BACTERIAL CELLULOSE PRODUCTION WITH GLUCONOACETOBACTER XYLINUS FROM HAZELNUT WASTE

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ABSTRACT

BACTERIAL CELLULOSE PRODUCTION WITH GLUCONOACETOBACTER XYLINUS FROM HAZELNUT WASTE

Bacterial cellulose (BC) is a natural biopolymer with significant potential in areas such as the biomedical, cosmetics, and electronics fields. BC features high surface area, permeability, biodegradability, and modifiability. This study used *Gluconacetobacter xylinus* to produce BC, optimizing various environmental conditions and media. The focus was on sugars obtained from hazelnut shells pretreated with acid and alkaline solutions. Chemical analysis of hazelnut shells showed lignin, cellulose, and hemicellulose contents of 42.09%, 21.84%, and 22.99%, respectively. Different pretreatments were evaluated for sugar release, with alkaline treatments being more effective. Pretreatment with 1% potassium hydroxide and 3% sodium hydroxide yielded the highest sugar concentrations at 37.8 and 39.0 g/L, respectively. Optimization involved examining inoculation rate, pH, duration, and ethanol presence. The highest BC yield (2369 mg/L) was achieved with 10% inoculation, 10 days, pH 6, and 1% ethanol concentration in the medium. Ethanol addition reduced water retention capacity. FT-IR results confirmed the purity and structural integrity of all samples. This study identified optimal conditions for BC production and demonstrated that hazelnut shells can serve as a sustainable sugar source.

ÖZET

FINDIK ATIKLARINDAN GLUCONOACETOBACTER XYLINUS İLE BAKTERİYEL SELÜLOZ ÜRETİMİ

Bakteriyel selüloz (BC), biyomedikal, kozmetik ve elektronik gibi birçok endüstriyel alanda önemli potansiyele sahip olan bu doğal polimerin özellikleri arasında yüksek yüzey alanı, sıvı ve gaz geçirgenliği, biyobozunabilirlik ve modifiye edilebilirlik bulunur. Bu çalışmada bakteriyel selüloz üretimi gluconacetobacter xylinus tarafından gerçekleştirilmiştir. BC üretimini optimize etmek için çeşitli çevresel koşullar ve besiyerleri test edilmiştir. Bu çalışmada, fındık kabuklarının asit ve baz kimyasal ön işlemleri ile elde edilen şekerlerin kullanımına odaklanılmıştır. Fındık kabuğunun kimyasal içeriği belirlenmiş ve lignin, selüloz ve hemiselüloz oranları sırasıyla %42,09, %21,84 ve %22,99 olarak bulunmuştur. Bu içerikler, farklı ön işlem yöntemlerinin etkinliğini değer- lendirmek için kullanılmıştır. Fındık kabukları, farklı kimyasallar ile ön işlem- den geçirilmiş ve şeker salınımı açısından değerlendirilmiştir. Alkali ön işlemler, şeker salınımı açısından daha etkili bulunmuştur. Özellikle, %1'lik potasyum hidroksit ve %3'lük sodyum hidroksit ile ön işlem gören numuneler sırasıyla 37,8 ve 39 g/L en yüksek şeker konsantrasyonuna sahip olmuştur. Optimiza-syon çalışmaları kapsamında inokülasyon oranı, pH değeri, gün ve etanol varlığı gibi parametreler incelenmiştir. %10 inokülasyon oranı, 10 gün ve pH 6 ile en yüksek BC verimi elde edilmiştir. Kıyaslanan kültürlere bakıldığında %1 potasyum hidroksit ile ön işlem görmüş sonrasında da besiyeri içerisinde %1'lik etanol bulunan besiyerinde 2369 mg/L' de en çok bakteriyel selüloz üretildiği belirlenmiştir. Farklı kültürlerden alınan bakteriyel selülozlarda etanol ilavesinin su tutma kapasitesini azalttığ görülmüştür. FT-IR sonuçları bütün örneklerin saf olarak elde edildiğini ve yapısal bütünlüğün korunduğu göstermiştir. Bu tez, BC üretimini optimize etmek için gerekli olan koşulları belirlemekte ve fındık kabuklarının sürdürülebilir bir şeker kaynağı olarak kullanılabileceğini göstermektedir.

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CHAPTER 1

INTRODUCTION

1.1. Cellulose Structure

Cellulose is a homopolymer that consists of 3000 or more glucose monomers which bind to each other with β 1-4 glycosidic bonds. A linear structure occurs when the glucose monomers bind. Interchain hydrogen bonding between hydroxyl groups and oxygens provides linearity due to stabilizing the linkage. This stabilization results in the crystallinity of cellulose (Sampath et al. 2016). The crystallinity of cellulose is a natural property related to its affinity for water, mechanical strength, and accessibility to chemical reagents (Goldstein et al. 2004). Cellulose takes the form of a bundle by the binding of three hydroxyl groups to each other. This structure is called a microfibril (Thomas et al., 2013). Cellulose microfibrils are arranged in different ways. The most common arrangement is the parallel arrangement. Parallel arrangements are called crystalline regions. Cellulose microfibrils can be also arranged diagonally and unordered. Diagonal arrangements are called amorphous regions. Thus, microfibrils can have crystalline and amorphous regions as shown in Figure 1.1.

Figure 1.1. Cellulose microfibrils' crystalline and amorphous regions (Source: Jasmania, 2018).

While the crystalline region of cellulose provides high tensile strength, the amorphous region provides elasticity. Cellulose has seven polymorphs (cellulose Iα, Iβ, II, III1, III2 IV1, and IV2) that have different crystal geometry (Nazir et al., 2019). These are shown in Figure 1.2.

Cellulose allomorph	Crystal geometry
Alpha cellulose (I_α)	Triclinic, one parallel chain
Beta cellulose (I_β)	Monoclinic, two parallel chains
Cellulose II	Monoclinic, two antiparallel chains
Cellulose III_I	One monoclinic, parallel chain
Cellulose III_{II}	Two monoclinic, antiparallel chains
Cellulose IV_I	Two orthorhombic, parallel chains
Cellulose IV_{II}	Two orthorhombic, antiparallel chains

Figure 1.2. Cellulose allomorphs' crystal geometry (Source: Nazir et al., 2019).

Three polymorphs of cellulose exist in nature (Rajangam, 2008). Cellulose I (natural cellulose) is known as a crystallographic form of cellulose which has parallel glucan chains, in a microfibrous structure. Cellulose I has two forms. Cellulose Iα is the algal and bacterial cellulose structure, while Cellulose Iβ is found in plants. Cellulose Iα and Iβ are present in natural cellulose but in different ratios because these ratios are related to crystal packing, molecular conformation, and hydrogen bonding. Cellulose I has parallel glucan chains and strong intramolecular hydrogen bonds. Cellulose I also has the same directional reducing end point of chains. Cellulose polymorphs can be converted into other cellulose polymorphs by thermochemical pretreatments as shown in Figure 1.2. Thermochemical pretreatments are applied for changing crystallinity. Cellulose II is obtained by dissolving it in an alkaline solution (mercerization) and washing it with water. The water-washing process helps to turn Cellulose I into cellulose II without dissolving (Y. Song et al., 2015). Cellulose II has antiparallel glucan chains and extra hydrogen bonds per glucose residue. These extra hydrogen bonds provide thermodynamic stability for cellulose II. Cellulose III1 and III2 are obtained by treating cellulose Iα, Iβ and II with liquid ammonia or some amines. Cellulose IV1 and IV2 are formed by heating cellulose III1 and cellulose III2 at 260°C in glycerol, respectively (Rajangam, 2008).

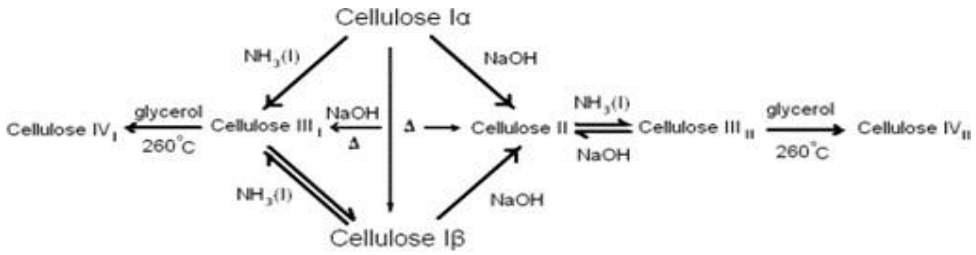

Figure 1.3. Cellulose polymorphs (Source: Rajangam, 2008).

1.2. Bacterial Cellulose (BC)

Bacterial cellulose is an exopolysaccharide produced by some bacterial strains such as Agrobacterium spp, Acetobacter spp, Azotobacter spp, Sarcina, Alcaligenes, and Pseudomonas. It has easy moldability, scalability, high biocompatibility, and easy tailoring for specific uses (Horue et al., 2023, Lahiri et al., 2021). Bacterial cellulose (BC) is superior to plant cellulose (PC) due to the absence of lignin and its higher crystallinity, higher water-holding capacity, and higher purity, as given in Table 1.1 (Wang et al., 2019, Coseri, 2021).

Table 1.1. Comparison of BC and PC properties.

Property	**BC**	**PC**	**References**
Water-holding capacity (%)	>95	25-35	Rebelo et al., 2018
Tensile Strength (MPa)	20-300	25-200	Feng et al., 2015
Purity (%)	>99	<85	Klemm et al., 2005
Porosity (%),	>85	<75	Al-Shamary and Darwash, 2013

BC also has the following desired properties: liquid sorption, mechanical strength, non-allergenicity, transparency, and moldability (de Andrade Arruda Fernandes et al., 2020). Due to these properties, BC is used in many fields such as food, medical, cosmetics, and textiles industries.

1.2.1. BC in the Food Industry

A safe and adequate food supply is vital for all heterotrophic organisms including humans. According to FAOSTAT, over 800 million people die from hunger per year. Precautions about food quality and safety have to be taken to prevent hunger, illness and disease. BC has been described as generally recognized as safe (GRAS) by the FDA, 2024 since 1992. BC has an absorptive capacity such that colors, and flavors from the surrounding culture medium are transmitted to the BC. These properties enhance the sensory qualities of food. The addition of cellulose in food provides a superior mouthfeel due to the water uptake property of cellulose. One product which takes advantage of this property is Nata de Coco, shown in Figure 1.4. Nata de Coco is a local dessert of the Philippines.

Figure 1.4. Nata de Coco (Source: Sharmin & Ahmed, 2021).

Nata de Coco is prepared by fermenting coconut water to yield a one- centimeter-thick cellulose gel sheet. Then it is immersed in sugar syrup. Nata de Pina is another product that is produced in the same way using pineapple juice. Teekvass (kombucha), made in northeastern China da Silva Júnior et al., 2022, is a fermented tea that is made by the cocultivation of yeast, bacteria, and lichen. BC is produced at the top of the tea culture, shown in Figure 1.5 This tea includes gluconic acid, hyaluronic acid, chondroitin sulfate, mucoitin sulruic acid, B1, B2, B3, B6 and B12 vitamins, lactic acid, and usnic

acid. Using Gamgyul (a kind of mandarin) instead of coconut water for BC production is used for fighting obesity in Jeju Gamgyul Center of the Rural Development Administration in South Korea (Choi et al., 2022).

Figure 1.5. Kombucha tea with BC (Source: Miranda et al., 2016).

BC is a non-caloric product. BC shows higher lipid blood and cholesterol-lowering effects than PC. It was thought that BC could only be used in oily products like mayonnaise or margarine but BC's texture does not resemble cream. Thus, BC can be used as a fat replacer in some foods, especially emulsified meat products such as patties, hamburgers, and sausage. BC provides this without changing taste and texture. According to the U.S. Department of Agriculture (USDA) standards, products that have total calorie counts that are reduced by 25% or more can be labeled as low-calorie. Using BC and beef extract instead of 1/3 of the quantity of beef, the calorie of the patty is decreased from 258 kcal to 194 kcal per 100 g meat. Also, there were no differences in mildness and juiciness. The fat amount of the patty using BC was 10% satisfying the standards of a 'low-fat hamburger' as recommended by the USDA (Iguchi et al., 2000). The same effect occurred when bacterial cellulose was added to sausage. Applying bacterial cellulose provided sausage with 12% fat and reduced calories by about 25% (Chau et al., 2008).

K. W. Lin and Lin, 2004 showed that addition of 10% BC to Chinese meatballs, did not affect the meatballs' texture, shelf stability and sensory qualities. Thanks to the suitable texture of products with BC, BC is a promising food additive. K. W. Lin and Lin, 2004 showed reduced rigidity of mahi mahi (dolphinfish) surumi and, an increased water-uptake capacity of mahi mahi surumi, when 5% alkali treated BC was added. Akoğlu et

al., 2015 examined the effects of addition of different percentages of BC to fat-reduced mayonnaise (none and from 0.25% to 2%). No differences were found in smell, aftertaste, oilness and thickness. Soy protein isolate (SPI) can be used instead of using fat, in ice cream making. SPI degrades at room temperature due to protein aggregation. Therefore, Guo et al., 2018 investigated the thermal stability and emulsifying properties of a SPI-BC complex as a fat replacer in ice cream. Their results showed that the BC-SPI complex provided enchanced stability. Addition of BC to also ice cream also reduces calories and enhances melting resistance and textural properties. The texture of BC gel varies from a hard texture like bone to the texture of mollusks and squid. For increasing the water content of BC, alginate, sugar alcohol or calcium chloride can be added and a chewable texture can be obtained. These characteristics enable its utilization in innovative culinary creations such as low-calorie desserts or salads (Choi et al., 2022).

In addition to its use as a food additive, BC is promising for food packaging. According to Geyer, 2020, approximately 8300 million metric tonnes of virgin plastics were produced until 2017. By 2015, about 6300 million metric tons of plastic waste had been produced. Of this amount, roughly 9% had been recycled, 12% had been burned, and 79% had been either disposed of in landfills or ended up in the natural environment. This situation causes concern about nature. One of the many uses of plastic is food packaging. Polyethylene, polypropylene, polystyrene, and polyvinyl chloride are most commonly used in food packaging. Production of these polymers is inexpensive but these polymers are not biodegradable (Choi et al., 2022). Because of this issue, sustainable packaging material is getting more popular. BC is a promising candidate among renewable polymers. The porous and thin reticulated structure of BC filters dust, microorganisms and fungus in the air. Due to this filtration property, BC is a promising packaging material for storage and to extend the shelf-life of food. Çoban et al., 2021 has reported that BC provides extended shelf life compared to petrochemical materials in sausage. In this study, sausages were wrapped with petrochemical material, BC, or left unwrapped. Unwrapped sausage had 1.0×10^6 cfu (colony forming unit)/ml microbial load, sausage wrapped with petrochemical material had 2.7 × 105 cfu/ml, and BC-wrapped sausage had 1.2 × 104 cfu/ml microbial load. Thus, the results indicate the usefulness of BC in food packaging.

1.2.2. BC in the Healthcare Industry

Good living conditions depend on the development of medicine and the healthcare industry. The growing human population also emphasizes the importance of the medical sector. The population of the world is believed to reach 9 billion people by 2050 (Khan & Ghosh, 2005). Moreover, the elderly population will increase threefold by 2050 (Goodman, 2007). Healthcare systems become more essential as the Earth's population ages. Growing medical needs can be met by innovative approaches. In this context, usage of eco-friendly and material of biological origin such as bacterial cellulose, in health systems can provides innovative perspectives (Navya et al., 2022). BC has a nanoporous structure with abundant free hydroxyl residues. These properties make BC a suitable candidate for wound healing (Horue et al., 2023). For example, Raut et al., 2023 showed that BC reduced skin irritation scores and prevented wound infection by doing experiments with rabbits. In other work, chronic venous limb ulcers of patients were healed in 180 days. In this work, 19 patients treated with BC and an untreated control group were compared for the effectiveness of BC coating on lesion healing. This study showed that BC stimulated the epithelization of lesions (Silva et al., 2021). BC's effect on arterial ulcer patients was also examined. In this study, 13 individuals were treated with BC while 11 were used as control. When the patient's ischemic wounds were examined, a wound size reduction of 55% was observed in the BC-treated group after a 30-day period, while in the control group, there was a reduction of 48.5%. After 90 days, 50% of the BC-treated group had completely healed, whereas this rate was 18.2% in the control group. Using a BC-based scaffold provides increased skin extracellular matrix deposition, supressing excessive inflamation in wound dressing. Modification of BC to carboxymethyl cellulose (CMC) also improved cell adhesion and proliferation (Cherng et al., 2021).

BC has been applied for tissue engineering as an appropriate scaffold material to stimulate cell growth and tissue development (Raut et al., 2023). Bacterial cellulose has been used especially in the production of bone, cartilage, vascular, nervous and cardiac tissues (Jadczak & Ochędzan-Siodłak, 2023). BC has desirable properties in tissue engineering, such as biocompatibility, high porosity, high surface area, and mechanical strength. An important obstacle in bone tissue engineering is the lack of osteogenic

activity. Gold nanoparticle-incorporated BC hydrogels overcome the lack of osteogenic activity. The controlled release of gold nanoparticles (GNPs) from hydrogels stimulates the development of bone-forming cells (hBMSCs) sourced from human bone marrow by triggering a process known as autophagy (Huang et al., 2023). For bone tissue engineering, BC-calcium phosphate composite can be applied. This composite allows cell adhesion and bone tissue regeneration (Busuioc et al., 2022). The structure of BC, such as controllable pore size and reshaping properties, promotes neocartilage regeneration (Xun et al., 2021). Also, lotus root starch, agarose porogen templating, and hydroxyapatite deposition modified BC showed enhanced cell growth, chondrocyte distribution, and alkaline phosphate activity (Wang et al., 2019). These results indicate the efficiency of using BC scaffold in cartilage tissue engineering. Because of its special qualities, BC is also used in applications involving nerve tissue. In order to facilitate nerve regeneration following transection, BC can be molded into hollow tubes that direct nerve axons. Moreover, BC-based electrodes are extremely robust and appropriate for neural interfacing applications, enabling in vivo recording of brain electric activity. These electrodes' Young's modulus is comparable to that of brain tissue. These results demonstrate the variety of uses of BC in nerve tissue engineering, including guiding nerves (Raut et al., 2023); (Yang et al., 2018).

In addition to its use in tissue engineering, BC is a good candidate for a drug delivery system to fight against cancer. According to WHO, 2008, 20 million new cancer cases and 9,7 million deaths happened in 2022. Furthermore, 53,5 million cancer patients lived with cancer in 2022. WHO, 2008 estimates that 35 million new cancer cases will be revealed in 2050. Considering all of this, the importance of fighting against cancer is clear. BC is a suitable and applicable product thanks to its improving therapeutic effectiveness, decreasing chemical dosage, high surface area, easy modification, relatively high permeability to liquid and gases, and controllable drug-release properties (Shahriari- Khalaji et al., 2021). J. H. Lin et al., 2011 showed that BC provided controllable antibody release, was biocompatible, and lacked cytotoxic effects. Doxorubicin-embedded BC inhibited tumor growth (TGI) from 85, 5% to 62, 4% for an hour in the mice gastric cancer model (Ando et al., 2021).

1.2.3. BC in the Cosmetic Industry

Cosmetics have a significant impact on people's lives due to their ability to help people look better, feel more confident, and improve their well-being. The cosmetic industry is worth billions of dollars and generates significant foreign exchange earnings, and job opportunities (Surya & Gunasekaran, 2021); (JOHN, 2014). The cosmetic industry is interested in BC due to its properties such as natural origin, biodegradability, and superior quality for skincare products (Oliveira et al., 2022). These superior qualities are water retention capacity, porosity, supporting the integration of active compounds, and improving moisturizing effects. BC can be applied as a mask-forming ingredient in the cosmetic industry (Choi et al., 2022) . Some brands such as Mary Kay, Bio Enzymes, and Leaders, produce facial masks containing BC. These masks are enriched with secondary compounds that provide hydration, antioxidant action, and revitalization of facial tissues (de Andrade Arruda Fernandes et al., 2020).

1.2.4. Bioremediation Potential of BC

Pollution is described as the release of energy and substances by humans that cause harm to health and ecological systems. These substances can be chemicals, heavy metals, pesticides, plastics, and biological substances which have negative effects on animal life and welfare. Pollution is a complex phenomenon because it can affect ecosystems, change natural processes, and cause dead zones in water bodies as a result of excessive nutrient discharge from pesticides, herbicides, and fertilizers. To mitigate pollution, one must decrease exposure to dangerous agents and clean up contaminated surroundings. One must also understand the scientific principles governing the transit and fate of pollutants (Brusseau et al., 2019); (Banner, 1999). BC has tremendous bioremediation potential since it can adsorb pollutants and convert polluting substances into non-toxic composites. This potential relies on its porous structure, large surface area, and abundant hydroxyl groups. The adsorption capacity of BC with different heavy metal pollutants is given in Table 1.2. Modified and unmodified BC have different heavy metal

adsorption capacities. Different modifications of BC provide higher heavy metal adsorption capacity. BC- graphene oxide composite has the highest adsorption capacity of lead, 303.30 mg lead per gram of cellulose. BC- graphene oxide can be used for treatment of lead contamination. These results indicate that BC is a good candidate for bioremediation.

Table 1.2. Adsorption capacities of various modifications for different pollutants.

Modification	**Pollutant**	**Adsorption capacity**	**Reference**
p-aminobenzoic groups	Ni^{2+}	110.34 mg/g	Gustava et al. 2004
Poly(acrylic acid-co-acrylamide)	Ni^{2+}	171.80 mg/g	Lin et al. 2016
Amidoximated	Cu^{2+}	84.00 mg/g	Chen et al., 2009
Graphene oxide	Pb^{2+}	303.30 mg/g	Mensah et al., 2019
Graphene oxide- Magnesium aluminum phyllosilicate	Cu^{2+}	150.79 mg/g	S. Song et al., 2020
Graphene oxide- Magnesium aluminum phyllosilicate	Pb^{2+}	217.80 mg/g	S. Song et al., 2020
Cu	Cyclohexane	66.4 g/g	Nguyen et al. 2022
Fe- Metal Organic Gel	Arsenate	5 ppm	H. Li et al., 2021
-	Pb^{2+}	87 mg/g	Mohite and Patil, 2014
Amidoximated	Pb^{2+}	67 mg/g	Chen et al., 2009

1.2.5. BC in the Textile Industry

Textiles have been integrated into human existence since the beginning of life. Two or more millennia ago, textiles were used to cover body parts to heat the body and defend against insects, etc. With the development of societies, clothing came to have more functions than just covering body parts. Clothing can be measure of attractiveness and also a sign of power (Mitchell, 2004). The textile industry's priorities are lower cost, variety, and more functionality. Also, textile products are made from plants and animals.

Some animals are victims for fashion fue to the demand for products like leather and fur. In this context, Suzan Lee who is a fashion designer, has integrated BC into the fashion sector successfully. Her idea was to ‘grow your clothes’. She has shown the production of BC and usage of BC in clothing making. Her products are shown in Figure 1.6.

Figure 1.6. BC clothes with dye. (Source: "Suzanne Lee: Grown Your Own Clothes". TED: Ideas Worth Spreading, TED, 2011.).

1.3. BC Production

Some bacteria can produce bacterial cellulose. Producing bacterial cellulose is a natural behavior for these bacteria. *Rhizobium* and *agrobacterium* species produce cellulose to attach to the plant. *Acetobacter* species produce cellulose to maintain the existence of an aerobic environment. Also, other bacteria species such as *Achromobacter, Aerobacter, Alcaligenes,* and *Pseudomonas* species produce BC to allow flocculation. Furthermore, *sarcina* produce BC but the biological role of BC in this bacteria is not known (Jonas & Farah, 1998). The genera *komagataeibacter* and *gluconacetobacter* are modal organisms for BC production. Producing BC from these genera can take place in industry. These genera are members of the acetic acid bacteria. With some phylogenetic studies, some strains of *komagataeibacter*, such as *komagataeibacter hansenii, komagataeibacter cocois, komagataeibacter maltaceti*, and *komagataeibacter pomaceti* are reclassified as *novacetimonas*. but *komataeibacter xylinum* is also known as *gluconacetobacter xylinus* (Brandão et al., 2022). BC can be produced in the presence of

xylose, glucose, or fructose. For all substances, there are different interrelated pathways used to produce bacterial cellulose as shown in Figure 1.7.

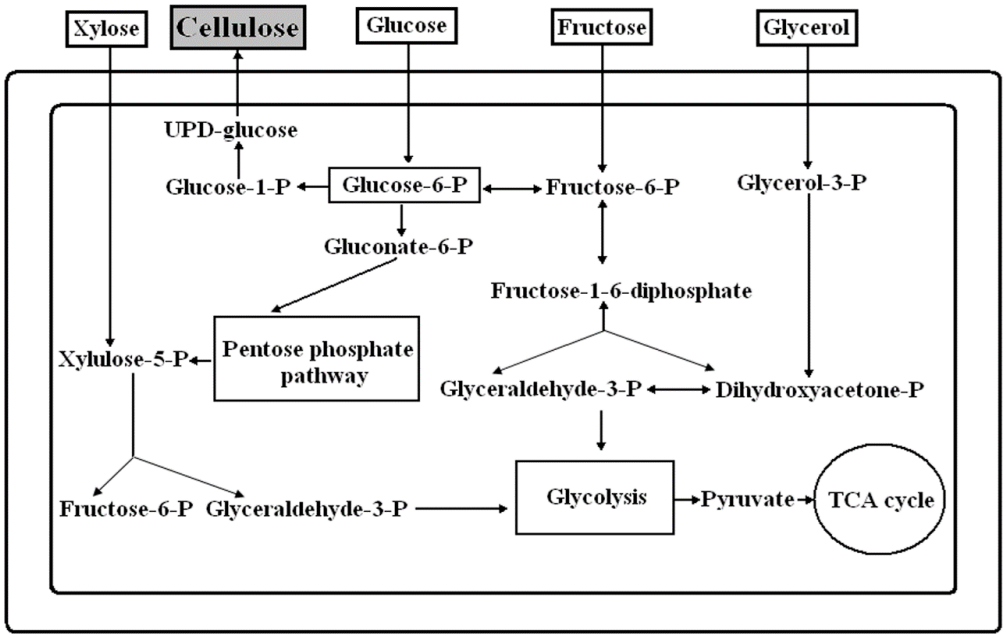

Figure 1.7. Metabolic pathway of producing bacterial cellulose in *komagataeibacter xylinum* (Source: Stepanov & Efremenko, 2018).

Most of cellulose-producing bacteria cannot synthesize cellulose from xylose. Only a few bacteria such as the *komagataeibacter xylinus*, an *acetobacter*, can synthesize cellulose from xylose, as shown in Figure 6. For utilization of xylose, xylose has to be converted to fructose-6-phosphate. Then, fructose-6-phosphate be turned into glucose-6-phosphate. Glucose-6-phosphate can be converted into glucose-1-phosphate by phosphoglucomutase. Glucose-1- phosphate is then turned into uridin diphosphate (UPD)- glucose by UDP-glucose pyrophosphorylase. At the end of pathway, cellulose is produced by converting UDP-glucose to glucose. Glucose monomers are assembled in the form of cellulose (Stepanov & Efremenko, 2018).

1.4. BC Producing Conditions

Acetic acid bacteria such as *gluconacetobacter xylinus* and *komagataeibacter hansenii*, are mostly used for BC production. The medium required for BC production was determined by Hestrin and Schramm in 1954. This medium includes important substances for bacterial growth such as disodium phosphate, citric acid monohydrate, peptone, yeast extract, and glucose. Disodium phosphate provides phosphate and pH stability (Zheng et al., 2019). Citric acid is an intermediate substance in the tricarboxylic acid (TCA) cycle. The TCA cycle is responsible for energy production in aerobic organisms. Peptone is the primary nitrogen source. Yeast extract is a complex substance which includes vitamins and nitrogen sources. Glucose is a carbon source for obtaining energy. To sum up, each ingredient in the medium is necessary for acetic acid bacteria. It has been devised to meet the bacterium's needs (X. Li et al., 2015). Research indicates that xylose transporters in *Escherichia coli* enable the simultaneous consumption of glucose and xylose, effectively overcoming the glucose-induced inhibition often seen in fermentation processes (Zhu et al., 2022). Strains adapted to xylose utilization have demonstrated improved co-utilization of sugars, leading to higher sugar consumption rates and increased ethanol production, which are beneficial for BC production (Dev et al., 2022). Studies have shown that using mixed carbon sources, including xylose, results in higher BC titers compared to using glucose alone. Metabolic analyses have revealed that xylose contributes to better ATP production and enhanced enzyme activity, which are crucial for efficient BC production (Wang & Zhong, 2022).

Substances used in the medium can be obtained from cheaper sources for integration into industry. In this context, agricultural wastes are a good source to replace carbon sources. The growing need for food and other agricultural products makes agricultural waste an important by-product that should be utilized. Plant wastes are inert lignocellulose products with common components such as lignin, cellulose, and hemicellulose (Pocha et al., 2022). Lignin binds to hemicellulose and cellulose and covers them. Lignin resists chemical or microbial destr uction (Prado et al., 2022). There are many lignocellulosic material sources that exist in the World. Some of them are shown in Figure 1.8. According to FAOSTAT data, 765,000 tons of hazelnuts were produced in Turkey in 2022. The shell constitutes 50% of the weight of hazelnuts, and this shell cannot

be actively used in industry. Due to its high-calorie properties, its use as fuel has become common but is harmful to the environment. Bacteria use glucose as a carbon source, but some bacteria can also use xylose as a carbon source. Therefore, cellulose and hemicellulose in the structure of agricultural waste can be broken down into monomers and used as a carbon source in bacterial cultures. In this way, products that can be used in the energy sector, such as bioethanol and biogas, can be produced. At the same time, hydrolysis of agricultural wastes can be used in the production of high-value-added products such as bacterial cellulose, which can be used in areas such as the food industry, textiles, and health. In addition, microbial proteins that form the content of animal feed, polyhydroxyalkanoates (PHA) used in bioplastic production, chitin and chitosan used in water purification and food packaging can be produced with agricultural wastes.

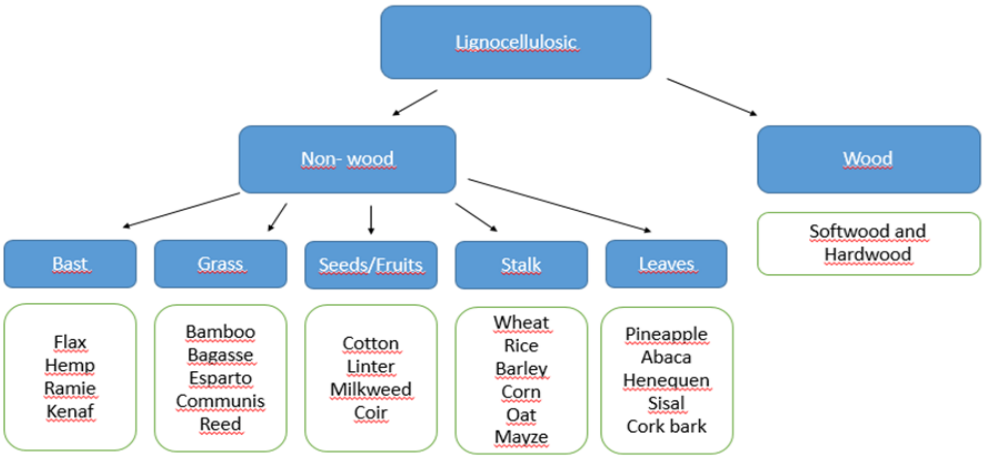

Figure 1.8. Lignocellulosic sources (Source: Lobo et al., 2021).

The production of bacterial cellulose from agricultural waste occurs through the stages of pre-treatment of the waste, hydrolysis of sugars, fermentation, and purification of the resulting product. The pretreatment is done to remove the lignin in the lignocellulosic waste and make the cellulose more accessible to the enzyme. From the waste where some lignin has been removed, smaller molecular weight sugars such as glucose and xylose are released through enzymatic digestion. These released sugars are added to the bacterial culture and the fermentation phase begins.

Table 1.3. Comparison of pretreatment methods (Sasmal and Mohanty, 2018).

Pretreatments	**Methods**	**Advantages**	**Disadvantages**
Chemical Pretreatments	Dilute Acid	Operation time is less and high yield of pentose sugar	Acid recovery and formation of furfural
	AFEX (Ammonia Fiber Extension)	High yield of pentose sugar and no inhibitory compounds	Recovery of ammonia is not effective, less effective process with increasing lignin content
	Lime	No inhibitory compounds	Operation time is more
	Organosolvsis	High yield of pentose sugar	Solvent recovery is expensive
Physical Pretreatments	Milling	Operation time is less	The overall yield is poor, energy requirement is high
	Irridation	High yield of sugar, no inhibitory compounds	Need special design of equipment and process
	High- pressure	No inhibitory compounds	Maintaining high the pressure itself is a challenge
Biological Pretreatments	Microorganism	Low energy requirement, no production of inhibitory compounds, mild operation conditions	The rate of reaction is slow

While the bacteria continue their vital activities, bacteria provide the desired bacterial cellulose to the culture as a by-product (Arenas-Cárdenas et al., 2017). Depending on the source of the lignocellulosic material, the ratio of lignin, hemicellulose and celullose change (Lobo et al. 2021). This ratio plays a critical role in choosing pretreatment and is related to applied technology. Pretreatment can be classified as physical, chemical, biological, and their combinations. When pretreatment is determined, economic and environmental issues have to be considered. Pretreatments have different advantages and disadvantages. Some of the advantages and disadvantages are shown in Table 1.3. When the pretreatments are compared, the yield of the pentose sugar is high in general but dilute acid pretreatment has a disadvantage for acid recovery and formation of furfural. Also, after the AFEX, recovery of ammonia is not effective and AFEX is less effective with increasing lignin content. Furthermore, Organosolvosis is an effective method but also is quite expensive. Physical methods have different advantages and disadvantages. Milling does not take too much time but it requires significant energy and the yield of milling is quite low. Irradiation provides a high yield of sugar and does not release inhibitory compounds. The energy requirement of biological pretreatment is quite low and after the biological pretreatment, inhibitory compounds are not released. However, this pretreatment method is slow (Sasmal & Mohanty, 2018). Biological pretreatments are done using enzymes. Cellulases are responsible for the cellulose fraction's enzymatic hydrolysis. The complex group of enzymes known as cellulases is mostly made up of three distinct hydrolase enzyme types with varying specificities. Internal β-1,4-glucosidic linkages are randomly hydrolyzed by endo-1,4-β-D-glucanases, while cellulose is transformed into cellodextrins by cellobiohydrolases I and II (CBH). Additionally, cellobiose and cellodextrins are hydrolyzed to glucose by 1,4-β-D-glucosidases. These enzymes work in concert with one another and can support one another. Enzymatic or acidic hydrolysis can also be used to transform the hemicellulose portion into monosaccharides. The hemicellulose fraction can be dissolved with cellulose in the enzymatic hydrolysis stage, contingent on the circumstances and the pretreatment option used. Raw sources rich in xylans include sugarcane bagasse and maize stover. Enzymes called xylanase, derived from bacteria and fungi, may hydrolyze the β (-1,4)-D-xylopyranosyl link, liberating monomers of xylose and xylooligosaccharides. Among the xylanases, β-xylosidases hydrolyze short-chain xylooligosaccharides, while endo-β-1,4-xylanases hydrolyze the xylan backbone oligosaccharide, resulting in

depolymerization. Following the hydrolysis stage, the sugars that are liberated from the polysaccharides will be fermented to yield various bioproducts (Prado et al., 2022).

1.5. Aim of the Study

Hazelnut is produced widely at 684,000 tons per year in Turkey. While hazelnut kernels are consumed or processed, hazelnut shells are considered a waste product. Due to the high-calorie content of hazelnut shell, it is often burned. Burning hazelnut shells harms the environment. Thus if hazelnut shells can be used in new areas, environmental protection is encouraged. This work aimed to use hazelnut shells in a new way and also produce value-added production in the form of BC. To achieve this, hazelnut shells from the Hazelnut Research Institute in Giresun were used with pretreatment by testing different acids or bases for obtaining sugar. Optimized pretreatment methods provide a more eco-friendly approach. The sugar obtained from the treatment was used instead of D-glucose in the Hestrin-Schramm media for the growth of Gluconacetobacter xylinus. To determine the optimal growth conditions of G. xylinus, the effects of factors such as day, incubation rate, and pH on BC production were analyzed. Thus, this study provides an optimized protocol for eco-friendly usage of hazelnut shells and improved production of BC.

CHAPTER 2

MATERIALS AND METHODS

2.1. Biological Material

Gluconacetobacter xylinus, BC producer bacterium, was obtained from ATCC (American Type Culture Collection). Hazelnuts were obtained from Hazelnut Research Institute, Giresun.

2.2. Methods

2.2.1. Preparation of Hazelnut Shell

To prepare material for pretreatment, the crude hazelnut shell was ground with a Grinding Mill/Knife grinder (Emir Endüstriyel Mutfak Ürünleri, EMR-Ö-01). The ground hazelnut shell was sieved by fractionated sieve until particle size was 0.5 mm (Figure 2.1) All of the subsequent analyses with the powdered hazelnut shells were perfomed in triplicate.

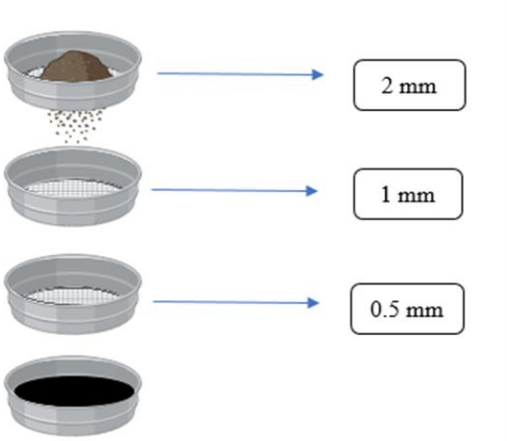

Figure 2.1. Use of fractionated sieve to prepare hazelnut shell samples (Biorender).

2.2.1.1. Determination of Moisture Content

One g ground samples were dried in an oven (Binder) at 80°C and weighed after drying. Moisture content was determined by subtracting the weight of the dried sample from the weight before drying (1 g).

2.2.1.2. Determination of Ash Content

One g dried samples were burned in a furnace at 550°C and weighed after burning. Ash content was determined by subtracting the final weight from the weight before burning.

2.2.1.3. Determination of Total Fat Content

Determination of total fat content was done according to (Yeddes et al., 2012). For this, 1 g hazelnut shells were incubated in 140 mL n-hexane using a Soxhlet device

(Buchi Fat-Extractor, E-500). The solvent was removed by evaporation at room temperature. Samples were weighed after evaporation. Total fat content was determined by subtracting this weight from the initial weight.

2.2.1.4. Determination of Protein Content

The total nitrogen and protein content of the samples were determined using the Kjeldahl method. The Kjeldahl method involves several steps. In the first step, digestion, each 1 g sample was placed in a digestion flask, to which concentrated sulfuric acid (H_2SO_4) was added. A catalyst, a mixture of potassium sulfate (K_2SO_4) and copper sulfate ($CuSO_4$), was also included to accelerate the digestion process. The flask was then heated to break down the organic matter, converting nitrogen in the sample into ammonium sulfate. After digestion, the mixture was allowed to cool before neutralization. The solution was diluted with distilled water and transferred to a distillation apparatus. An excess of sodium hydroxide (NaOH) was added to neutralize the acid, converting the ammonium sulfate into ammonia gas. During distillation, the ammonia gas was distilled off and absorbed in a boric acid (H_3BO_3) solution, forming an ammonium borate. The ammonium borate solution was then titrated with a standard acid solution (HCl) to determine the amount of ammonia, and thus the amount of nitrogen present in the sample. The total nitrogen content was calculated based on the amount of acid used in the titration. The total protein content was then derived from the nitrogen content using a conversion factor appropriate for Hazelnut Shell as 6.25.

2.2.1.5. Determination of Extractive Content

Determination of extractives was done according to Ayeni et al., 2015. For this, 2 g hazelnut shells were incubated in 120 mL acetone at 90°C for 2 h. Samples were dried in an oven at 95°C until the weight was constant. Extractive content was determined by subtracting the final weight from the initial weight.

2.2.1.6. Determination of Hemicellulose Content

Determination of hemicellulose was done according to Ayeni et al., 2015. One g extractive-free sample was mixed with 0.5 M 150 mL NaOH. The mixture was heated at 80°C for 3.5 h. It was then washed with deionized water until the pH was neutral. The sample was dried at 95-105°C until the weight was constant. Hemicellulose content was determined by subtracting the final weight from the initial weight.

2.2.1.7. Determination of Lignin Content

Determination of lignin was done according to Ayeni et al., 2015. For this, 0.5 g extractive-free sample was incubated with 3 mL 72% H_2SO_4 for 2 h with 30-min intervals mixing. The sample was heated at 121°C for 1 h after adding 84 mL of distilled water. The sample was filtered after cooling. The dried sample was burned at 575°C until the weight was constant. Lignin content was determined by subtracting the ash weight from the solid weight remaining after incubation with acid.

2.2.1.8. Determination of Cellulose Content

Determination of cellulose was done according to Ayeni et al., 2015. Cellulose content was calculated by the assumption of lignocellulosic content consisting of extractives, hemicellulose, lignin, and cellulose. Therefore, cellulose percentage was calculated by subtracting total lignin, hemicellulose, and extractive percentages from 100%.

2.2.2. Chemical Pretreatments

Two different chemical pretreatment methods, dilute acid pretreatment and alkaline pretreatment were tested. Sodium hydroxide and potassium hydroxide were used as alkalis.

Table 2.1. Naming of pretreated samples.

Name	**Used chemicals and concentrations for pretreatment**
NA1	Nitric acid 1% v/v
NA2	Nitric acid 2% v/v
NA3	Nitric acid 3% v/v
NA4	Nitric acid 4% v/v
HA1	Hydrochloric acid 1% v/v
HA2	Hydrochloric acid 2% v/v
HA3	Hydrochloric acid 3% v/v
HA4	Hydrochloric acid 4% v/v
PA1	Phosphoric acid 1% v/v
PA2	Phosphoric acid 2% v/v
PA3	Phosphoric acid 3% v/v
PA4	Phosphoric acid 4% v/v
SA1	Sulfuric acid 1% v/v
SA2	Sulfuric acid 2% v/v
SA3	Sulfuric acid 3% v/v
SA4	Sulfuric acid 4% v/v
SH1	Sodium hydroxide 1% v/v
SH2	Sodium hydroxide 2% v/v
SH3	Sodium hydroxide 3% v/v
SH4	Sodium hydroxide 4% v/v
PH1	Potassium hydroxide 1% v/v
PH2	Potassium hydroxide 2% v/v
PH3	Potassium hydroxide 3% v/v
PH4	Potassium hydroxide 4% v/v

Sulfuric acid, hydrochloric acid, phosphoric acid, and nitric acid were used as acids. Different concentrations of acids and bases were used and pretreated samples were named as shown in Table 2.1 Hazelnut shells were air-dried and ground to fine dust before pretreatment. The resulting material was aliquoted in plastic bags and stored at 4°C for later use.

2.2.2.1. Dilute Acid Pretreatment

Each 1 g sample was mixed with 20 mL acid such as H_2SO_4, HCl, H_3PO_4, and HNO_3 [0.5, 1, 2, 4 % (v/v)] and then incubated at 121°C for 1 h. The mixture of chemicals and hazelnut was filtered after cooling down. The remaining solids were washed with distilled water 3 times.

2.2.2.2. Alkaline Pretreatments

Each 1 g sample was mixed with 20 mL alkaline chemical such as NaOH and KOH [0.5, 1, 2, 4 % (v/v)] and incubated at 121°C for 1 h. The mixture of chemicals and hazelnut shells was filtered after cooling down. The remaining solids were washed with distilled water 3 times.

2.2.2.3. Enzmatic Hydrolysis

Chemical-pretreated hazelnut shells (1/10 w/w) were incubated in 50 mM citrate buffer as described below (pH= 4.8) at 50°C for 20 min as shown in Figure 2. To prepare citrate buffer, 42 g citric acid monohydrate, 150 mL deionized water, and 10-12 g sodium hydroxide were mixed and diluted to 1 L. pH was adjusted to 4.5 with HCl or NaOH. Citrate buffer was stored as 1M. When the buffer was used, dilution was done to decrease

the molarity of the buffer to 0.05M. Then pH was checked and adjusted to 4.8 with HCl or NaOH before use. After treatment with citrate buffer, Celtic Ctec2, a cellulase enzyme blend, was added to the mixture of hazelnut shell and citrate buffer. Enzyme/mixture volume was 1/15 v/v. Samples were incubated at 50°C for 3 d at 150 rpm.

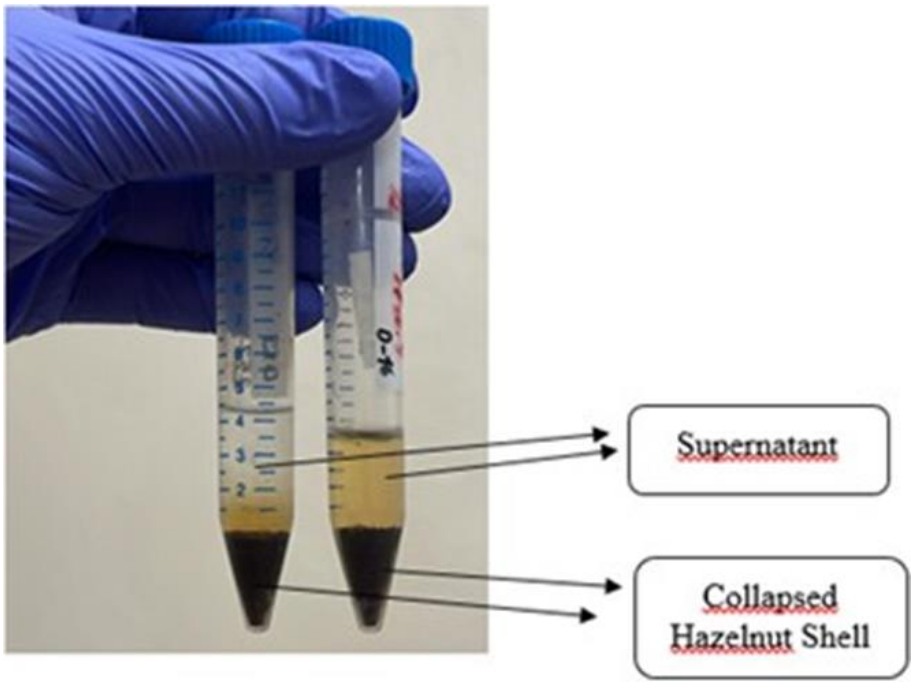

Figure 2.2. Chemically pretreated and enzymatic hydrolysed hazelnut shells.

2.2.2.4. Evaluation of Releasing Sugar

Releasing sugar concentration was determined by using DNS (3,5-dinitro salicylic acid) assay and then results were calculated from concentration to weight for determining yield. DNS provides a color change in the presence of free carbonyl groups. Free carbonyl groups are called reducing sugars.

To perform the DNS assay, citrate buffer and DNS-reagent were prepared before the experiment. Citrate buffer was prepared as described in 1.2.2.3. For the DNS-reagent, the DNS reagent was prepared according to NREL 2.12 g 3,5-dinitro salicylic acid, 3.96 g sodium hydroxide, and 283.2 mL distilled water were mixed. After dissolving, 61.2 g

sodium potassium tartrate (Rochelle salt), 1.52 mL Phenol (melted at 50°C), and 1.66 g sodium metabisulfite were added.

For spectrometric analysis, D-glucose standards were prepared from 1 g/L to 7 g/L. Supernatants as shown in Figure 2, were diluted with distilled water because samples had to be between 1 g/L and 7 g/L. After dilution, each standard or sample (0.5 ml) was mixed with 1 ml citrate buffer, and 3 ml DNS reagent in a 15 ml falcon tube. Falcon tubes were incubated at 95°C for 10 min. After incubation, samples were vortexed. Then, 0.2 ml of these samples were placed in centrifuge tubes with 1 ml distilled water. Centrifuge tubes were homogenized. Then samples were loaded to the 96-well plates as shown in Figure 2.3.

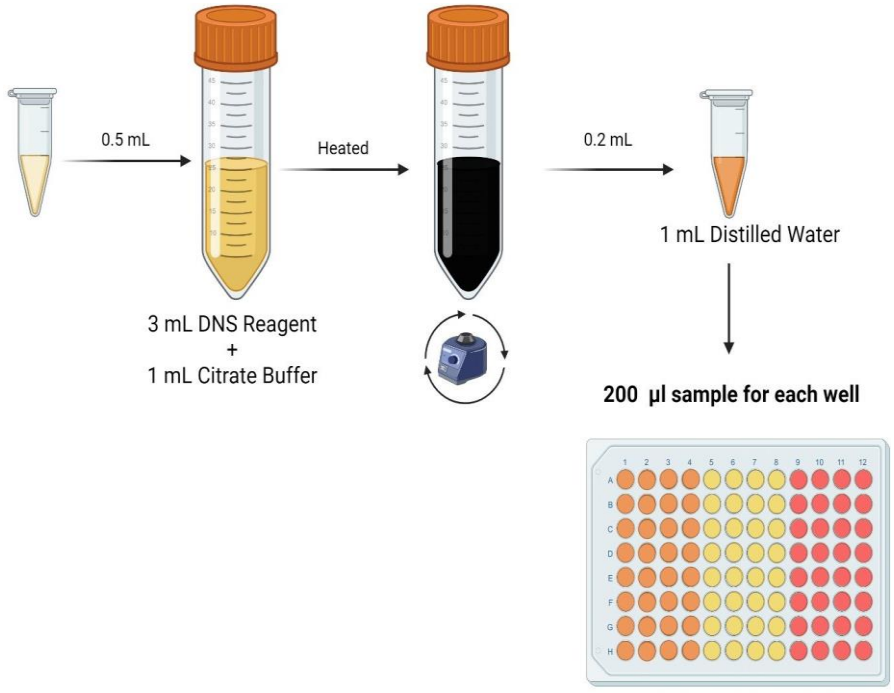

Figure 2.3. Chemically pretreated and enzymatic hydrolysed hazelnut shells.

2.2.3. BC Production and Production Optimization

Lyophilized Gluconacetobacter xylinus ATCC® 23770 ™ was diluted with 1 mL Mannitol Broth (5 g/L yeast extract, 3 g/L peptone, 25 g/L mannitol) before transfer to 5

mL of the same medium at 28°C. The cultures were incubated until turbidity was visible. At this point, they were plated on mannitol agar (5 g/L yeast extract, 3 g/L peptone, 25 g/L mannitol, 15 g/L agar) which was defined by Klemm et al., 2005. Bacteria on the agar were incubated at 28°C for 48-72 h until single colonies were visible. Single colonies were incubated in 40 mL HS (Hestrin-Schramm) (20 g/L d-glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L disodium phosphate, 1.15 g/L citric acid monohydrate) in 250 mL jars at 28°C for 5 d. After 5 d, BC in the medium was separated and the medium was centrifuged at 4500 rpm for 8 min. After centrifugation, the liquid part was discarded and the pellet was dissolved in the HS medium. To make bacterial stock solutions, bacterial culture samples were placed in cryo tubes containing 7% DMSO (dimethyl sulfoxide) and stored at -20°C.

2.2.3.1. Determination of Optimized Inoculation Rate in HS Medium

Stock bacteria were inoculated in HS agar for 3-4 days. Single colonies were inoculated in 5 mL HS for 3 d at 28°C. Bacteria in 5 mL media were inoculated to 20 mL of the same medium with inoculation rates of 1, 4, 7, 10, 13% and grown for 3 d at 28°C. Bacteria in the 20 mL samples were then inoculated to 100 mL of the same medium at 28°C in the jars. After 10 d, BC was collected from the medium and soaked in 0.1 M NaOH for 2 h at 80°C. Then BC was dried in a freeze drier for 3 d. After 3 d, dried BC was weighed.

2.2.3.2. Determination of Optimized pH for HS Medium

Stock bacteria were inoculated to HS agar for 3-4 d at 28°C. Single colonies were inoculated in 5 mL HS media for 3 d at 28°C. Bacteria in 5 mL media were inoculated to 20 mL of HS medium at 28°C. The 100 mL medium samples were adjusted to pH 4, 4.5, 5, 5.5, 6, 6.5, and 7 with HCl or NaOH. Bacteria in 20 mL media were inoculated to 100 mL medium of different pH in the jars. After 10 d, BC was collected from the medium

and soaked in 0.1 M NaOH for 2 h at 80°C. Then BC was dried in a freeze drier for 3 d. After 3 d, dried BC was weighed.

2.2.3.3. Determination of Optimized Incubation Time in HS Medium

Stock bacteria were inoculated in HS agar for 3-4 d at 28°C. Single colonies were inoculated in 5 mL HS media for 3 d at 28°C. Bacteria in 5 mL medium were inoculated to 20 mL HS medium at 28°C. Inoculation rates and pH were determined as pH:6 and 10 days according to results from 2.2.3.1 and 2.2.3.2. Bacteria in 20 mL medium were inoculated to 100 mL medium at 28°C in the jars. BC was collected from the different medium after 4, 7, 10, 14, 21 d and soaked in 0.1 M NaOH for 2 h at 80°C. Then BC was dried in a freeze drier for 3 d. After 3 d, dried BC was weighed.

2.2.3.4. Alternative Medium Containing Hazelnut Waste

Stock bacteria were inoculated in HS agar for 3-4 d at 28°C. Single colonies were inoculated in 5 mL HS media for 3 d at 28°C. Bacteria in 5 mL media were inoculated to 20 mL HS medium.

Table 2.2. Alternative media for producing BC.

Name	**Sugar Source**	**Ethanol Addition**
HS0	Pretreated Hazelnut Shell	None
HS1	Pretreated Hazelnut Shell	1%
HS2	Pretreated Hazelnut Shell	2%
E0	D-glucose	None
E1	D-glucose	1%
E2	D-glucose	2%

Inoculation rates, pH, and incubation times were determined as pH:6, 10% inoculation rate and 10 d according to results from 2.2.3.1, 2.2.3.2, and 2.2.3.3. Bacteria in 20 mL media were inoculated to 100 mL alternative medium which was made with pretreated hazelnut shells instead of d-glucose, in the jars. Chao et al. (2015) showed that ethanol had a positive effect on xylose utilization by G. xylinus. Due to this impact, media were prepared with different ethanol concentrations and sugar sources. Media were named as shown in Table 2.2. After 10 d, BC was collected from the medium and soaked in 0.1 M NaOH for 2 h at 80°C. Then BC was dried in a freeze drier for 3 d. After 3 d, dried BC was weighed.

2.2.4. BC Characterization

The produced BC was removed from the medium with forceps. BC was soaked in 0.1 M NaOH at 80°C for 2 h. NaOH was decanted and distilled water was added. BC was incubated with distilled water for 1 d. Then BC was dried in a freeze drier for 3 d. All of the subsequent analyses with the lyophilized BC were performed in triplicate.

2.2.4.1. Water Holding Capacity

Water holding capacity was measured according to Ul-Islam et al., 2013. Dried cellulose was incubated in distilled water until it was swollen. Water on the surface of the BC was removed by shaking. BC was dried at 50°C for 1 d. Dried BC was weighed. Water holding capacity was determined by subtracting the wet weight from the dried weight. Water holding capacity was calculated by dividing this value by the dried weight.

2.2.4.2. SEM (Scanning Electron Microscopy)

SEM analysis was done according to Morais et al., 2013 and Azzaoui et al., 2017 to view the surface of BC. Freeze-dried BC was sprayed with a thin evaporated gold layer before screening for prevention of electrical charge. SEM images were taken at 25 kV voltage and 5000 K magnification using the Zeiss Evo MA10 at Iztech T.A.M. (Tümleşik Araştırma Merkezi).

2.2.4.3. FT-IR (Fourier Transform Infrared Spectroscopy)

FT-IR was performed to characterize the molecular bonds of the BC samples and to determine the functional groups in the structure of BC. This analysis was used to determine whether the BC collected from different media were the same, the state of the bonds in the structure, the binding sites and whether the structure was aromatic or aliphatic. Scans were done at 4000-400 cm-1 wavelength. FT-IR was done according to Ciolacu et al., 2011 and Azzaoui et al., 2017 using the FT-IR spectrometer (PerkinElmer, Spectrum Two) at Iztech T.A.M. (Tümleşik Araştırma Merkezi).

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Chemical Content of Hazelnut Shell

Lignin, cellulose and hemicellulose percentages of lignocellulosic materials may differ in different types of the same materials, even in the same material collected at different times. Considering the existence of these differences, it was necessary to determine the content of the hazelnut shell. The content of the hazelnut shell was determined for evaluating pretreatment effectiveness. The lignin content of the hazelnut shell was measured as 42.09%. Studies by Yanik et al., 2008 and Demirbaş, 2005, found almost the same percentage of lignin in the hazelnut shell: 41.83% and 42.1%, respectively. Lignin was the most abundant substance in the hazelnut shell. Cellulose and hemicellulose contents were lower than lignin content with 21.84% cellulose in the hazelnut shell. Uzuner et al., 2017 and Demirbaş, 2005 found that the content of cellulose in hazelnut shells was 24.2% and 25.2% respectively. In the current work, the hemicellulose content of the hazelnut shell was found to be 22.99%, very similar to the amount of cellulose. Hassan et al., 2018 found that the hemicellulose content of hazelnut shells was 23%. In addition to these substances, the hazelnut shell included 1.62% ash, 10.57% fat, and 2.34% protein as shown in Table 3.1. When all these results were compared, it was determined that approximately 45% of the hazelnut shell could be converted into monosaccharides. Considering the high amount of lignin, more effective pretreatment methods were applied.

Table 3.1. Chemical content of hazelnut shell.

Substance	**Percentage**
Moisture	9.03 ± 0.61
Ash	1.62 ± 0.02
Total Fat	10.57 ± 0.9
Protein	2.34 ± 0.45
Extractives	13.08 ± 0.27
Lignin	42.09 ± 1.49
Hemicellulose	22.99 ± 0.11
Cellulose	21.84 ± 1.28

3.2. Chemical Pretreatment and Enzymatic Hydrolysis

Hazelnut shells were pretreated with different chemicals in different ratios. Chemically pretreated samples were incubated with Celtic Ctec2 cellulase enzyme to release sugar. After incubation, total sugar content was determined with the DNS-assay. As a control group, biologically pretreated hazelnut shell was used. Statistical analysis was performed using LSD methods via SPSS. When untreated (UT) was compared with acid pretreated samples, there was no significant difference between the acid pretreated samples. Despite pretreatments with four different acids (H_2SO_4, HCl, H_3PO_4, and HNO_3) at four different concentrations, all of the samples treated with acid had approximately 24% total sugar. The reason for not observing a significant difference between acid pretreated samples and UT was hypothesized resulting from the conversion of sugars to 5-hydromethylfurfural (HMF) at high temperatures and in an acidic environment. Rahmawati et al., 2020 showed the effect of higher temperature and acidic concentration on converting glucose to HMF. Unlike the acid pretreated samples, those treated with alkaline solutions (list bases here) showed significant differences with the UT sample. Different alkali treatments also differed from each other. PH1 and SH3 samples had the highest sugar concentrations of approximately 38%. These treatments resulted in significantly more sugar than all but one other treatment (SH2).. SH1 had the lowest sugar

concentra- tion among the alkaline pretreated samples (32%), however, this treatment still resulted in a higher total sugars than the best acid pretreatment. Considering all of these results, it was determined that SH3 or PH1 treatment should be used for the experiments aimed at optimizing BC production.

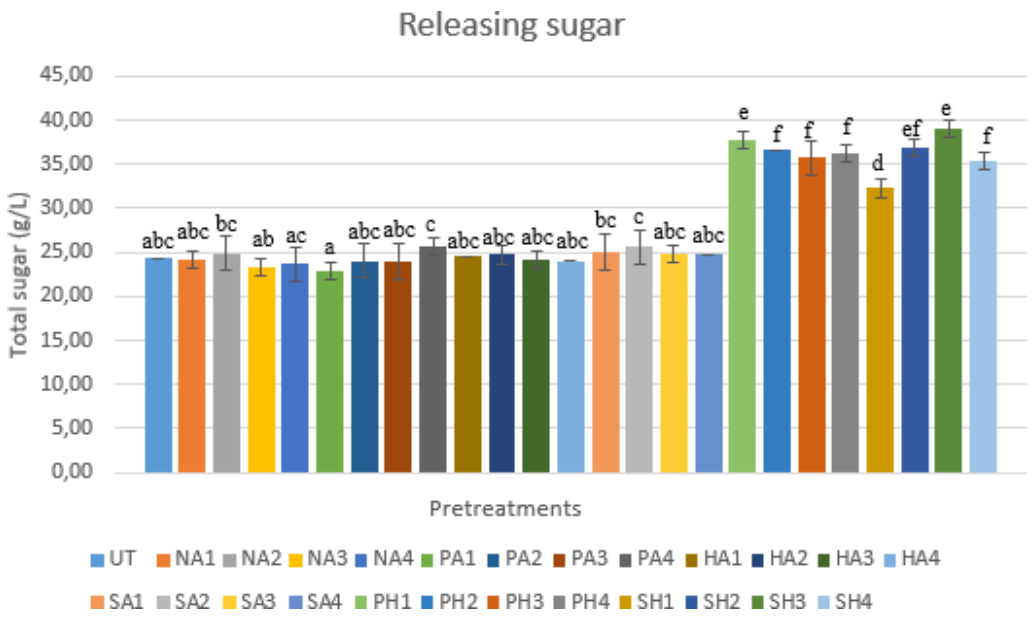

Figure 3.1. Measured sugar concentration after enzymatic hydrolysis.

3.3. Optimization of BC Production

In order to improve BC production, optimal conditions must be provided to the bacterium by making changes to growth conditions. While making these changes, the environmental impact of the BC production system was taken into consideration. Some optimizations were made to HS medium, which is a widely-used growth medium for the bacteria. These optimizations were applied to the medium using pretreated hazelnut shells as a sugar source. The decisions on optimal conditions were made based on the BC weights produced. When the bacteria are transferred from the old medium to fresh medium, a certain amount is taken from the old medium and transferred to the fresh medium. The ratio of this transferred volume to the total volume is called the inoculation

rate. Studies have found different optimum inoculation rates for *gluconacetobacter xylinus*. Chandrasekaran et al., 2017, R. Jaramillo et al., 2013, and Xiao et al., 2010 found the optimum inoculation rate was 10% for Gluconacetobacter xylinus. Farrag et al., 2019 found the best inoculation rate to be 8%, while Zhou et al. 2010 found it to be 12%. In studies on other BC-producing bacteria, Tang WeiHua et al., 2009 worked with Gluconacetobacter oboediens and found the optimum inoculation rate for this bacterium to be 8%. In another study by Farrag et al., 2019 , this value was found to be 7% for Gluconacetobacter hansenii. This difference in the studies made it necessary to test inoculation rate for optimization in our system. Thus in order to determine optimum inoculation rate, 1%, 4%, 7%, 10%, 13% inoculation rates were tried. The least yield was obtained in the experiment performed with 1% inoculation rate, and significant differences were found between this yield and those obtained with rates of 7% and higher. When these data were examined, 10% inoculation rate was clearly the optimal value.

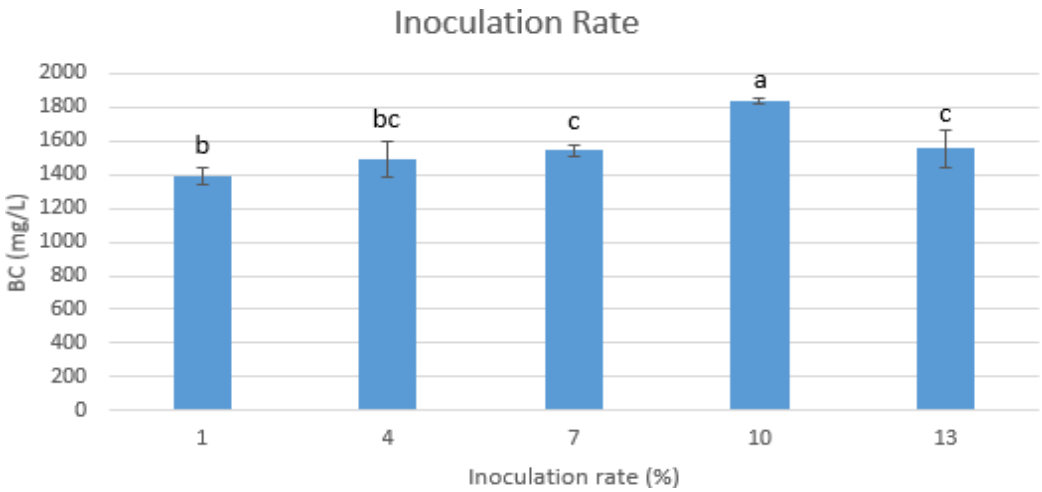

Figure 3.2. The inoculation rate effect on BC producing.

pH plays a critical role in metabolic activities and there is an optimal pH value at which each microorganism can grow. Some studies have been carried out to determine the optimum pH in BC production, and as a result, the optimum pH range has been delimited between 4.32 and 7 as shown in Table 3.2. It must be taken into account that these previous results are dependent on the many different parameters used in each study.

By examining all these data, it was deemed appropriate to carry out pH studies between 4.5 and 7 at 0.5 unit intervals.

Table 3.2. Optimal pH for various microorganisms.

Microorganism	**Optimal pH**	**Reference**
Gluconacetobacter xylinus TJU-D2	4.32	Du et al., 2020
G. xylinus ATCC 23768	4.5	J. H. Lin et al., 2011
Acetobacter xylinum from MARDI (Malaysia Agricultural Research and Development Institute)	5.15	(Nazeri, 2012)
Gluconacetobacter xylinus IFO 13693	5.6	(R. Jaramillo et al., 2013)
Glucoacetobacter xylinum BC-11	6	Zhao et al., 2018
Gluconacetobacter xylinus C18	6.5	Singh et al., 2017
Acetobacter senegalensis MA1	7	(Aswini et al., 2020)
Gluconacetobacter xylinus CH001	7.3	Xiao et al., 2010

When BC obtained from incubation at different pHs were examined, the maximum amounts (approximately 1750 mg/L) were obtained at pH 6 and pH 7. When these two values were compared statistically, there was no significant difference between them, however, they were significantly greater than the yields obtained at other pH levels. The amount of BC produced generally decreased with the decrease in pH. Moreover, with the decrease in pH, it became difficult to remove BC from the culture without structural deterioration. Thus, as a result of these analyses, it was determined that low pH caused significant decreases in BC yield and quality. The pH measured before pH adjustment was found to be around 6.25. For this reason, 6 was chosen as the optimum pH because it can adjust the pH using less chemicals.

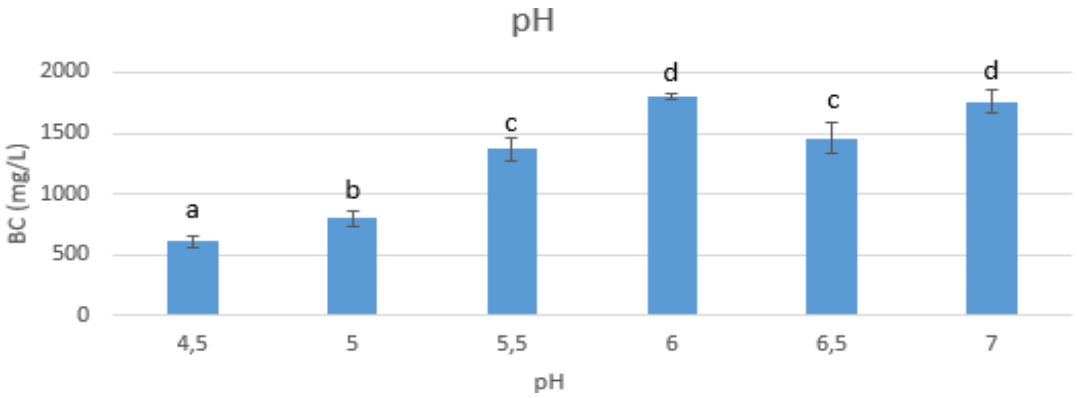

Figure 3.3. The pH effect on BC producing.

Unless fresh medium is added regularly, the bacteria begin to die and cannot produce BC. It is important to find the time at which BC production is discontinued or reduced because time is one of the most valuable things in the industry. Therefore, the day on which BC production stopped and the optimum length of culture had to be determined. In studies on BC production, the optimum number of culture days has been determined to be between 7 and 16 days. Singh et al., 2017 and Feng et al., 2015 found the optimum day to be 7 in their studies. Alemam et al., 2021 and Du et al., 2020 determined the optimum day as 8 in their studies. While Raiszadeh-Jahromi et al., 2020 determined the optimum day as 10, Farrag et al., 2019 determined the optimum day as 12 days, R. D. Jaramillo et al., 2014 determined it as 14 days, and Zhang et al., 2021 determined it as 16 days. When the culture times were compared in the current experiment, the least BC production occurred on the 4th and 7th days, and significant differences were found between the BC amounts produced on the other days (3.4). When the BC production on the 10th day was compared with the yield from longer culture, no significant difference was found. Considering these data, the optimum length was determined as 10 days, and the difference was emphasized by dividing the amount of BC produced for each day by the number of days. As a result of these calculations, the lowest efficiency was seen on the 4th day with 143 mg/day, and the highest efficiency was seen on the 10th day with 179.7 mg/day. When we look at this efficiency calculation, a decrease is observed starting from the 10th day.

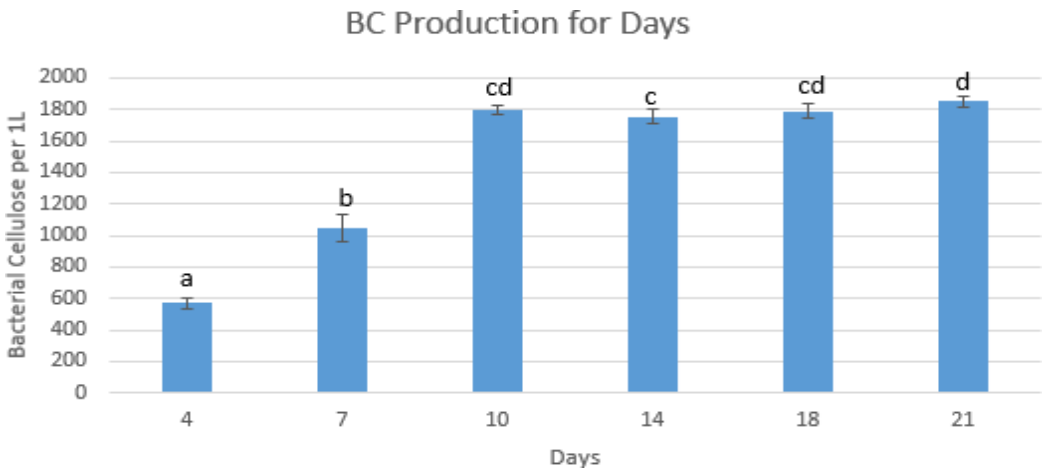

Figure 3.4. The incubation time effect on BC producing.

In addition to culture time, inoculation rate, and pH parameters optimized on HS medium, the medium was prepared using hazelnut shells in line with the results obtained from acid and base chemical pretreatments and these conditions were applied. It was determined that xylose coming from hemicellulose would also be present in the total sugar obtained from chemical pretreatments, so it was aimed to prepare alternative cultures in which xylose could be used efficiently by the bacteria. The hazelnut shells, which were pre-treated with 1% potassium hydroxide, were then incubated with the Celtic Ctec2 cellulase enzyme to ensure that the sugars were soluble in the buffer solution. The amount of reducing sugar was determined by the DNS method and added to the medium at a rate of 20 grams of sugar per liter. Other components were completed with the same volume of distilled water as the HS medium. In order to observe the effect of the presence of ethanol in the prepared medium on BC production, media without ethanol, 1% ethanol and 2% ethanol were also prepared. When cultures grown in these different media were examined, it was determined that there was no significant difference between HS2 and E0 and that all other cultures had significant differences between each other (Figure 3.5). Based on the yield of BC, HS1 medium had the highest efficiency.

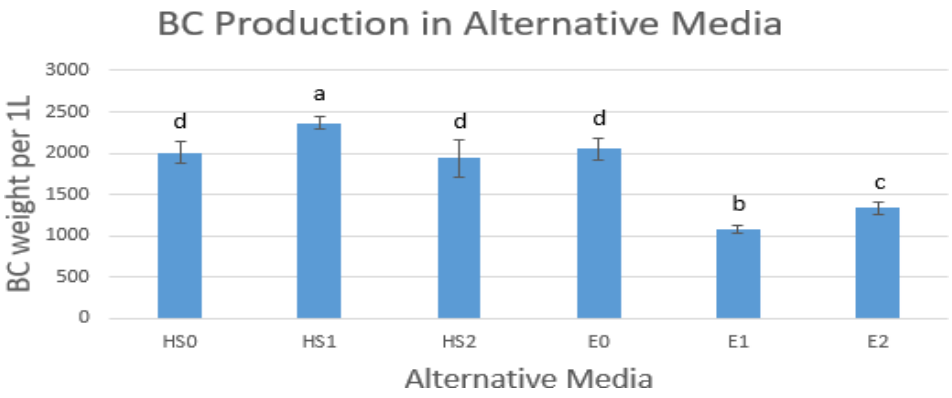

Figure 3.5. The effect of alternative media on BC producing.

According to the obtained data, the highest BC yield (2369 mg/L) was achieved with 10% inoculation, 10 days, pH 6, and 1% ethanol in the medium.

3.4. Characterization of BC

The results of alternative cultures created under optimum conditions were evaluated based on BC weights. Characterization studies were carried out on BC to determine the relationship between the weights of BC samples and other physicochemical properties. The first of these studies was water retention capacity. While determining the water retention capacity, the amount of water each gram of BC holds was determined (Figure 3.6). This equation was calculated as (liquid weight-dry weight/dry weight) x100. When the water retention capacities of bacterial celluloses taken from different cultures were compared, significant decreases were observed in the cultures to which ethanol was added (HS1, HS2, E1, E2). Bacterial cellulose produced by bacteria growing in their own medium without ethanol (E0) had the highest water retention capacity and this value was significantly higher than those obtained from the other media. Characterization of the molecular bonds in the BC was performed with FT-IR to identify functional groups. It was determined that the samples from different culture conditions had similar spectral properties to each other. Results are shown in Figure 3.7.

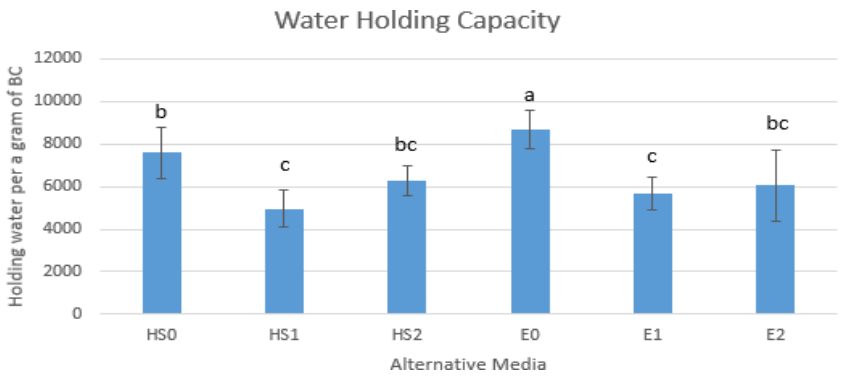

Figure 3.6. Water holding capacities of BC.

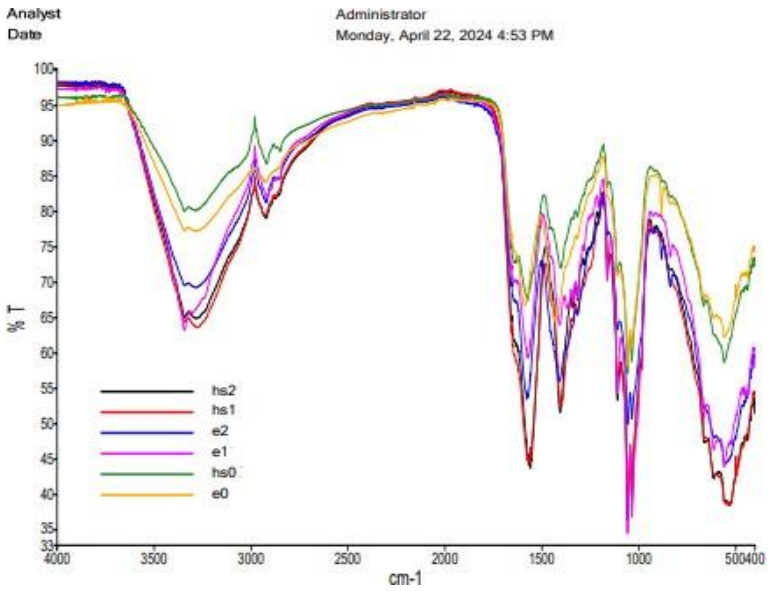

Figure 3.7. FT-IR results of BC.

C-O-C stretching at 1100 cm^{1-} was detected in all samples, indicating that structural integrity was preserved. The C-O-C stretch shows the ether bond between glucose units. While the peak seen at 1050 cm^{-1} determines purity, the C-O-C stretch can vary between 1020 and 1160 cm^{1-}. As seen in the examples, a value of 1100 cm^{-1} was obtained with the BC obtained from hazelnut shells. These samples show the abundance of the crystalline region. The water absorption peak (1640 cm^{1-}) is similar in all samples. This peak, based on the interaction of water and the OH ion, is a measure of the moisture

content and water holding capacity of the sample. This shows that the water retention capacities of the different samples are similar. Looking at the peaks at 2900 cm^{1-} that occur in the presence of the CH group, the carbon skeleton has similar density and structural features are preserved among the samples. The peaks at 3400 cm^{1-}, which occurred in the presence of the OH group, appeared broad and distinct in all samples. The broad appearance of these peaks shows that the amorphous region is reduced and confirms the results from the peaks formed in C-O-C stretching. It was determined that the samples were of high purity due to the clear nature of the peaks formed as a result of OH and CH.

The FT-IR results of BC and plant cellulose produced from Nata De Coco by Halib et al., 2012 and BC obtained by Surma-Slusarska and Matejak, 2009 were compared with each other and with pure cellulose as shown in Figure 3.8. When the peaks in this graph were examined and compared with the hazelnut shell results, it was determined that the BC produced from Nata De Coco and hazelnut shells had similar structures.

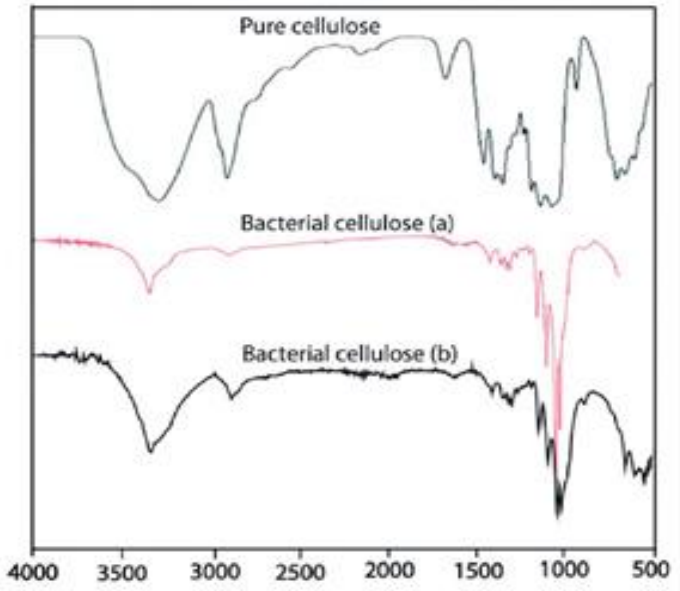

Figure 3.8. Comparison of FT-IR results of different types of cellulose BC (a) from Surma-Ślusarska et al. (2008) and BC (b) from nata de coco.

In Figure 3.9. reveal the structural differences of bacterial celluloses produced in different media. While the cellulose obtained in the hazelnut shell medium (A) exhibited

a dense and compact structure, in the medium containing hazelnut shells and 1% ethanol (B), the gaps between the fibers increased and a looser, irregular structure was observed. In the medium (C) containing hazelnut shells and 2% ethanol, the gaps between the fibers became wider and the structure became more open and loose. Cellulose produced in standard medium (D) has a tightly knit and dense structure, and there are no obvious gaps between the fibers. With the addition of 1% ethanol to the standard medium (E), the gaps between the fibers increased and the structure became looser and irregular.

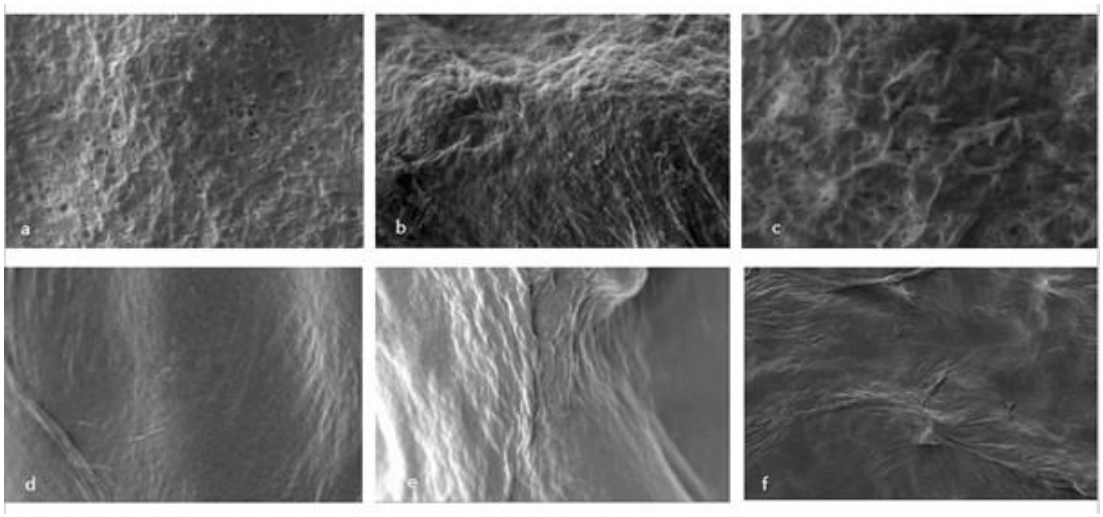

Figure 3.9. SEM Images of BC a) Hazelnut shell, b) hazelnut shell with 1% EtOH, c) hazelnut shell with 2% EtOH, d) HS medium.

With the addition of 2% ethanol to the standard medium (F), the fibers exhibited a very loose and irregular structure, the voids increased significantly and the fiber density decreased. In general, the addition of ethanol caused the fibers to have a looser and irregular structure, while the hazelnut shell medium created a more dense and compact structure than the standard medium. These structural differences reveal the effects of medium components and ethanol on bacterial cellulose production.

CHAPTER 4

CONCLUSION

In this study, hazelnut shells were successfully utilized as a sustainable and eco-friendly source for bacterial cellulose (BC) production using *Gluconacetobacter xylinus*. The optimal conditions for BC production were identified, including a 10% inoculation rate, pH 6, a 10-day incubation period, and the addition of 1% ethanol in the medium. Among the various pretreatment methods tested, alkaline pretreatment with 1% potassium hydroxide (KOH) and 3% sodium hydroxide (NaOH) yielded the highest sugar concentrations, demonstrating the effectiveness of these methods in releasing sugars from hazelnut shells. The study confirmed that hazelnut shells, which are typically considered agricultural waste, can be repurposed as a valuable raw material for BC production. The findings suggest that the use of hazelnut shells not only provides an alternative carbon source for BC production but also contributes to waste valorization and environmental sustainability. Additionally, the BC produced under the optimized conditions exhibited high purity and structural integrity, making it suitable for potential applications in biomedical, cosmetic, and food industries. 2.4 g/L BC was produced in the end of the this study. Similarly, Yang et al. (2024) enhanced BC production from 2.2 g/L to 6.8 g/L by overexpressing the *bcsc* and *bcsd* genes in *Gluconacetobacter xylinus* under optimized conditions using an enriched HS medium ($MgSO_4$ 5.7 g/L and trisodium citrate 20 g/L), pH 5.4-5.8, and a 10-day incubation period at 30°C. Zhao et al. (2018) also produced 1.117 g/L BC using carbon sources from polysaccharide fermentation wastewater in combination with HS medium, incubating *Gluconacetobacter xylinum BC-11* at pH 7.0 and 30°C for 10 days. Gomez et al. (2013) reported a yield of 0.85 g/L in 4 days using *Gluconacetobacter sacchari* in HS medium with carbon from dry olive mill waste at pH 5.0 and 30°C under static conditions. Additionally, Hyun et al. used makgeolli sludge as a carbon source, achieving 1.67 g/L BC in 7 days by incubating *Gluconacetobacter xylinus* at pH 5.0 and 30°C. These studies highlight the effectiveness of alternative carbon sources like agricultural and industrial waste in optimizing BC production. The findings

suggest that BC production can be significantly improved through genetic engineering, optimized growth conditions, and the use of cost-effective feedstocks, offering promising opportunities for sustainable and scalable BC production. Future studies could explore further optimization of the production process, including the use of different microbial strains and pretreatment techniques, to enhance BC yield and quality. Moreover, the potential for scaling up this process for industrial applications should be investigated, as it holds promise for large-scale production of BC from renewable and low-cost feedstocks like hazelnut shells.

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