic hydrolysis may have been affected by all factors discussed above. However, it is evident that the factors which contribute to the results given above were mostly the product inhibition of cellulases by glucose and cellobiose; and also substrate saturation which gives rise to unproductive binding of cellulases to cellulose. Since the starting dry matter amount is the same for each enzymatic condition and they are formed in the same acidic pretreatment batch, residual (remaining after washing of the solid material) lignin and lignin derived phenolics which interfere with the enzymatic activity must be identical. Therefore, the inhibition caused by these compounds should be identical for each enzymatic reaction. On the other hand, glucose and cellobiose should be naturally generated in larger amounts in enzymatic reactions with higher enzymatic concentrations, which in turn act as inhibitors of β-glucosidases, cellobiohydrolases and endoglucanases. Similarly, higher cellulase concentration at the same amount of cellulose would result in greater inhibition by enzymes which ineffectively occupy cellulose and prevent the binding of active enzymes.

Consequently, it can be concluded that while more reducing sugars were formed as the enzyme amount increased, some parts of them were lost by the inhibition, which correspondingly explains the nonproportional rise of reducing sugar concentration.

In respect of agitated conditions in enzymatic hydrolysis, shear stress is worth to mention. Indeed, the deactivation of enzymes due to shear stress caused by agitation is broadly studied. Particularly in the case of cellulases, it was shown that deactivation is more triggered with higher agitation speed regardless of the presence of substrate. All components of cellulases, namely endoglucanases, exoglucanases and β-glucosidases showed decreasing activiy with increasing agitation, however, at varying degrees for each enzyme (Gunjikar et al., 2001).

Therefore, it can be presumed that obtained reducing sugar amount in each enzymatic reaction was lower in the case of agitation compared to the static condition due to shear stress.

3.3.2.2 High pressure liquid chromatography (HPLC) analysis of the reducing sugars after enzymatic reaction

With the HPLC analysis, the presence of galactose, glucose, mannose, arabinose, xylose, rhamnose, lactose, fructose, maltose and cellobiose in enzymatic hydrolysates was investigated and shown in Figures 3.13 to 3.17.

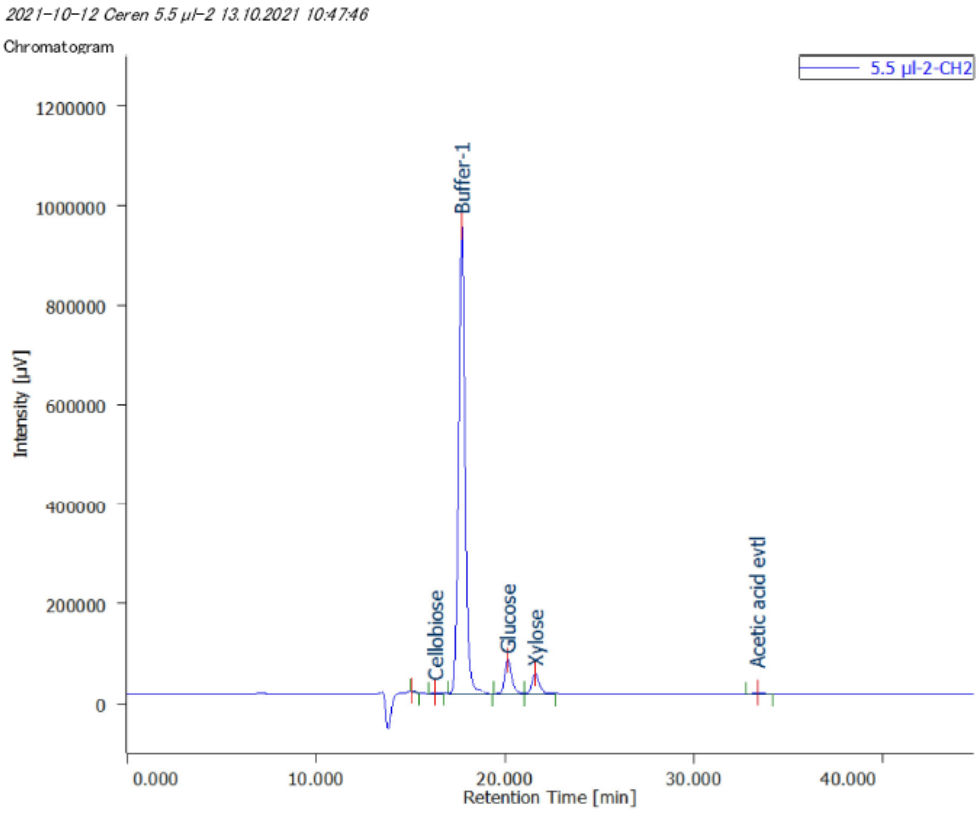
In Figure 3.18, the amount of sugars (%) in each sample is indicated. Both glucose and xylose+fructose yield increased as the enzyme amount increased. Nevertheless, glucose amount was much more affected by enzyme concentration. The yield of glucose and xylose+fructose was 7 %, and 4.3 % respectively for ENZ 5.5. In the hydrolysate ENZ 11, the glucose amount was 11.5 % while xyl+fru was 5.1 %. In the hydrolysate ENZ 22, the values were 12.5 % and 5.6 %; for ENZ 55, 22.9 % and 6.8 %, and finally for ENZ 110, 29.2 % and 7.5 %, respectively for glucose and xyl+fru. In the case of xyl+fru, the increase in sugar amount with increasing enzyme was not as high as in the case of glucose. A 22-fold rise in enzyme amount (from ENZ 5.5 to ENZ 110) increased xylose only by 3.5 %, while the same amount of enzyme provided a 22.2 % increase for glucose.

Although the amounts vary between samples, the dominant sugar in the enzymatic solutions is glucose, which is followed by xylose as shown in Figures 3.13 – 3.17. This finding supports that the enzymatic solutions could be used as growth media due to their high content of glucose, as glucose has been shown to be the most preferable carbon source for BC producing microorganisms, while i.e. xylose cannot be metabolized effectively (De Muynck et al., 2006; Keshk and Sameshima, 2005; Ishihara et al., 2002).

3.4 Bacterial Cellulose Production in Enzymatic Hydrolysates

The growth medium with hydrolysate ENZ 110 was prepared with the addition of yeast extract, peptone and Na_2HPO_4 to use the reducing sugars coming from the enzymatic reaction as carbon source. Since the citric acid concentration in the enzyme buffer was already higher than the concentration of those in the Hestrin-Schramm medium, no

additional citric acid was included. Using this medium, no BC was obtained. Therefore, the toxic effect of citric acid and inhibitory compounds generated by pretreatment was investigated.



(a)

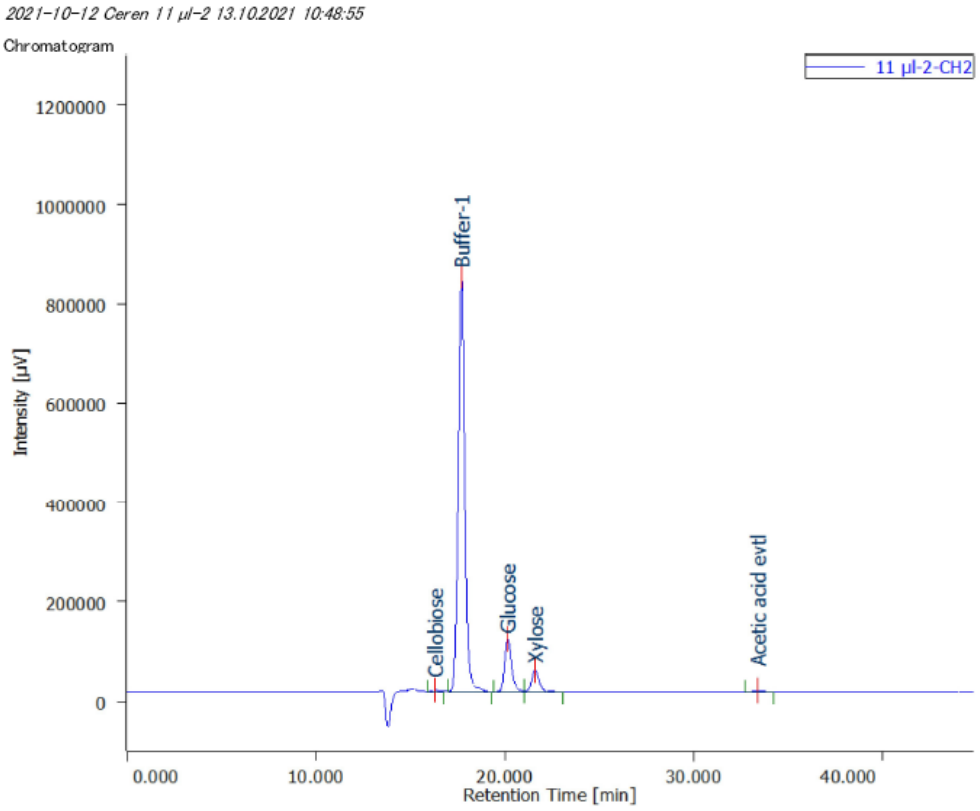
Channel & Peak Info.Table

Chromatogram Name	5.5 µl-2-CH2
Sample Name	
Channel Name	RI
Sampling Interval	500 [msec]
Peak Method	Integration1

#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Quantity
2	Cellobiose	2	16.308	40035	1766	0.156	N/A
3	Buffer-1	2	17.725	22609977	941702	88.221	N/A
4	Glucose	2	20.150	1776910	67870	6.933	N/A
5	Xylose	2	21.608	1090433	40519	4.255	N/A
6	Acetic acid evtl	2	33.367	92862	2677	0.362	N/A

(b)

Figure 3.13: (a) HPLC Chromatogram (b) Peak info table of ENZ 5.5.



(a)

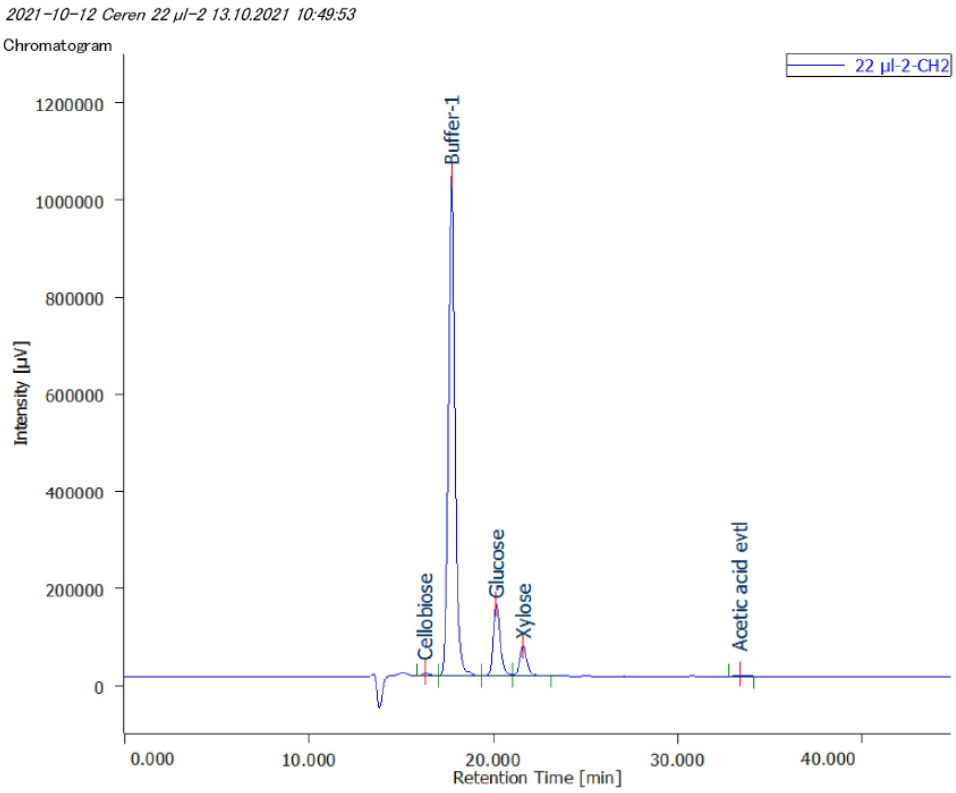
Channel & Peak Info.Table

Chromatogram Name	11 µl-2-CH2
Sample Name	
Channel Name	RI
Sampling Interval	500 [msec]
Peak Method	Integration1

#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Quantity
1	Cellobiose	2	16.308	64739	2852	0.267	N/A
2	Buffer-1	2	17.717	20054176	834491	82.800	N/A
3	Glucose	2	20.150	2775061	105476	11.458	N/A
4	Xylose	2	21.600	1223972	43825	5.054	N/A
5	Acetic acid evtl	2	33.375	102094	2904	0.422	N/A

(b)

Figure 3.14: (a) HPLC Chromatogram (b) Peak info table of ENZ 11.



(a)

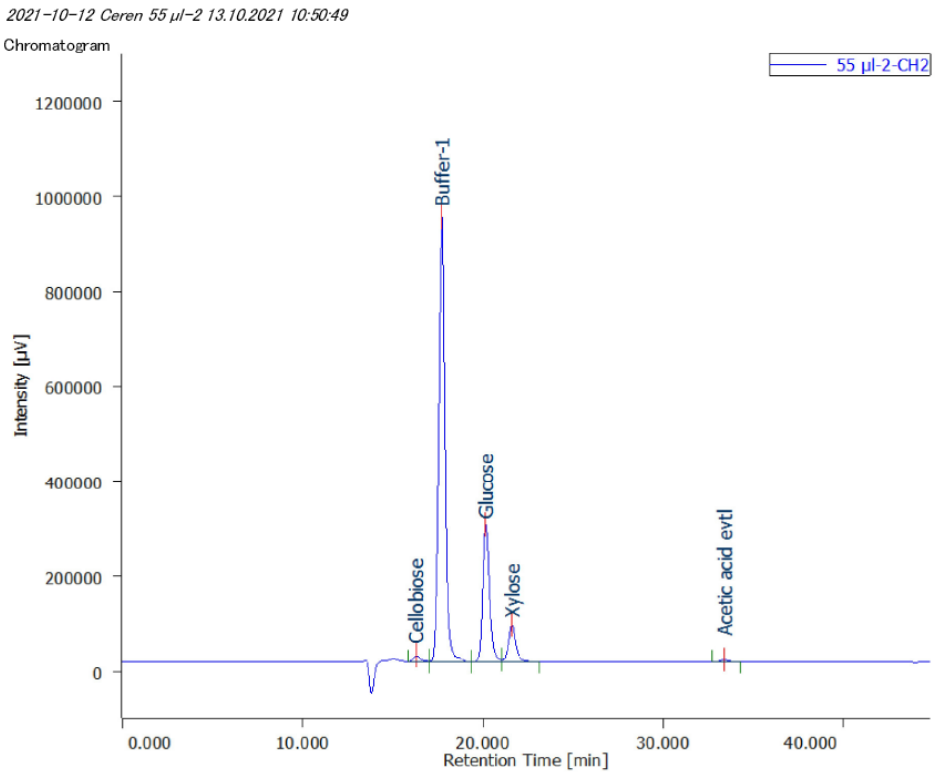
Channel & Peak Info.Table

Chromatogram Name	22 µl-2-CH2
Sample Name	
Channel Name	RI
Sampling Interval	500 [msec]
Peak Method	Integration1

#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Quantity
1	Cellobiose	2	16.300	160729	6130	0.515	N/A
2	Buffer-1	2	17.742	25344637	1029006	81.137	N/A
3	Glucose	2	20.158	3903234	147460	12.496	N/A
4	Xylose	2	21.608	1749244	61871	5.600	N/A
5	Acetic acid evtl	2	33.367	78845	2314	0.252	N/A

(b)

Figure 3.15: (a) HPLC Chromatogram (b) Peak info table of ENZ 22.



(a)

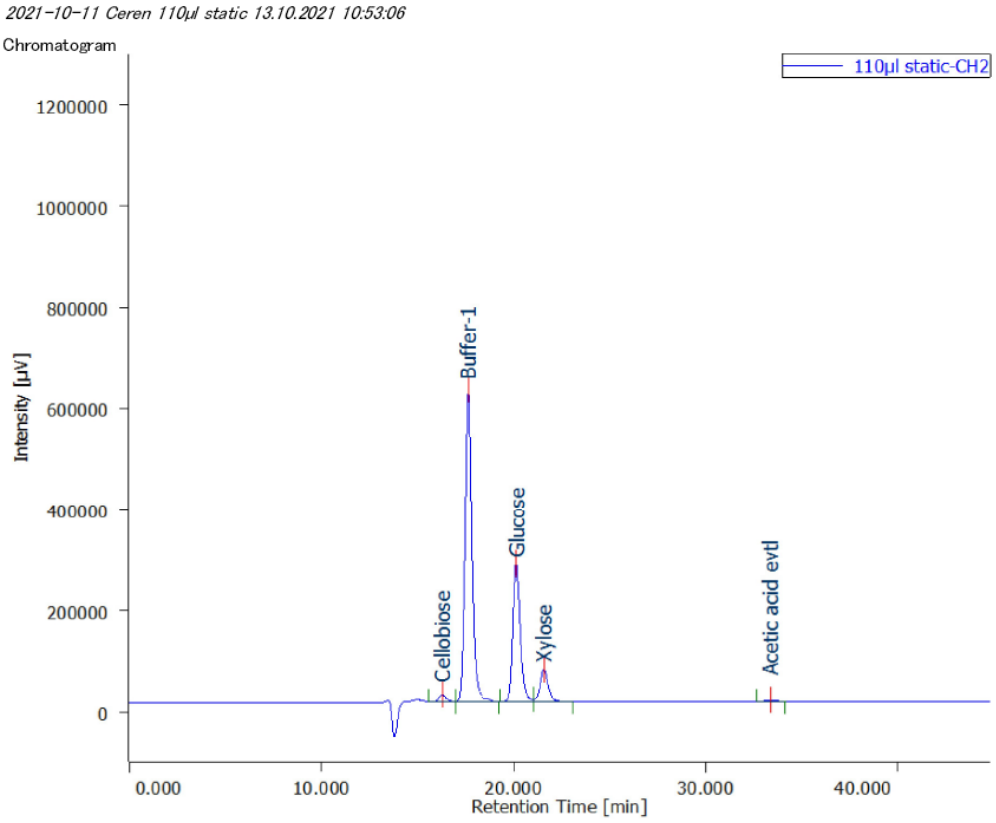
Channel & Peak Info.Table

Chromatogram Name	55 µl-2-CH2
Sample Name	
Channel Name	RI
Sampling Interval	500 [msec]
Peak Method	Integration1

#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Quantity
1	Cellobiose	2	16.300	290381	11451	0.893	N/A
2	Buffer-1	2	17.725	22449898	937840	69.030	N/A
3	Glucose	2	20.158	7432548	285892	22.854	N/A
4	Xylose	2	21.608	2196607	77204	6.754	N/A
5	Acetic acid evtl	2	33.383	152365	4449	0.469	N/A

(b)

Figure 3.16: (a) HPLC Chromatogram (b) Peak info table of ENZ 55.



(a)

Channel & Peak Info.Table

Chromatogram Name	110µl static-CH2
Sample Name	
Channel Name	RI
Sampling Interval	500 [msec]
Peak Method	Integration1

#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Quantity
1	Cellobiose	2	16.275	361914	13695	1.434	N/A
2	Buffer-1	2	17.650	15537743	615658	61.565	N/A
3	Glucose	2	20.125	7351276	271113	29.128	N/A
4	Xylose	2	21.575	1895677	63596	7.511	N/A
5	Acetic acid evtl	2	33.350	91239	2622	0.362	N/A

(b)

Figure 3.17: (a) HPLC Chromatogram (b) Peak info table of ENZ 110.

3.4.1 Growth media preparation with enzymatic hydrolysates – role of citric acid

The effect of citric acid concentration on microbial inhibition was investigated as explained under the title 2.5.1. BC formation in wells on the 10th day of incubation is shown in Figure 3.19. BC dry weights after 21 days of incubation are given in Table 3.7.

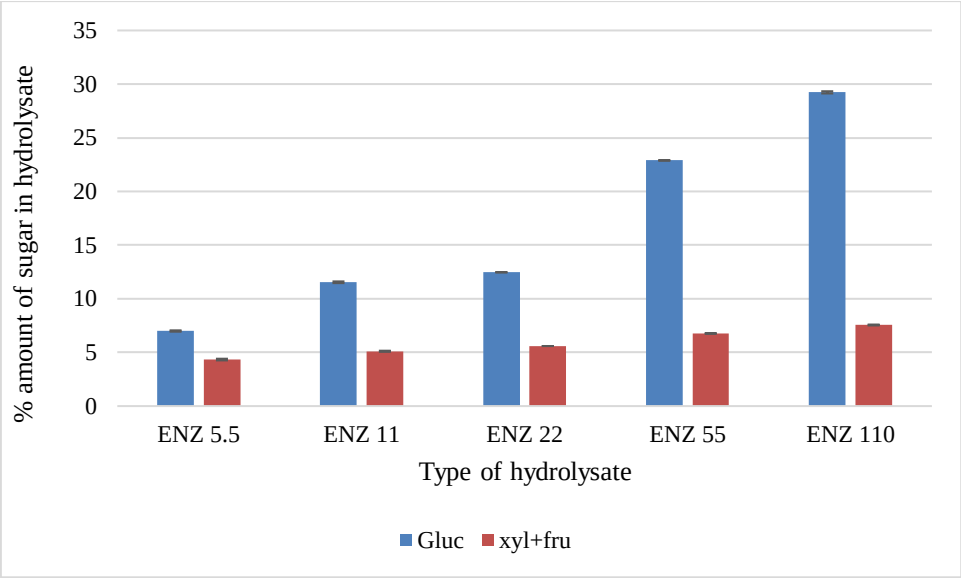


Figure 3.18: Sugar composition of hydrolysates obtained by enzymatic hydrolysis of OP.

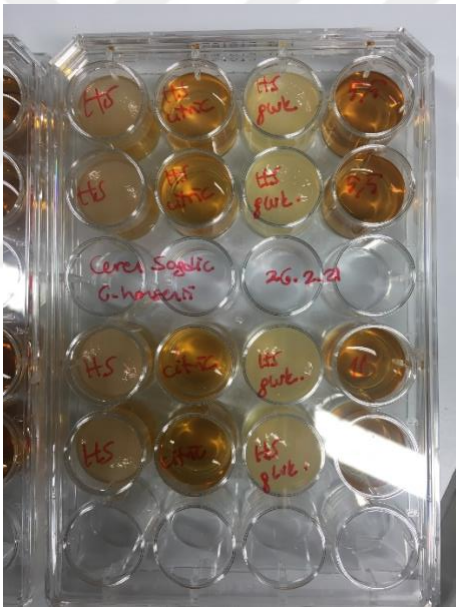


Figure 3.19: HS: Hestrin-Schramm medium; HS citric ++: HS media including the same concentration of citric acid found in the sodium citrate buffer (4.5 g/l); HS gluc: HS media containing the same concentration of glucose as the reducing sugar concentration of the related enzymatic solution.

Table 3.7: Amount of BC produced due to the growth media type.

Growth media	Amount of BC produced (g/l)
HS	5.125
HS citric ++	4.125
HS gluc (5.28 g/l)	1

The results show that the citric acid concentration of buffer does not significantly affect the BC production. These results support the findings of Yassine et al. (2016) that stated that even 2.6 M citric acid (499. 5 g/l) did not inhibit BC formation, which is much higher compared to 4.5 g/l in the enzyme buffer.

On the other hand, as shown in Figure 3.19, BC formation took longer in the HS citric ++ wells compared to the HS wells. More specifically, from the 3rd day of incubation, BC formation started to be observed in wells where citric acid concentration was 1.15 g/l. However, in wells with 4.5 g/l citric acid concentration, BC formation occurred on day 7. This could be explained as the adaptation period of bacteria to a relatively less favourable environment. Similarly, it is indicated that lower medium pH retards carbon source utilization compared to the standard medium (Yassine et al., 2016).

3.4.2 Comparison of methods for the detoxification of enzymatic hydrolysates

As stated before, treatment of lignocellulosic material leads to the production of compounds that inhibit microorganismal growth. To overcome this inhibition, many strategies have been suggested. On the other hand, these applications may lead to some sugar loss in the treated solution. The impact of each method depends on the pretreatment strategy and the pretreated material. Thus, a preliminary assay was conducted to investigate the most appropriate procedure which does not cause considerable sugar loss in this assay. $Ca(OH)_2$, NaOH and activated charcoal were used as detoxifying agents. After blank correction, absorbance values were obtained as shown in Table 3.8. According to the result, activated charcoal and $Ca(OH)_2$ were shown to affect the glucose concentration the least. Therefore, detoxification of the enzymatic hydrolysates was conducted with these 2 methods.

Table 3.8: DNSA assay of detoxification methods-absorbance differences in accordance with the blank.

Detoxification method	Absorbance difference in accordance with the blank
Activated charcoal	0
$Ca(OH)_2$	0.14
NaOH	0.9

The findings were in line with the work by Guo et al. (2013) which indicates that glucose concentration dropped by 4 % and 13 % upon activated charcoal and $Ca(OH)_2$ treatments, respectively, in spruce hydrolysate. However, in the same study, NaOH

treatment caused a decrease in glucose only by 3 %. On the other hand, Larsson et al. (1999) reported that both $Ca(OH)_2$ and NaOH treatments led to a 4 % sugar decrease in spruce hydrolysate. In another study by Persson et al. (2002), it was shown that higher pH (10) led to less sugar loss compared to low pH (5.5) when lignocellulose hydrolysates were treated with $Ca(OH)_2$. Therefore, it can be concluded that along with the treatment strategy and the ion included in the mechanism, pH plays a key role in the loss of sugars of lignocellulosic hydrolysates.

3.4.3 Bacterial cellulose generation in detoxified hydrolysates

The amount of BC produced in each condition is shown in Table 3.9. Contrary to the findings of Guo et al. (2013), there was no BC production in activated charcoal wells. However, BC production in wells treated with $Ca(OH)_2$, BC amount was close to the one in the control medium with the same amount of glucose (HS gluc), which can be evaluated that most of the inhibitors in enzymatic hydrolysates were removed by this method. Therefore, $Ca(OH)_2$ treatment was determined as the most convenient detoxification method for this study.

Table 3.9: Amount of BC produced in control and detoxification wells.

Condition	Amount of BC produced (g/l)
HS	5.25
HS gluc	2.5
Act. charcoal	0
$Ca(OH)_2$	1.875

Differently, it was reported by Guo et al. (2013) that activated charcoal led to 88 % removal of total phenols, and 94 % removal of HMF and furfural; while $Ca(OH)_2$ gave rise only to 14 %, 45 % and 35 % removal of the same compounds in spruce hydrolysate.

Indeed, overliming (calcium hydroxide) treatment has been shown as one of the most effective detoxification methods. It is also convenient with its relatively lower cost compared to newly emerging technologies such as genetic/metabolic engineering and biological detoxification (Jönnson et al., 2013). The mechanism of detoxification by overliming was hypothesized by Van Zyl et al. (1988) as the precipitation of inhibitors, while Persson et al. (2002) stated that furan aldehydes and phenolic compounds convert into non-toxic substances by alkali treatment.

3.4.4 Bacterial cellulose generation in overlimed hydrolysates

BC produced in HS, HG and enzymatic hydrolysate media are shown in Figure 3.20. It can be more clearly seen from samples in water that the structure of the BC produced in enzymatic hydrolysate medium is weaker and thinner compared to the others (BC produced in HS is not shown), due to a lower amount of carbon source which could be converted to cellulose by bacteria.

BC amount obtained from each growth medium is shown in Figure 3.21. BC production among HG growth media increased as the amount of glucose increased in the medium (from HG5.5 to HG110). Nevertheless, in the case of enzymatic hydrolysates, while BC production followed a rising trend until ENZ22, the amount was reduced in ENZ55 and to a higher degree in ENZ110 media, albeit with higher sugar concentration. It is stated by previous studies that BC production is dependent on the sugar concentration along with the inhibitor quantity (Hong et al., 2011; Kiziltas et al., 2015). Thus, it could be conferred that the reduction in BC amount is caused by the higher amount of inhibitors in ENZ55 and ENZ110 growth media due to the increased amount of enzyme.

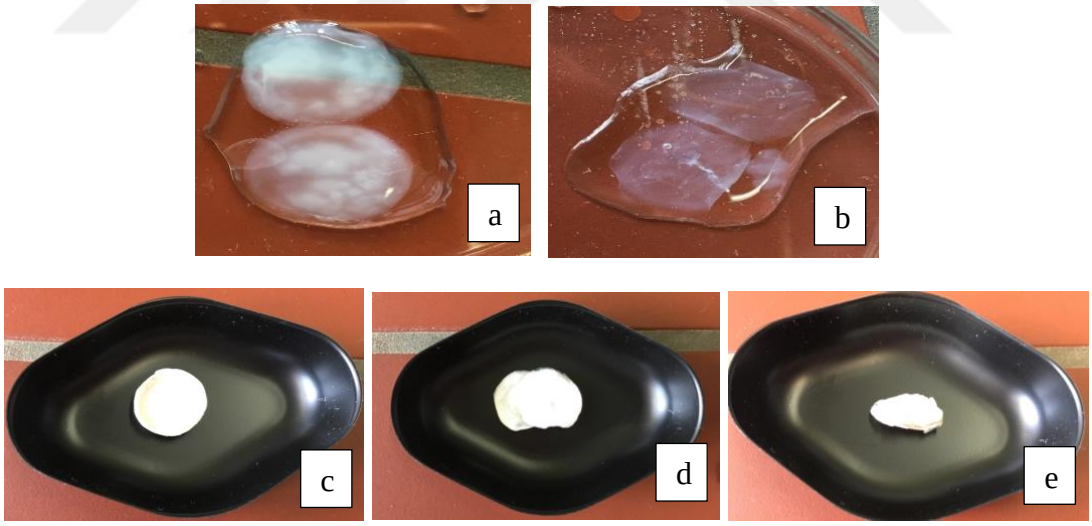


Figure 3.20: BC produced in (a) HG medium, (b) enzymatic hydrolysate medium, (in water); BC freze-dried from (c) HS medium, (d) HG medium, (e) enzymatic hydrolysate medium.

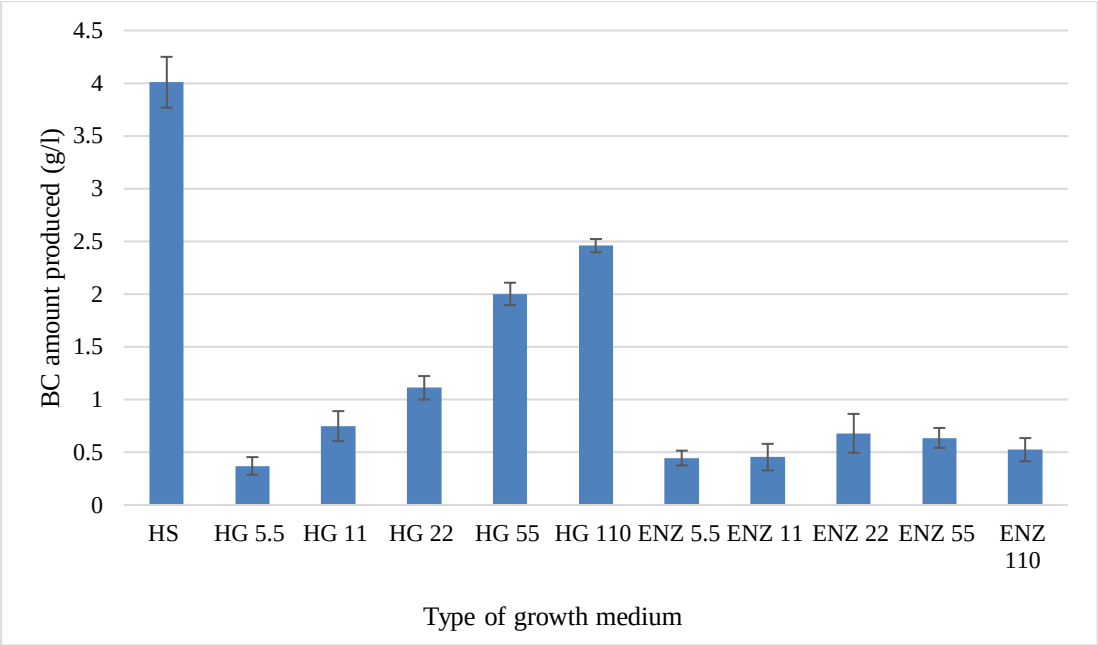


Figure 3.21: Bacterial cellulose amounts produced in various media (g/l). HS: Hestrin-Schramm medium; HG: HS media containing the same concentration of glucose as the reducing sugar concentration of the related enzymatic solution, ENZ: media prepared with enzymatic hydrolysates, enzyme amount stated for each condition.

Pretreatment of lignocellulosic biomass generates microbial inhibitors among which are aliphatic acids, furan aldehydes, phenolics and other aromatic compounds, inorganic ions and bio-alcohols.

Hydrolysis of lignocellulose leads to the formation of aliphatic acids such as acetic acid, formic acid and levulinic acid. More specifically, the degradation of acetyl groups of hemicellulose generates acetic acid. Besides, formic acid is produced by both furfural and 5-hydroxymethylfurfural (HMF) hydrolysis; levulinic acid is a product of HMF degradation only (Ulbricht et al., 1984). While acetic acid can be directly formed by hemicellulose hydrolysis, formic acid and levulinic acid can also be produced indirectly from hemicellulose. Larsson et al. (1999) demonstrated the toxicity of these aliphatic acids on *S. cerevisiae* as acetic acid < levulinic acid < formic acid. The inhibition is caused by the dissociation of acids upon entering the cell. While these acids are undissociated in the hydrolysate, they become dissociated due to the neutral pH value of the cytoplasm when diffused into the cell. This gives rise to a decrease in the cytosolic pH, which may result in cell death (Pampulha et al., 1989).

Amphiphilic molecules, also known as uncouplers, are a different class of substances that disturb cells in a different way. Eukaryotic cell inner mitochondrial membrane is

broken down by these molecules. As a result, the mitochondria lose their capacity to produce ATP from ADP, and the cell starves to death. P-hydroxybenzoic acid and salicylic acid are a couple of the uncouplers present in lignocellulose hydrolysate (Verduyn et al., 1992).

On the other hand, hemicellulose can be degraded to glucose, mannose, galactose, xylose and arabinose, all of which are potent aldehyde precursors. HMF originated from hexoses, and furfural from pentoses impede microbial growth by disrupting protein synthesis and sugar metabolism (Banu et al., 2022). In addition, HMF may also be hydrolysed to xylose and arabinose which could be transformed into furfural. As stated before, both HMF and furfural can lead to formic acid formation (Figure 3.22). These furan aldehydes are also known to inhibit *S. cerevisiae* growth and diminish ethanol production (Jönsson et al., 2013). The toxicity of aldehydes is dependent on their electrochemical, space site resistance, solubility and molecular weight properties (Jayakody and Jin, 2021).

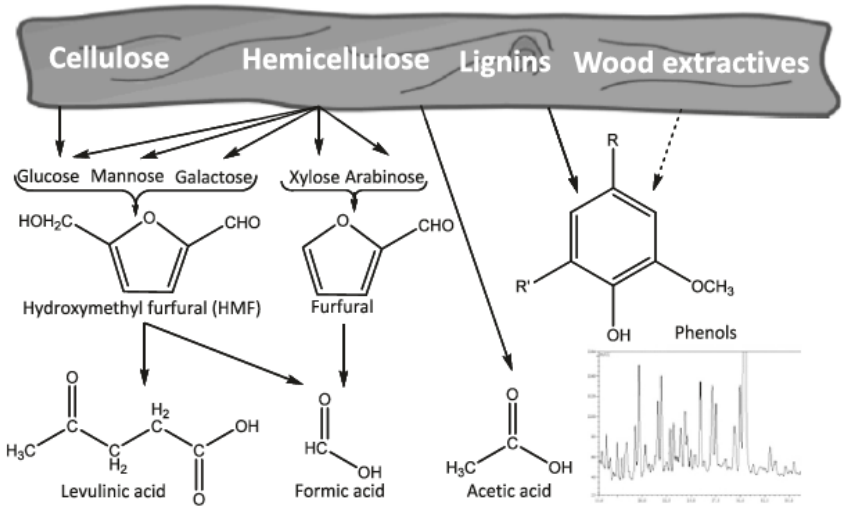


Figure 3.22: Inhibitor formation upon pretreatment of lignocellulose (Jönsson et al., 2013).

Aromatic compounds are another group of microorganism inhibitors generated by lignocellulose hydrolysis and unlike other inhibitors, they are produced even if the catalysis is not done by an acid. The inhibition effects seem to be dependent on the specific functional groups such as hydroxyl and methoxyl substituents (Ando et al., 1986; Larsson et al., 2000). Keweloh et al. (1990) suggest that phenolics negatively affect the membrane integrity by altering the protein-lipid proportion and thus perturbing the membrane functions.

Basic phenolic compounds such as syringic acid, vanillin, ferulic acid, p-hydroxybenzoic acid, syringaldehyde, vanillic acid, trans-cinnamic acid, and coumaric acid are generated by lignin hydrolysation and known to drastically inhibit microorganismal activity even at low amounts (Ko et al., 2015). Larsson et al. (2000) reported that approximately 0.2 g/l concentration of coniferyl acid hindered the growth and ethanol production in *S. cerevisiae*. Similar to aldehydes, it was revealed that microbial inhibition of phenols is related to the molecular weight of the compound; the lower the molecular weight, the easier for the phenolics to enter the cell, thus breaking down the cellular structure (Ghimire et al., 2021).

Though most of the studies in microbial inhibition are conducted with yeast, it is also shown by several research that these compounds are toxic to BC producing bacteria too. For example, the necessity of a detoxification step was demonstrated in hydrolysates of wheat straw and konjac powder which were used to produce BC (Hong et al., 2011; Hong et al., 2008). Moreover, Zhang et al. (2014) compared several phenolic substances in terms of BC production by *Gluconacetobacter xylinus*. The results showed that coniferyl aldehyde causes the strongest inhibition compared to vanillin, ferulic acid, and 4-hydroxybenzoic acid.

As shown in Figure 3.21, BC production followed an increasing trend throughout HG growth media with increasing glucose concentrations, apparently due to a lack of inhibitors arising from pretreatment. However, the case is quite different for ENZ media; while BC production improved with increasing reducing sugar concentration and reached the highest value at ENZ 22, it decreased stepwise at ENZ 55 and ENZ 110.

By these results, it can be concluded that media prepared with ENZ 5, ENZ 11 and ENZ 22 hydrolysates did not have high concentrations of microbial inhibitors due to a relatively low amount of enzymes. However, since ENZ 55 and ENZ 110 hydrolysates contained at least 2 fold of enzyme amount compared to the former ones, they involved more reducing sugars along with more aliphatic acids, furan aldehydes and phenolics derived from cellulose, hemicellulose and lignin degradation. Considering the complex mechanism of transformations and reversible reactions between pentose / hexose sugars, furan aldehydes and aliphatic acids, increased sugar amounts would always result in greater inhibitor concentrations. It can be deduced that detoxification which was applied prior to medium preparation with enzymatic hydrolysates was not

sufficient to eliminate the high amount of inhibitors in ENZ 55 and ENZ 110, while it was efficient enough in ENZ 5, ENZ 11 and ENZ 22.

However, it should be noted that the principle inhibition for these enzymatic reactions is likely to emanate from cellulose and lignin degradation since most of the hemicellulose is washed away after the pretreatment with the liquid phase (Ko et al., 2015a). Nevertheless, as well as mannose, galactose, xylose and arabinose, which are hemicellulose hydrolysis products, glucose, originating from cellulose is a precursor of HMF. As stated before HMF, which is a strong microbial inhibitor itself, may also lead to the formation of furfural by being hydrolysed to arabinose and xylose. Therefore, even if the pentose sugars (xylose, arabinose) and furfural do not derive from hemicellulose, higher glucose production by enzymatic reaction seems to have generated much more aldehydes and aliphatic acids in ENZ 55 and ENZ 110. Needless to say, ENZ 110 had the most severe inhibition, thus the lowest BC production.

It can be hypothesized that inhibitor concentrations might be minimized by adding more $Ca(OH)_2$ during detoxification. However, overliming binds inhibitors at the expense of reducing sugar loss. Therefore, an appropriate analysis must be done to eliminate inhibitors with the minimum sugar deprivation.

3.4.5 Sugar consumption in Hestrin-Schramm and enzymatic media

To determine the sugar content of ENZ 110 and HS media, another HPLC analysis was performed. Besides, the sugar consumption of *N. hansenii* during the incubation time was also monitored in both media. While the glucose amount remained almost constant during the first 2 days in ENZ 110 media, it sharply decreased from day 2 on and was completely consumed at the end of day 5, as can be seen in Figure 3.23. On the other hand, the xylose+fructose amount was almost steady until day 4; however, it significantly diminished between day 4 and day 5 and was depleted at the end of day 5, albeit a relatively low amount. This may be due to the need for bacteria to use an alternative carbon source when growth medium lacks glucose, which corresponds with the previous studies (Ishihara et al., 2002; Keshk and Sameshima, 2005).

Besides, glucose in HS media followed a rather constant trend in terms of glucose consumption by bacteria as shown in Figure 3.24. Contrary to the ENZ 110 media, glucose was not consumed until the end of day 8, apparently due to the higher initial concentration.

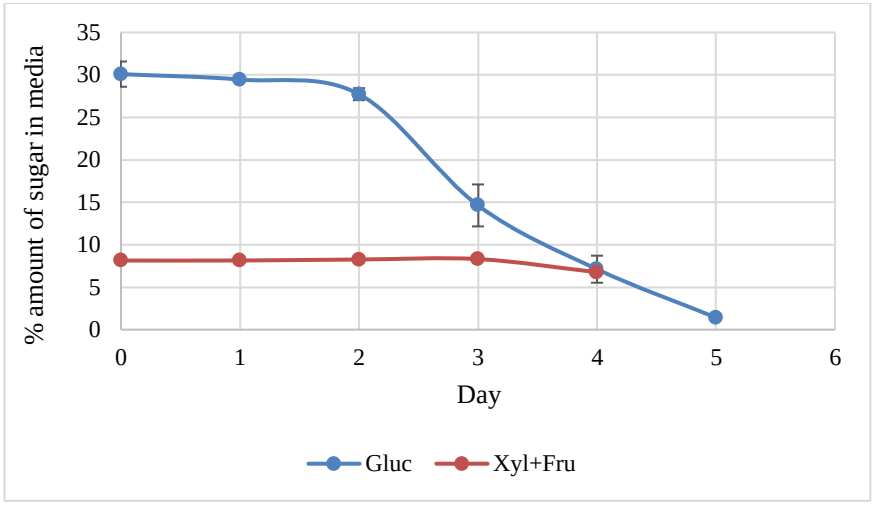


Figure 3.23: Monosaccharide consumption during BC growth in ENZ 110.

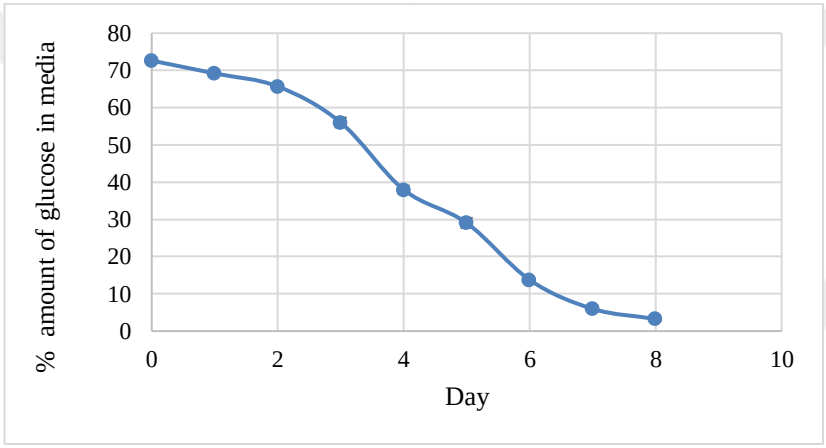


Figure 3.24: Glucose consumption in HS medium.

3.4.6 Bacterial cellulose production efficiencies in Hestrin-Schramm and enzymatic media

For the calculation of substrate conversion ratio, BC production rate and BC production yield of ENZ 110 and HS media, HPLC data from the previous step were employed. As shown in Table 3.10, at the end of day 5 of incubation, substrate conversion ratio (α) was 95 % in the ENZ 110 medium, while (α) was 60 % in the HS medium. It can be concluded that the HS medium still contained glucose, whereas almost all the glucose was consumed in ENZ 110 medium after 5 days. Therefore, the substrate conversion rate of ENZ 110 medium seemed to be higher compared to the HS medium, due to the total consumption of glucose. However, the BC production rate of HS medium was higher than ENZ 110 medium upon the superior amount of BC production.

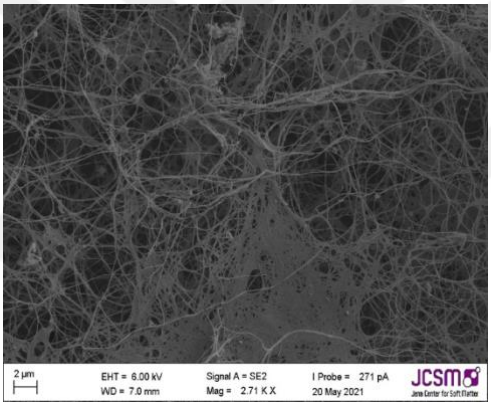
Table 3.10: Substrate conversions, BC production rates and yields for the enzymatic and HS media at the end of day 5.

Type of growth medium	Substrate conversion ratio - α (%)	BC production rate – r_{BC} (mg ml^{-1} day^{-1})	BC production yield - $Y_{BC/S}$
Enzymatic	95.11	0.14	9.6
HS	60.01	0.84	2.53

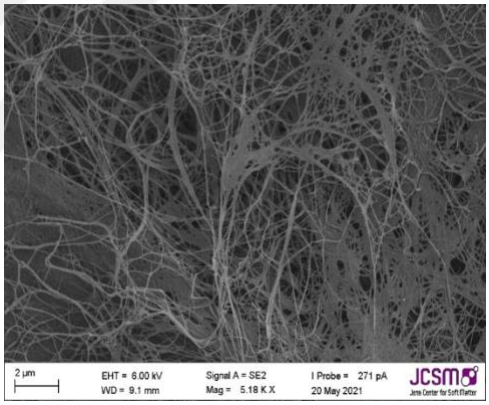
3.5 Characterization of Bacterial Cellulose

3.5.1 Scanning electron microscopy (SEM)

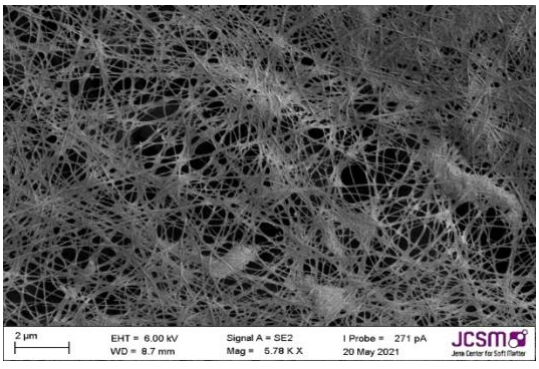
SEM results indicate that BC samples produced in enzymatic hydrolysates of olive pomace have a nano-sized fibrillar structure as shown in Figure 3.25. These findings are in accordance with the previous studies of BC production conducted with agricultural waste materials (Lin et al., 2014; Yang et al., 2014; Li et al., 2015).



(a)



(b)



(c)

Figure 3.25: (a) SEM image of the BC produced in HS standard medium, (b) SEM image of the BC produced in HG medium (9.637 g/l glucose), (c) SEM image of the BC produced in enzymatic medium. (EHT: 6.0 kV; Magnification: 2.71, 5.18, 5.78 10^3 respectively).

3.5.2 FT-IR analysis

By FT-IR spectroscopy analysis, it was shown that the absorption peaks at 3344 cm^{-1}, 2895 cm^{-1}, 1654 cm^{-1} and 1032 cm^{-1} correspond to O-H, C-H, C-O-C and C-O stretching, respectively, which are the characteristical FTIR spectrum of cellulosic material as presented in Figure 3.26. The results are consistent with former studies (Yang *et al*., 2014; Kiziltas *et al*., 2015; Jahan *et al*., 2018). IR bands corresponding to impurities could not be detected.

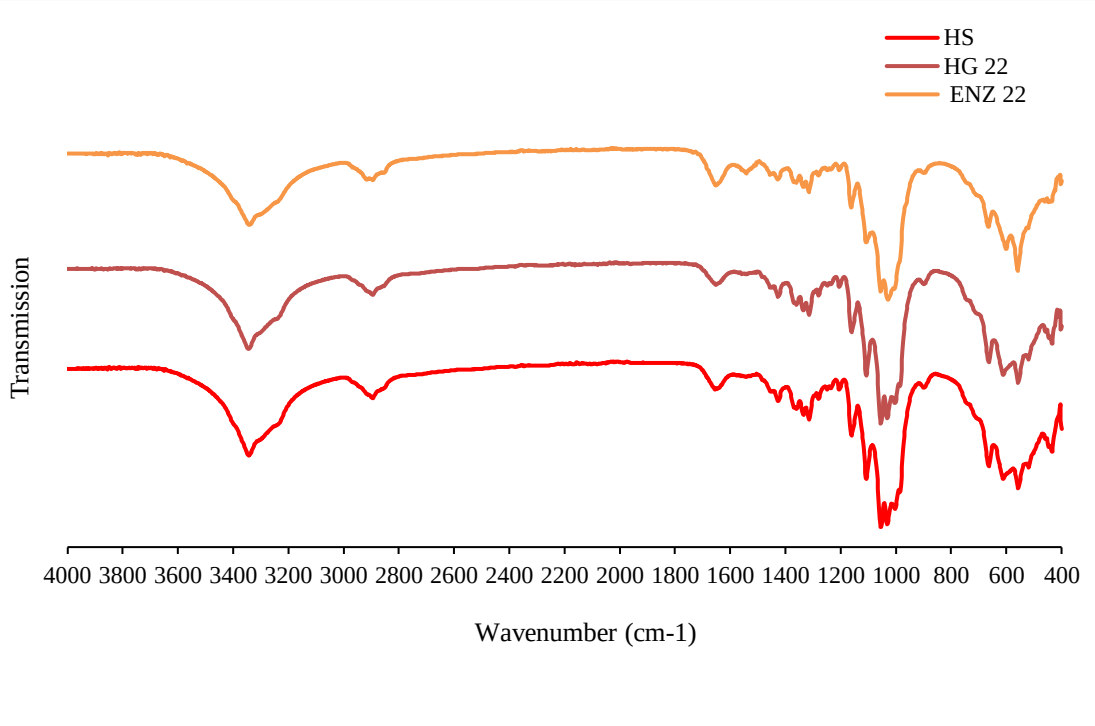


Figure 3.26: FT-IR spectra of HS-BC (red), HG-BC (orange) and ENZ-BC (green) samples.

3.5.3 X-ray diffraction analysis

The X-ray diffraction pattern of the BC obtained from HS standard medium is shown in Figure 3.27 and the signal degrees are in Table 3.11. According to the data, it was concluded that the samples showed a high resolution scattering pattern, the structural type was cellulose-I (no amorphous background was detected), and the degree of crystallinity was above 80 %. Moreover, no phase indicating any impurity was observed.

On the other hand, it can be estimated by evaluating XRD- and EDX-data, BC samples obtained from enzymatic media may contain at least one foreign substance derived

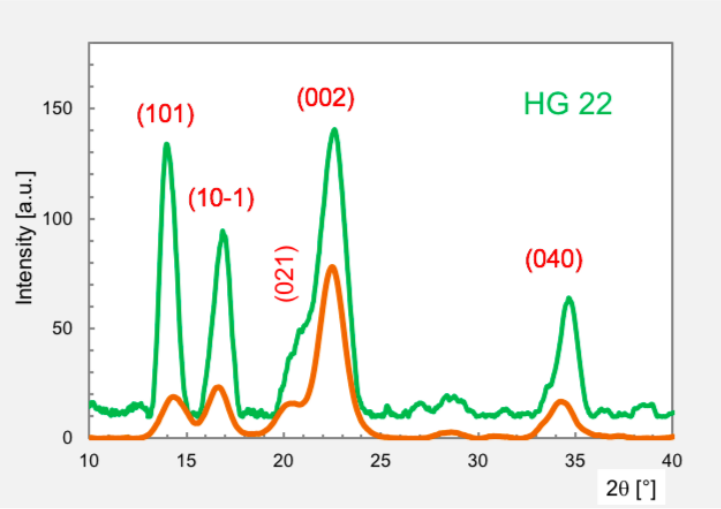


Figure 3.27: X-ray diffraction pattern of BC produced in HS medium.

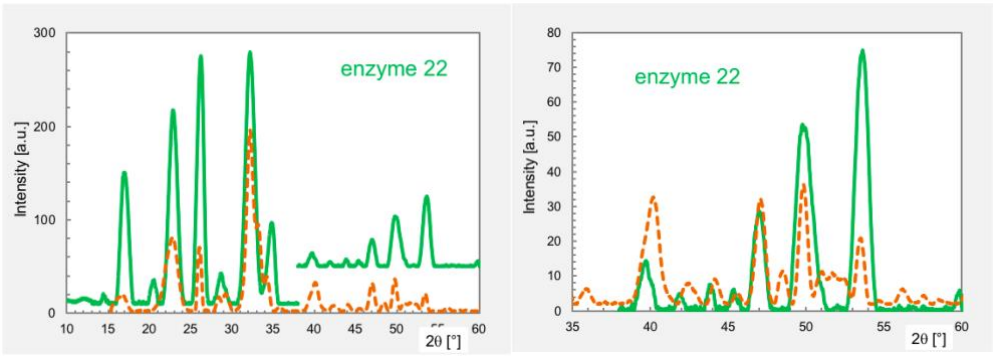
Table 3.11: Signal degrees determined according to the diffraction plane of BC produced in the HS medium.

(hkl)-plane	set value	found
(101)	14.7 °	14.0 °
(10-1)	16.5 °	16.8 °
(021)	no separate signal	
(002)	22.6 °	22.7 °
(040)	34.6 °	34.7 °

from the ternary system $CaO/P_2O_5/H_2O$. To reconstruct conceivable reaction products that might have formed in the sequence of BC-preparation, calcium hydroxide (1.7 g, 0.023 mol) was dispersed in 1.2 l water, whereupon 1.87 g (0.014 mol) potassium-dihydrophosphate dissolved in 0.5 l water was added dropwise. The suspension was allowed to settle overnight, decanted and dried. The solid cake was washed with hot water several times and finally dried at 105 °C.

The obtained white powder was identified to consist of $Ca_3(PO_4)_2$. $Ca(OH)_2$ = Hydroxyapatite (HAP) as shown in Figure 3.28. The residual WAXS-scan of the sample, where HAP signals are deducted is demonstrated in Figure 3.29. Signals obtained due to diffraction planes are presented in Table 3.12.

To compare the results achieved by transmission mode, another WAXS-scan was employed in reflection mode to analyze BC produced in the enzymatic medium. The diffraction pattern is shown in Figure 3.30. Upon partial peak deconvolution, it was demonstrated that when 2θ is between 30 ° and 36 °, a slight signal around 34° (red



(a) (b)

Figure 3.28: Diffraction plot of BS produced in enzymatic medium (transmission mode) (a) complete scanning range range; (b) narrowed scanning range (- - - - HAP).

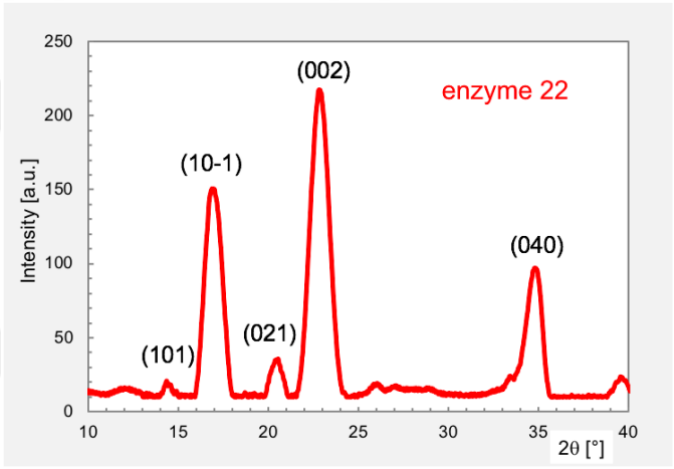


Figure 3.29: Residual WAXS-scan diffratogram of the BC produced in enzymatic medium (transmission mode, HAP-signals subtracted).

Table 3.12: Signal degrees determined according to the diffraction plane of BC produced in the enzymatic medium.

(hkl)-plane	set value	found
(101)	14,7 °	14,5 °
(10-1)	16,5 °	16,9 °
(021)	20,6°	20,4°
(002)	22,6°	22,8°
(040)	34,6°	34,8°

curve) is caused by cellulose and other grey dashed signals represent HAP reflections. In narrowed scanning range, however, when 2θ is between 5 º and 27 º, high scattering intensity of cellulose suppresses HAP signals (Figure 3.31). When 2θ is between 30 º and 36 º, HAP signals suppress the cellulose signals.

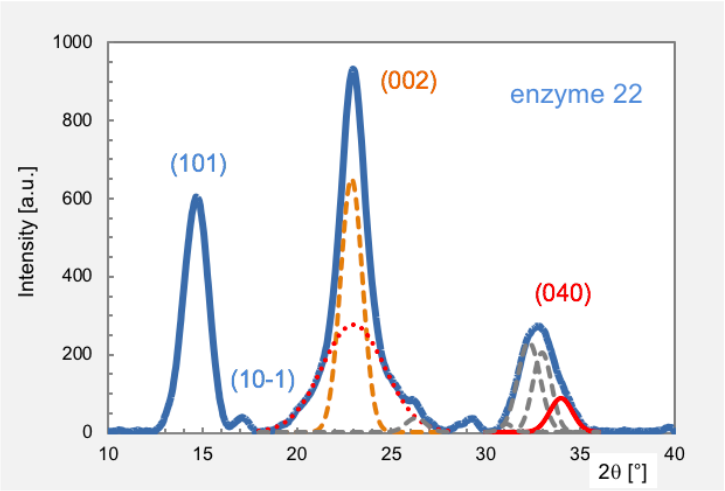


Figure 3.30: WAXS-scan diffractogram of the BC sample produced in enzymatic medium (reflection mode).

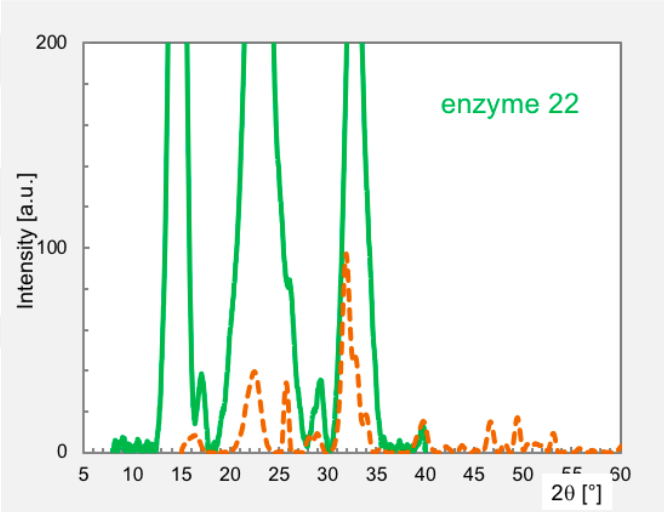


Figure 3.31: WAXS-scan diffractogram of the BC sample produced in enzymatic medium in narrowed range (reflection mode).

Based on the WAXS-results it was confirmed, that all samples under investigation were exclusively composed of cellulose-I. The scattering patterns are well-resolved and the crystallinity can be estimated to exceed 80 % owing to the lack of an amorphous background.

Nevertheless, BC samples produced in the enzymatic medium were detected to have been contaminated with a small quantity of hydroxyapatite which is known to generate high signal intensity even at very low concentrations. Further, it was concluded that scanning in reflection mode generates an intensive (101)-signal at 2θ = 14,6 °, which is almost invisible when the scan is taken in transmission mode and vice versa. In case scattering patterns show significant differences between both modes (e.g. missing

signal or inverse signal intensities), then the sample is expected to possess anisotropic properties (i.e. preferred crystallite orientation) as recently described by Aleshina et al. (2019).

4. CONCLUSIONS

Cellulose, which was discovered in 1838 by Payen is an organic polymer with hydrophilic property and is not soluble in water (Payen, 1838). When treated with acidic or alkaline chemicals, it can be hydrolysed to its glucose monomers. Nevertheless, the β-acetal linkages necessitate the presence of special enzymes to be degraded, of which the physiological environment is deprieved. Cellulose constitutes the principal ingredient of the cell wall of most plants with various distributions among species. For example, while cotton fiber is composed of 90 % cellulose, the proportions are 40 – 50 % and 60 % for dried hemp and wood, respectively (Wang et al., 2019). This makes cellulose a very important industrial agent in fields such as textile, paper-making, and pulp production. Moreover, cellulose can also be generated by some genera of bacteria, fungi and algae. Structure, morphology and function may differ according to the microorganism which produces the cellulose. G. xylinum is the most widely used bacterial species due to its high bacterial cellulose (BC) production yield. Compared to plant cellulose (PC), BC has a number of superior characteristics such as high purity, degree of polymerization, water holding capacity and tensile strength. With these excellent properties, BC has been an attractive biopolymer in a wide variety of application areas among which artificial meat, high quality paper, high fidelity speaker, biosensors, wound dressing, drug delivery systems and nanomaterial composites can be counted. For BC cultivation, several standard media have been proposed such as Hestrin-Schramm (HS) and Luria-Bertani. However, due to their high price, studies have been fronted to find alternative sources which in turn may reduce the production cost. Pineapple juice (Lestari et al., 2014), waste beer yeast (Lin et al., 2014), cocunut and citrus pulp water (Cao et al., 2018) are some of the proposed alternative media.

Lignocellulose, on the other hand, is the most abundant material in the world and may serve as a renewable reserve for production of biopolymers and biofuels. Lignocellulosic feedstock comprises agricultural and forestry residues, as well as crops and biorefinery byproducts. Since the fossil resources pose environmental problems as well as sustainability concerns, a large interest in the application of lignocellulosic

material has been emerged. Lignocellulosic biomass can substantially support the energy demand without contesting the food requirement on the cultivated lands. In fact, it can be foreseen that the specialties which make use of lignocellulosic potential will be more and more essential in the future. Nevertheless, lignocellulose has a complex structure which renders it difficult to break down. A pretreatment method, i.e. dilute acid hydrolysis, which separates lignin from cellulose and hemicellulose is required for further derivation of fermentable sugars. Another obstacle in lignocellulose utilization is the formation of inhibitors during pretreatment, which in turn negatively affect the enzymatic and the microbial activity.

In this study, use of a lignocellulosic material, olive pomace (OP) as carbon source for *N. hansenii* BC production is aimed. OP is the main byproduct of olive oil industry, which has a significant economical impact in Mediterranean countries including Turkey. OP possess a high potential with 30.0 to 41.6 % lignin and 35.3 to 49.0 % cell wall polysaccharide content, though much of this feedstock is incinerated for energy production (Sagdic-Oztan et al., 2023). Therefore, a more favourable application of the material is obvious.

Following the preliminary experiments to determine the bacterial strain and culture conditions with higher BC production yields, utilization of OP as an alternative growth medium was studied. Since BC produced in the medium with OP and lactose (from whey) was too low in amount and much weaker compared to the control BC (produced in HS), elementary analysis of both OP and the BC produced in the OP medium was conducted. The results showed that OP had very little nitrogen content which cannot support the medium as a nitrogen source. Besides, the BC produced in the OP medium was detected to have protein contamination which took longer to remove by washing, which is an undesirable feature concerning the time and chemical amount, together with the fragile structure. However, since the carbon potential of OP was evident with high lignocellulosic content, OP was decided to be processed to use as carbon source for microbial medium.

For the pretreatment of lignocellulose in OP, 1 % H_3PO_4 was added to OP in a high pressure reactor at 170 ^oC in stirring condition. At the end of the cooling process, the liquid and solid parts are collected separately. The solid part was further subjected to enzymatic hydrolysis with varying enzyme:substrate proportions for 72 h at 50 ^oC, either at static or agitating conditions. Among all trial conditions, 1:3 enzyme:substrate

ratio (ENZ 110) at static condition was found to lead to the highest reducing sugar concentration with 9.31 g/l, which was determined by DNSA assay. In addition, enzymatic samples were scanned with HPLC for their monosaccharide content, of which glucose was the main sugar and cellobiose the inferior.

In the next step, enzymatic hydrolysates were prepared as carbon sources of the growth media where nitrogen sources and salts, stated in the ingredients of HS medium were added only. Without any detoxification of the enzymatic hydrolysates, no BC production occured, obviously due to the inhibitor compounds resulting from the acid pretreatment, which undermine the microbial growth. Therefore, several detoxification methods were applied namely, activated charcoal, overliming [$Ca(OH)_2$], and NaOH treatments. Although the detoxification is essential for the microbial activity, this step causes sugar loss in the hydrolysates, which leads to less microbial activity and so less polymer production. To determine the method with the least sugar deprivation, the mentioned techniques were compared with a priliminary experiment. Since overliming resulted in minimum sugar loss, this method was used to detoxify the enzymatic hydrolysates used for BC production.

At the end of the incubation period, BC were collected, washed and freze-dried to observe the differences of BC production among different hydrolysates. Moreover, BC production rates and yields in HS and new medium were determined by tracking the monosaccharide comsumption during a stated cultivation period. Unexpectedly, the highest BC production in enzymatic hydrolysates was in ENZ 22 medium (0.68 g/l), instead of ENZ 110, although the latter had higher reducing sugar concentration. As mentioned in detail in “Results and Discussion” section, this was most probably caused by the increased amount of monosaccharadies, which can further transform to microbial inhibitors.

Finally, the characterization of the BC produced in the medium prepared with OP enzymatic hydrolysates was realized by SEM, FTIR and X-ray diffraction methods. The newly produced material was shown to possess all characteristics of BC. This study demonstrates a new approach for BC production by the enzymatic hydrolysation of lignocellulosic biomass. Considering that the principal use of lignocellulose enzymatic hydrolysation is to generate bioethanol and biofuels, this work presents a novel procedure by which BC is produced. Using the fermentable sugars resulting from enzymatic degradation, carbon source is obtained and utilized as a growth

medium ingredient for *N. hansenii*. Nevertheless, BC yield is much lower in comparison with the HS medium, which is a common drawback of alternative media trials. The medium can be supported with additional carbon sources, however at the expense of increasing the production cost. Another strategy may be to search for a detoxification method, which would more effectively eliminate the high amount of inhibitors in the hydrolysates with higher enzyme concentrations.

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Publications

- **Sagdic-Oztan, C.,** Koschella, A., Heinze, T., Karaguler, N.G., Tuter, M. (2023). Preparation of bacterial cellulose using enzymatic hydrolysate of olive pomace as carbon source. *Bioresources* 18(2), 4168-4181. DOI: 10.15376/biores.18.2.4168-4181

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