INVESTIGATION OF BACTERIAL CELLULOSE PRODUCTION INCLUDING A MOLECULAR APPROACH UNDER DIFFERENT STRESS CONDITIONS

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ABSTRACT

INVESTIGATION OF BACTERIAL CELLULOSE PRODUCTION INCLUDING A MOLECULAR APPROACH UNDER DIFFERENT STRESS CONDITIONS

Bacterial cellulose has many unique, fascinating, and desirable properties such as high crystallinity, high mechanical strength, high degree of polymerization, high water holding capacity, flexibility, elevated purity, mouldability in different shapes and unique nanostructure. Therefore, bacterial cellulose has a wide range of applications in food industry, electronic and biomedical fields. In this study, two different types of stress conditions, which are cold shock and sonication, were applied during bacterial cellulose production. Structural and characteristic properties of produced bacterial cellulose and behavior of K. xylinus were investigated. Both sonication and cold shock stress conditions have similar effects on both cell behavior and differences in produced bacterial cellulose characteristics. For bacterial cellulose production, sonication 60 seconds stress applied group yielded 1.3 fold greater than control group with 2.9 g/L BC production and cold shock stress applied group yielded 1.6 fold greater than the control group with 3.3 g/L. The application of both cold shock and sonication stress, resulted in the production of stronger and more durable bacterial celluloses. 2.1 fold grater ultimate strength than the control group for sonication stress and 2.0 fold grater ultimate strength than the control group for cold shock stress. Applied stress resulted in a rougher surface structure, higher tensile strength, more thermostable structure with 1.73 fold less mass loss per temperature change than the control group for sonication stress, 1.8 fold less mass loss per temperature change than the control group for cold shock stress, and preserved molecular structure and conformation of bacterial celluloses along with stronger structure. For sonication stress, gene expression levels for bcsA, ccpAx genes were investigated. For 30 seconds, gene expression reached higher levels than the control group for bcsA gene as 1.4 fold of the control group on the 4^{th} day and 1.7 fold of the control group on the 7^{th} day. While ccAx gene is 0.8 fold of the control group on the 4^{th} day, reached 1.4 fold on the 7^{th} day of the fermentation. For 60 seconds, higher expression levels were reached when compared with 30 seconds on the first days. When compared to control group, 1.8 fold greater for bcsA gene and 2.4 fold greater for ccpAx gene on the 4^{th} day. However continuous stress resulted in expression levels to drop 0.8 fold for bcsA gene and 0.6 fold for ccpAx gene when compared with the control group.

ÖZET

FARKLI STRES KOŞULLARI ALTINDA BAKTERİYEL SELÜLOZ ÜRETİMİNİN MOLEKÜLER SEVİYEDE İNCELENMESİ

Bakteriyel selüloz, yüksek kristallik, mekanik mukavemet, su tutma kapasitesi, esneklik, saflık, farklı şekillerde kalıplanabilirlik, yüksek derecede polimerizasyon, ve benzersiz nanoyapı gibi birçok benzersiz, büyüleyici ve arzu edilen özelliklere sahiptir. Bu nedenle bakteriyel selüloz gıda endüstrisi, elektronik ve biyomedikal alanlarda geniş bir uygulama alanına sahiptir. Bu çalışmada, bakteriyel selüloz üretimi sırasında soğuk şok ve sonikasyon olmak üzere iki farklı stres koşulu uygulanmıştır. Stres koşullarının üretilen bakteriyel selüloz üzerindeki ve K. xylinus'un davranışı üzerindeki etkileri araştırılmıştır. Sonikasyon ve soğuk şok streslerinin benzer etkileri olup, K. xylinus davranışında ve üretilen bakteriyel selüloz özelliklerinde de farklılıklara sebep olmuştur. Bakteriyel selüloz üretimi için, 60 saniyelik stres uygulanan grup, 2,9 g/L BC üretimi ile kontrol grubuna göre 1,3 kat daha fazla ürün vermiştir ve soğuk şok stresi uygulanan grup, 3,3 g/L ile kontrol grubuna göre 1,6 kat daha fazla verim sağlamıştır. Hem soğuk şok hem de sonikasyon stresi, daha güçlü ve daha dayanıklı bakteriyel selülozlarının üretilmesiyle sonuçlanmıştır. Sonikasyon stresinde, kontrol grubuna göre 2.1 kat daha fazla nihai mukavemet ve soğuk şok stresinde kontrol grubuna göre 2.0 kat daha fazla nihai mukavemet elde edilmiştir. Sonikasyon stresi, kontrol grubuna göre sıcaklık değişimi başına 1.73 kat daha az kütle kaybı, soğuk şok stresi için kontrol grubuna göre sıcaklık değişimi başına 1.8 kat daha az kütle kaybı ile daha termostabil yapı, daha pürüzlü bir yüzey yapısı, daha yüksek çekme mukavemeti, daha güçlü yapı ile birlikte bakteriyel selülozların korunmuş moleküler yapısı ve konformasyonu ile sonuçlandı. Sonikasyon stresi için bcsA, ccpAx genlerinin gen ekspresyon seviyeleri araştırıldı. Sonikasyon 30 saniye stresinde bcsA geni için gen ekspresyonu 4. günde kontrol grubunun 1.4 katı ve 7. günde kontrol grubunun 1.7 katı olarak kontrol grubuna göre daha yüksek seviyelere ulaştı. CcpAx geni 4. günde kontrol grubunun 0.8 katı iken, fermantasyonun 7. gününde 1.4 katına ulaştı. Sonication 60 saniye için, ilk günlerde 30 saniye ile karşılaştırıldığında daha yüksek ekspresyon seviyelerine ulaşıldı. 4. günde kontrol grubu ile karşılaştırıldığında bcsA geninde 1.8 kat, CcpAx geninde 2.4 kat daha fazla artış görüldü. Ancak sürekli stres, kontrol grubu ile karşılaştırıldığında, ekspresyon düzeylerinin bcsA geni için 0,8 kat ve ccpAx geni için 0,6 kat düşmesine neden olmuştur.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

BC	Bacterial cellulose
°C	Centigrade degrees
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
GRAS	Generally recognized as safe
GSR	General stress response
ORF	Open reading frame
RT-qPCR	Real-time quantitative polymerase chain reaction
SEM	Scanning electron microscope
σ	Sigma
TGA	Thermogravimetric analysis
XRD	X-ray diffraction

1. INTRODUCTION

1.1. CELLULOSE

Cellulose is a natural polymer that is the most abundant one on the earth with $1.5x10^{12}$ tons of production and decomposition annually [1]. It is also the most common and demanded sustainable and renewable resource due to its carbon neutrality, biodegradability, exhibiting low environmental risks along with low health and safety risks and nonpetroleum-based property along with unique properties as hydrophilicity, biocompatibility and nontoxicity [2,3]. In miscellaneous applications, as a biodegradable and renewable raw material, cellulose usage provided a solution to recycling problems and environmental concerns. Due to its versatile structuring that can be manipulated with chemical and physical methodologies, various possible applications are present, such as viscosity regulators, fillers, papers, sorption media, laminates, optical films, advanced functional materials building and coating materials, and textiles [4].

1.1.1. Sources of Cellulose

Cellulose sources in nature can be listed as plants, bacteria, minerals, fungi, algae, and animals. In plants, 40percent of the carbon fraction is composed of cellulose which has an important role as a structuring element. Plant cellulose is usually found in an impure form due to accompany of lignins, hemicelluloses, and extractives. In addition to plants, cellulose is present in the outer membrane of some marine animals such as ascidians. Green algae such as *Chaetamorpha melagonicum* and *Valonica ventricose* can produce cellulose. The synthesis of bacterial cellulose can be performed by bacteria from the genera of *gluconacetobacter*, *rhizobium*, *pseudomanas*, *agrobacterium* and *sarcina* with the use of various carbon sources. Bacterial cellulose is synthesized as a fibrous network directly and does not contain hemicelluloses, pectin, lignin and different biogenic product, therefore it is considered as pure along with its high degree of polymerization and highly crystalline properties [4].

1.1.2. Structure and Properties of Cellulose

Cellulose is biodegradable, insoluble in water and most of the organic solvents, chiral, odorless and a hydrophilic natural polymer with a 20-30percent angle of contact with water [5]. It has also unique desirable properties such as high thermal stability, biocompatibility, high modulus and high tensile strength [6]. The molecular structure of cellulose consists of units of D-glucopyranose rings, in $^4C_1$-chair configuration, that link one another with β-1,4-glycosidic bonds which produces a form of the cellulose chains in an alternate turning along the axis with 180 degrees of angle, independently from the cellulose source. $^4C_1$-chair configuration of D-glucopyranose ring units constitutes the conformation with the lowest energy. Repeating cellulose chains with at least 1.3nm length are considered cellobiose. Each anhydroglucose unit (AGU) in the cellulose chain contains three reactive hydroxyl groups: a primary group at C6 and two secondary groups in the plane of the ring at C2 and C3. Polymer formation with polycondensation results in the cellulose chain ends are different from each other chemically. A cellulose chain consists of one reducing and one nonreducing end. The nonreducing end includes an anomeric carbon atom that is linked with a glycosidic bond, and the reducing end which is the other end of the chain includes a D-glucopyranose unit with aldehyde function in equilibrium. Structural changes in cellulose at the molecular level emerge from oxidation or hydrolysis resulting reactions of the chain which occurs generally in the amorphous regions or on the fibril surfaces [7]. D-glucopyranose ring found on the cellulose chains contains hydroxyl groups, these groups forms hydrogen bonds with neighboring chains and provide firmly held chains together and produce microfibrils that have high tensile strength [8].

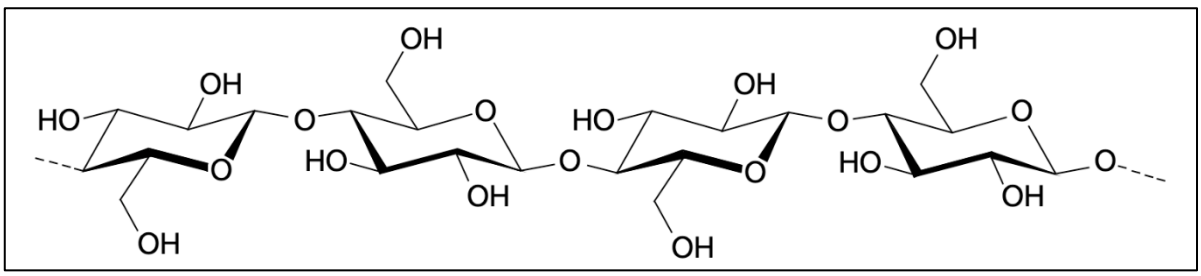

Figure 1.1. Structure of cellulose molecule

1.2. BACTERIAL CELLULOSE

Recently bacterial cellulose has gained more attraction in many fields such as biological and biomedical applications due to its outstanding physicochemical characteristics [9]. When the morphology of bacterial cellulose is investigated, it can be said that it is very pure when compared with plant cellulose by considering that it is not containing any lignin and hemicelluloses, however it contains only in very small amount of carboxyl and carbonyl moieties [10]. Bacterial cellulose consists of a basic fibril structure that includes β-1→4 glucan chain with the molecular formula of $(C_6H_{10}O_5)n$. Hydrogen bonds among chains and in a single chain keep the total structure together and provide well-arranged three dimensional nanofibers with the properties of high porosity and high surface area [11]. Bacterial cellulose possesses a 3D network of ultrafine cellulose fibrils that consist of 80 to 150 nanometers of diameter, ranging with the ability of containing water up to 99percent when in the never dried state and high degree of polymerization values up to 10000. Bacterial cellulose exhibits exceptional properties such as very high water absorption capacity, extraordinary mechanical strength, high tensile strength and high crystallinity with more than 80percent and in addition, has the advantage of moldability during biosynthesis. The ability of in situ moldability on bacterial cellulose in other words to be able to shape during biosynthesis is a crucial advantage [7]. Bacterial cellulose contains glucose monomers only, therefore it possesses impressive properties which are, high crystallinity, high mechanical strength, high degree of polymerization, high water holding capacity, hydrophobicity, flexibility, elevated purity, mouldability in different shapes and unique nanostructure. Due to these excellent properties, bacterial cellulose has a wide range of applications in food industry, electronic and biomedical fields. In addition, bacterial cellulose has many crucial applications in medical field such as implants for cartilage and bone repair, coverings in nerve surgery, artificial blood vessels, dura mater prosthesis, electronic platforms, hemostatic material, wound dressing, topical covering for severe wounds, engineering of artificial skin [12,13].

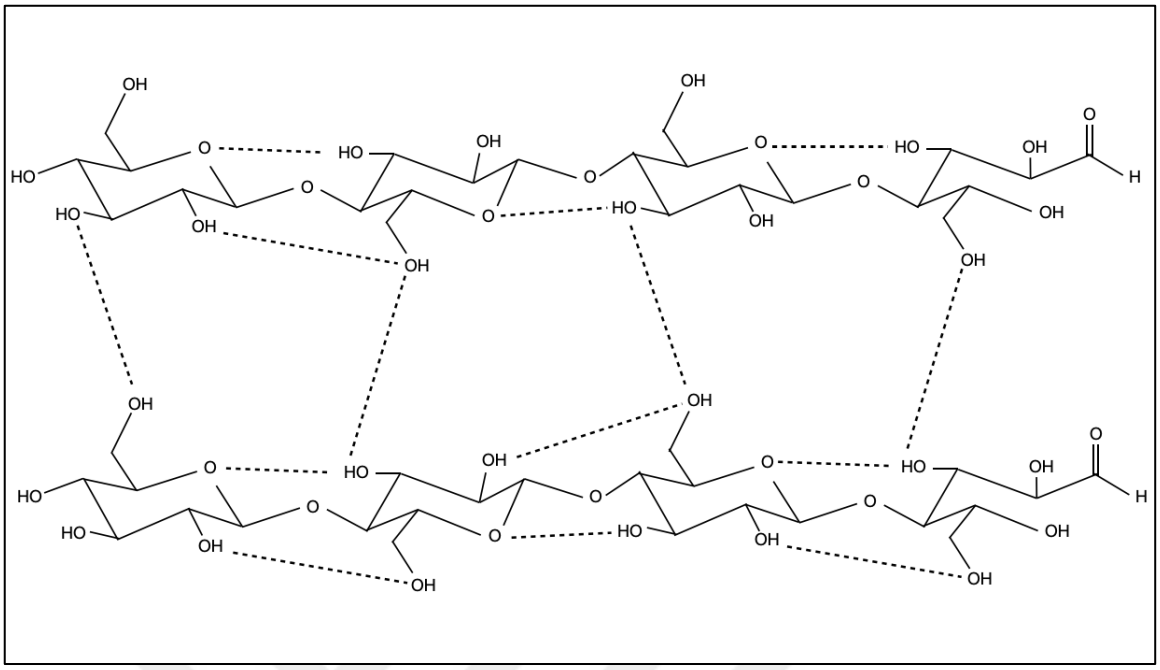

Figure 1.2. Intra and inter hydrogen bonds of bacterial cellulose (shown with dotted lines)

Bacterial cellulose is synthesized with oxidative fermentation by acetic acid bacteria. *Komagataeibacter* (formerly *Gluconacetobacter* and *Acetobacter*) *xylinus* is the most efficient producer of bacterial cellulose, therefore considered and studied as a model organism for bacterial cellulose production. It has the ability to produce of bacterial cellulose in large amounts by cellulose in liquid culture by utilizing a variety of carbon and nitrogen sources. *K. xylinus* produces a high-quality bacterial cellulose in terms of biocompatibility, water holding capacity, mechanical strength, crystallinity, and biodegradability [7]. *K. xylinus* is a rod shaped, gram-negative bacteria that is strictly aerobic. Bacterial cellulose that is produced by this microorganism extracellularly, exhibits a nanofibrillar structure and in liquid culture, is produced as a pellicle structure with a gelatinous layer at one side and a denser surface at the opposite side [8].

1.2.1. Production of Bacterial Cellulose

In furtherance of production of bacterial cellulose efficiently, a bacterial strain that is stable and efficient is required accompanied with growth requirements are not too expensive and possible to scale up to industrial settings conveniently. After the production process, produced cellulose is mainly isolated from the fermentation medium easily and modified

later to be used for numerous medical or other applications by using various approaches. In bacterial cellulose production three most important aspects can be considered which are bacteria, technology, and modifications for various applications. The selection of bacterial strain that will be used for the bacterial cellulose production should be selected from bacterial strains that have high production capacity of bacterial cellulose, optimization of growth conditions such as supplied oxygen amount, temperature, pH, and growth factors should be arranged properly, and search for cheap natural growth media should be achieved. The production technology, such as static or agitated production, different types and scales of bioreactors, proper aeration, is also an important aspect for production. Modifications, chemical or physical, are another important aspect that can be applied according to the usage are for the produced bacterial cellulose [13].

Bacterial cellulose is a polysaccharide in a nanofibrillar structure, that is produced by various bacteria extracellularly. Production may occur when bacteria are growing in static conditions or in shaking culture. Bacterial cellulose is produced by bacteria, on media with a variety of carbon sources, albeit the efficiency of bacterial cellulose synthesis varies significantly between growth substrates. During the high energy-consuming cellulose synthesis pathway for the cell, the substrate provides energy to bacterial metabolism. In principle, every carbon unit that the bacterial cell converts into glucose may be utilized to produce cellulose [14,15]. Bacterial cellulose production ability is ubiquitous among bacteria, however the most notable and known bacterial cellulose producer is the acetic acid bacterium species *Komagataeibacter xylinus* which is an acetic acid bacterium. Acetic acid bacteria are gram-negative bacteria that are strictly aerobic, and they are classed as α-Proteobacteria [16,17]. For many years, the species was known as *Acetobacter xylinum*, but it was later categorized as *Gluconacetobacter xylinus* and then reclassified as *Komagataeibacter xylinus* owing to additional taxonomic modifications. Other acetic acid bacteria species with a high potential for cellulose synthesis include *Komagataeibacter rhaeticus, Komagataeibacter medellinensis, Komagataeibacter saccharivorans, Komagataeibacter pomaceti, Komagataeibacter hansenii, Komagataeibacter nataicola,* and *Komagataeibacter oboediens* [18,19,20,21,22]. The fact that acetic acid bacteria are food grade or GRAS bacteria is a crucial component of employing them for cellulose manufacture which means generally recognized as safe. Bacterial cellulose is derived from nucleotide activated glucose in the bacterial membrane. Bacteria then transport bacterial cellulose via cell

membrane pores as fibrils made of D-glucose units connected by β-1,4-glycosidic linkages. Produced chain is extruded from the cell and in linear form. Then, through all accessible hydroxyl groups, unidirectional and lateral aligned chains create inter and intra hydrogen bonds. As a result, the chains combine to form insoluble nanofibrils up to 25 nanometers wide and 1 to 9 μm long, representing glucose residues from 2000 to 18,000 units [23,24]. Produced and extruded nanofibrils then assemble into ribbon-shaped fibrils with 100nm width, which provide a unique mix of qualities to bacterial cellulose, such as mouldability, crystallinity, hydrophobicity, and high-water holding capacity. Although practically all hydroxyl groups in cellulose polymers are engaged by hydrogen bonds, therefore one end of each cellulose polymer has an unaltered Carbon4-hydroxyl group and the opposite end contains a vacant Carbon1-hydroxyl group, which together offer potential locations for cellulose chemical modification [25].

In the cytoplasm of bacteria, nucleotide-activated glucose is synthesized. If glucose is the initial substrate, uridine diphosphate glucose which named shortly as UDP-glucose is synthesized in three steps: glucokinase phosphorylation, phosphoglucomutase isomerization of glucose-6-phosphate into glucose-1-phosphate, and uridylyltransferase (UTP)-glucose-1-phosphate synthesis. Lastly, cellulose synthase attaches UDP-glucose glucosyl residues to the newly forming β-D-1,4-glucan chain. Cellulose synthase is a glycosyltransferase with two or three subunits that is embedded to the membrane. The catalytic subunit of cellulose synthase is a fundamental contributor of the chemical and physical characteristics of bacterial cellulose, which means that various bacterial species can produce cellulose of varying lengths [26,27]. A comparison of genomes of acetic acid bacteria indicated that acetic acid bacteria can have additional operons for cellulose synthesis, and that the composition of operons varies. These changes are extremely likely to effect cellulose synthesis, cellulose transport to the cell surface, and/or fibril ribbon construction [14,17,28].

Acetic acid bacteria secrete more than one extracellular polysaccharide, including bacterial cellulose. Acetan and levan which are other well recognized extracellular polysaccharides, are water soluble [17,29,30]. Acetan was initially discovered in the bacterium *Komagataeibacter xylinus*. Acetan is a branching acidic heteropolysaccharide which is similar to cellulose. Ishida et al. discovered reduced cellulose synthesis in a mutant that did not produce acetan. However, cellulose production may be resumed by adding acetan to the medium, indicating that the synthesis of both polymers is not genetically linked [31,32].

In bacterial cellulose production, with synthetic media, various carbon sources can be used such as glucose, galactose, mannitol, xylose, cellobiose, fructose, maltose etc. In addition to carbon source, growth factors are also required and generally peptone and yeast extract are used for this purpose. Generally, glucose proved to be the optimum energy source for bacteria; in addition to that, glucose may be employed as a direct precursor for cellulose formation from glucose units [19]. Due to yeast extract and peptone are not cheap, the researchers have been looking for low-cost raw materials with high quantities of sugars to use as substrates for bacterial cellulose synthesis. In order to obtain cheaper substitutes for yeast extract and peptone, raw materials such as corncob acid hydrolysate [33], corn steep liquor [34], beverage industrial waste [35], waste beer yeast [36], tobacco waste extract [37], cheese whey media, sugar beet molasses [38], distillery effluent [39], litchi extract [40], corn stalks [41], fruit juice [42] and wine production waste can be considered [43].

During bacterial cellulose production, used carbon source influence the properties of produced bacterial cellulose such as: polymerization degree, crystallinity index ranging from 67percent to 96percent), water holding capacity, intrinsic viscosity, porosity, oxygen, and water vapor transmission rates, mean crystallite size ranging from 5.7nm to 6.4 nm, molecular weight, surface area and mechanical properties. According to Molina-Ramrez et al. addition of acetic acid and ethanol into the growth medium improves yield of bacterial cellulose production however decreases degree of polymerization by 36percent, maximum rate of degradation temperatures by 4.96percent and crystallinity index by 9.2percent. In addition, bacterial cellulose production in the presence of ascorbic acid, crystallinity index decreased with notable alteration in d-spacing [44,45]. Nonetheless, according to a recent study of Wang et al. bacterial cellulose that has similar microfibrillar structure and morphology that produced with different carbon sources indicates that bacterial strain that is selected has a major effect on produced bacterial cellulose therefore bacterial strain and product should be checked before scaling up to large scales [15].

Bacterial cellulose production may be easily executed in containers that preferably has large surface area in order to supply the required oxygen and allow the formation of large cellulose sheets which will be formed on the surface. Various technological approaches may be utilized, in order to improve the bacterial cellulose production efficiency and to obtain bacterial cellulose with desired characteristics. Methods that can be used for bacterial cellulose production can be listed as: shaking culture, static, in rotating disc bioreactor, in

airlift bioreactor, in trickling bed reactor [46]. Static production is the most common method that is used in the laboratory scale production. This process can be up to two weeks long and obtained cellulose sheets are in the hydrogel form. In addition, produced bacterial cellulose has the shape of the surface of the vessel that the production was performed [47]. Production in shaking culture provide better oxygen supply to the bacterial cells. However, might be resulted in lower bacterial cellulose production and reduced genetic stability for bacteria. Bacterial cellulose production can be achieved as different particle sizes, obtained cellulose is mainly in spherical shape. This method can be suitable for production in economic scale [48,49]. Bacterial cellulose production in airlift bioreactor is considered efficient supply of oxygen with low power supply. Produced bacterial cellulose with this production method is obtained in pellet [49,50]. Production with the utilization of rotating disc bioreactors provide a homogenous cellulose product and the bacterial cellulose yield is comparable to the static production process [51]. In bacterial cellulose production with trickling bed reactor, low shear force is applied, and high oxygen concentration is provided. Produce bacterial cellulose sheets are in irregular form [52].

1.2.2. Applications and Modifications of Bacterial Cellulose

Bacterial cellulose is used in many areas: in food industry as low calorie and low cholesterol products, food additive and dietary aid, and food packaging materials; in paper industry as special functional paper, high retaining water paper, packaging paper; in textile industry, in electrical and electronic industries as electrical display device, OLED technology, fuel cell, flexible supercapacitor, stereo headphones and monitors; and in biomedical industry as wound dressings, cartilage tissue engineering, bone tissue engineering, dental implants, artificial blood vessels and vascular grafts, urethral implants, artificial cornea and retina, nerve implants, delivery of drugs and bioactive agents and cell-enzyme immobilization with BC scaffolds [53]. At static conditions, bacterial cellulose is three dimensionally structured within an interwoven, gelatinous, transparent and nano-fibrous network of linear polysaccharide polymers. In compared to plant based cellulose sources, bacterial cellulose exhibits extraordinary mechanical qualities such as flexibility and soft-tissue behavior mimicking behavior of stress strain, as well as high water holding capacity and crystallinity. Bacterial cellulose is a highly pure substance that lacks typical cellulose companions such as hemicellulose and lignin. Consequently, it is regarded as non-genotoxic,

non-cytotoxic and exceedingly biocompatible. Nevertheless, bacterial cellulose appears to lack the suitable functions to initiate cell attachment and regulate porosity, and it degrades relatively slowly. For the purpose of overcome these, bacterial cellulose has been changed chemically that changes in functionalities and chemical structure, and physically that changes in fiber density, crystallinity and porosity using a variety of ex situ and in situ approaches. In situ adjustments are accomplished by changes in growth conditions, carbon sources, and the addition of other materials, whereas ex situ modifications are accomplished through chemical and physical manipulation of produced bacterial cellulose [54-56].

For various applications, produced bacterial cellulose are modified in order to gain properties that relevant with its purpose of use. For biomedical applications: modification of bacterial cellulose to produce BC/PHEMA hydrogen matrice results in properties such as increased tensile strength, tissue replacement and wound healing, in situ UV radical polymerization, nontoxicity, rat mesenchymal stem cell proliferation [57]. For tissue engineering applications: modification of bacterial cellulose with tuned porosity results in higher pore size when compared with native bacterial cellulose that allows muscle cell ingrowth and includes small decrease in mechanical strength [58]. For suture biomaterial applications: BC nanocrystals/Regenerated Chitin fibers (BCNC/RC) results in in vivo cell proliferation promotion by chitin, adjustable degradation rate depending on varying BCNC concentration, possible enzymatic degradation and biocompatible surgical sutures with increased mechanical strength provided by BCNC/RC filaments [59]. For wound dressing applications: topography modification of bacterial cellulose that results in cell alignment improvement, in wound bed deposition of new collogen and fibroblast infiltration promotion [60]; vaccarin impregnation that results in dense new tissue formation including hyperplastic fibrous connective tissue and collogen fibers, neovascularization, stratified squamous epithelium [61]; BC/ε-poly-L-Lysine nanocomposite resulted in broad spectrum antimicrobial activity along with no effect on beneficial mechanical and structural properties, *S. epidermidis* growth is inhibited by ε-poly-L-Lysine on the membranes and modification with a nontoxic biopolymer is achieved [62]; BC/ZnO nanocomposite resulted in significant healing in 15 days by 66percent and antimicrobial activity effective on *Staphylococcus aureus, Escherichia coli, Citrobacter freundii, Pseudomonas aeruginosa* [63]; BC/Ag nanoparticle composite resulted in sustained release of Ag, prolonged and effective antibacterial activity for *Staphylococcus aureus* and this modification can be

considered as environmentally facile and benign approach [64]; BC/TiO_2 nanocomposite that results in antimicrobial activity for *Staphylococcus aureus* and *Escherichia coli* [65]; 2,2,6,6-Tetramethylpiperidinyloxy (TEMPO)-Oxidized bacterial cellulose with silver nanoparticles results in a 12.2percent per day silver ion release rate in 3 days at 37°C including antimicrobial activity and its biocompatibility [66]; BC/Silymarin (SMN)-zein nanoparticle nanocomposite resulted properties are antimicrobial and antioxidant activity, slowing down the lipid oxidation of air dried SMN-zein/BC nanocomposite and change of swelling and wettability [67]. For drug delivery applications: BC/Octenidin/Poloxamer hybrid system and BC/CMC/Methotrexate results in octenidine release in a controlled way in long term, antimicrobial and mechanical property improvements, Poloxamer-loaded bacterial cellulose is ready to use to treat infected wounds in an advance way, nontoxicity, DC-CMC effect on methotrexate loading, psoriasis treatment, elastic modulus decrement as CMC's degree of substitution (DS) increased [68,69]. For artificial cornea: BC/Hyaluronic acid (HA) and BC/PVA composite results in improved visible light transmittance when compared with plain bacterial cellulose [70,71]. For blood vessels: BC/iron oxide nanoparticles result in corresponding young's modulus values for blood vessels and introduction of magnetic domains [72]. For retinal pigment epithelium: BC/urinary bladder matrix results in improved proliferation and adhesion of retinal pigment epithelium cells, and closer recapitulation of cell phenotype when compared with uncoated bacterial cellulose [73].

1.3. BACTERIAL CELLULOSE SYNTHESIS BY *K. XYLINUS*

The synthesis of bacterial cellulose occurs on the inner membrane's cytoplasmic part and released to the extracellular environment after the transportation through the periplasmic space. For bacterial cellulose synthesis and export, there are terminal complexes are present in *K. xylinus* cells along the longitudinal axis of them. During the exportation process, this structural pattern provides hydrogen bonds to be formed between the adjacent glucan chains, also allowing the crystallization, translocation, and elongation of bacterial cellulose. Four protein subunits, which are BcsA, BcsB, BcsC, and BcsD, consist the *K. xylinus* bacterial cellulose synthesis terminal complex. Bacterial cellulose synthesis operon, named bcsABCD, encodes these four protein subunits. The location of BcsA subunit is on the inner membrane, facing the cytoplasm and also includes a domain of catalytic β-1,4-

glycosyltransferase which polymerizes the β-1,4-glucan chains that forms cellulose from units of UDP-glucose (uridine diphosphoglucose). In the periplasm, the protein subunit BcsB binds to the subunit BcsA and stabilizes it along with guiding the glucan chains through the periplasmic space. The function of BcsC, which is believed to produce a channel in the outer membrane of *K. xylinus* based on its structure, is thought to enable bacterial cellulose extrusion to the extracellular environment from the periplasm. BcsD, a cylindrical octameric periplasmic protein with four spiral channels that permits hydrogen bonding of four glucan chains during export through BcsC, is responsible for crystallization of bacterial cellulose. CmcAx is located upstream of the bcs operon and encodes an endo-β-1,4-glucanase with the activity of hydrolysis of cellulose. This implies that CmcAx's activity of hydrolyzing cellulose may have a regulatory influence on the biosynthesis of bacterial cellulose. BC biosynthesis. C ellulose-complementing protein (CcpAx) encoding CcpAx is essential for in vivo BC production, notably crystallization, is located in the same upstream locus as cmcAx. CcpAx and BcsD are co-localized horizontally on the cell membrane facing the extracellular side, indicating that CcpAx is an important component of the terminal complex of bacterial cellulose synthesis. BglAx, which encodes β-glucosidase, is located downstream of the operon of bacterial cellulose. This enzyme has a role of hydrolyzation of oligosaccharides that is greater than residues resultin in production of single β-D-glucose units [74].

1.4. STRESS CONDITIONS AND POSSIBLE EFFECTS ON BACTERIA

Stress is prevalent in living beings, and microorganisms without exception. Being able to recognize and adapt to changes in the environment in a reasonable time is one fundamental to survival and having the necessary capabilities to handle a variety of environmental difficulties makes bacteria exceptionally robust. Changes in pH and temperature, as well as exposure to reactive oxygen species, all cause environmental stress that the bacteria must adjust to maintain homeostasis. Stress responses of bacteria range from post translational modification to gene expression regulation to and toxin remediation [75].

Bacteria have successfully colonized widely on the globe, from the soil-dwelling gram-positive *Bacillus subtilis* to gram negative *Escherichia coli* discovered in mammalian lower intestines to *Deinococcus radiodurans*, which achieved to survive in nuclear reactors and

can endure the doses of radiation that is enough to kill all other living beings. Bacteria are subjected to radically varying environmental stressors in this large variety of various habitats, including fluctuations in osmolarity, pH, temperature, radiation, and toxin and nutrient concentrations [76]. In furtherance of surviving difficulties, bacteria may move to more favorable areas using flagellum which is their molecular motor, or the bacteria may adjust to differences in their surrounding context by reacting to the applied stress. Changes in gene expression patterns required for the genes that the outputs are necessary to battle the detrimental aspect of the stress achieve the reaction to the imposed stress. The stimulation of transcription factors associated with RNA polymerase to regulate gene expression results in the upregulation of transcription of stress-responsive genes. The sigma factor, an RNA polymerase component that is important for transcription start by playing a vital role in promoter recognition, is one family of transcription factors having a role in stress resistance. Each of the cell's sigma factors is necessary for the transcription of a certain group of genes/operons inside their regulon [77,78]. The σ^B regulon in *B. subtilis*, for example, consists of 200 open reading frames which is the 5 percent of the genome, the products of which impart overall cell's stress tolerance, although the majority of the other 17 sigma factors identified in bacteria individually control less than 50 ORFs. Because sigma factors can control a significant subset of ORFs, modulating their activity can be quite complex for example, the *B. subtilis* sporulation mother-cell-specific σ^F, which has a known regulon of 19 ORFs, is governed by a network of proteins, so even though σ^B has many regulators that work together to provide tight control. The accessibility of some substitutive sigma factors such as σ^B and σ^F for building productive complexes with the utilization of RNA polymerase is controlled entirely by a binding associate described as an anti-sigma factor, which can be modulated by targeted proteolysis or phosphorylation. Complementary stress detection responses are likewise phosphorylation dependent and are regulated by two component regulatory frameworks consisting narratively of a sensory kinase anchored in the membrane and a regulator of response. When a stress signal is received, the kinase autophosphorylates a conserved histidine before transporting the phosphoryl group to an unchanging aspartate in its corresponding response regulator, activating its latent biological activity. Response regulators are transcriptional activators that bind upstream of the promoters of the ORFs, they control and accelerate transcription initiation by associating with sigma-bound RNA polymerase [79].

General stress response, which is abbreviated as GSR, of bacteria is a conceptual model for how one pathway incorporates a wide variety of environmental stimuli and how a specialized mechanism with wide molecular responses is organized to ensure to survive via complimentary pathways. Environmental stress signals including as pH, osmotic stress and heat variations are detected by sensory proteins, which stimulate the sigma factor, Sig B, to control over 200 genes via a signaling cascade. Furthermore, the GSR is critical in stress exposure, which promotes bacterial fitness to independent future stressors such as oxidative chemicals. Whereas the GSR is associated with oxidative stress, the reason for the stimulation is unclear, implying that responses and sensors for environmental and oxidative stress interact to regulate antioxidant actions. Metabolomes, proteomes, and transcriptomes which are cellular responses of stressed bacteria against stress, as well as single-cell analyses, might offer insight on the regulated processes that minimize, repair and protect from, damage in dynamic environments [75].

1.5. AIM OF THE STUDY

Aim of this study was to gain an understanding of the effects of cold shock and sonication stress on bacterial cellulose production including the investigation of structural and characteristic features of produced cellulose under stress conditions and behavioral changes of *K. xylinus* under stress such as growth, cellulose production and expression of genes related with cellulose production.

2. MATERIALS AND METHOD

2.1. MICROORGANIMS, CULTURE MEDIA AND CHEMICALS

Komagataeibacter xylinus FC01 strain that was previously isolated and identified with 16S rRNA sequencing, during former studies at Yeditepe University laboratories. Two different culture media were used named as M1A1P6 and M1A05P5. Composition of M1A1P6 includes: 10 g/L glucose, 10 g/L yeast extract, 7 g/L peptone (bacteriological), 10 mL/L ethanol and 1.5 mL/L acetic acid. pH was adjusted to 6 with 6M NaOH. M1A05P5 was composed of 10 g/L glucose, 10 g/L yeast extract, 7 g/L peptone (bacteriological), 5 mL/L ethanol and 1.5 mL acetic acid [80]. pH was adjusted to 5 with 6M NaOH. After fermentation, 2M NaOH was used at 80°C for 1 hour, in order to remove bacterial cells. 3,5-Dinitrosalicylic acid was used as reagent for glucose assay. All chemicals that were mentioned above, were purchased from Sigma Aldrich St. Louis, Missouri, United States.

2.2. EXPERIMENTAL METHOD

2.2.1. Fermentation and Stress Conditions

K. xylinus FC01 was inoculated from stock to M1A1P6 medium, which is for bacterial growth, with 3percent inoculation ratio and incubated at 30°C as a static culture for 2 days. After bacterial growth and cellulose fibers were observed, fermentation was started. For fermentation, M1A05P5 medium, which is production medium, was used with 3percent inoculation of *K. xylinus* FC01 that was inoculated from stock to M1A1P6 medium 2 days earlier. M1A05P5 medium with 5 mL/L ethanol concentration was used for production due to providing the maximum yield of bacterial cellulose at pH 5 [80]. Ethanol in the production medium results in the bacterial cellulose yield enhancement by the reduction of the citric acid cycle's primary by product along with providing an additional carbon source [81] Fermentation was performed into 84 separate sample containers with 50 mL total volume capacity as 30 mL total fermentation volume with 3percent inoculation ratio of *K. xylinus* FC01. Sample containers were sealed by using cotton discs that was attached with paper tape and sample containers were left at room temperature which is 25°C for fermentation.

During fermentation two different types of stress were applied which are sonication as 30 seconds and 60 seconds, and cold shock as 30 minutes and 60 minutes. A control group with no stress applied was also performed. Therefore, 5 groups were formed as cold shock 30min, cold shock 60min, sonication 30sec, sonication 60sec and control group. Treatments, which are applied stress, were started to be applied after 2 days from fermentation start. Related treatments were applied daily to all groups excluding the control group for all sets. Sample collection was started to be performed daily after 3 days from fermentation start due to the cellulose formation time, in other words the day after first treatment application. Sample collection was achieved by collecting one set of each group daily, shown in Figure 3.

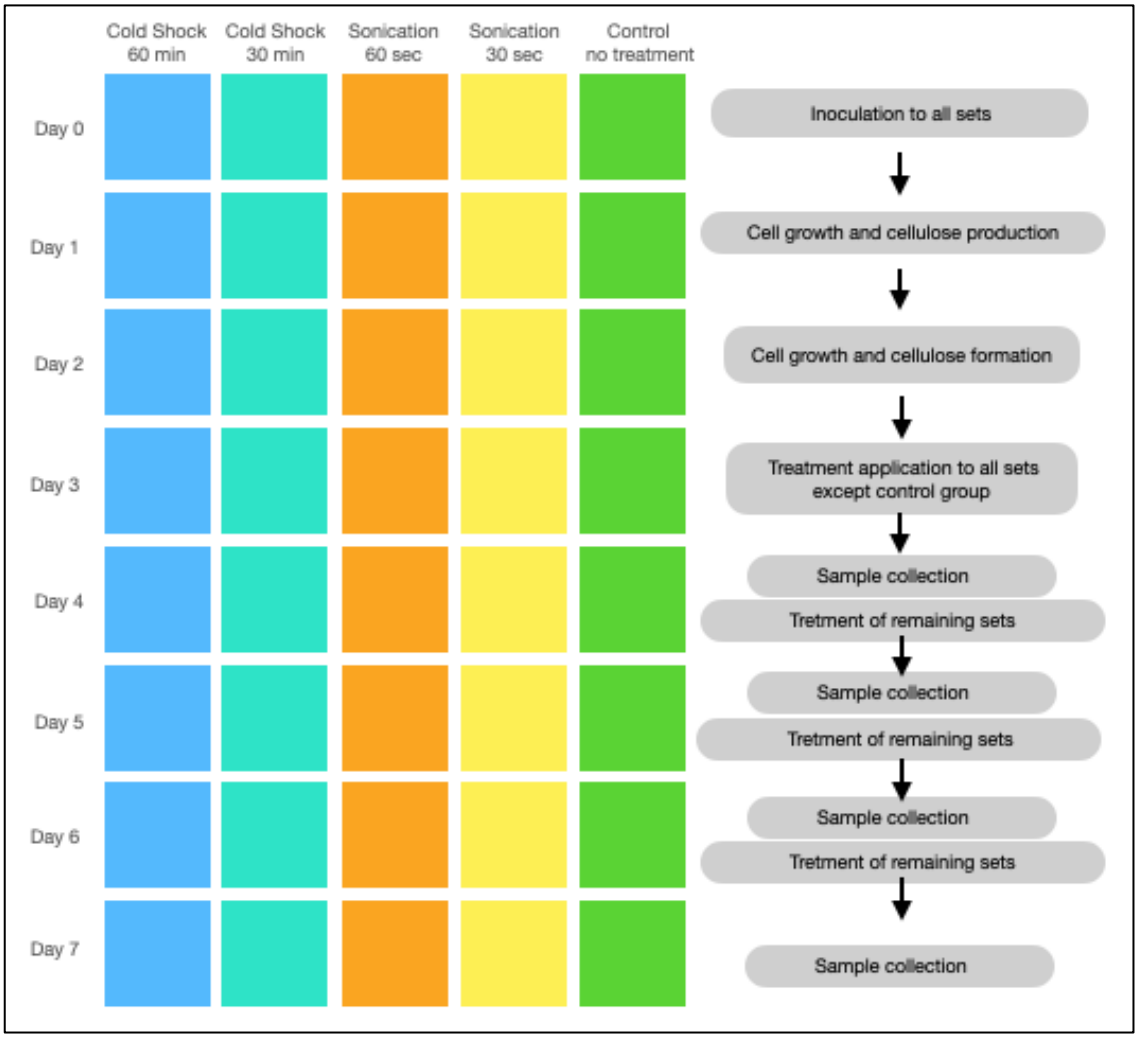

Figure 2.1. Schematic representation of experimental method

As mentioned before, two different stress types, which were cold shock and sonication, were applied to the bacterial cells during fermentation while cellulose production was occurring.

Cold shock stress was applied by transferring sample containers into a cold room with 4°C temperature. At 4°C, water is in the densest form, and it has the maximum density of 1g/cm^3 in pure water. The temperature change weather higher or lower than the 4°C, the density will be lower than 1g/cm^3 [82]. Cold shock stress was applied as two different time periods which are 30 minutes and 60 minutes by keeping samples in the cold room for 30 minutes and for an hour. Sonication was applied also to the samples in two different time periods by using ultrasonic water bath (Fisherbrand) at room temperature (25°C) as 30 seconds and 60 seconds. Ultrasonic water bath was filled with pure water as water level reaches one third of the sample containers, which have 50 mL total volume and 30 mL inoculation volume, to prevent containers to fall over.

For the reason that each set contain 4 replicates, first two which are sample 1 and 2 of the set, were used for several analyses as repeats of each other and last two which are 3 and 2 of the set, were used for several analyses as repeats of each other. Therefore, different treatments were applied to them after collection, due to the requirements of the analyses that will be performed on them, such as drying. Collected samples were firstly separated as after fermentation liquid and cellulose by centrifugation at 4°C, 4000rpm for 20 minutes and removing the supernatant to a tube. After the separation of celluloses was performed, 1st and 2nd samples of each set were freeze dried; separated celluloses, of 3rd and 4th samples of each set, were washed with distilled water then treated with 2M NaOH for an hour at 80°C water bath. After the treatment, celluloses were washed again with distilled water.

2.2.2. Determination of Cell Dry Weight and Bacterial Cellulose

Collected celluloses were placed into pre weighed falcon tubes, freeze drying was applied after kept overnight at -80°C, falcon tubes were weighted again and from the difference between two measurements, weigh of bacterial cellulose and bacterial cells were obtained. BCs were then treated with 2M NaOH for an hour at 80°C water bath for the removal of bacterial cells and remaining medium components [83]. BCs were kept overnight at -80°C and freeze dried again then weigh measurement was performed again, and weigh of BCs were obtained. By the difference between BC and cell weighs and BC weighs, cell dry weigh and weigh of bacterial cellulose were obtained.

2.2.3. Determination of Glucose Concentration

After centrifugation, supernatants of samples were analyzed for glucose concentration, in order to determine glucose consumption of bacterial cells, with glucose assay by using 3,5-Dinitrosalicylic acid reagent, which binds to reducing sugars and changes color [84]. At the end of the glucose assay, absorbance at 540nm was measured for all samples and glucose concentrations were calculated according to the glucose standard curve [85].

2.2.4. Scanning Electron Microscopy (SEM) Imaging

Bacterial cellulose samples that were produced under different stress conditions were analyzed for their structural properties under scanning electron microscope (SEM). Freeze dried bacterial cellulose samples were prepared in mounted and coated with gold before the imaging. Carl Zeiss EVO-40 microscope was used for this imaging at high potential, which is 10kV, and under vacuum.

2.2.5. Tensile Strength Testing

Tensile testing machine Instron 5900 was used for tensile strength and Young's modulus analysis of produced BC. This analysis was performed for both freeze dried and wet state BC. Testing was performed at room temperature and with 10 N/min. After the tensile strength data were obtained from the analysis, that was performed by using tensile testing machine, Young's modulus values were calculated by using the formula 2.1. The maximum stress that the testing material can withstand before it breaks is known as ultimate tensile strength [86].

$$Young's\ modulus = \frac{stress}{strain} = \frac{F/(w \times t)}{\Delta L/L_0} \quad (2.1)$$

F: force (N), w: film width (mm), t: film thickness (mm), ΔL: length of extension under force (mm), L_0: initial film length (mm)

2.2.6. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is a chemical analysis technique that is used for the determination of molecules that are present and the concentrations of the present molecules in the sample. By using FTIR an infrared spectrum is obtained, and high-resolution spectral data is collected in a broad spectral range. Produced infrared absorption spectrum can be used for the identification of chemical bonds in a molecule [87]. Fourier transform infrared spectroscopy was used for determination of molecular structure and present chemical bonds of freeze-dried bacterial cellulose samples in order to clarify of the changes resulting from applied stress by using Thermo Scientific Nicolet iS10 FT-IR Spectrometer. Bacterial cellulose films were scanned in a frequency range of 400 to 4000 cm^{-1}.

2.2.7. X-Ray Diffraction (XRD)

Crystalline structure was identified using X-ray diffraction (XRD) system of RD-Bruker AXS D8 Advance at Cu-Kα radiation in the 2θ range of 10-60º and samples that were cut into 2 cm x 2 cm pieces were used.

2.2.8. Differential Scanning Calorimetry (DSC)

Thermal property analysis of bacterial cellulose samples were performed by using a differential scanning calorimeter of SETARAM Instrumentation Keep Technologies model DSC 131. Freeze dried bacterial cellulose samples was used during the analysis and temperature was changed from 20°C to 500°C with 10°C per minute heating rate.

2.2.9. Thermogravimetric Analysis (TGA)

Determination of thermal stability of produced bacterial cellulose samples were achieved by thermogravimetric analysis by using Perkin Elmer Pyris 1 TGA device. Dried bacterial cellulose samples were used for this analysis and analysis was performed with the temperature range of 20°C to 400°C.

2.2.10. RNA Purification, Quality Control, and First-strand cDNA Synthesis

At the end of the fermentation, produced bacterial cellulose samples was removed and samples were centrifuged at 4°C, 4000rpm for 20 minutes. After the centrifugation supernatant was discarded and cell pellet was used for RNA isolation. Total RNA extraction and purification was performed with Macherey-Nagel NucleoSpin RNA Purification Kit. Quality of purified RNA was determined by agarose gel electrophoresis. Concentration and purity of RNA was spectrophotometrically determined by using Shimadzu BioSpec-nano UV-VIS-NIR Spectrophotometer. A_{260}/A_{280} values were evaluated for purity. RNA isolates were diluted to same concentration and used for cDNA synthesis. cDNA synthesis was performed in a thermocycler by using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit with random hexamers. For negative control, in order to assess gDNA contamination, reactions were performed that contain all components except reverse transcriptase.

2.2.11. Primer Design and Reverse-transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

For selected genes that involves in bacterial cellulose production (*bcsA, ccpAx*) in *K. xylinus* and also for a reference gene (*23SrRNA*), primer design was performed. Primers were designed as reverse and forward primers by using Integrated DNA Technologies Primer Quest Tool. In primer design, conditions such as having melting temperature to between 55-65°C, having primer size between 18-24 base pairs, having GC content between 40-60percent, amplicon size between 100-200 base pairs and having hairpin delta G value greater than -3 kcal/mol were taken into consideration.

Expression levels of selected genes after treatment with stress conditions were determined by using Roche LightCycler 480 Instrument. Reaction of RT-qPCR included 2 µl cDNA of each template, 1X iTaq™ Universal SYBR® Green Supermix and 500 nM for each primer in a total volume of 20 µl. Reaction conditions were determined according to the iTaq™ Universal SYBR® Green Supermix Kit with slight changes. Reaction conditions that were applied in RT-qPCR were 95°C for 30 seconds for preincubation as 1 cycle; 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 60 seconds in amplification step as 40 cycles;

95°C for 10 seconds, 62°C for 60 seconds, and 97°C for 1 second for melt curve analysis as 1 cycle. Samples were loaded on a 96 well plate as triplicates per treatment.

2.3. STATISTICAL ANALYSIS

Statistical analysis was performed for the obtained data during this study by using Microsoft Office Excel program and evaluated with paired Student's t-test. Statistical significance was interpreted according to p values $p \leq 0.05$ and $p \leq 0.1$.

3. RESULTS AND DISCUSSION

Bacterial cellulose production under two different stress conditions, which are cold shock and sonication, was investigated. Including the assessment of changes in cell growth, behavior, and production of bacterial cellulose; the structural and characteristic differences of bacterial cellulose that was produced under stress and the analysis of gene expression differences for selected genes that responsible in production of bacterial cellulose in *Komagataeibacter xylinus*.

3.1. SONICATION STRESS

3.1.1. Cell Dry Weight and Dry BC Weight Analysis

For the evaluation of bacterial cellulose production amount under sonication stress, dry BC weight was measured for BC produced by bacteria that exposed to stress daily as sonication stress for 30 seconds and 60 seconds. Also, BC produced by a control group that exposed to no stress was also measured.

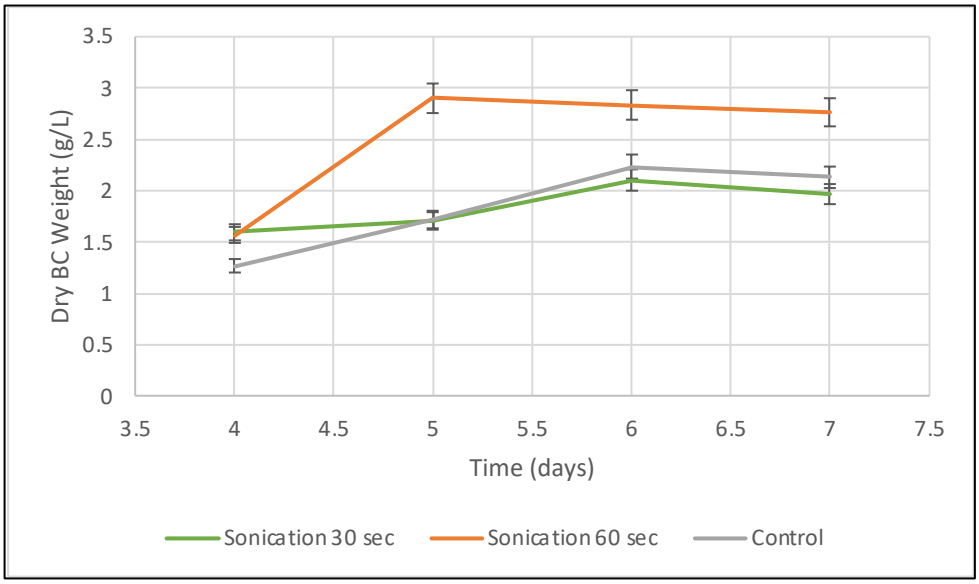

Figure 3.1. Weight of dry BC that were produced under sonication stress 30 and 60 seconds and control group (no stress applied)

After the inoculation, 2 days were waited for bacterial cells to grow and start to produce cellulose, 3rd day first stress was started to be applied and continued to be applied daily. The first sample was collected 24 hours after from the stress application, which is 4th day, in order to observe the effects of the stress. After first sample collection samples were continued to be collected daily. Therefore, samples were collected as 4th,5th, 6th and 7th days. In Figure 3.1. produced bacterial cellulose amount by *K. xylinus* that exposed to stress which is sonication 30 seconds and 60 seconds and control group with no stress applied. On the 4th day, produced bacterial cellulose amount was observed higher for both sonication 30 seconds and sonication 60 seconds when compared with control group. For the following days, produced BC amount by sonication 60 seconds stressed bacteria was observed higher than both sonication 30 seconds and control group. The increment was observed faster from 4th day to 5th day than remained stable. Sonication 30 seconds and the control group showed similar BC amounts and similar increment rates. For bacterial cellulose, sonication 60 seconds stress applied group yielded 1.3 fold greater than control group on the 6th day of the fermentation. When bacterial cellulose production analyzed statistically, a significant difference was observed in the bacterial cellulose production amounts for sonication 60 seconds group with a p value of 0.035 as $p<0.05$ and for sonication 30 seconds group with $p<0.1$ when compared with the control group.

In order to determine the effect of stress on microbial growth, cell dry weight was measured for sonication 30 seconds and 60 seconds and for control group with no stress applied. As shown in Figure 3.2. application of stress has a negative effect on microbial growth. On the 4th day of the fermentation cell dry weight values were observed as close to each other. On the following days difference in the microbial growth can be observed. Control group has higher microbial growth when compared with both sonication 30 second and 60 seconds stressed applied groups. In the bacteria that sonication 60 seconds stress applied, a larger decrement was observed on the 5th day, however an increment between the 5th day to 7th day is observed and the microbial growth is getting closer to the control group's value at 7th day. This may result from bacterial response to stress that provides bacteria to be less affected from stress [88]. When the microbial growth of sonication 30 seconds stress was considered, the curve is observed more stable. There are no sudden increments and decrements. This may be resulting that, due to stress application the cell dry weigh remained lower when compared to control group, however sonication 30 seconds stress may not be enough to cause

same stress response that provides bacteria to be less affected from stress in growth when compared with sonication 60 seconds.

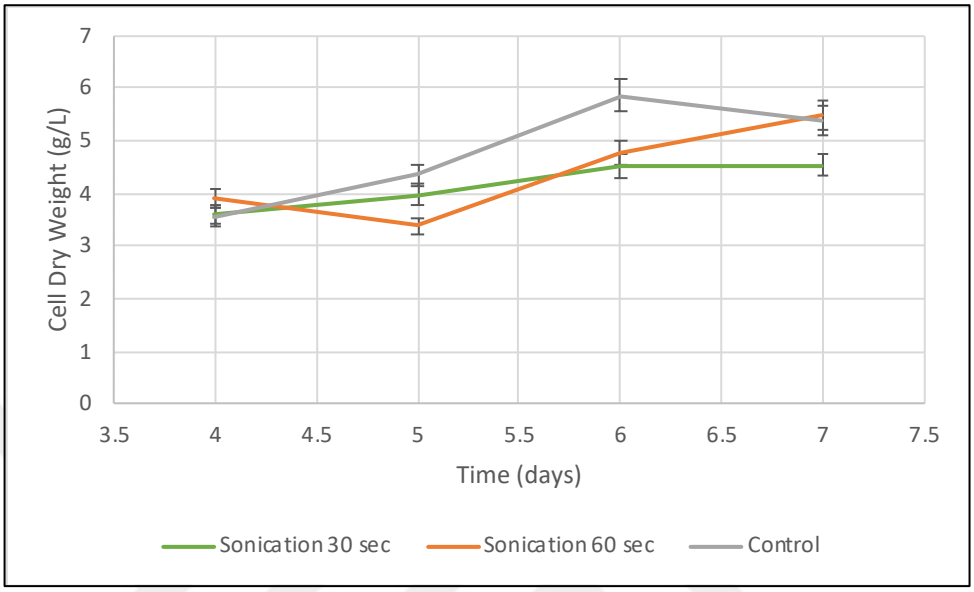

Figure 3.2. Cell dry weight of sonication stress 30 and 60 seconds and control group (no stress applied)

Therefore, applied stress caused lower microorganism growth while causing higher bacterial cellulose production. This may be reasoning from the microorganisms trying to protect itself against applied stress. However, there is no statistically significant difference in the microbial growth for both sonication 30 seconds and 60 seconds when compared with the control group.

3.1.2. Evaluation of Normalized Cell Dry Weight and Dry BC Weight Analyses

Normalization of cell dry weight and dry bacterial cellulose amount graphs for sonication 30 seconds and 60 seconds stress groups and the control group for samples of 4^{th}, 5^{th}, 6^{th} and 7^{th} days. Normalized graphs of dry bacterial cellulose and cell dry weight were plotted in order to understand the changes in cell growth related with the applied stress, and changes in the bacterial cellulose production due to the stress conditions that bacteria were exposed to during the fermentation.

In Figure 3.3. normalized weight of dry BC that were produced under sonication stress 30 and 60 seconds and cell dry weight of sonication stress 30 and 60 seconds and control group were shown. Both for cell growth and bacterial cellulose production, sonication 30 minutes and control group have similar change rates. Therefore, it is observed that sonication 30 seconds stress was not affected the growth rate. For sonication 30 seconds, BC production is observed as slowed down at the end of the fermentation and cell focused on growing due to exposure to stress for a while. For sonication 60 seconds growth was negatively affected at the beginning while cell is more focused on BC production, on the following days cells focused on growth.

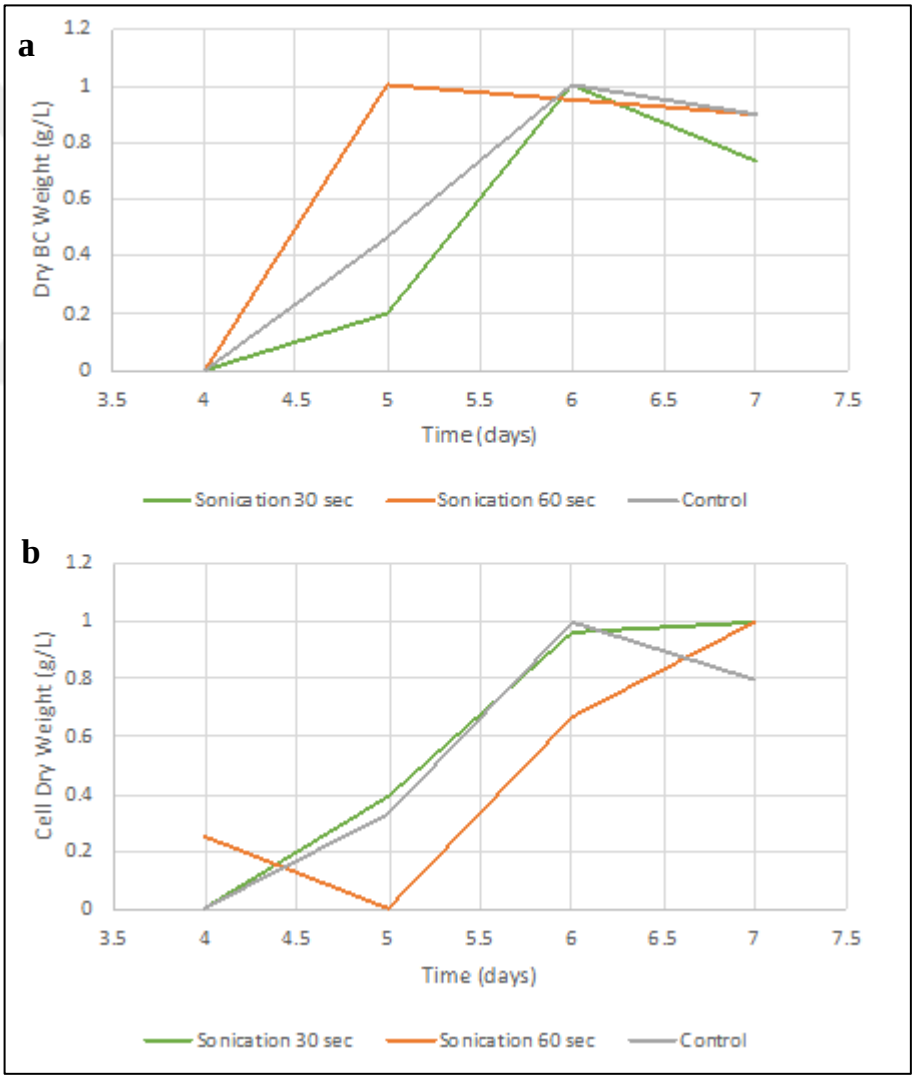

Figure 3.3. Normalized (a) weight of dry BC that were produced under sonication stress 30 and 60 seconds, (b) cell dry weight of sonication stress 30 and 60 seconds and control group

The effect of stress can be clearly seen on sonication 60 seconds stress group as causing lower microorganism growth while causing higher bacterial cellulose production when compared with sonication 30 seconds and the control group which are close to each other. When gene expression levels were considered (see part 3.1.10), for sonication 60 seconds group, expression levels were observed high compared with control group at the beginning, however expression levels were dropped at the end which is very similar with the BC production of sonication 60 minutes shown in Figure 3.3.b.

3.1.3. Glucose and Protein Concentration Changes During Fermentation

Glucose and protein concentrations were measured during fermentations in order to investigate the change in cell behavior during fermentation under stress. At the end of the fermentation, which is 7th day, glucose and protein concentration were measured in order to compare with the initial values. Bacterial cells were supplied with 10 g/L glucose at the beginning of the fermentation. In Figure 3.4. glucose consumption of groups sonication 30 seconds and 60 seconds and control group were shown. It is clearly observed that bacteria that were exposed to stress conditions consumed more glucose when compared with control group. When sonication 30 seconds and 60 seconds were compared with each other, it can be said that glucose consumption is between them close to each other.

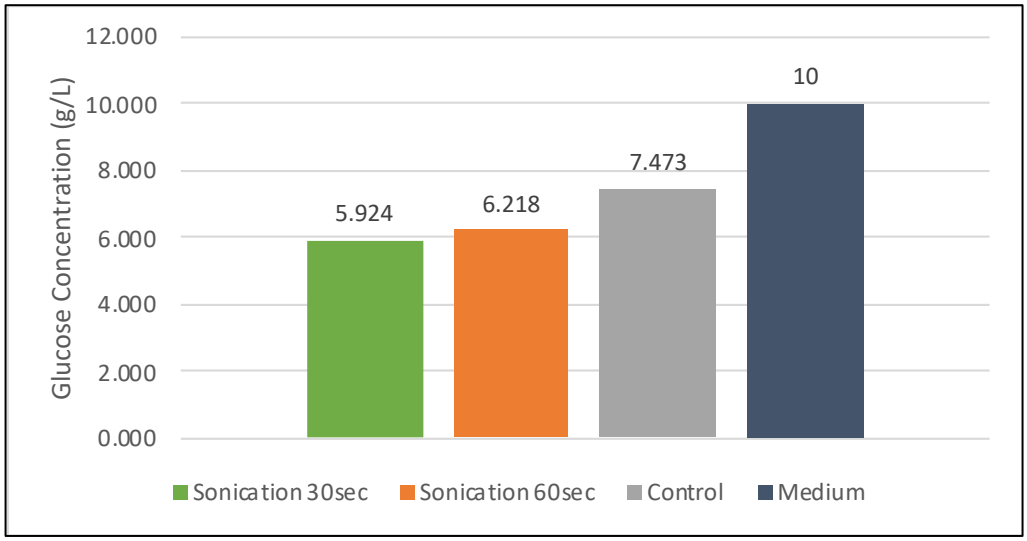

Figure 3.4. Measured glucose concentration during fermentation under stress, sonication 30 and 60 seconds and control group (no stress applied)

Glucose consumption rates of sonication 30 seconds, sonication 60 second and control group are 40.8percent, 37.8 and 25.3percent of the supplied glucose concentration respectively.

Protein concentration was also measured for sonication 30 second and 60 minutes and control group in order to observe stress effect on fermentation. For all groups, measured protein amount was higher than provided protein sources. Protein increment during fermentation may be resulting from either biomass accumulation or due to concentration of protein with the decreasing carbohydrates due to consumption [89].

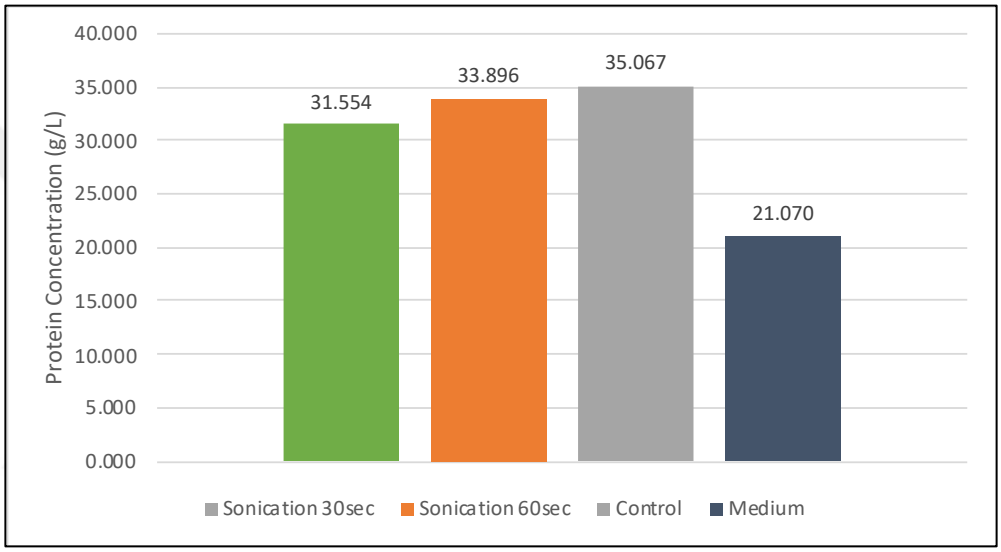

Figure 3.5. Measured protein concentration during fermentation under stress, sonication 30 and 60 seconds and control group (no stress applied)

As shown in Figure 3.5. all groups have more protein concentration than provided in the medium at the beginning of the fermentation. Control group has the highest protein concentration when compared with the sonication 30 seconds and 60 seconds, the reason may be the cell amount of control group was higher than both stress groups. Sonication 60 seconds has more protein concentration than sonication 30 seconds. The reason for this can be the cell amount of sonication 60 seconds was higher on the 7th day.

3.1.4. Surface Characteristics of BC

SEM imaging was performed in order to investigate the morphologies of bacterial celluloses produced under sonication 30 seconds and 60 seconds stress and control group with no stress.

In Figure 3.5., 4th and 7th day bacterial cellulose samples were shown for sonication 30 seconds, 60 seconds and control group.

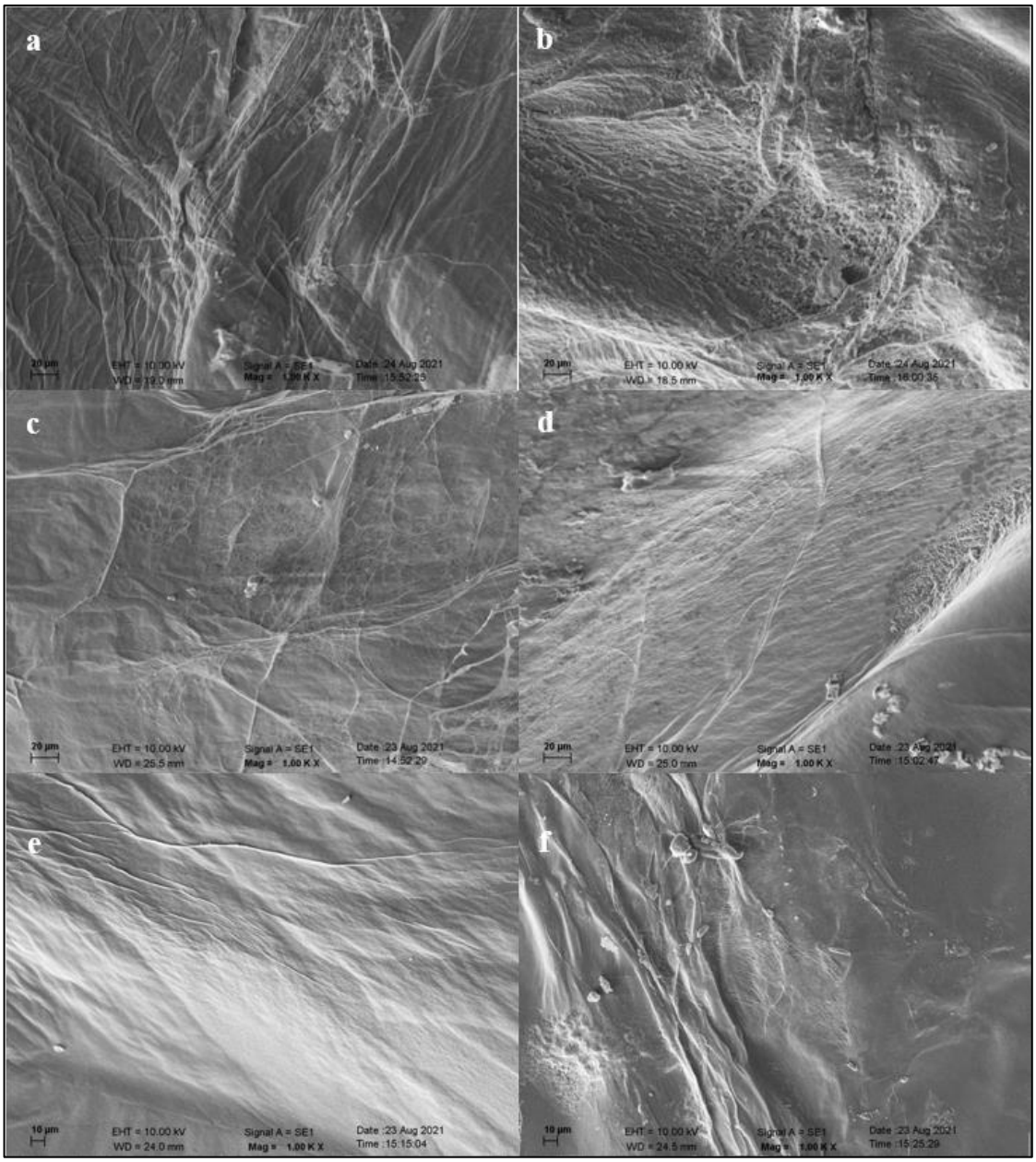

Figure 3.6. SEM images of bacterial cellulose of (a) sonication 30 seconds day 4, (b) sonication 30 seconds day 7, (c) sonication 60 seconds day 4, (d) sonication 60 seconds day 7, (e) control no stress applied day 4, (f) control no stress applied day 7

When SEM micrographs were assessed, it can be said that control group has more smooth surface morphology for both 4th and 7th day. In sonication 30 seconds and 60 seconds samples surface is observed that a rougher, fibrillar and porous structure is observed when

compared with the control group with no stress applied. had a stable production with no movement resulted in a smoother surface morphology. When bacterial celluloses that were produced under stress conditions are assessed, for both sonication 30 seconds and sonication 60 seconds, 7th day bacterial cellulose samples have rougher surface when compared with 4th day. Therefore, sonication stress for both 30 seconds and 60 seconds had an effect on surface morphology of bacterial cellulose as rougher surface that observed as more fibrillar and porous structure. The rough surface structures of BC that produced under stress may be reasoning from more irregular accumulation of fibrils during the polymer formation due to applied stress while BC produced by the control group. As can be seen in the FTIR analyses, shown in part 3.1.8, from stressed groups more durable and strong bacterial celluloses were obtained. Therefore, different morphology of stressed groups which is rougher surface when compared with the control group.

3.1.5. X-Ray Diffraction

X-ray diffraction was performed for bacterial celluloses produced under sonication 30 seconds and 60 seconds stress and control group with no stress in order to assess crystallinity of BC.

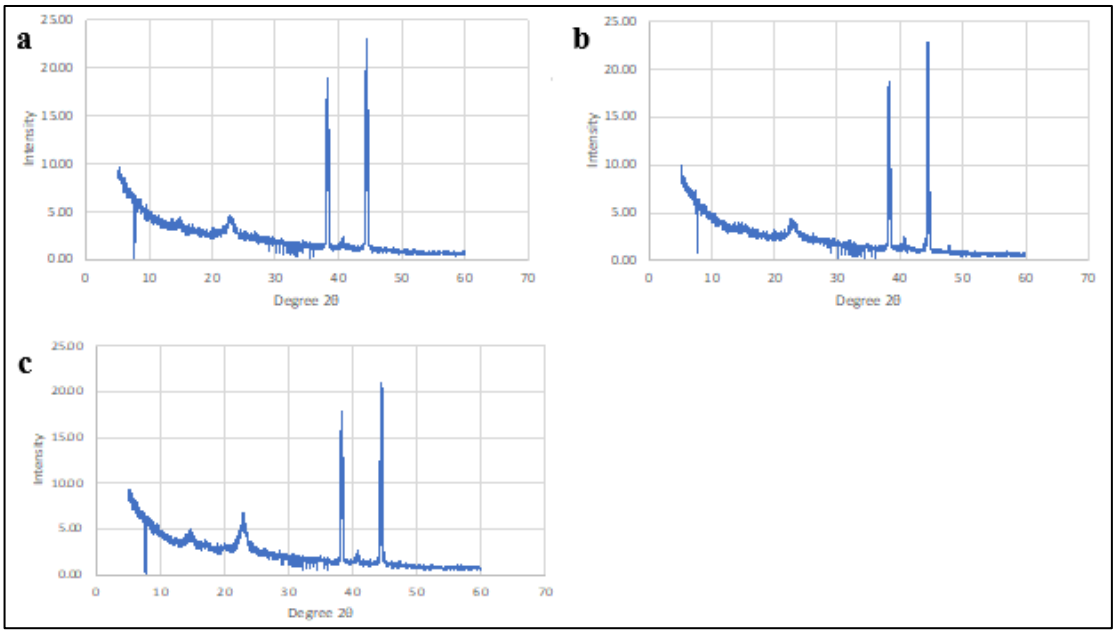

Figure 3.7. X-ray diffractograms of (a) sonication 30 seconds, (b) sonication 60 seconds, (c) control (no stress)

X-ray diffractograms of sonication 30 seconds, sonication 60 seconds and control group were shown in Figure 3.7. For sonication 30 seconds, sonication 60 seconds and control group, diffractogram presents peaks at 2 θ = 38.2, 44.4. The x-ray diffractograms and crystallinity of produced BC by sonication 30 seconds, sonication 60 seconds and control groups are very similar to each other. Therefore, sonication stress has no effect on crystallinity of BC.

3.1.6. Differential Scanning Calorimetry

Differential scanning calorimetry was performed for bacterial celluloses produced under sonication 30 seconds and 60 seconds stress and control group with no stress. An endotherm event was observed around 100°C, this may be reasoning from intra or intermolecular bonded cellulosic chains or residual moisture [90]. The second event occurred between 230-350°C that is in collaboration of the mass loss in the ΔTGA spectra that shown in Figure 3.8. which may be resulting from the cellulose degradation [91]. Beyond the temperatures from 270°C a depolymerization process or cellulose decomposing is indicated by the heat flux [92].

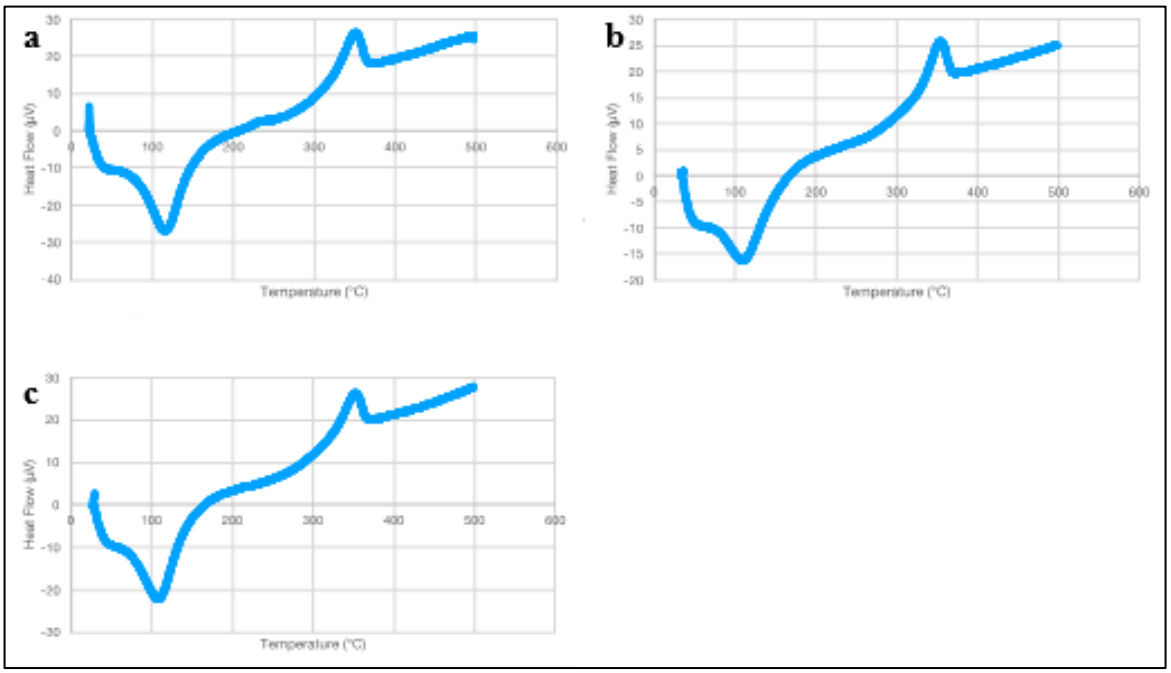

Figure 3.8. Differential scanning calorimetry (DSC) curves of (a) sonication 30 seconds, (b) sonication 60 seconds, (c) control (no stress)

Glass transition temperature (T_g) was not observed, this situation may occur in some polymers such as cellulose if the decomposition occurred just before or occurred parallel to the glass transition temperature [93].

Differential scanning calorimetry curves of sonication 30 seconds, sonication 60 seconds and control group were shown in Figure 3.8. The first event which is the first peak around 100°C, indicates crystallization temperature (T_c) which is 120°C for sonication 30 seconds, 111.5 for sonication 60 seconds and 111.1 for control group. Second event which is the second peak in the curve, indicates melting temperature (T_m) which is 358.6 for sonication 30 seconds, 359.5 for sonication 60 seconds and 358.4 for control group. For all groups DSC curves appeared similar to each other, T_c and T_m values were very closed to each other. Therefore, sonication stress has no clear effect on amount of heat required to increase the temperature. This situation indicates that the molecular structure and conformation of bacterial cellulose was preserved.

3.1.7. Thermogravimetric Analysis

Thermogravimetric analysis was performed for sonication 30 seconds, sonication 60 seconds groups and control the group that no stress applied. Thermogravimetric curves (TGA) and their derivatives (ΔTGA) for bacterial cellulose that were produced under sonication 30 seconds, 60 seconds stress and no stress (control group), shown in Figure 3.9. According to Figure 3.9., decomposition of bacterial cellulose can be divided into three main events for control group. The first event occurred between 0-200°C, this event indicates volatilization of components with low molecular weights and loss of water. The second and third events, indicates that the decomposition of polymeric chains of the bacterial cellulose which is shown in spectra as two mass loses [93]. At the first event control group lost 12.5percent, sonication 30 seconds and sonication 60 seconds lost 6.8percent of their weight. Second event occurred at 200-300°C for control group, 220-320°C for sonication 30 seconds and 220-330°C for sonication 60 seconds with mass loses 60.8percent, 59.3percent 56.3percent respectively. The third event occurred at 330-400°C for all groups and with mass losses of 7.2percent, 7.1percent 5.4percent respectively. Consequently, bacterial celluloses that were produced under stress conditions of sonication 30 seconds and 60 seconds were observed as more thermostable when compared with control group.

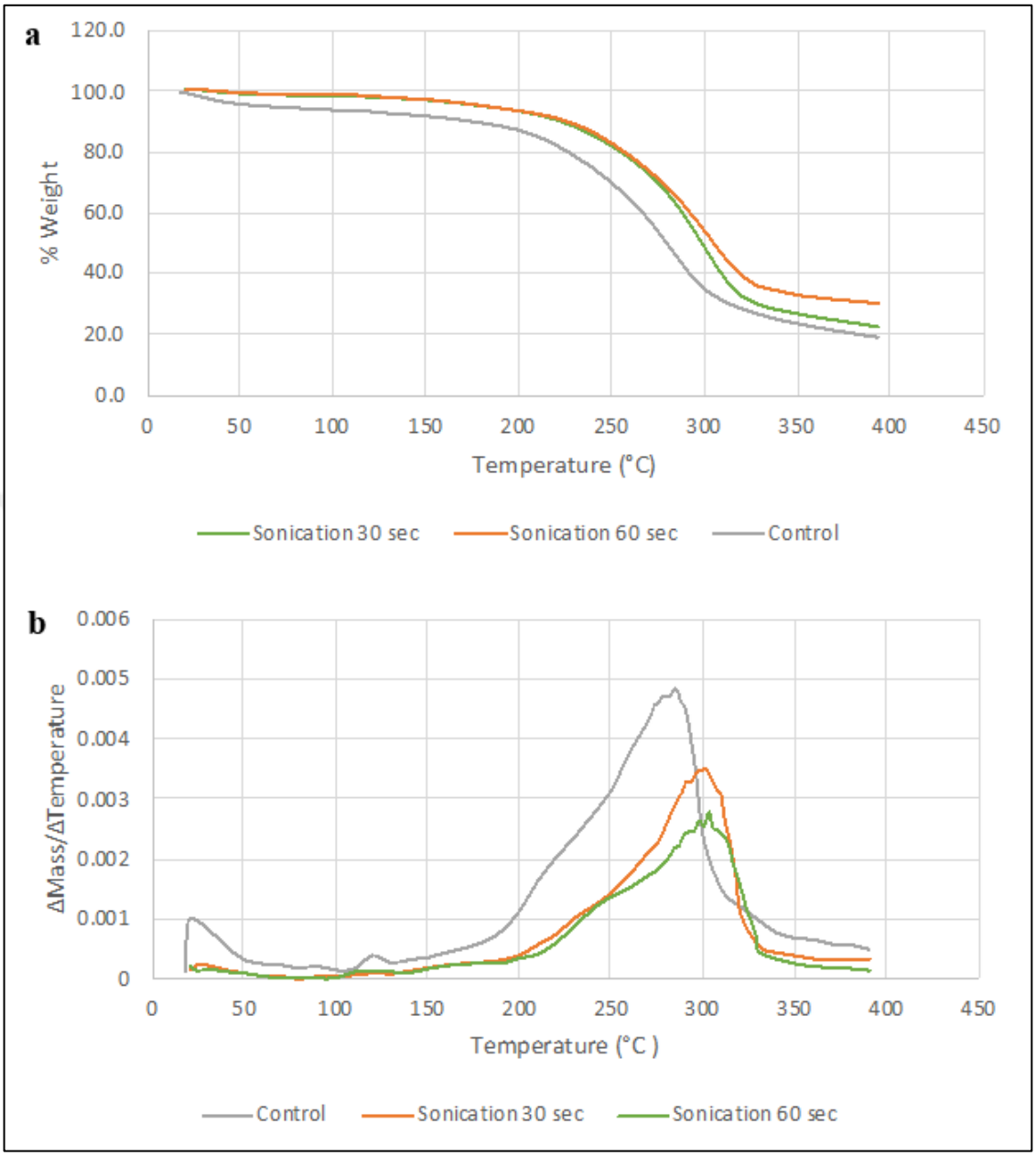

Figure 3.9. (a) TGA spectra of BC samples of sonication 30 seconds, sonication 60 seconds and control group (b) ΔTGA spectra BC samples of sonication 30 seconds, sonication 60 seconds and control group

When sonication 30 seconds and 60 second were compare it can be said that sonication 60 seconds lost a smaller percentage of its weight however weight loss was more rapid. Both sonication 30 and 60 decomposed at higher temperatures when compared to control group. Therefore, applied sonication stress provided a better thermal stability to produced bacterial celluloses.

3.1.8. Fourier-Transform Infrared Spectroscopy

Fourier Transform-Infrared Spectroscopy was performed for freeze dried bacterial cellulose samples of sonication 30 seconds, sonication 60 seconds groups and the control group that no stress applied in order to determine molecular bonds and structural differences that may be resulting from stress application. In Figure 3.10. FTIR spectra of bacterial cellulose samples for sonication 30 seconds, sonication 60 seconds groups and the control group were shown.

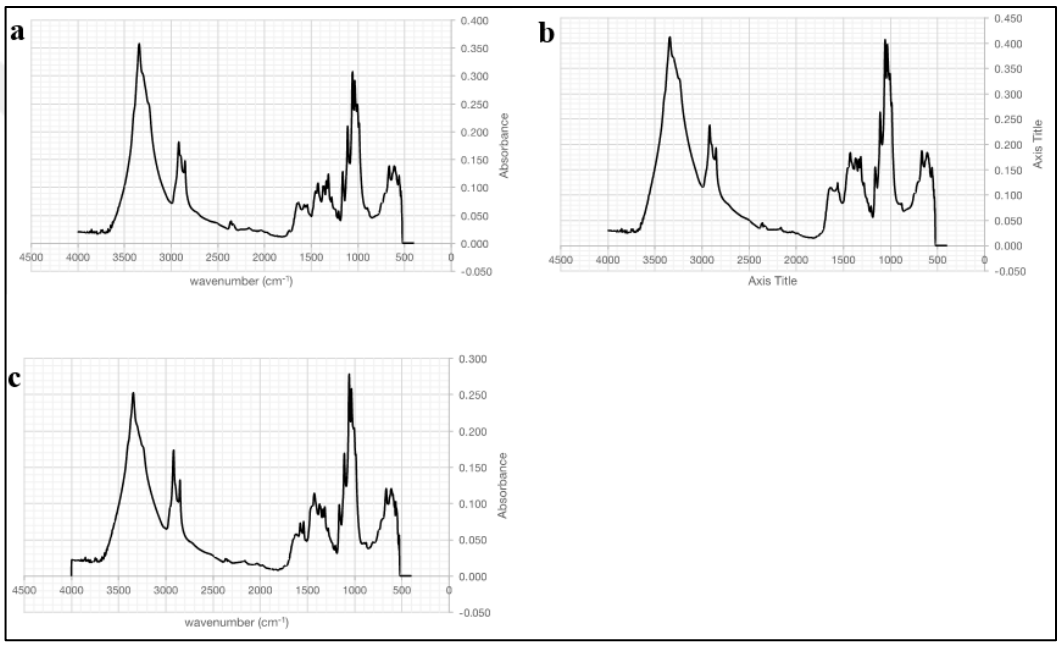

Figure 3.10. FTIR spectra of 7th day BC samples of (a) sonication 30 seconds, (b) sonication 60 seconds (c) control group

For cellulose, infrared band assignments were shown at Table 3.1. [94]. During FTIR analysis obtained peaks for bacterial cellulose samples of sonication 30 seconds, 60 seconds, and control, were shown in Table 3.2. As shown in Figure 3.10. three intense peaks for all groups can be observed. One of the most intense peaks was obtained at ~1060 cm^{-1} that assigned to C-O-C stretching vibrations originating from the cellulose main chain parallel to the molecular chain axis. Another intense peak was obtained at ~1003 cm^{-1} which is derived from C3-O3 stretching vibrations that is considered as main bonding that forms a cross

Table 3.1. Infrared band assignments for cellulose

Frequency (cm^{-1})	**Assignment**
~ 3340	3-OH•••O-5
~ 2900	CH_2
~ 1726	C=O
~ 1600	C=C
~ 1420	CH_2
~ 1250	CH+COH
~ 1162	CH
~ 1110	C-2•••O-2
~ 1060	C-O-C
~ 1003	C-3•••O-3
~ 895	CH/COC
~ 650	RING

Table 3.2. Measured frequencies of peaks of BC samples of sonication 30 seconds, 60 seconds and control group

Frequency (cm^{-1}) Sonication 30 sec.	**Frequency (cm^{-1}) Sonication 60 sec.**	**Frequency (cm^{-1}) Control**
3336	3342	3344
2847	2916	2891
1423	1424	1425
1312	1312	1313
1167	1159	1160
1107	1106	1107
1055	1054	1056
1033	1031	1032
1003	1003	1003
663	662	664

linking structure. And another intense peak was observed around 3340 cm^{-1} which indicates intra molecular hydrogen bonds (3-OH•••O-5). More hydrogen bonds indicate more durable structure for the stressed groups. These three peaks were observed more intense in stress applied groups when compared with control group. Among sonication 30 and 60 seconds, sonication 60 seconds group has more intense peaks than sonication 30 seconds. Another peak was observed at the area of 2900 cm^{-1}, which indicates CH_2. For this peak control group

has more intense peak when compared with stress applied groups. Overall, the most significant difference between stressed groups and the control group is the hydrogen bond intensity which provides a stronger structure which approves the tensile strength results in part 3.1.9. Applied stress caused the formation of more hydrogen bonds that resulted in a stronger structure.

3.1.9. Tensile Strength Testing

Tensile stress testing was performed for both wet and dry bacterial cellulose samples of sonication 30 seconds, sonication 60 seconds groups and a control group that no stress applied in order to characterize and determine the mechanical properties.

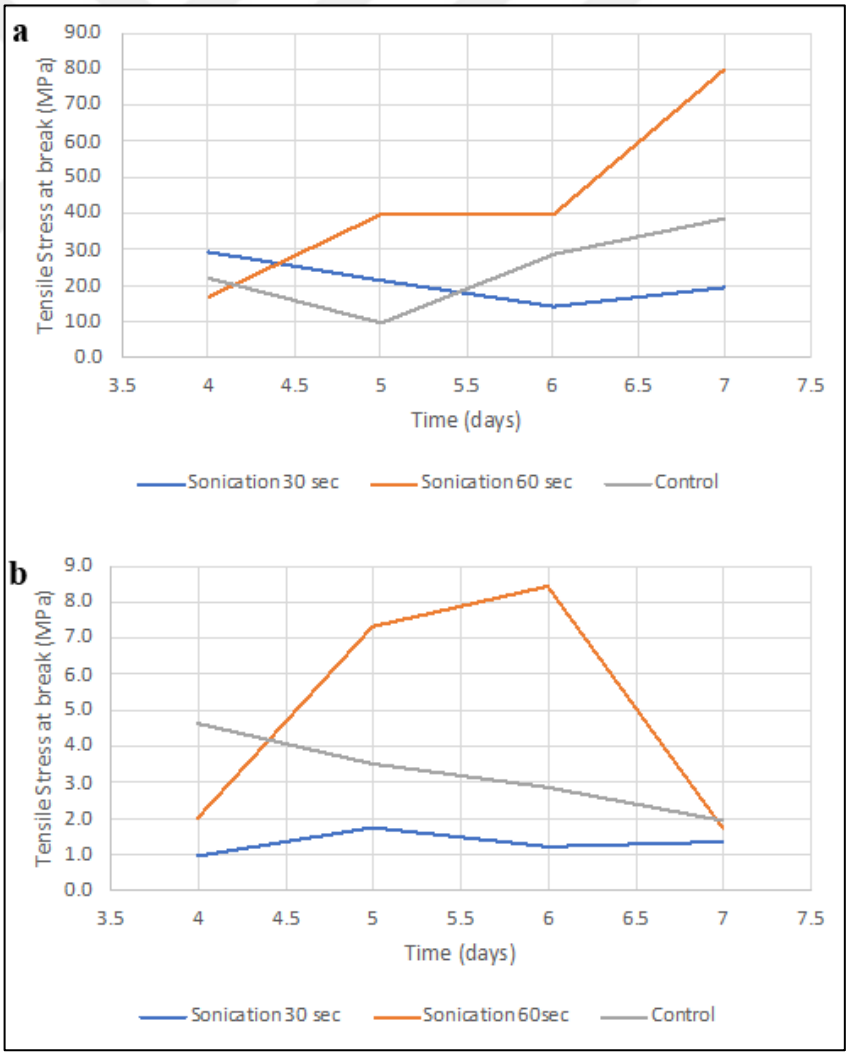

Figure 3.11. Tensile stress at break for (a) dry, (b) wet BC samples for sonication 30 seconds, sonication 60 seconds and control group

In figure 3.11. tensile stress at break values were shown for daily collected (4^{th} day to 7^{th} day) bacterial cellulose samples of sonication 30 seconds, sonication 60 seconds groups and the control group. Tensile stress at break values, which is in other words the ultimate strength that the samples can resist, indicates that resistance of bacterial cellulose samples against applied force. According to the figure 3.11., bacterial cellulose samples that were produced under sonication 60 seconds stress has the highest ultimate strength in MPa followed by sonication 30 seconds and lastly the control group. Therefore, this outcome approves the FTIR results (see part 3.1.8) that suggests stronger bacterial cellulose production under sonication stress conditions. A considerable difference between dry and wet bacterial cellulose samples is present. Dried bacterial cellulose samples were observed as more durable when compared with wet samples. This situation is reasoning from the friction force created by the randomly accumulated fibrils in the dry form of the cellulose, in the wet state the water in the structure removes the friction force that provides the strength.

3.1.10. Gene Expression

RNA isolation was performed for 4^{th} and 7^{th} days of sonication 30 seconds, sonication 60 seconds groups and the control group that no stress applied samples in order to synthesize cDNA and continue to RT-qPCR. For the determination of concentration and purity of isolated RNA, spectrophotometric measurement was performed, shown in Table 3.3. For pure RNA, the optical density for the 260/280nm ratio value is equal to ~2.2-2.3 [96]. Therefore, according to the Table 3.3. purity of purified RNA samples can be considered as pure. Concentration measurements were used in cDNA synthesis for the usage of same concentration during the procedure.

Table 3.3. Isolated RNA concentration and OD260/280 for purity assessment

Sample	Nucleic Acid Conc. (ng/µL)	OD260/280
Sonication 30 sec day 4	235.72	2.24
Sonication 60 sec day 4	270,99	2.27
Control day 4	133,23	2,21
Sonication 30 sec day 7	665.75	2.32
Sonication 60 sec day 7	410.4	2.33
Control day 7	218.01	2.31

Cq values known as values of quantification cycle. A higher Cq value indicates a lower expression. The comparison of Cq values of two samples allows a comparison for gene expression [95]. Cq values for 4th and 7th day samples of sonication 30 seconds and 60 seconds groups and the control group for bcsA, ccpAx genes and 23s rRNA gene, which is used as a reference gene, that were obtained during RT-qPCR were shown in Table 3.4. It is clear that all groups including stressed and control groups have lower gene expression for bcsA, ccpAx genes, which have function as glycosyltransferase/BC synthesis and BC crystallization/regulation respectively, when compared with the reference gene.

Table 3.4. RT-qPCR Cq values for bcsA, ccpAx and 23s rRNA genes

Sample	**bcsA**	**ccpAx**	**23s rRNA**
Sonication 30 sec 4th day	35.983	36.970	7.437
Sonication 30 sec 7th day	35.847	37.670	6.863
Sonication 60 sec 4th day	36.247	38.487	7.253
Sonication 60 sec 7th day	35.350	37.185	7.437
Control (no stress) 4th day	35.903	37.747	7.790
Control (no stress) 7th day	36.473	38.665	8.240

Cq values for 4th and 7th day samples of sonication 30 seconds and 60 seconds groups and the control group for bcsA, ccpAx genes and 23s rRNA gene, which is used as a reference gene, that were obtained during RT-qPCR were shown in Table 3.4. It is clear that all groups including stressed and control groups have lower gene expression for bcsA, ccpAx genes, which have function as glycosyltransferase/BC synthesis and BC crystallization/regulation respectively, when compared with the reference gene.

According to Table 3.4. Cq values of target genes, which are bcsA and ccpAx genes, were observed very close to each other that indicates similar gene expression levels. Therefore, the stress application seems no clear effect on gene expression. However, for a better evaluation and a better understanding of changes in the expression, normalization of Cq values relative to control group also using the reference gene Cq values were shown in Table 3.5. that were calculated by using Pfaffl method [98].

Table 3.5. Relative normalized expression of 4th and 7th day samples of sonication 30 seconds and 60 seconds for bcsA and ccpAx genes to the control group

Sample	bcsA	ccpAx
Sonication 30 sec 4th day	1.350	0.746
Sonication 30 sec 7th day	1.682	1.303
Sonication 60 sec 4th day	1.840	2.423
Sonication 60 sec 7th day	0.801	0.626
Control (no stress) 4th day	1.000	1.000
Control (no stress) 7th day	1.000	1.000

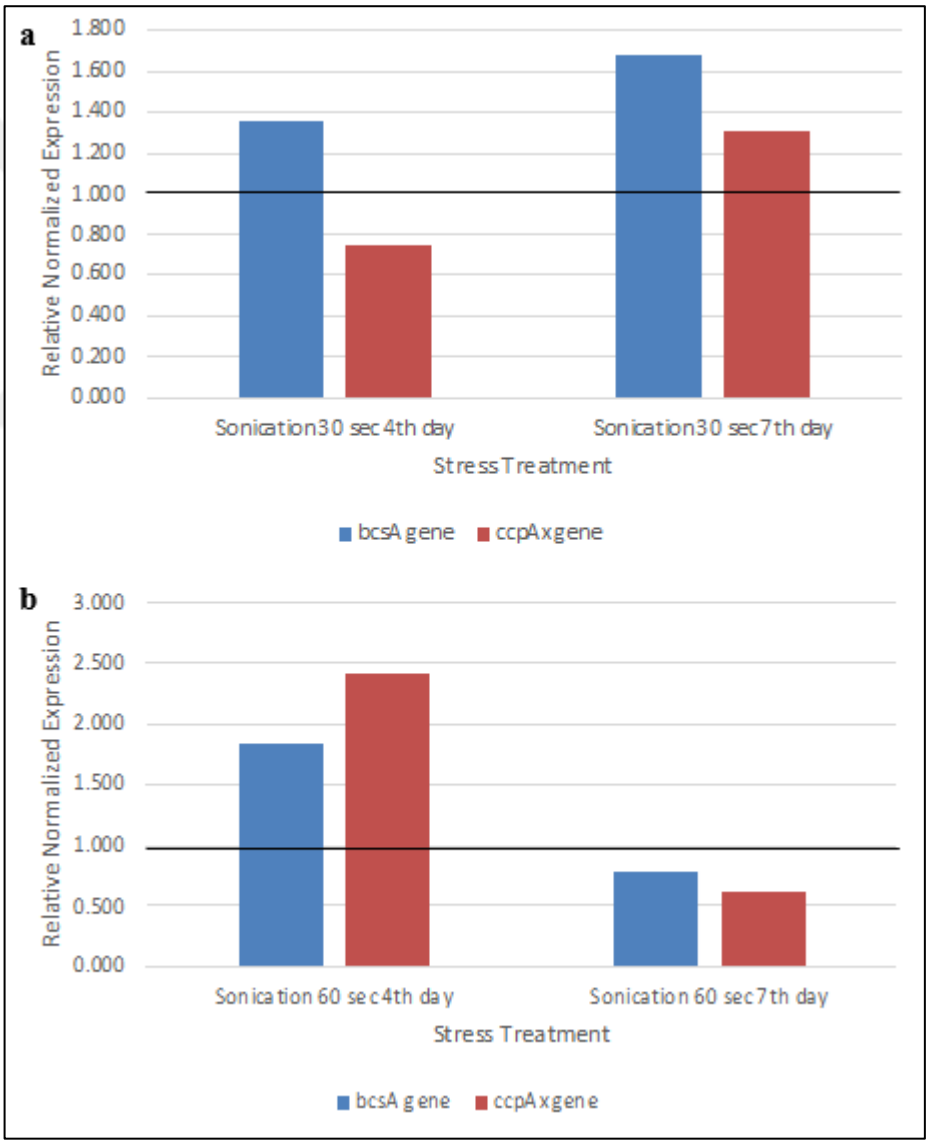

Figure 3.12. Relative normalized expression of 4th and 7th day samples of sonication 30 seconds (a) and 60 seconds (b) for bcsA and ccpAx genes to the control group

In Figure 3.12. relative normalized expression of bcsA and ccpAx genes is visualized with the indication (black line) at the expression level of the control group. According to the Figure 3.12., sonication 30 seconds treatment group expressed the bcsA gene 35percent higher and ccpAx gene 25percent lower when compared with the control group for the 4th day of the fermentation, while on the 7th day of the fermentation, expression of bcsA gene is 68percent higher and ccpAx gene is expressed 30percent higher when compared to the control group. For sonication 60 seconds treatment group expressed the bcsA gene 84percent higher and ccpAx gene 142percent higher when compared with the control group for the 4th day of the fermentation, while on the 7th day of the fermentation, expression of bcsA gene is 20percent lower and ccpAx gene is expressed 27percent lower when compared to the control group. According to these results it can be said that sonication 30 seconds stress has a positive effect on expression of bcsA and ccpAx genes overall, while sonication 60 seconds stress promoted higher expression levels on the 4th day, levels dropped significantly on the 7th day, decrement in the expression levels may be reasoning from the longer exposure to stress. According to this result it can be said that microorganism tolerated the sonication 30 seconds stress and the sonication 30 second stress less effected the gene expression. The effect of the elevated expression levels in the sonication 60 seconds can be also seen on the BC production amounts (see part 3.1.1) by high amounts of production at the beginning but remaining at the same level later as the expression levels dropped. Therefore, it can be said that sonication 60 stress is more effective than sonication 30 seconds stress for the BC amount that was produced however continuous exposure to sonication 60 seconds stress drops the expression levels and produced BC amounts did increase after the 5th day.

3.2. COLD SHOCK STRESS

3.2.1. Cell Dry Weight and Produced Bacterial Cellulose Amount

For the evaluation of bacterial cellulose production amount under cold shock stress, dry BC weight was measured for BC produced by bacteria that exposed to stress daily as cold shock stress for 30 minutes and 60 minutes. Also, BC produced by a control group that exposed to no stress was also measured. After the inoculation 2 days were waited for bacterial cells to grow and start to produce cellulose, 3rd day first stress was started to be applied and continued to be applied daily. First sample was collected 24 hours after from the stress application, which is 4th day, in order to observe the effects of the stress. After first sample collection samples were continued to be collected daily. Therefore, samples were collected as 4th,5th, 6^{th,} and 7th days of the fermentation. Dry bacterial cellulose weighs were shown in Figure 3.13. that was measured daily for cold shock stress for 30 minutes and 60 minutes and control group with no stress application.

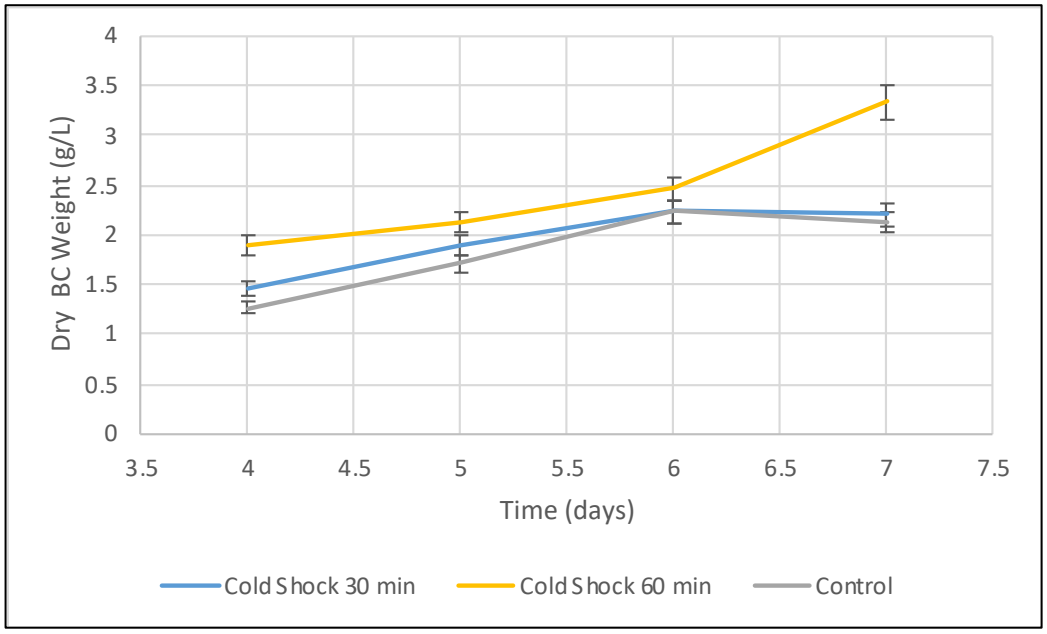

Figure 3.13. Weight of dry BC that were produced under cold shock stress 30 and 60 minutes and control group (no stress applied)

Bacterial cellulose amount that was produced by bacteria that exposed to cold shock stress for 60 minutes, was observed higher than cold shock 30 minutes and the control group. All three groups had similar curves on the days between 4^{th} and 6^{th} days. However, between 6^{th} and 7^{th} day, the bacterial cellulose amount in cold shock 60 minutes group was increased when compared with cold shock 30 minutes and the control group with no stress applied. Although the bacterial cellulose amount that was produced by bacteria that was exposed to cold shock stress for 30 minutes had slightly higher bacterial cellulose amount, control group and cold shock 30 minutes group had very similar curves. For bacterial cellulose, cold shock 60 seconds applied stress applied group yielded 1.6 fold greater than the control group on the 7^{th} day of the fermentation. When bacterial cellulose production analyzed statistically, a significant difference was observed in the bacterial cellulose production amounts for cold shock 60 minutes group with p value of 0.059 that near $p \leq 0.05$ and for cold shock 30 minutes group with $p \leq 0.1$ when compared with the control group.

In order to determine the effect of stress on microbial growth, cell dry weight was measured for cold shock stress for 30 minutes and 60 minutes and control group with no stress application. As seen in Figure 3.14. on the 4^{th} day, cell dry weight values can be listed from higher to lower as control group, sonication 30 minutes and sonication 60 minutes as well as being close each other. Between the 4^{th} and 5^{th} days all three groups shown a similar growth. However, cell dry weight between the 5^{th} and 6^{th} days cold shock 60 minutes was increased while cold shock 30 minutes was decreasing. This increment is similar with the pattern of the control group's increment with being slightly higher than the control group. The reason behind the increment and closing to the control group behavior of cod shock 60 minutes, may be reasoning from the bacterial response to the cold shock stress. In cold shock stress, it is observed that bacteria developed mechanisms to provide growth in low temperatures involving modifications such as membrane lipid composition change to an increased proportion of unsaturated and shorter fatty acids, in order to maintain structural integrity and membrane fluidity [94].

Due to the formation of a response in the cold shock 60 minutes group growth was observed however in cold shock 30 minutes, either stress was not enough to produce a response the response was produce later at the 7^{th} day. If so, this situation can be an explanation to the cell dry weight value of 7^{th} day for cold shock 30 minutes stress group.

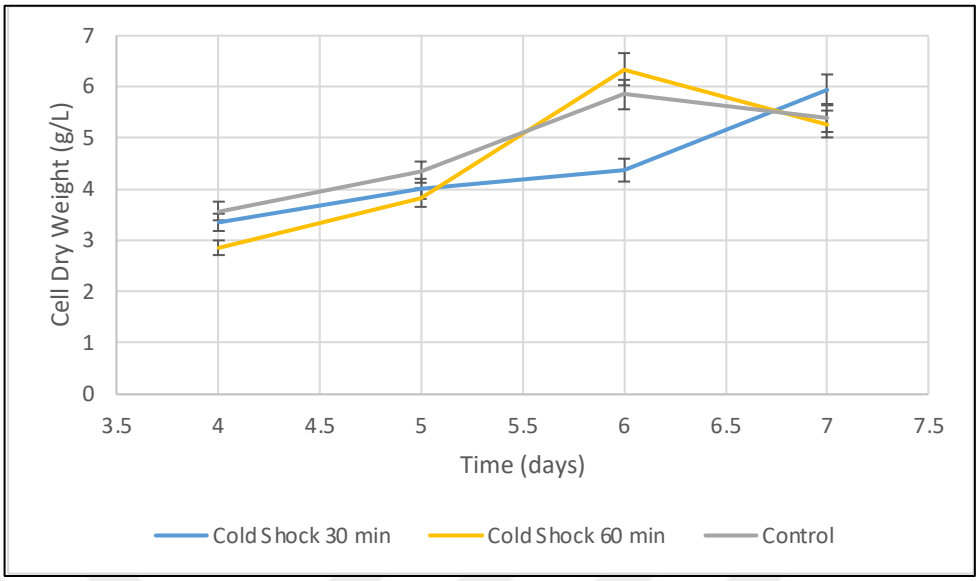

Figure 3.14. Cell dry weight of cold shock stress 30 and 60 minutes and control group (no stress applied)

3.2.2. Evaluation of Normalized Cell Dry Weight and Dry BC Weight Analyses

Normalization of cell dry weight and dry bacterial cellulose amount graphs for cold shock 30 minutes and cold shock 60 minutes stress groups and the control group, for samples of 4^{th}, 5^{th}, $6^{th,}$ and 7^{th} days. Normalized graphs of dry bacterial cellulose and cell dry weight were plotted in order to understand the changes in cell growth related with the applied stress, and changes in the bacterial cellulose production due to the stress conditions that bacteria were exposed to during the fermentation.

In Figure 3.15. normalized weight of dry BC that were produced under sonication stress 30 and 60 seconds and cell dry weight of cold shock stress 30 minutes and 60 minutes and control group were shown. For bacterial cellulose production, cold shock stress 30 minutes and control group have similar change rates. Therefore, it is observed that cold shock 30 minutes stress was not affected the production. However, cold shock 60 minutes stress affected cell growth negatively. In cold shock 30 min focused on cellulose production more than cellular growth, and BC production is observed as slowed down at the end of the fermentation and cell focused on growing due to explosion to stress for a while. For cold shock 60 minutes growth was negatively affected at the beginning while cell is more focused on BC production, on the following days cells focused more on growth.

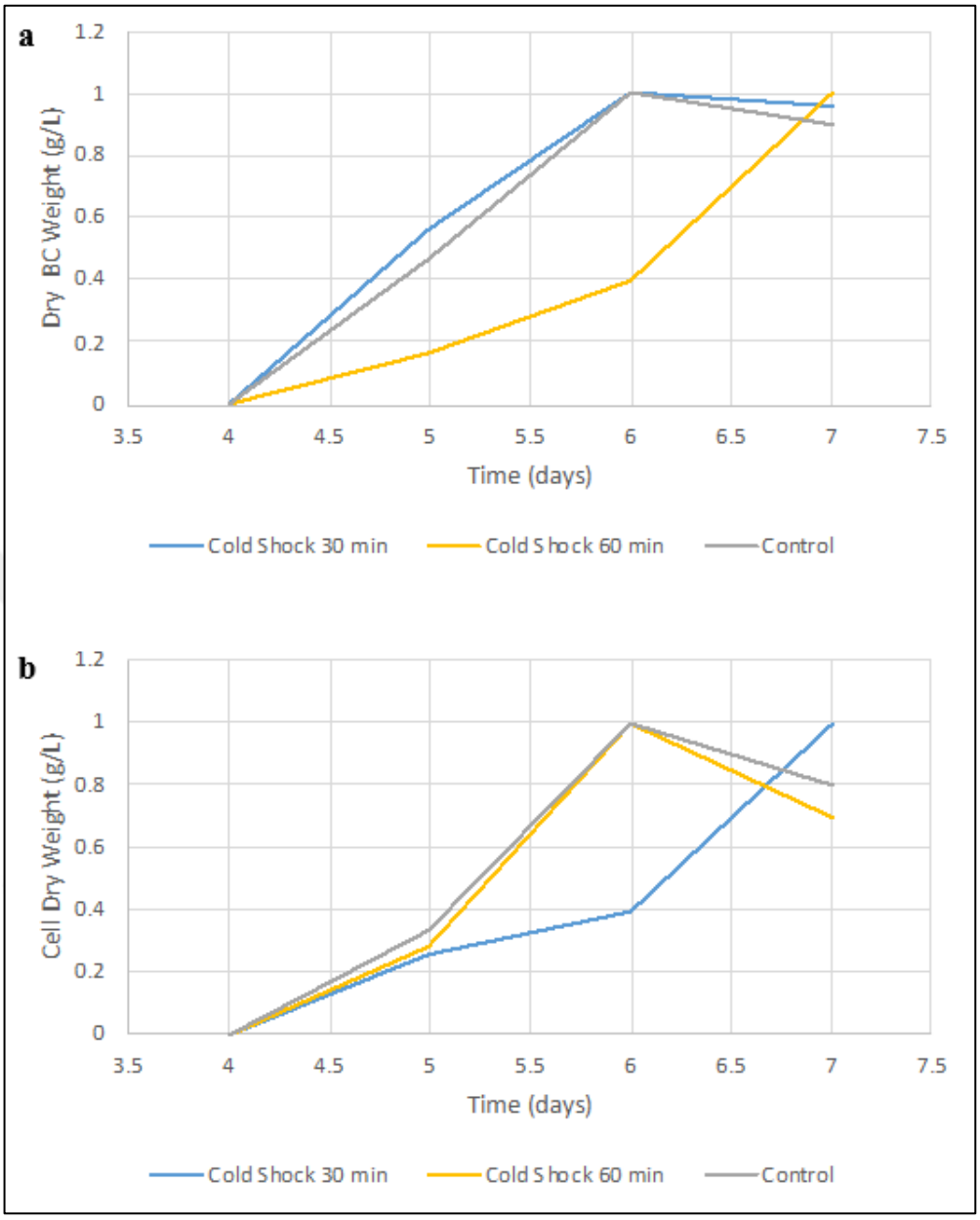

Figure 3.15. Normalized (a) weight of dry BC that were produced under cold shock stress 30 minutes and 60 minutes, (b) cell dry weight of cold shock stress 30 minutes and 60 minutes and control group

3.2.3. Glucose and Protein Concentration Changes During Fermentation

Glucose and protein concentrations were measured during fermentations in order to investigate the change in cell behavior under stress. At the end of the fermentation, which is 7^{th} day, glucose and protein concentration were measured in order to compare with the initial

values. Bacterial cells were supplied with 10 g/L glucose at the beginning of the fermentation. In Figure 3.16. glucose was consumption of groups cold shock 30 minutes, 60 minutes and control group were shown. It is clearly observed that bacteria that were exposed to stress conditions consumed more glucose when compared with control group. When cold shock 30 minutes and 60 minutes were compared with each other, it can be said that bacteria that have exposed to stress longer consume more glucose.

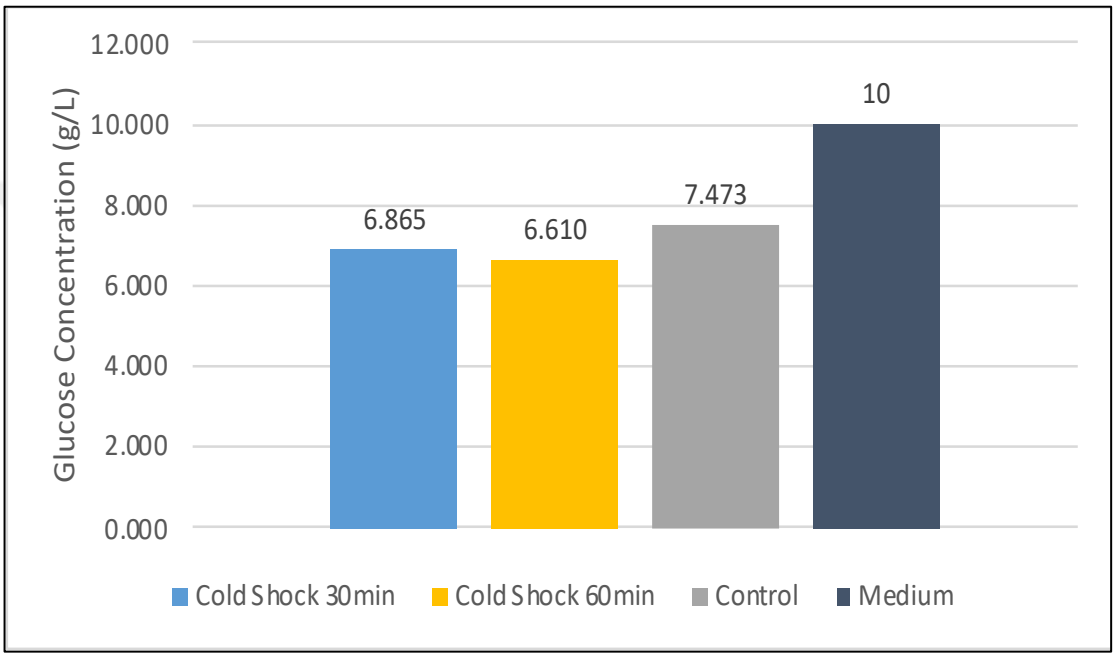

Figure 3.16. Measured glucose concentration during fermentation under stress, cold shock 30 and 60 minutes and control group (no stress applied)

Protein concentration was also measured for sonication 30 and 60 seconds and control group in order to observe stress effect on fermentation. For all groups, measured protein amount was higher than provided protein sources. Protein increment during fermentation may be resulting from either biomass accumulation or due to concentration of protein with the decreasing carbohydrates due to consumption [89].

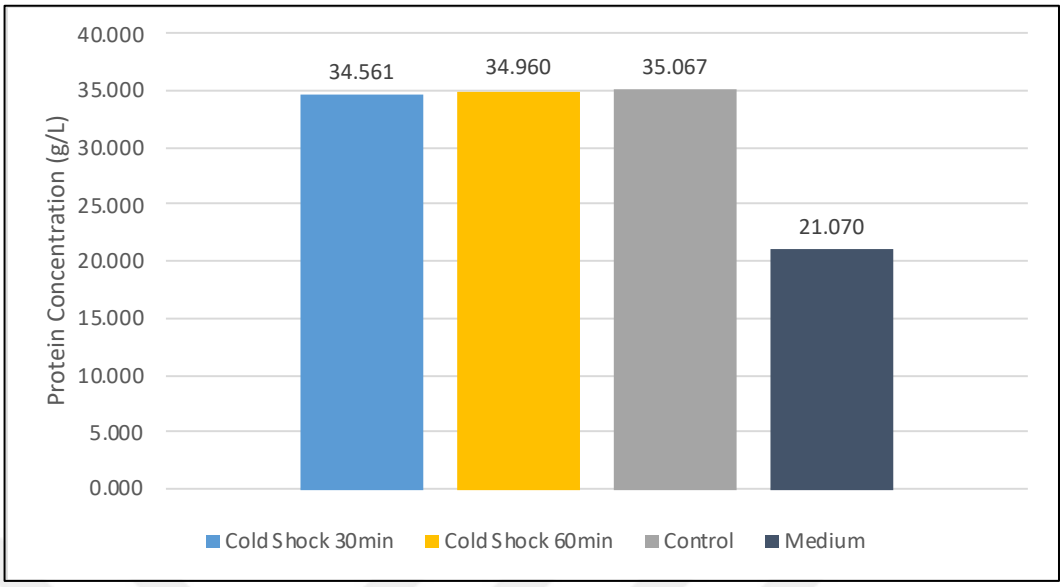

Figure 3.17. Measured protein concentration during fermentation under stress, sonication 30 and 60 seconds and control group (no stress applied)

As shown in Figure 3.17. protein concentration for both stress groups and control group is higher than the protein concentration that was supplied with the medium at the beginning of the fermentation. However, protein concentrations of cold shock 30 minutes and 60 minutes and control group has protein concentration very close to each other with 34.6 g/L, 35 g/L and 35.1 g/L respectively.

3.2.4. Surface Characteristics of BC

SEM imaging was performed in order to investigate the morphologies of bacterial celluloses produced under cold shock 30 minutes and cold shock 60 minutes stress and control group with no stress. In Figure 3.18., 4^{th} and 7^{th} day bacterial cellulose samples were shown for cold shock 30 minutes, cold shock 60 minutes and control group When SEM micrographs were assessed it can be said that control group has a smooth surface morphology for both 4^{th} and 7^{th} day. Also, bacterial cellulose that was produced under cold shock 30 minutes stress has similar smooth surface morphology with control group that indicates cold shock 30 minutes stress had not affected the morphology of the produced bacterial cellulose for both 4^{th} and 7^{th} day.

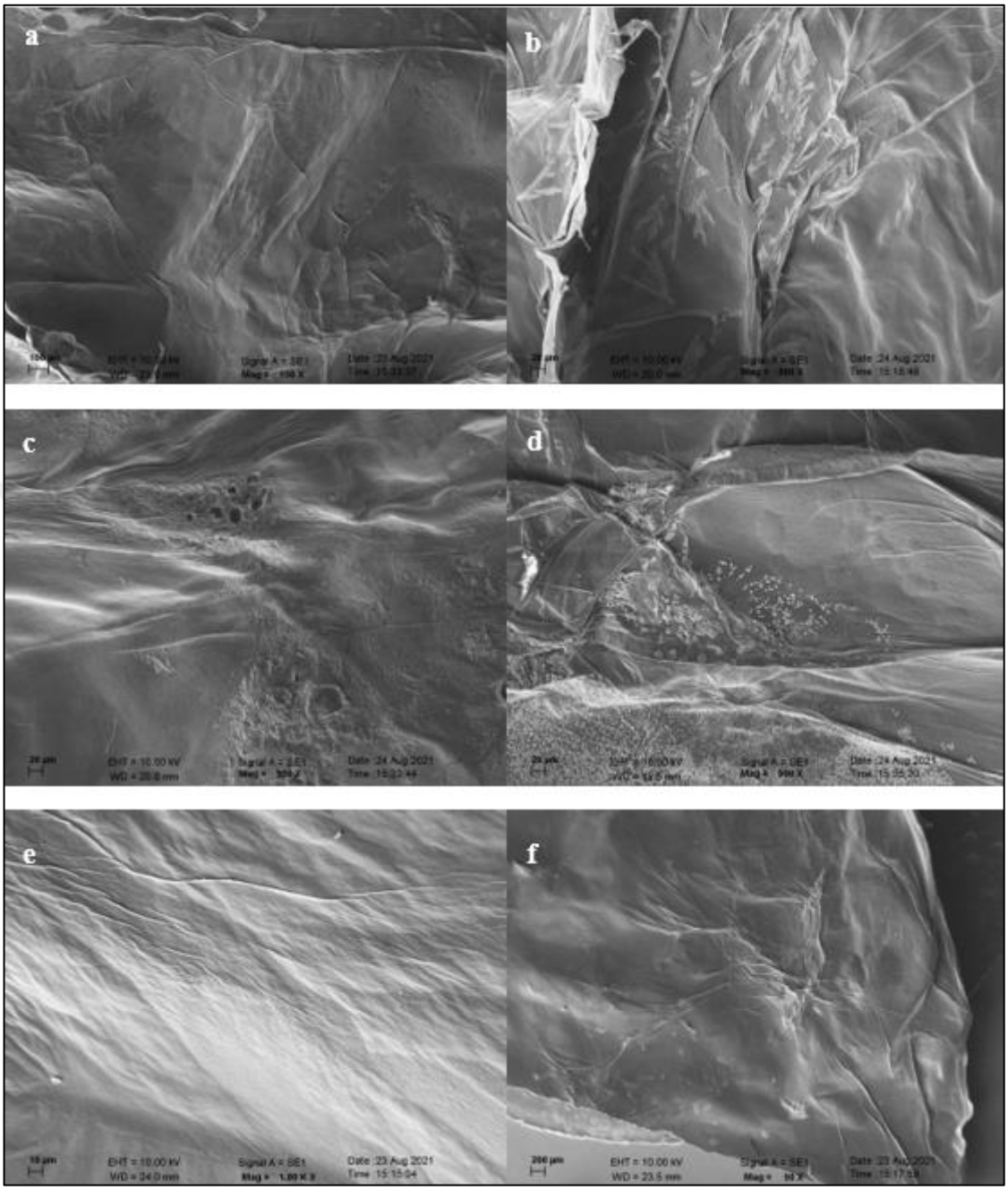

Figure 3.18. SEM images of bacterial cellulose of (a) cold shock 30 minutes day 4, (b) cold shock 30 minutes day 7, (c) cold shock 60 minutes day 4, (d) cold shock 60 minutes day 4 day 7, (e) control no stress applied day 4, (f) control no stress applied day 7

However, when morphology of bacterial cellulose that were produced under cold shock 60 minutes stress, it is observed that they had a rougher surface when compared with cold shock 30 minutes stress and control group. The rough surface structures of BC that produced under

stress may be reasoning from more irregular accumulation of fibrils during the polymer formation due to applied stress while BC produced by the control group.

3.2.5. X-Ray Diffraction

X-ray diffraction was performed for bacterial celluloses produced under cold shock 30 minutes and cold shock 60 minutes stress and control group with no stress in order to assess crystallinity of BC.

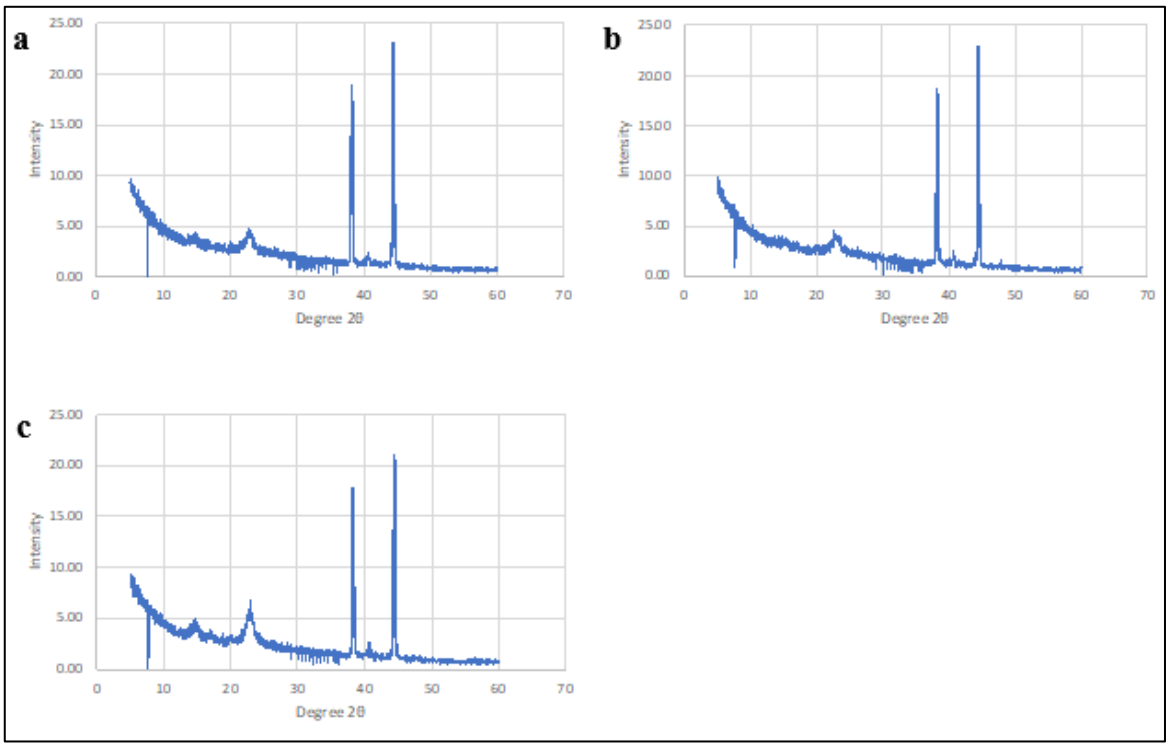

Figure 3.19. X-ray diffractograms of (a) cold shock 30 minutes, (b) cold shock 60 minutes, (c) control (no stress)

X-ray diffractograms of cold shock 30 minutes and cold shock 60 minutes and control group were shown in Figure 3.19. For cold shock 30 minutes and cold shock 60 minutes and control group, diffractogram presents peaks at 2 θ = 38.2, 44.4. The x-ray diffractograms and crystallinity of produced BC by cold shock 30 minutes and cold shock 60 minutes and control groups are very similar to each other. Therefore, cold shock stress has no effect on crystallinity of BC.

3.2.6. Differential Scanning Calorimetry

Differential scanning calorimetry was performed for calorimetry bacterial celluloses produced under cold shock 30 minutes and cold shock 60 minutes and control group with no stress. An endotherm event was observed around 100°C, this may be reasoning from intra or intermolecular bonded cellulosic chains or residual moisture [90]. The second event occurred between 230-350°C that is in collaboration of the mass loss in the ΔTGA spectra that shown in Figure 3.9. which may be resulting from the cellulose degradation [91]. Beyond the temperatures from 270°C a depolymerization process or cellulose decomposing is indicated by the heat flux [92]. Glass transition temperature (T_g) was not observed, this situation may occur in some polymers such as cellulose if the decomposition occurred just before or occurred parallel to the glass transition temperature [93].

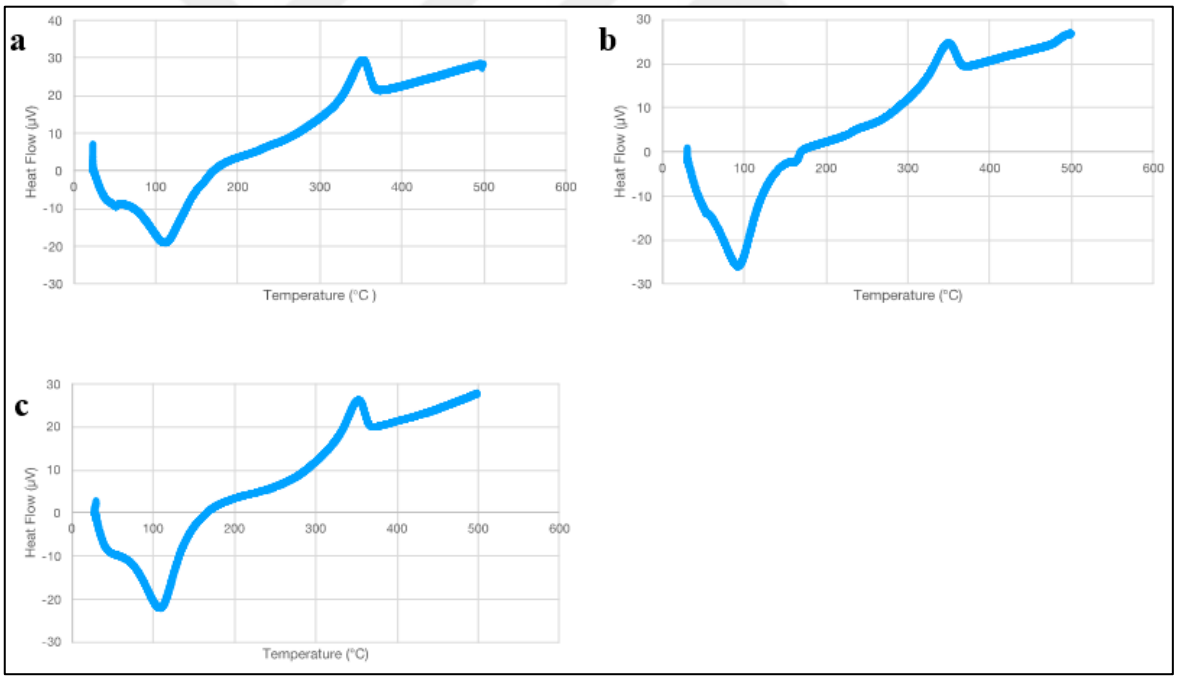

Figure 3.20. Differential scanning calorimetry (DSC) curves (a) cold shock 30 minutes, (b) cold shock 60 minutes, (c) control (no stress)

Differential scanning calorimetry curves of cold shock 30 minutes and cold shock 60 minutes and control group were shown in Figure 3.20. The first event which is the first peak around 100°C, indicates crystallization temperature (T_c) which is 115.6°C for cold shock 30 minutes, 97.2 for cold shock 60 minutes and 111.1 for control group. Second event which is

the second peak in the curve, indicates melting temperature (T_m) which is 358.9 for cold shock 30 minutes, 356.6 for cold shock 60 minutes and 358.4 for control group. For all groups DSC curves appeared similar to each other, T_c and T_m values were very closed to each other. Therefore, cold shock stress has no clear effect on amount of heat required to increase the temperature. This situation indicates that the molecular structure and conformation of bacterial cellulose was preserved.

3.2.7. Thermogravimetric Analysis

Thermogravimetric analysis was performed for cold shock 30 minutes and cold shock 60 minutes groups and the control group that no stress applied. Thermogravimetric curves (TGA) and their derivatives (ΔTGA) for bacterial cellulose that were produced under shock 30 minutes and cold shock 60 minutes and no stress (control group), shown in Figure 3.21.

According to Figure 3.21., decomposition of bacterial cellulose can be divided into three main events. The first event occurred between 20-200°C, this event indicates volatilization of components with low molecular weights and loss of water. The second and third events, indicates that the decomposition of polymeric chains of the bacterial cellulose which is shown in spectra as two mass loses [92]. At the firs event that occurred at 20-200°C for control group, cold shock 30 minutes and cold shock 60 minutes loss of weight are 17.3percent, 9percent and 9percent respectively.

Second event occurred at 200-310°C for control group, 220-310°C for cold shock 30 seconds and 220-320°C for cold shock 60 minutes with mass loses 51.5percent, 56.7percent and 56.2percent respectively. The third event occurred at 330-400°C for all groups and with mass losses of 11.9percent, 7.3percent 8.8percent respectively. Consequently, bacterial celluloses that were produced under stress conditions of cold shock 30 minutes and cold shock 60 minutes were observed as more thermostable when compared with control group.

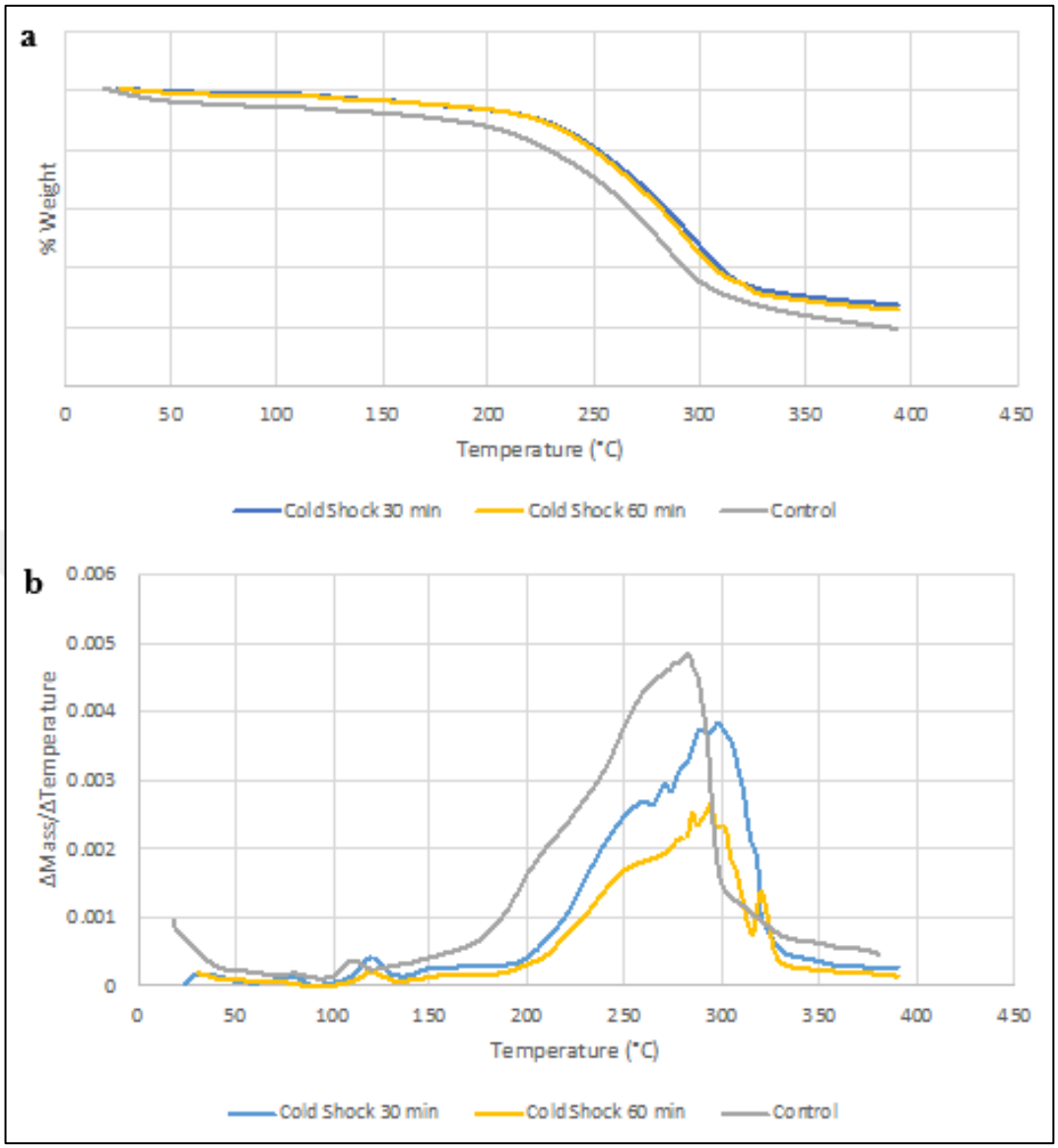

Figure 3.21. (a) TGA spectra of BC samples of cold shock 30 minutes, cold shock 60 minutes and control group (b) ΔTGA spectra BC samples of cold shock 30 minutes, cold shock 60 minutes and control group

When cold shock 30 minutes and 60 minutes were compared, it can be said that their TGA curve is very similar to each other. However, when DTGA curve was assessed, it is seen that cold shock 60 minutes is more thermostable when compared to cold shock 30 minutes. Both cold shock 30 and 60 minutes decomposed at higher temperatures when compared to control group. Therefore, applied cold shock stress provided a better thermal stability to produced bacterial celluloses.

3.2.8. Fourier-Transform Infrared Spectroscopy

Fourier Transform-Infrared Spectroscopy was performed for bacterial cellulose samples of cold shock 30 minutes and cold shock 60 minutes groups and the control group that no stress applied in order to determine molecular bonds and structural differences that may be resulting from stress application. In Figure 3.22. FTIR spectra of bacterial cellulose samples for cold shock 30 minutes, cold shock 60 minutes groups and the control group were shown.

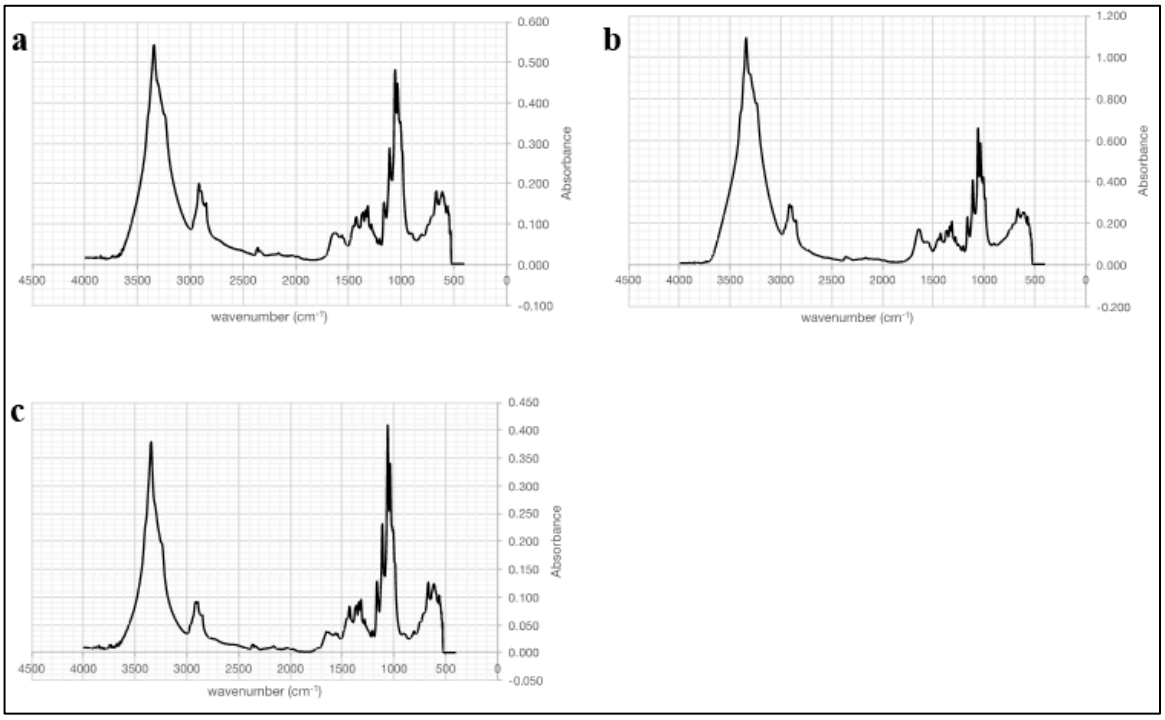

Figure 3.22. FTIR spectra of 7th day BC samples of (a) cold shock 30 minutes, (b) cold shock 60 minutes (c) control group

For cellulose, infrared band assignments were shown at Table 3.1. [94]. During FTIR analysis obtained peaks for bacterial cellulose samples of cold shock 30 minutes, 60 minutes and control group, were shown in Table 3.4. As shown in Figure 3.22. three intense peaks for all groups can be observed. One of the most intense peaks was obtained at ~1060 cm^{-1} that assigned to C-O-C stretching vibrations originating from the cellulose main chain parallel to the molecular chain axis. Another intense peak was obtained at ~1003 cm^{-1} which is derived from C3-O3 stretching vibrations that is considered as main bonding that forms a cross linking structure. And another intense peak was observed around ~3340 cm^{-1} which

indicates intra molecular hydrogen bonds (3-OH•••O-5). Both cold shock 30 minutes and 60 minutes groups have more intense peaks when compared with control group and more hydrogen bonds indicates more durable structure of the stressed groups. When two stress groups are compared with each other, cold shock 60 minutes group's ~3340 cm^{-1} peak is significantly intense than cold shock 30 minutes.

Table 3.6. Measured frequencies of BC samples of cold shock 30 minutes, 60 minutes, and the control group

Frequency (cm^{-1}) **Cold Shock 30 min.**	**Frequency (cm^{-1})** **Cold Shock 60 min.**	**Frequency (cm^{-1})** **Control**
3340	3340	3344
2909	2900	2891
1417	1420	1425
1312	1312	1313
1132	1158	1160
1104	1106	1107
1052	1054	1056
1029	1031	1032
1001	1002	1003
664	658	664

These three peaks were observed more intense in stress applied groups when compared with control group. Among cold shock 30 minutes and 60 minutes groups, cold shock 60 minutes group has more intense peaks than cold shock 60 minutes. Another peak was observed at the area of 2900 cm^{-1}, which indicates CH_2. For this peak control group has less intense peak when compared with stress applied groups. Overall, due to intense hydrogen bonds the most durable one is cold shock 60 minutes followed by cold shock 30 minutes and lastly the control group. Applied stress caused the formation of more hydrogen bonds that resulted in a stronger structure.

3.2.9. Tensile Strength Testing

Tensile stress testing was performed for both wet and dry bacterial cellulose samples of cold shock 30 minutes, cold shock 60 minutes groups and the control group that no stress applied in order to characterize and determine the mechanical properties.

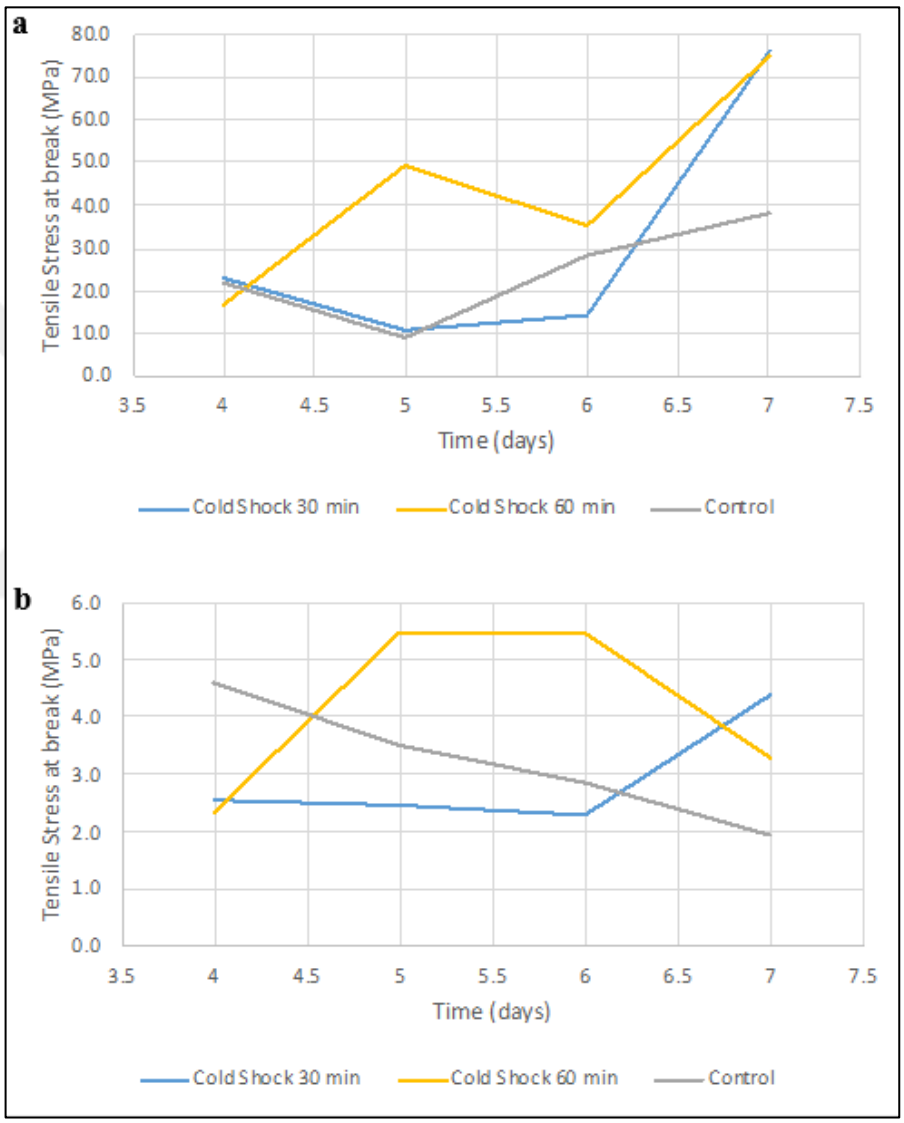

Figure 3.23. Tensile stress at break for (a) wet and (b) dry BC samples for cold shock 30 minutes, cold shock 60 minutes and control group

In Figure 3.23. tensile stress at break values were shown for daily collected (4^{th} day to 7^{th} day) bacterial cellulose samples of cold shock 30 minutes, cold shock 60 minutes groups and the control group. Tensile stress at break values, which is in other words the ultimate strength

that the samples can resist, indicates that resistance of bacterial cellulose samples against applied force. According to the Figure 3.23., bacterial cellulose samples that were produced under cold shock 30 minutes and 60 minutes stress both have the high ultimate strength in MPa when compared with the control group. However, cold shock 60 minutes had high values during the fermentation, while cold shock 30 minutes reached high values at the end of the fermentation. Therefore, the outcome that indicates stressed groups produced more durable bacterial cellulose, approves the FTIR results (see part 3.2.8) that suggests stronger bacterial cellulose production under cold shock stress conditions. A considerable difference between dry and wet bacterial cellulose samples is present. Dried bacterial cellulose samples were observed as more durable when compared with wet samples. This situation is reasoning from the friction force created by the randomly accumulated fibrils in the dry form of the cellulose, in the wet state the water in the structure removes the friction force that provides the strength.

4. CONCLUSIONS

Bacterial cellulose production under cold shock and sonication stress affects cell behavior of *K. xylinus* by altering growth rate, bacterial cellulose production amount, glucose consumption. Both sonication and cold shock stress conditions has effects on bacterial cellulose characteristics. As the result of the stress application for both cold shock stress and sonication stress, resulted in the production of stronger and more durable bacterial cellulose due to random accumulation of cellulose fibers, having more hydrogen bonds and more thermostable structure. Also, differences in gene expression levels were detected.

When cell behavior is considered, for both sonication and cold shock stress it is observed that intense stress which is sonication 60 seconds and cold shock 60 minutes resulted in slightly higher levels of bacterial cellulose production. In addition, under stress *K. xylinus* is observed to focus more on BC production rather than cell growth. Therefore, BC production is elevated under stress when compared with the control group. Glucose consumption is elevated under stress application along with the BC production increment. Produce BC under stress is observed stronger when compared with the control group that no stress applied. Morphologic changes also were observed in the stressed groups as having rougher surface while the control group has smoother surface. Rough surface is reasoning from the random accumulation of fibrils due to stress applications. In addition, according to Fourier transform infrared spectroscopy results hydrogen bond content is higher of the bacterial celluloses that were produced under stress. High hydrogen bond content indicates a stronger structure and tensile strength testing results confirms that stressed groups produced stronger bacterial cellulose. Friction force resulting from randomly accumulated fibrils due to applied stress resulted in a stronger structure. Moreover, molecular structure and conformation is preserved in bacterial celluloses of stressed groups according to the DSC analysis. Bacterial cellulose that was produced under stress conditions are obtained as more thermostable according to thermogravimetric analysis. Overall, under stress BC production was promoted with an effect of slightly stronger structure of produced bacterial cellulose. Sonication 60 seconds stress was observed as more effective for production amounts in BC however long exposure had negative effects.

Gene expression investigation was performed for sonication stress and showed stress conditions effect the expression levels of bcsA and ccpAx genes, which have function as glycosyltransferase/BC synthesis and BC crystallization/regulation respectively. Sonication 60 second stress promotes higher expression levels however longer exposure to stress negatively effects the expression levels. Sonication 30 seconds stress has lower expression levels when compared to sonication 60 however the increment was observed in long exposure unlike sonication 60 seconds. Therefore, the effect of the elevated expression levels in the sonication 60 seconds can be also seen on the BC production amounts by high amounts of production at the beginning but remaining at the same level later as the expression levels dropped. Therefore, it can be said that sonication 60 stress is more effective than sonication 30 seconds stress for the BC amount that was produced however continuous exposure to sonication 60 seconds stress drops the expression levels and produced BC amounts did increase after the 5^{th} day.

5. FUTURE PROSPECTS

As future works, different stress conditions along with different stress application intervals, can be investigated due to the knowledge of these effects on both cell behavior and the differences in the obtained product. In addition to that, a wider range of genes can be investigated for the expression levels related to the applied stress. Investigation of gene expression of more genes may provide a better understanding of the stress application outcomes.

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