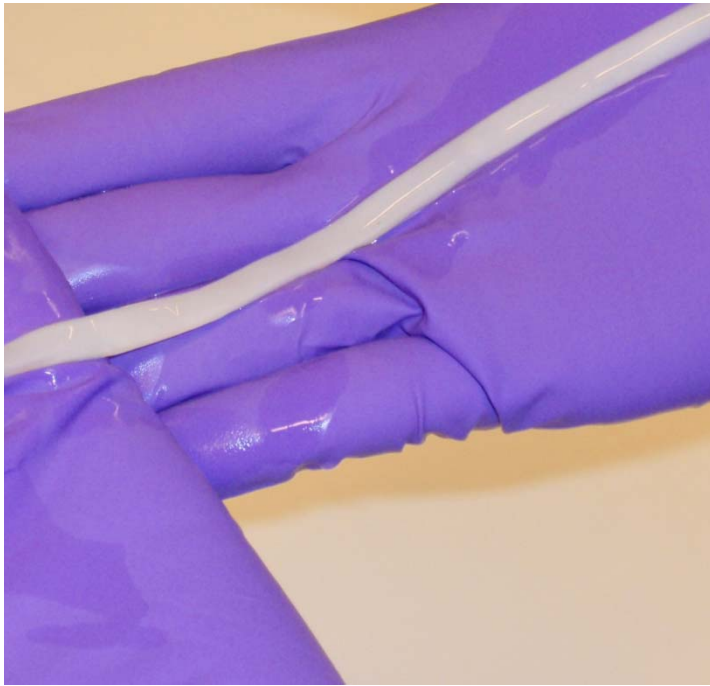# CHALMERS

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Master of Science Thesis in the Master Degree Program, Biotechnology

JEFFREY PHAN
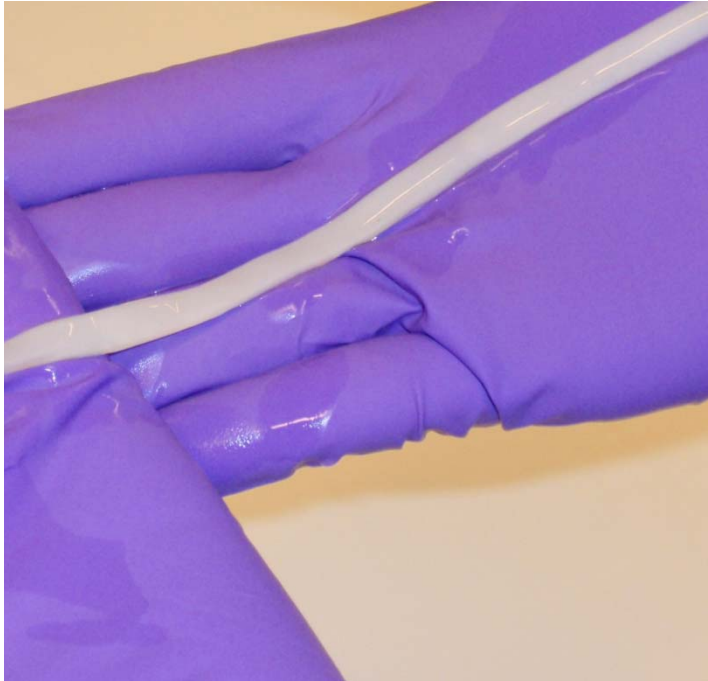
Department of Chemical and Biological Engineering

Division of Industrial Biotechnology

CHALMERS UNIVERSITY OF TECHNOLOGY

Göteborg, Sweden, 2010

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ABSTRACT

In the Western societies, the largest cause of mortality is atherosclerotic vascular disease. Bypass surgery is one of the most common today treatments. However, bypass surgery in which smaller blood vessels are to be replaced often fails because of occlusion. To date, there is no clinically suitable bypass material, for smaller blood vessels based on synthetic fabrics. Cellulose can be biosynthesized extracellularly by a microbe called *Acetobacter xylinum*. Presently, Arterion AB utilizes a novel technique that consists of using bacterial cellulose as a raw material for producing small vascular grafts, which are to replace small-diameter vessels in bypass surgery.

The current bioreactor set-up for the production of bacterial cellulose grafts is in pilot stage. The mass profiles of the produced grafts are not homogenous alongside the graft. A request from the surgeons is that thicker ends are preferred, in order to sew the graft together with the native blood vessels more easily. The aim of this master thesis project is first to acquire a more homogenous mass profile. Secondly to achieve a mass profile that suits the surgical requests. In order to analyze the produced grafts, radial tensile tests were performed along with scanning electron microscopy. In each produced graft several rings were cut out that represented the different areas of a produced graft to illustrate the mass profile of a single graft. In between each ring samples for the scanning electron microscopy were cut out as well.

The project was divided into two improvement areas which in total resulted into three protocols that were evaluated. Two of these protocols resulted in a more homogeneous mass profile while the third protocol established a novel concept of a modified fermentation set-up.

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BACKGROUND

The largest cause of mortality today in the Western societies is atherosclerotic vascular disease, such as coronary artery disease and peripheral vascular disease [1]. Already in 1952 began the speculation of treatments began when Voorhees *et al*. proposed to replace diseased blood vessels with synthetic fabrics [1]. This has lead to widespread use of grafts made of polyethylene terephthalate (PET) and expanded polytetra-fluoroethylene (ePTFE) in cardiovascular surgery, where bypass of occluded arteries is one of the treatments. However, bypass surgery in which smaller blood vessels (graft diameter ≤6 mm) are to be replaced by PET or ePTFE fails swiftly because of occlusion [1]. Bypassing of these vessels requires small-caliber grafts with unique properties; of critical importance are the antithrombotic and the visco-elastic properties of the graft [2]. Thus, the current standard for these procedures is autologous vascular tissue, such as the saphenous vein, the internal mammary artery and the radial artery [3, 4]. As a result of disease or previous surgery these vessels are not always available and removal of these grafts is undesirable. As for now there is no clinically suitable bypass material for the smaller blood vessels based on synthetic fabrics [2]. For the synthetic grafts there is still the problem of inducing low-level foreign body reaction, chronic inflammation and as artificial materials there is the possibility of microbial infection [1]. Nonetheless, the biggest concern is the reactivity between material surface and blood components. Blood components, such as proteins, will eventually adhere to a very inert surface, and start the process that will eventually lead to blockage [5]. The natural vessels have living endothelial cells lined along the luminal vessel surface, which makes the blood vessel non-reactive. Therefore, in order to develop a blood vessel substitute, it is required to synchronize the mechanical graft properties with the surrounding tissue, but also to assess the possibility of growing endothelial cells onto the vessel wall.

Cellulose, which is one of the most abundant biopolymers on Earth, is utilized in many commercial applications, such as paper, clothes and food additives [6]. This natural biopolymer has also shown to hold several attractive properties, such as being stable within a wide range of temperatures and different pH levels, as well as its mechanical properties [5]. Cellulose is insoluble in water and degradation of cellulose in animals is limited, because of the lack of enzymes (cellulases) that can degrade cellulose [7]. Cellulose (β-1,4-glucan) is biosynthesized as structural material in the walls of plants [8] but can also be produced extracellularly by a microbe called *Acetobacter xylinum* [9]. *A. xylinum* is a gram-negative bacterium that has been studied as a model organism of cellulose biosynthesis, since it produces large amounts of cellulose called "bacterial cellulose" (BC) [6, 10]. The produced BC differs from the plant-derived cellulose in that it has high crystallinity, mechanical strength, water retention and wet strength, as well as great elasticity and unique conformability in the wet state [9]. Furthermore, the BC is free from lignin and hemicelluloses that are present in plant-derived cellulose [9] and the BC production does not involve any other polymers or proteins. The resulting hydrogel holds approximately 99% water [11]. The unique conformability makes the hydrogel possible to shape into any form and size during its biosynthesis [9].

Presently, Arterion AB is carrying out research in this particular area. The unique novel technology consists of using BC as a raw material for producing small vascular grafts. These artificial blood vessels are then to replace small-diameter vessels in both peripheral and coronary applications [12].

The current fermentation/production process set-up is in a pilot stage, in which the BC vascular graft is built-up in a radial direction and several microlayers are synthesized on top of each other. It is also at the same time crosslinked, which turns it into the desired hydrogel. The BC is synthesized by *A. xylinum* fermentation utilizing oxygen and a carbon source. A schematic illustration of the fermentation set-up can be visualized below in figure 1.
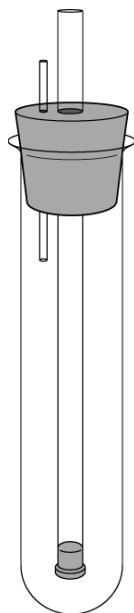
Figure 1. Schematic illustration of the currently used bioreactor. It is made up of a test tube with a rubber stopper that has two holes, one for an oxygen gas inlet and one for inoculation of bacteria. Connected to the stopper is a silicone tube, which is also attached to a weight at the bottom. The silicone tube is submerged in culture medium that provides the carbon source needed for fermentation.

The BC grafts are produced in bioreactors possessing a gas inlet that is located at the top of the bioreactor. The gas inlet will supply the necessary oxygen through a silicone tube and distribute oxygen to the bacteria by membrane diffusion. At the bottom of the silicone tube there is a weight that keeps the silicone tube straight, and also acts as a stopper for the incoming gas. On the top there is a stopper with two holes, one for the gas inlet and one for the inoculation of bacteria. The test tube will also contain media that provides the carbon source required for fermentation.

PROBLEM STATEMENT

It has been noted that the mass profile of the BC grafts produced are not homogenous along the silicone tube. This has also been confirmed by the observation that each produced graft is unique in itself. The surgeons that have used these grafts also noted that it sometimes is hard to sew the graft together with the blood vessels. Preferably the surgeons would want to have more rigid or thicker ends to sew with.

Furthermore, as the grafts are uniquely produced, a bioreactor that produces more similar grafts is wanted, i.e. a bioreactor that has a more reproducible process. Preferably, the process should create grafts that are replicas. The main hypotheses result in two ways of improving the bioreactor system in which one improvement is described in protocol 1 and 2, while the second improvement is described in protocol 3.

AIMS AND SCOPE

The purpose of this project is to examine the current pilot fermentation set-up and attempt to develop an improved bioreactor. An improved bioreactor in the sense of producing less variable grafts and preferably with a mass profile that suits the surgeon. Results from this project will also aid Arterion in its aim to industrialize the production of BC tubes and provide the solution for revascularization treatments for small blood vessels.

The aim of this project is to further develop the current production process. In order to achieve the reproducible process that can provide uniformly produced grafts. There are two focuses:

One of the project focuses will be on the mass profile on how to produce grafts that has a more homogeneous mass profile. This will result in a better product reproducibility and improved production process of the BC tubes. In order to fulfill this, alterations on the bioreactor properties will be set to enhance better control of the bioreactor and the production of the grafts. The second focus is on controlling the mass profile of the grafts. This might result in better surgical preferences of the vascular grafts, which are homogeneous in the middle and a bit thicker at each end. Thus, the surgical handling will be enhanced after surgeon requests. To achieve this, a remodel of the current bioreactor set-up into a new novel bioreactor set-up will be investigated.

There are several development projects running simultaneously with the aim to improve the fermentation process. Due to that, this project will be focused solely on the bioreactor set-up. Investigating how to get a better reproducibility and more robust process as well as getting a more homogeneous BC distribution axially along the tube. Other areas of improvement, which are not included in the aims of this project, are the culture medium, the inoculation procedure, and the radial stratification. Thus, the project will use standardized variables, which are not to be changed regardless of results gained from the other simultaneously running projects. The method for evaluation will also be limited. The finishing product will only be evaluated with microscopy and biomechanical testing called tensile test. Thus, no investigation will be made on the biochemistry of the cellulose production or on the true oxygen profile of the fermentation.

THEORETICAL BACKGROUND

The following sections contain the basic knowledge that will help the understanding of tissue engineered blood vessels, improvements of the bioreactor and the utilization of bacteria *A. xylinum*. The sections that are covered involve native blood vessels, history of tissue engineered blood vessels, fermentation, bacterial cellulose and bioreactors.

NATIVE BLOOD VESSELS

The cardiovascular system is a system that distributes and transports blood to every part of the body from toe to head. The blood carries and delivers resources such as oxygen, nutrients and hormones and it also has the task of getting rid of wastes. The transport of blood is achieved by the blood vessels that constitute the circulatory routes. These routes make their way from the heart to all the body parts and back to the heart again.

There are five types of blood vessels:

- Arteries
- Arterioles (small arteries)
- Capillaries
- Venules (little veins)
- Veins

The functions of blood vessels can be summarized into a system of tubes that takes blood from the heart in arteries. The blood is then transported in arterioles, capillaries and venules through the tissue of the body until it returns back again to the heart by the veins. In the capillaries substance exchange occurs between the blood and the body tissue cells. Nutrients and oxygen are diffused into the tissue from the blood through the interstitial fluid [13]. In the opposite direction waste products and carbon dioxide is diffused back to the blood. Furthermore the blood vessels have the function of controlling the blood flow when reaching different parts of the vessels. High pressure can be seen in the arteries and due to the decreasing size that occurs from arteries to arterioles to capillaries the pressure concurrently drops [13, 14]. The blood will then arrive at regions of lower pressures in venules and veins and eventually the blood will be collected back by the heart. Therefore the blood flow is not only affected by for example heart rate, blood volume, blood viscosity and hormones it is also dependent on the architecture or the configuration of the vessels such as length, diameter and branching angle [13, 14].

The arteries, arterioles, venules and veins have essentially the same basis of structure. They consist of three layers (tunics) that surround the lumen. The proportion of the different layers changes depending on which blood vessel type it is. The three layers are divided into the inner layer (*tunica intima*), the middle layer (*tunica media*) and the outer layer (*tunica adventitia*). The inner layer is composed of an endothelium, a flat single layer of cells, connected to a basement membrane. The basement membrane or basal lamina separates the endothelium from the other outer layers that line the entire vascular system, from big arteries to small capillaries [15]. The middle layer consists of smooth muscle tissue mixed with elastic tissue. The smooth muscle tissue is connected to the nervous system that in combination with stimulation will cause the smooth muscle to contract or relax. The contraction will decrease the diameter while the relaxation will increase the diameter of the lumen adjusting the blood flow rate [13]. The amount of elastic tissue (fibers) in the middle layer has an effect as well. The vessels with high proportions of elastic fibers help to push

the blood onwards. When blood is flushing in the highly elastic walls will stretch out and the recoiling of those walls will then force the blood onwards [13]. The third and last outer layer is composed of collagen and elastic fibers. Compared to the arteries the veins have thinner inner and middle layers. The lumen of the veins is larger in diameter than the arteries. A special feature of the veins is that in some parts there are preformed valves in the vessels that prevent backflow of blood.

The capillaries only consists of an endothelium with basement membrane, due to its thin layer it is possible for diffusion of substances between blood and tissue. The exchange of substances varies depending on how tight the endothelial cells are, i.e. degree of permeability. They make up the transition from arteries to veins and function as exchange vessels with an extensive network at body tissues that have high metabolic activity.

ARTERIOSCLEROSIS

The chronic disease with the signature symptoms of thickened, hardened and stiff arterial walls is called arteriosclerosis [16]. Arteriosclerosis is the result of weakened or damaged blood circulation. The most common form of arteriosclerosis is atherosclerosis, it is characterized by atherosclerotic plaques that are deposited within the arterial walls. These plaques result in a decrease or a stop of the blood flow. The deposition of plaques will reduce the cross-sectional area, which in turn will result in a higher blood flow resistance and proportionally reduce the flow in the vessel. While slowing down the blood flow it will allow more plaques and other biological materials (such as proteins, clotting factors) to interact and after a long time it will eventually occlude the vessel [16]. Accumulation of plaques in a particular area is called a clot. Another scenario of occlusion that could occur is when a clot breaks away from where it was established. The clot will travel through the vascular system until it gets trapped at a blood vessel smaller or with the same size and consequently occlude that blood vessel. The result of the obstruction may lead to myocardial infarction (heart attack) or stroke and sudden death [16]. Infarction is the death of a tissue area due to stopped blood supply, thus myocardial infarction means the death of heart tissue. The reduced blood flow from a decreased cross-sectional area could lead to myocardial ischemia often leading to hypoxia (reduced oxygen supply) that may weaken cells without killing them [13].

HISTORY

Atherosclerotic vascular disease is one of the greatest causes of death in Western societies today [1]. Currently, there are three treatments for these diseases. First, one can remove a portion of the diseased blood vessel and replace it with a vascular graft, also called bypass surgery. The vascular graft could be a segment from an unoccluded native blood vessel (autologous vessel or autograft) taken from the saphenous vein (leg/foot), the internal mammary artery (chest cavity) or the radial artery (forearm) [3,4]. The second treatment is the insertion of a stent, which is a stiff, annular framework that holds the vessel open while inside the occluded vessel [16]. The third treatment is balloon angioplasty that makes use of a balloon that is inflated inside the narrowed vessel. This causes the balloon to press and flatten out the blockage to clear the lumen of the vessel [16]. In occurrence of diseased or damaged blood vessels the use of autologous vessels is the preferred treatment. But replacement vessels are often unavailable, as a result from disease or previous surgery. As a consequence, it is often undesirable to remove them. In those cases synthetic vascular grafts are used as replacements. The replacement of diseased blood vessels with synthetic fabrics

was speculated already in 1952. This particular treatment was proposed by Voorhees *et al.*, which later has lead to the widespread use of synthetic grafts [1, 17, 18]. The implanted material is often made of polyethylene terephthalate (PET) or expanded polytetra-fluorothylene (ePTFE), of which ePTFE is the most widely implanted material for synthetic vascular grafts [16, 17]. Although synthetic grafts work well for the replacement of larger blood vessels, they fail swiftly in bypassing of smaller blood vessels (inner graft diameter ≤6 mm). When smaller blood vessels are replaced by PET or ePTFE they stop working due to occlusion of the graft [1, 16, 17, 19]. Unique properties are needed for small-caliber grafts, during bypass surgeries.

Native vessels have rather different mechanical and surface properties from grafts composed of for example synthetic material or a segment portion from another blood vessel [16]. Especially the antithrombotic and the viscoelastic properties are of great importance for the replacement graft [2]. Currently, there is no clinically suitable bypass material for smaller blood vessels based on synthetic fabrics, due to the issue of reactivity between material surface and blood components [2]. The process that will lead to blockage is caused by blood components, such as proteins that will adhere even to a very inert surface [5, 17]. The synthetic grafts have the problem of inducing low-level foreign body reaction, chronic inflammation and, as artificial materials, possibly microbial infection [1, 19]. The native vessels have living endothelial cells lined along the luminal vessel surface, which makes the blood vessel non-reactive. The endothelium is the source of chemical agents, agents that prevent blood coagulation, control vascular tone, prevent or promote epithelial and smooth muscle cell proliferation, induce inflammation and degrade extracellular matrix [16].

In order to mimic native blood vessels, efforts in tissue engineering have been conducted. Early *in vitro* tissue engineering attempts involved seeding the lumen of synthetic grafts with endothelial cells. The pioneer of this technique was the team of Herring (1987) [17]. The aim of the seeding was to create a more biocompatible surface with less risk of occlusion. However, problems occurred regarding the culturing of a confluent layer of endothelial cells, and the issue on how to receive cells from a source for widespread clinical application remains problematic [17]. A different attempt was the hybrid construction of blood vessel grafts using synthetic and biological materials in a co-joint method. In 1986, Weinberg and Bell were able to develop a blood vessel *in vitro* that contained the three layers corresponding to natural blood vessels [17]. In the technique a PET mesh was used externally to provide further mechanical support. In 1995, Matsuda and Miwa made use of a similar hybrid technique. An artificial scaffold composed of polyurethane was created. The scaffold was later seeded with smooth muscle and endothelial cells. The interesting part with the Matsuda and Miwa construction was that when implanted in canines, remodeling occurred *in vivo* [17]. The smooth muscle cells and the endothelial cells became oriented in certain directions, and the implants were successful for up to one year. Several other scientists have followed in the same direction using native cells in combination with different synthetic polymers as support. The *in vitro* tissue engineering holds promising results. However, the use of the patient's own cells require months of preparations in advance, due to cell harvesting and tissue culturing, and would not be suitable for acute emergencies.

Another promising technique is *in vivo* tissue engineering. The idea is that the remodeling takes place inside the body after implantation, in order to form a "neoartery" [17]. The use of a bioresorbable material that will be replaced by new tissue is the general idea. The main issue will be to find a good enough balance between the graft resorption/degradation and the tissue ingrowth. It is of utter importance that the structural stability does not collapse or falter. There are endless of options for maintaining structural stability; it could e.g. be the

utilization of nonresorbable materials that act as mechanical support or the combination of materials with different resorption rates. A collagen-based biomaterial has been investigated as a potential vascular graft. The biomaterial was obtained from arteries that were cleansed and enzymatically processed into a matrix by the group of Wilson *et al.* in 1990 [17]. The generated matrix, composed of collagen, elastin and glycosaminoglycans was later inserted as a graft in a bypass operation. Instead of using collagen from arteries, the collagen can be derived from the intestines, something that was investigated by scientists at Organogenesis [17]. In a similar way the collagen is prepared and cleansed not only with chemical means but by the use of mechanical processes as well. The special process for producing collagen tubes retains the desired native collagen structure while it also secures the mechanical properties that are needed. It also provides the possibility of remodeling through cellular infiltration (ingrowth), while also maintaining hemostasis. The aim is to provide a collagen scaffold that will be remodeled *in vivo* into a new "*neoartery*".

FERMENTATION

The word fermentation comes from the Latin word "*fervere*", which is a word that describes the action of yeast or malt when encountering sugar or fruit extracts and grain [20]. Fermentation is in strict terms a process in which organic molecules act both as electron donors and electron acceptors. 6000 B.C., the Sumerians and the Babylonians already had the knowledge of producing alcohol from yeast [20]. Another known historical fermentation is the glycerol production from alcohol fermentation during World War I for the manufacturing of explosives that introduced an industry fermentation process for the production of 35 tons of glycerol a day by the Germans [20]. When growing cells on a large scale it is called industrial fermentation. The growth is often performed in a bioreactor that controls aeration, pH, temperature etc. There exist several fundamental fermentation processes, for example ethanol- and vinegar production. The production of yoghurt and cheese is achieved through lactic acid bacteria on milk, other bacteria, moulds and enzymes are also used for cheese production. Nowadays, there are many bioprocesses or fermentation processes used in the industry. Among them are high quality products such as antibiotics, organic acids (of different kinds), alcohols, chemicals, lipids and proteins [20, 21]. Microorganisms that make use of an organic source and form a product during the cells exponential growth phase are producing primary metabolites. An example of a primary metabolite is ethanol. Secondary metabolites are products formed during the stationary phase, an example of a valuable secondary metabolite is penicillin.

Fermentation processing or bioprocessing requires knowledge that integrates biochemistry, microbiology and engineering science [20]. Utilizing microorganisms and cultured tissue cells in an industrial process in order to achieve specific products is known as bioprocessing. Therefore, the focus of bioprocessing is the ability to cultivate quantitative amounts of organisms and fermentation products. The means of doing is to make benefit from vessels known as fermentors or the more used term bioreactors. The original term fermentor was used for describing fermentation in anaerobic conditions though most fermentors are used for aerobic cultivation. Thus, the term bioreactor was introduced that involved both aerobic and anaerobic processes.

As discussed earlier the major products from fermentation are within the area of chemicals, pharmaceuticals, energy, food and agriculture. Most of these products can be categorized into three types, biomass, cell products and modified compounds (biotransformation). The production of biomass or masses of cells is more or less the production of cells such as microbes, yeast and fungi. An example of a commercial production of biomass is the baker's

yeast production. Cell products are products referring to enzymes or metabolites. These products can be either intracellular or extracellular. There is a big industry around the production of enzymes, which can be extracted from animals or plants. The microbial enzymes on the other hand, can be produced quite easily in a standard procedure. Enzymes are utilized vastly by the industry in for example baking (amylase), coffee (pectinase and hemicellulase), dairy products (lactase, protease and catalase) and fruit juices (glucose oxidase) [20]. Biotransformation is the function of cells that convert an added compound into another compound. This can happen through different forms of enzymatic reactions such as dehydration, hydroxylation and oxidation. These reactions aid the transformation for production of for example steroids, antibiotics and prostaglandins [20]. In order to produce huge quantities in industry, a large amount of enzymes is required. Since the half-life of the enzymes is a critical factor, the use of whole cells is preferred in which the enzymes are simply immobilized. The use of whole cells further helps in the process of extraction of products, by separation or purification. The production of bacterial cellulose is included within the category of cell products, in which the bacterial cellulose is produced extracellularly. The traditional and classical view of fermentation is anaerobic, but the art of fermentation can also be described as the chemical transformation of organic compounds with the help of enzymes, thus the BC synthesis can be viewed as a fermentation process.

BACTERIAL CELLULOSE

Cellulose, which is one of the most abundant biopolymers on Earth, is utilized in many commercial applications, such as paper, clothes and food additives [6, 22]. Cellulose is a polysaccharide that is produced by plants and has the chemical formula $(C_6H_{10}O_5)_n$. The polysaccharide consists of several D-glucose units that are joined by glycosidic bonds in β-(1,4) linkages.

Cellulose acts as the structural component of plant cell walls [23]. About 33 percent of all plant matter is cellulose [24]. In wood, the cellulose content is about 50 percent since it is combined with other materials such as hemicelluloses and lignin. The cellulose content in cotton is as high as 90 percent [24]. The cellulose that is used industrially is obtained mainly from these two sources [23, 25]. Cellulose can also be produced by different types of microorganisms such as algae, fungi or bacteria [23, 26].

Cellulose is hydrophilic and insoluble in water. It is a chiral molecule and biodegradable [23]. The hydroxyl groups in the cellulose molecule make hydrogen bonds. The hydrogen bonds hold the chains together in a rigid structure which gives the high tensile strength of cellulose. Depending on the chain length, the number of glucose units that makes up one polymer molecule, the properties of cellulose will often change. Wood typically has a chain length of around 300 to 1'700 units while cotton and other plants fibers and bacterial cellulose as well have chain lengths ranging from 800 to 10'000 units [23]. Properties such as being stable within a wide range of temperatures and different pH-levels have made cellulose an attractive polymer [5]. Other attractive features of cellulose are its mechanical properties, ability to expand in water and biocompatibility [27].

Cellulose is synthesized and crystallized by a multimeric enzyme complex called the rosette terminal complex that contains the necessary enzymes needed to synthesize the cellulose [23, 28-30, 31]. On the other hand the breakdown of cellulose or cellulysis occurs through a hydrolysis reaction initiated by enzymes called cellulases [32]. Mammals do not have the ability to break down cellulose, but cows, sheep and certain other animals have bacteria in their intestines that produce cellulases that degrade cellulose [7, 33].

The bacterium *Acetobacter xylinum*, also known as *Gluconacetobacter xylinus*, produces cellulose extracellularly, a biomaterial that is called bacterial cellulose (BC). *A. xylinum* is a well-known model organism used for studies on biosynthesis and structural properties of BC [23, 31, 34, 35]. *A. xylinum* is a gram-negative bacterium that can produce high amount of BC, the synthesis of BC occurs within a few days [23, 27, 35, 36]. The bacterium may be cultured *in vitro* which gives unique possibilities to affect the production (and thus the properties) of BC. This can be done by changing cultivation conditions, medium and bacterial strain [23, 34]. *A. xylinum* is an obligate aerobic bacterium and is normally found on fruits, fruit products, vegetables, and in fruit juices, alcoholic beverages and vinegar [23, 35]. The bacterium produces a biofilm made of BC and embeds itself within it [23]. The biofilm has several functions; it serves as a protection against enemies, ultraviolet radiation, dryness and heavy metal ions and at the same time enables access of nutrients through diffusion and maintains an aerobic environment [23, 35]. The BC synthesized by *A. xylinum* is molecularly identical to the cellulose synthesized by plants. On the other hand, compared to the plant-derived cellulose which is normally contaminated with hemicelluloses, lignin, pectin and other substances, the microbial cellulose is pure with high water content [23]. The synthesized BC is extruded into the surrounding medium in the form of microfibrils and later bundled into ribbons [9, 23, 34, 35]. These ribbons and microfibrils form an ultrafine three-dimensional network that constitutes the biofilm utilized by the bacteria. The biofilm holds a high amount of water, about 99%, which makes it a hydrogel [11]. The microfibrils of BC differ from plant cellulose fibrils, they are about 100 times smaller in diameter [9]. This is believed to have an effect on the BC properties, resulting in a larger surface area that can hold a high amount of water, high cellulose crystallinity (60-80%) and an enormous mechanical strength [9, 23, 34]. Other properties that BC possesses in wet state are high modulus of elasticity, wet-strength and conformability [9, 23, 35]. The unique conformability gives the hydrogel an ability to be molded into any shape and size desired [9]. Due to its different qualities, including its biocompatibility, BC has been identified as a potential implant material [9, 23].

BIOREACTORS

In general, a bioreactor is used as a device where biological and/or biochemical processes develop (illustrated in figure 2) [37]. It is the heart of any biochemical process that utilizes enzymes, microbial, plant or mammalian cell systems for the production of any useful biological outcome [20, 37]. The bioreactor provides close monitoring and tightly controlled environmental and operating conditions (for example temperature, pressure, pH, nutrient supply and waste removal). The essential principles of biology and engineering are integrated in order to design a bioreactor [38]. The main function of a designed bioreactor is to provide a controlled environment in order to achieve optimal growth and/or product formation in the particular system used [37]. This also includes minimizing the costs of the process while retaining the quality wanted with the constraints from biology and technology [38]. The performance of the bioreactor can be varied dependent on many different factors, such as [20]:

- Concentration of biomass, nutrients
- Sterile conditions
- Effective agitation
- Heat maintenance
- Correct shear conditions

- Nutrient supply
- Product removal
- Product inhibition
- Aeration
- Metabolism/microbial activity

The high level of control, reproducibility and automation makes the bioreactor an ideal system for large-scale applications, manufacturing products in which specific criteria need to be followed, such as efficacy, safety, reproducibility and quality. When automated and standardized, the bioreactors could also reduce the production costs and thus be cost-effective. The most classical use of bioreactors is within industrial fermentation processing, food processing, wastewater treatment and production of pharmaceuticals and recombinant proteins (e.g. antibodies, growth factors, vaccines and antibiotics). In several of these applications the aim is to get a high yield of products thus the bioreactor is supposed to provide an environment that enhances production as much as possible.

In tissue engineering the main reason for using bioreactors is essentially the same as for microbial cultivation namely that of controlling the process parameters and the possibility of standardization for future scale-up. Additionally, the bioreactor has a role of providing a well organized environment that supports cell function and tissue development. In nature the architects for developing tissues and organs are the cells. Thus, the bioreactor is responsible for mimicking an environment similar to the native environment in order to facilitate the specific function or product that is wanted [39].
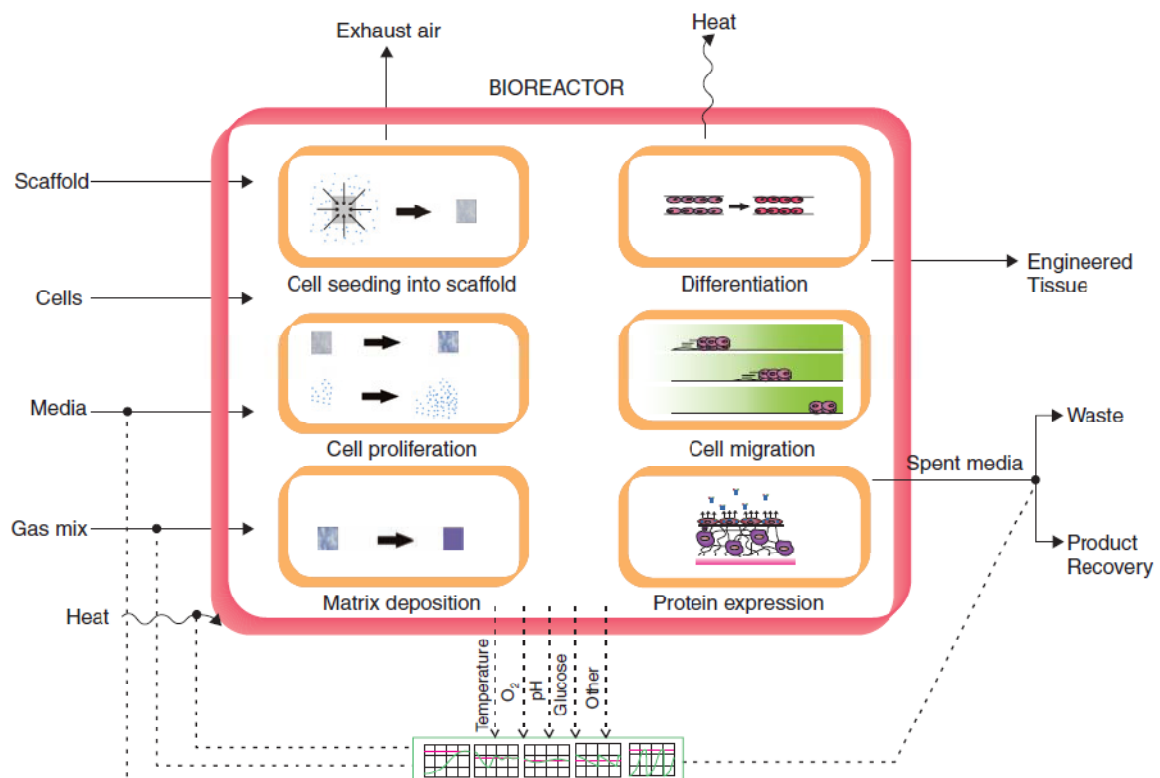

Figure 2. An illustration of what could occur within a bioreactor. All inputs are shown to the left, the outcome could be an engineered tissue. Waste and metabolic products, heat and exhaust air are typical outflows, and at the bottom different examples of controlling factors are described. Within the bioreactor (box) various processes are exemplified, which result in the outcome of products. [40]

BIOREACTOR MODES

There are typically three principal modes of bioreactor operation; batch, fed-batch and continuous. There are many types of bioreactors, they can vary in size, complexity and functional capabilities and the choice of bioreactor depends mostly on resources used by it and what purpose it has [39].

BATCH BIOREACTOR

The majority of all bioreactors utilized are batch based, and the most common batch reactor is the stirred tank reactor. The most characteristic feature of the batch reactor is that the process operates in a closed system, in other words there are no in- and/or outgoing flows. In summary it is a reactor or container available in various shapes and sizes, that is sterilized at first and then microorganisms along with its culture medium is inoculated to attain some specific product/result that is removed at the end. The reaction that occurs over time will be dynamic, and concentrations of different substances such as biomass, substrates and products will change. Mixing in the bioreactor will provide optimal blending and help to keep temperature and composition evenly in the whole reactor. Gas supply helps when dealing with an aerobic cultivation in a batch reactor and an exhaust will take care of the CO_2 that is created by removing it. When the reaction or the desired event has occurred it is stopped and the product is removed and a new batch is started.

FED-BATCH BIOREACTOR

Compared to the batch reactor the fed-batch has the distinguishing feature that there is only an ingoing flow and no outgoing flow. The fed-batch operation works through continuous or periodic feeding of nutrients to the current content in the reactor. This provides control over the amount of nutrients available, it gives control over the concentrations of nutrients. By monitoring the dissolved oxygen levels or exhaust gas composition, the rate and timing of the feeds can be determined [21]. Fed-batch culture is often used in bakers' yeast production in order to avoid catabolite repression and to control oxygen demand, it is also used commonly in penicillin production.

CONTINUOUS BIOREACTOR

The continuous reactor has an inflow that equals the outflow resulting in a constant volume. Just like the fed-batch, there is a feeding process that is continuous all the time. Simultaneously there is a continuous flow that draws out the product from the reactor. If the reactor is well mixed the effect is that the product stream has the same composition as the liquid inside the reactor. This mode of operation is used industrially in bioprocesses such as brewing and waste water treatment. The culture medium is sterile or that contains microorganisms is continuously fed into the reactor and product is fed out. This will cause the reaction that occurs inside to be maintained at steady-state due to concentrations in the reactor adjusting according to the feed rate [21]. All the parameters and variables remain steady which results in a reactor state that provides a continuous productivity and output. This mode works well in lab-scale but is rarely used industrially.

BIOREACTOR TYPES

There are several different types of bioreactors, three of these are the stirred tank, bubble column and airlift. All of them are aerobic bioreactors which are more common in industry, and most microbial processes in general require aeration and/or agitation of some kind [20]. The anaerobic processes are usually more used for traditional products of certain winemaking, beer brewing or cheese articles but also in wastewater treatment and in ethanol production [41].

STIRRED TANK REACTOR

Today, the most commonly used aerobic bioreactor is the stirred tank reactor. The stirred tank reactor can be modified internally into different configurations in order to design specific circulation patterns [37, 41]. One of its important features is the low capital and operating costs that is a result from incorporation of automation in the process [20, 38, 41]. The reactor vessel can be made in different sizes. For laboratory use it is typically made of glass and in industrial services it is constructed out of stainless steel. The design of the stirred tank is relatively simple which is shown in figure 3. The dimensions of the reactor are often a height that is equal to or greater than double its width [21, 38]. The stirrer in the tank is located at the bottom, sometimes additional one/or more stirrers can be equipped in the upper compartment(s) [37, 38]. The agitation system will not only be composed of stirrers but baffles (that can range from four to eight in number) will help for further or optimized mixing [20]. The baffles will also prevent a whirlpool to be formed in the vessel [20, 21, 38, 41]. Inside the baffles warm or cold water can be circulated, in order to either chill down or heat up the system [20, 41]. With this control overheating will never be a problem and an optimal temperature will be kept. Air supply is normally entered from the bottom beneath the stirrer. An air sparger is responsible for the aeration to occur. Bubbles that are created from the air supply will be broken up by the agitator as they travel upwards [41]. The agitator should be placed at a certain position in order to interact with the bottom to decrease power consumption [20, 38] and at a higher position there is a possibility of getting no bubbles from the gas inflow to recirculate down in the lower compartment [38]. With high agitation and aeration, there is a risk of getting problems with foaming which can be prevented by adding antifoaming agents though the agents may have inhibitory effects on the microorganism growth. If that is the case then rakes can be put on the stirrer shaft and on the surface of the liquid to prevent the foam formation. There is also a more intricate device, an antifoaming agent, which makes use of centrifugal forces to destroy the foam; the nutrients in the foam will be driven back in the liquid, while the air will be released from the vessel [20]. At the top of the bioreactor there is an exhaust for gases.
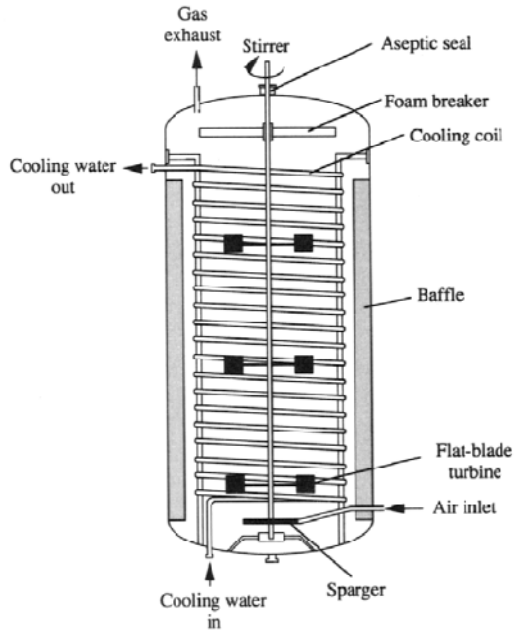
Figure 3. An example of an aerobically stirred tank reactor, containing several of the devices mentioned in the text. The stirrer is in this case is a flat-blade turbine combined with a cooling system. Other normal devices, such as foam breaker, gas exhaust, sparger and baffles are also depicted. [21]

BUBBLE COLUMN REACTOR

The bubble column reactor is a simple reactor which is an alternative to the stirred tank reactor with no mechanical agitation, represented by the schematics given in figure 4. More or less it is a tank with a sparger mounted in the bottom. The mixing and the aeration are accomplished by gas sparging. The sparger nozzles that bubbles gas will create certain flow patterns. In order to get a good mixing the nozzles will have to be distributed all over the bottom. Often the sparger in the bottom will have a ring form or a number of parallel pipes or a star-like construction of pipes [38]. These pipes are drilled with holes in it that serves as nozzles for the air supply. Industrially the bubble column reactor is used for applications such as production of bakers' yeast, beer, vinegar and wastewater treatment [21].
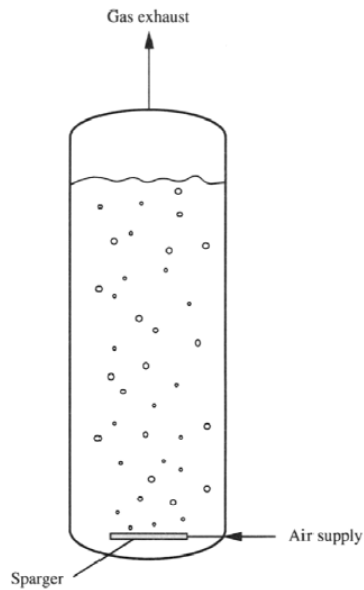
Figure 4. Illustration of a bubble column reactor; the air supply is placed at the bottom. The bubbles are created through a sparger and excessive gas will be exhausted at the top. [21]

AIRLIFT REACTOR

The airlift reactor, also known as a tower reactor, can be seen as a type of bubble column reactor. The mixing in the airlift reactor is accomplished without any mechanical agitation, only by aeration. It is mostly used for tissue cultures, since the cells are shear sensitive which render normal mixing impossible [20, 21, 37]. There exist many types of airlift bioreactors, but in general there are two main types of airlift systems, the internal loop reactor (containing a tube) and the external loop reactor shown in figure 5 a, b [21, 37, 38, 41]. The internal loop reactor can be seen as a reactor containing a draught tube which controls the circulation of the medium and gas. The external loop reactor has two "pipes" that are interconnected at the top and bottom.

The construction of the airlift and its purpose is that two different flows will be created. The first one entitled as "the riser" and the other one as "the downcomer". The sparger at the bottom supplies gas bubbles to the reactor, the riser flow, along the enclosed tube/line. The gas will rise through the reactor and eventually reach the top where it disengages. At the downcomer the degassed liquid will flow downwards and in most circumstances that stream does not contain any gas. Because of the density difference created between the riser stream and the downcomer stream an intensive liquid circulation will be formed [20, 21, 38].
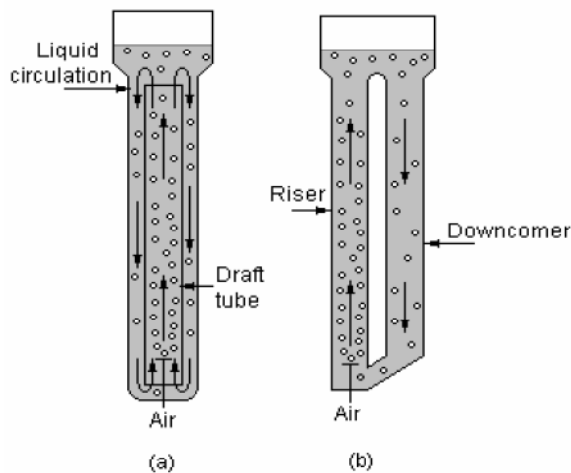
Figure 5. In this figure, the two configurations of airlifts are shown. (a) the internal loop reactor containing the draft tube, in which air rise through, the riser, and the downcomer which is located at the sides where degassed liquid is flowing. (b) shows the external loop reactor with two pipes that are differentiated by the riser and the downcomer. [37]

MIXING

The purpose of mixing is often defined as the process that decreases the inhomogeneity of a system, in this case the contents of a vessel [38]. It can also be described as the physical operation that eliminates any concentration, pH and temperature gradients thus creating uniformity in the fluids [20, 37]. An ideally mixed system occurs when the chance of finding a given component at a certain position is the same as for any place in the vessel. Thus, there is a homogeneous distribution of the system properties. Mixing is utterly important for bioprocessing, perhaps the most important characteristic, by supplying an efficient liquid mixing there will not only be a uniform liquid concentration but the dissolved oxygen concentration will be uniform as well. For cell growth it is important to provide an optimal environment in the bioreactor, with sufficient mixing the cell culture will have access to all substrates including oxygen in the bioreactor. Though emphasis is put on the concentration gradient, the pH and temperature gradient are of great importance as well. The bioreactors have to be able to operate at a constant temperature and pH. Thus keeping the heat transfer at bay and avoid pH changes, since mammalian cells in particular are very sensitive to pH changes. Conclusively, the mixing aim to circulate the fluid during a given time, to dissolve the gas (bubbles) in the liquid and to maintain consistent conditions for mass and heat transfer operations. Mixing becomes interestingly difficult as the scale of the bioreactor increases.

The mechanism by which mixing is applied to a bioreactor must be carefully adapted to the needs of the microbes and the type of bioreactor. Generally, there are three different mixing mechanisms; shear, exchange and diffusion. Mixing through shear occurs due to fluid layers that shear along each other. Exchange is the random process of particles that are continuously moved in respect of each others. Diffusion is the mixing process that is formed through molecular diffusion, mixing on a molecular scale.

Normally increased rate of the stirring will be better for the mixing but there are the bad effects of how the fluids and the cells will react to it. Foam creation is a factor that has to be considered but a very high agitation will contribute to high shear forces as well. These

forces might damage the cell wall and cause the cell to rupture [20]. Animal cells and plant tissue cultures are often shear sensitive and thus special configurations of the impellers are required [20, 37].

DEAERATION

Deaeration describes a process that removes gases (e.g. air bubbles, oxygen) from an aqueous liquid [42, 43]. There are several techniques utilized for deaeration, one of them is the use of an inert gas [42, 43]. The inert gas (preferably nitrogen gas or a noble gas such as helium) is provided to the aqueous liquid. It can be supplied through a sparger or by gas bubbling [42]. The insertion of gas will reduce the amount of unwanted gas dissolved in the liquid. The process follows Henry's law which described that the dissolved gas in a liquid is directly proportional to the partial pressure of that gas in that liquid, when in the state of equilibrium [44]. Preparations of beverages often use this kind of method for removal of oxygen, which results in longer shelf-life and less undesirable oxidations [45].

SILICONE MEMBRANE TUBE

A membrane can be used as a barrier that separates two compartments to various degrees; the process is achieved through differences in concentration, pressure or electrical potential [46]. The barrier is selective, in which one type of substance can pass more easily than others. Mostly the driving forces used are concentration and pressure gradients, but the type of membrane used could also have an effect on the membrane process [47]. A membrane can be made of porous glass, sintered metal or synthetic polymer (porous or non-porous) [47].

The use of dense-phase membranes to keep two distinct phases (in this case a gaseous and a liquid phase) separate has been utilized in bioreactor applications [48]. Two familiar types of applications in reactors are bubble-free aerated bioreactors and extractive membrane bioreactors (EMBs) [48]. The EMBs are used in wastewater treatment, separating a wastewater compartment from a biological compartment. Separation is achieved through diffusional transportation of organic substances, thus EMB can be used for direct detoxification of hostile wastewaters [48]. The bubble-free bioreactor is used to diffuse oxygen through a membrane into another compartment holding an aqueous biological phase. Dense gas-permeable material is used in order to make the oxygen diffusion bubble-free and maintained at high gas pressures [49, 50]. Silicone rubber is a commonly used membrane material. The silicone membranes have high oxygen permeability, and are resistant to both chemical and mechanical abrasion [51, 52]. The tubular silicone rubber membranes can be operated at high membrane pressures without any bubble formation [53].

Transportation of oxygen through the silicone membrane occurs first by dissolution in the membrane and then by diffusion of gas through the membrane, by the driving (pressure) force. For one-dimensional flux of dissolved O_2 in the silicone membrane Fick's equation can be used and may be simplified into [44]:

$$N_{Ar} = -D_{AB} \frac{dc_A}{dr} + \frac{c_A}{c}(N_{Ar}) \cong -D_{AB} \frac{dc_A}{dr}$$

N_{Ar} is the molar flux of compound A along the direction of r, the D_{AB} is the mass diffusivity or diffusion coefficient of compound A through component B, dc_A/dr is the concentration gradient along the direction of r across the membrane.

Henry's law relates the mole fraction of compound in the liquid to the partial pressure of the compound in the gas. A similar equation can be used for the interface of gas and solid [44]:

$$c_{A,solid} = S \cdot p_A$$

The $c_{A,solid}$ is the molar concentration of a compound A within the solid at the interface (kg mole/m3) and p_A is the partial pressure of gas phase species A over the solid in units of Pa. The partition coefficient S, also known as the solubility constant, has units of kg mole/m^3·Pa.

The permeability constant is a general formula for a gas, which is defined as the product of the diffusivity coefficient and the solubility coefficient for a certain gas through a certain material [51].

$$P = S \cdot D_{AB}$$

Thus by combining the equations with Fick's equation the one-dimensional gas flux through the membrane can be expressed as:

$$N_{Ar} = -P \frac{dp_A}{d_r} = -P \frac{p_{A2} - p_{A1}}{r_2 - r_1} = \begin{Bmatrix} r_1 = 0 \\ p_{A1} \geq p_{A2} \end{Bmatrix} = P \frac{p_{A1} - p_{A2}}{r_2}$$

Therefore the transportation of oxygen is mostly dependent on the pressure gradient, thus the transportation through the membrane can be solely relied on the pressure applied across the membrane thickness (p/r) and the gas permeability of the membrane (P or the product of D_{AB} and S).

By changing the pressure profile (an analog of the oxygen profile) throughout the length of the tubular membrane, the oxygen transportation may be controlled. Thus, the mass transfer will be proportionally changed with the applied pressure gradient.

The mass transfer of dissolved oxygen has to overcome several resistances in series, not only the membrane resistance. The overall mass transfer of dissolved oxygen in a membrane reactor setup is composed of a gas resistance, a membrane resistance and a liquid resistance [49, 53-57]. The gas resistance resides in the oxygen-silicone interphase, the membrane resistance in the silicone bulk and the liquid resistance in the silicone-medium interphase. In addition, biofilm that is created by bacteria will have an impact on the overall resistance [51]. During fermentation the impact of the biofilm resistance will increase with time.

ANALYZING METHODS

In order to assess and validate that the vascular grafts are comparable to native blood vessels, one could use mechanical analysis. Mechanical strength is of great importance, since the graft needs to withstand high blood pressures. Some *ex vivo* validation/verification tests are tensile, suture, peel, burst strength and compliance tests. To investigate the surface and network structure, Scanning Electron Microscopy (SEM) can be used. In this study, tensile testing and SEM are the analyses used.

TENSILE TEST

In a tensile test, cylindrical parts of the graft are cut out. They are preferably cut into small ring segments [58]. The analysis of the ring segments will give information on the mechanical and structural properties of the vessel walls. It will measure the strength of the material and how much it will elongate during a certain load. The data will generate to a stress/strain curve that has to match, or be comparable to, the native blood vessel curve. From the stress/strain curve, several things can be outlined; the strain, stress, Young's modulus and the yield point can be calculated. The strain indicates how much the material can be elongated before break, while the stress is the force needed for rapture. The Young's modulus is a measurement of the stiffness of the material. The yield point shows at which stress the material is deformed permanently, the strain where the material changes from elastic deformation to plastic deformation. The information gained will not only give mechanical data, but also on the network structure; the more dense material, the higher mechanical strength. The tensile test is performed radially; the ring segment is fixated around two pins that are attached to the tensile testing machine, see figure 6. During the start of the testing, the two pins will separate at a uniform speed and subsequently generate a stress/strain curve.
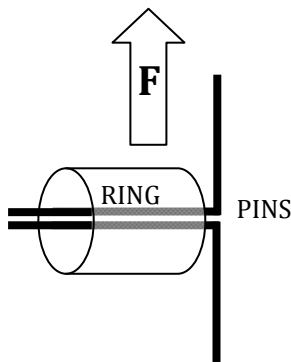

Figure 6. An illustration of how the radial tensile test is performed. Two pins fixate the ring that is to be tested and the pins will start to separate at a uniform speed. The force is read by the tensile testing machine and will generate a stress/strain curve.

The investigation of the surface and network structure is achieved through SEM analysis. A three-dimensional structure of the specimen surface is generated, which will give a visual appearance of the material. This will give information on how the material is structured. By detection of electrons that are scattered or emitted from the surface of the specimen, SEM can produce images. Before this occurs, the specimen has to be fixed and coated with a thin layer of heavy metal. The sample is thereafter scanned with a narrow beam of electrons, and the amount of electrons scattered or emitted from the metallic surface is measured. The highlights and shadows in the image, gives the three-dimensional appearance, which is an effect of the electron scattering that varies with the angle of the incoming beam. The technique is commonly used to study whole tissues and not smaller cellular components, due to the low resolution.

MATERIALS AND METHODS

Fermentation of BC tubes was performed according to standardized procedures and variables, with minor modifications depending on the experiments conducted. Minor alterations from the standardized protocols were also made, due to the laboratory conditions. The results from the two modifications were then evaluated with analyzing tensile testing (Instron 5565A) and SEM (Leo Ultra 55 FEG SEM)), which are compared with reference tests. The test of all prototypes from each improvement/trial will be collected and analyzed at the same time in order to save time and money. The tensile test will inform how much force that is needed to rupture the vascular graft. By separating the tensile test into areas along the BC graft and comparing the results, knowledge on how homogeneous the mass profile is and if the grafts are thicker at the end or not, will be obtained.

OXYGEN AND CARBON SOURCE

The growth of *A. xylinum* is dependent on oxygen and a carbon source. The oxygen supplied was industrial oxygen gas from AGA (AGA Gas Company). The medium containing the carbon source was prepared according to a recipe shown in table 1.

Culture Medium		Trace Metal Solution		Vitamin Solution	
Compounds	Amount (/L)	Compounds	Amount (/L)	Compounds	Amount (/L)
D-Fructose	40.00 g	EDTA	3.000 g	Inositol	400.00 mg
Yeast Extract	5.00 g	$CaCl_2 \cdot 2H_2O$	1.470 g	Pyrodoxine HCL	80.00 mg
$(NH_4)_2SO_4$	3.30 g	$FeSO_4 \cdot 7H_2O$	0.360 g	Niacin	80.00 mg
KH_2PO_4	1.00 g	$Na_2MoO_4 \cdot 2H_2O$	0.242 g	Thiamine HCL	80.00 mg
$MgSO_4 \cdot 7H_2O$	0.25 g	$ZnSO_4 \cdot 7H_2O$	0.173 g	p-Aminobenzoic Acid	40.00 mg
Corn Steep Liquor	20.00 ml	$MnSO_4 \cdot 5H_2O$	0.139 g	D-Pantothenic Acid	40.00 mg
Trace Metal Solution	10.00 ml	$CuSO_4 \cdot 5H_2O$	0.005 g	Riboflavin	40.00 mg
Vitamin Solution	5.00 ml			D-Biotin	0.40 mg
				Folic Acid	0.04 mg

Table 1. List of ingredients for the culture medium needed for the cultivation of *A. xylinum* and the fermentation of BC tubes. Every solution was mixed with MQ-water, and adjusted to a pH of 5 with the help of NaOH or HCl solution except the culture medium, which was adjusted to a pH of 5.5.

All compounds were mixed with MQ-water, and the pH was adjusted with the help of NaOH and HCl solutions. Both the vitamin and the trace metal solution were adjusted to a pH of 5, while the culture medium was adjusted to 5.5. The liquids were sterilized through filters and stored in sterilized flasks.

PRECULTIVATION OF *ACETOBACTER XYLINUM*

The bacterial strain used was *Acetobacter xylinum*, subspecies *sucrofermentas* BPR2001 (ATCC 1700178TM, American Type Culture Collection). The strain was precultivated and colonies were obtained and stored in cryo tubes containing glycerol, and frozen as aliquots at a temperature of -18°C. The aim of precultivation was to obtain a high bacterial concentration, containing cellulose-synthesizing bacteria whose growth had reached log-phase. Aliquots with *A. xylinum* were thawed and transferred into a cell culture flask containing 100 ml culture medium. The cell culture flask was sealed and then placed inside an incubator, at 30°C for 48 hours.

ASSEMBLY OF BIOREACTORS

The preparations before the fermentation of the BC tubes included the assembly of bioreactors, which provided a sterile environment that delivers a sterile microbial fermentation of cellulose. A bioreactor consisted of a test tube (~60ml) made of glass, a stopper with inlets for oxygen and inoculation, a silicone tube for oxygen to diffuse through, and a steel plug and a steel plate that stretched the silicone tube. Construction of the bioreactor involved assembly of all pieces, and finally sterilization of the bioreactor in an autoclave. The silicone tubings were cut to a certain length and the ends of the tubings were attached to plugs and weights. The other end of the silicone tubings were attached to oxygen inlets. The stopper/tubing constructs were placed inside and sealed the test tubes. The parts of the bioreactor that were most susceptible for contamination were wrapped with aluminum foil, for extra protection until use. The assembly of the bioreactors was modified to some extent, to achieve the adjustments that were required from each protocol and its purpose.

FERMENTATION AND PURIFICATION OF BC TUBES

The fermentation of the BC tubes included loading of culture medium up to around 80% of the holding volume. The preculture flask was then vigorously shaken, in order to liberate as much bacteria as possible. The liquid was transferred with a pipette, by loading of 2.5 ml of preculture through the inoculation pipe into the bioreactor. The remaining space in the bioreactor was filled with culture medium and the inoculation pipe was sealed with a 0.2 μm air filter (Acrodisc® 13mm syringe filter, Pall corporation). When each bioreactor was loaded, all bioreactors were placed in a test tube rack and transferred to an incubator. The fermentation took place at 30°C, with an oxygen flow of 100ml/min (measured when not attached to the silicon tube) for 7 days. Including the precultivation time of the bacteria, the whole fermentation process took 9 full days. The fermentation was followed by harvest and purification, since after fermentation the BC tubes were covered in culture medium and contained bacterial cells that were integrated in the polymeric network of BC. The cleaning process involved alkali treatment (NaOH), which caused the BC network to swell. The swelling allowed the embedded bacteria to be extracted into the base. Continuous change of NaOH removed the bacterial cells and dissolved culture medium, along with excess aqueous sodium ions. The BC tubes were placed into flasks filled with 0.1 M NaOH and left in room temperature over night, after the fermentation. The next day the liquid was poured out and replaced with new 0.1 M NaOH and placed in a shaker (100 rpm, 60°C) for 2 hours; the procedure were repeated for a total of 4 cycles. After the alkali treatment, the last liquid was replaced with water and, by using the same procedure placed in a shaker for 2 hours and repeated until 4 cycles had been achieved. The water treatment restored the previously

swelled BC network into its original form. The repeated exposure of water washed out the excessive water-solvated sodium ions (Na^+) and hydroxide ions (OH^-). The harvest and purification ended by placement of the purified BC tubes in plastic jars, filled with 100 ml of MQ-water, followed by autoclaving and storage in refrigerator. The harvest and purification added another 2 days, for a total of 11 days to conduct an experiment from beginning to end.

The fermentation procedures varied slightly between each protocol, mostly they differ due to the alterations and small adjustments carried out for the experiments. Except for these changes, the protocol follows the standard fermentation and purification as mentioned.

ANALYSIS PROCEDURE

After purification, parts were sliced out for both the tensile tests and the SEM analyses. For the tensile tests, rings were cut out with a length of 1 cm. In protocols 1 and 2, a total of 7 rings were cut out from each tube, which represented a tube from top to bottom. The rings from protocol 3 were produced in the same manner, but with a total of 8 rings obtained, due to slightly longer silicone tubes. The rings were then subjected to measurement in the tensile testing machine. The SEM analyses involved sliced parts of 0.5 cm in length, which were freeze-dried (Jouan LP3) and coated with gold particles (Edwards sputter coater S150B) and finally placed under the microscope for picture-taking.

RESULTS AND DISCUSSION

In this section, results and discussion from the two improvement procedures will be presented. With the aid of the previous analyzing methods, the conducted experiments will be compared to reference experiments.

PROTOCOL 1 AND 2

The experiments that were conducted to achieve a more homogeneous mass profile made use of two sets of protocols. The results from each improvement were compared to reference tests.

The first results are from radial tensile tests, which measure the stress and strain of the tubes. The compiled data shows the tensile stress at break (MPa) and is illustrated by box plots.
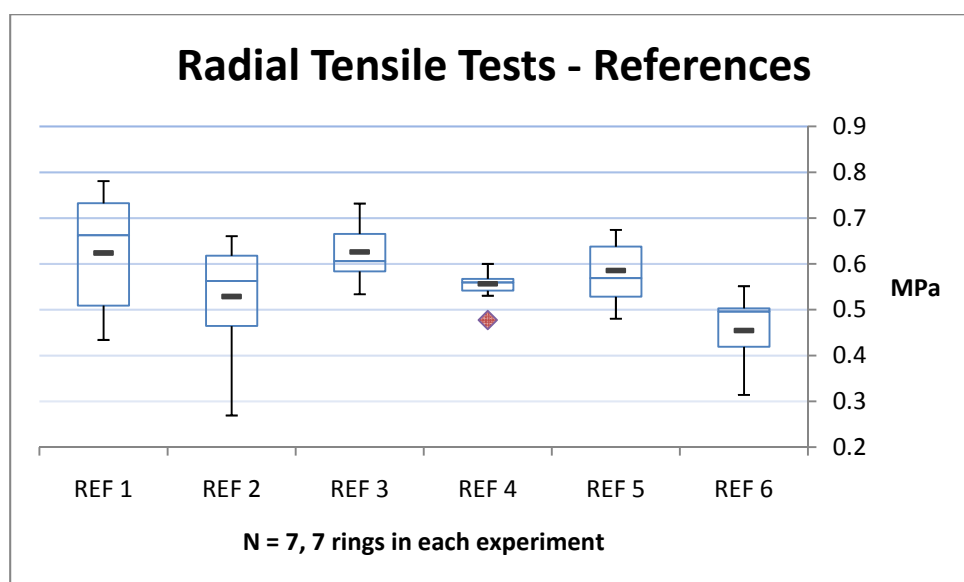
Chart 1. Box plot results from the reference tubes. The boxes display 25% (bottom of the box), 50% (the median, band in the middle) and 75% (top of the box) of the population. It also illustrates the average (thick small band) and the min and max value within 1.5 Interquartile range (IQR) and additional outliers if found. Each experiment contains seven tensile tests on rings that represent the tube from top to bottom. The reference tubes illustrate the varied results from the fermented BC tubes. Big boxes are evidence of big spread between the tensile tested rings in one experiment and the other way around for smaller boxes.

Chart 1 demonstrates the uneven characteristic production of BC tubes with the standard method. The method is not reproducible and will create dissimilar tubes. There are tubes with large box plots and tubes with smaller box plots and even a tube (REF 4) that has a tiny box plot that is desirable. The inconsistency of each tube is proof that the there is no uniform tube production with the old system.
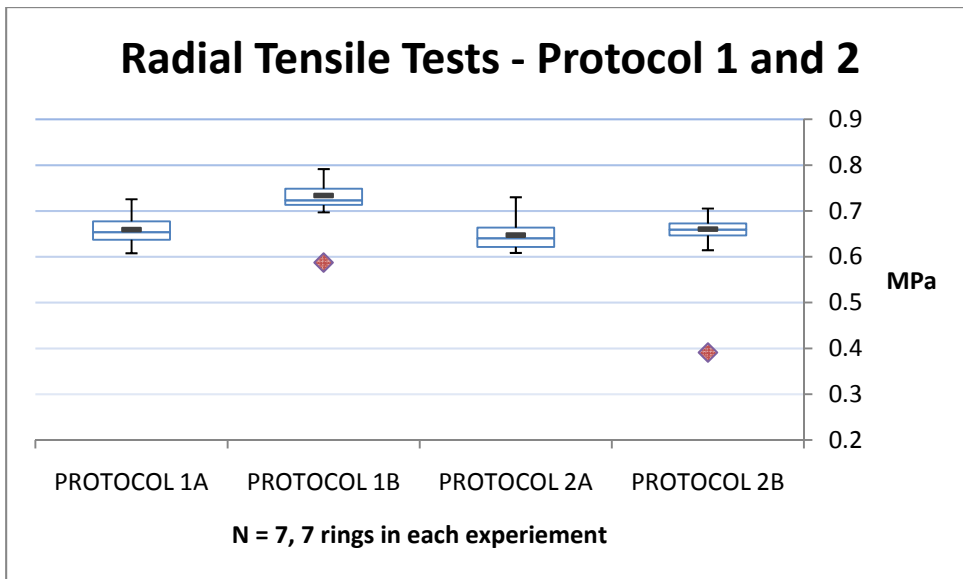
Chart 2. The chart illustrates the radial tensile tests performed on the tubes fermented with different set-ups. The tensile test results are in MPa, including all seven ring parts from one tube in one box plot. The set-ups demonstrate small boxes, which indicate a lesser spread between the ring parts of a single tube.

In chart 2, it is obvious that both improvements, which were hypothesized to contribute to a more even mass profile, also led to smaller box plots, which indicate more homogeneous tubes, compared to the reference. The chart shows that with these new methods to increase the control of the bioreactor will result in a reduced spreading. Additionally, the protocol 1 results indicate stronger mechanical properties and most likely a stronger and denser network, compared to the protocol 2 and the reference. However, the results from protocol 2 are more uniform except for one outlier.

Only two tubes of each improvement method were obtained, due to the high risk of infection during laboratory work. The experiments were conducted in an open space laboratory bench and not in a LAF (Laminar Air Flow)-bench, which contributed to the risk of infection. The several steps of preparation for fermentation increased the risk of infection in the open space laboratory bench. The long fermentation time also had an effect, as well as temperature and pH. Another factor that affected fermentation in protocol 2 was the use of equipment that were difficult to keep sterilized, sterilization occurred through ethanol wash due to that the equipment was non-autoclavable.

Charts 3-5 below illustrate the tensile tests of circumferential ring parts, from protocol 1 and 2 as well as the reference tube. Ring 1 of each chart represents the tube segment closest to the top and ring 7 represents, correspondingly, the tube segment closest to the bottom.
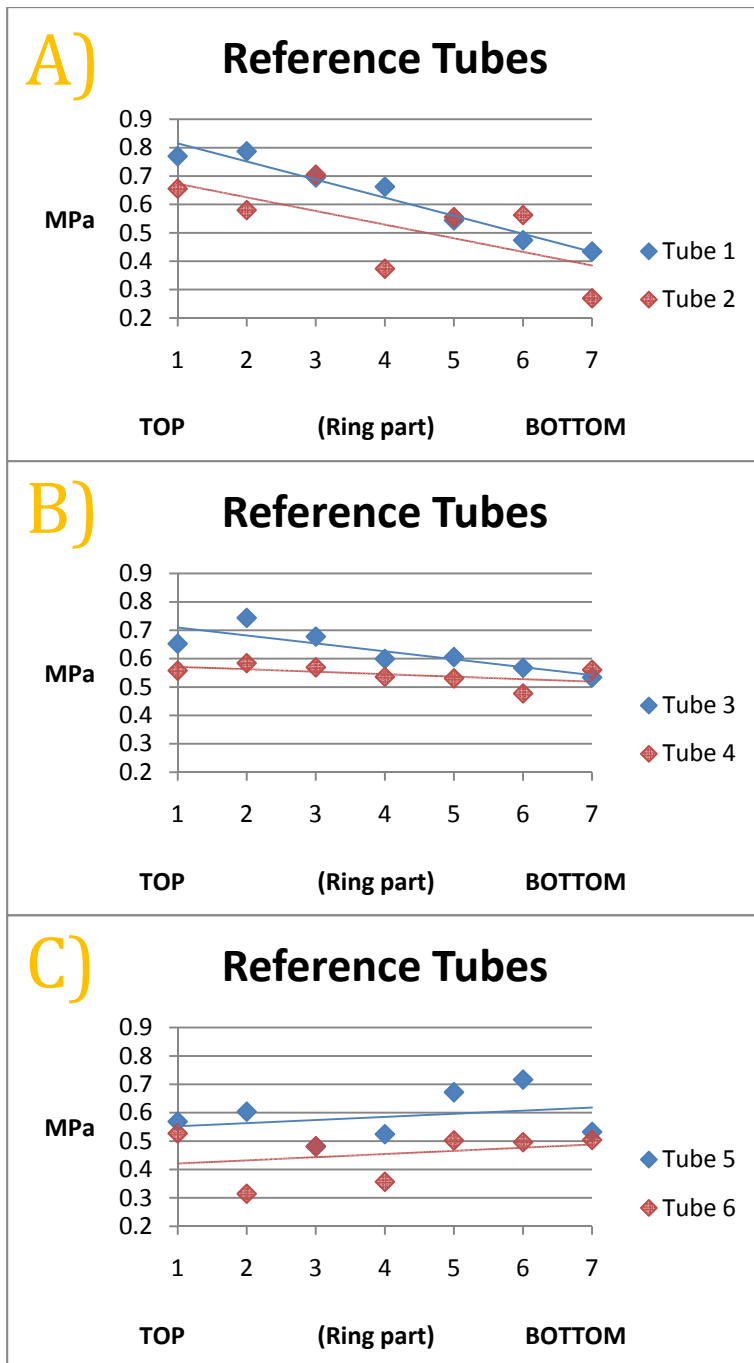
Chart 3. The charts show all individual tensile tests performed on each ring of the reference tubes. Ring 1 represents the top and ring 7 represents the bottom of the tube. The chart confirms the hypothesis that the strength between segments of a tube differs, and that it is highest at the top and decreases downwards along the tube in chart A and B (tubes 1-4). Chart C (tubes 5-6) does not follow the trend; however, the most important observations are the unevenness of all tubes and how widespread the results are.

Chart 3 demonstrates the problem of the uneven characteristics along the reference tubes; there is a slight trend where it is strongest at the top and weakens gradually to the bottom, shown in chart 3A and B by trend lines. There are a few tubes (chart 3C) in which the trend is reversed. Nonetheless, the important fact is that the BC tube production is not stable with the current set-up, which forms the basis of the first improvement hypothesis.
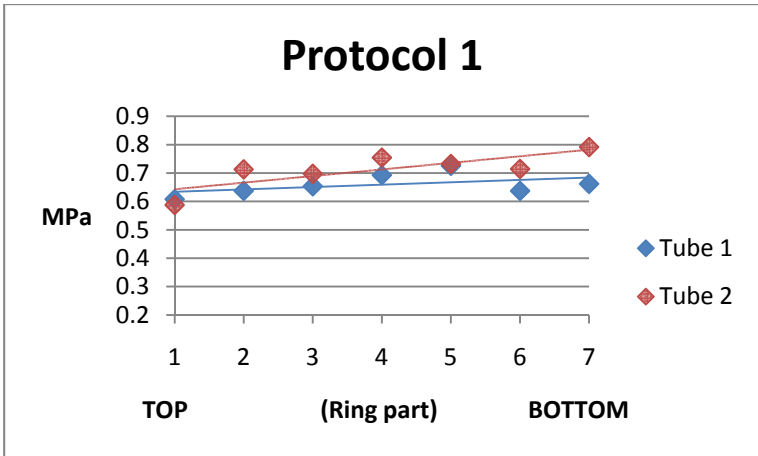
Chart 4. The chart exhibits the rings of two tubes from protocol 1; ring 1 equals a part from the top and ring 7 equals a part from the bottom of a tube. The results do not show the trend illustrated in chart 2. Instead, the trend lines are more horizontal.
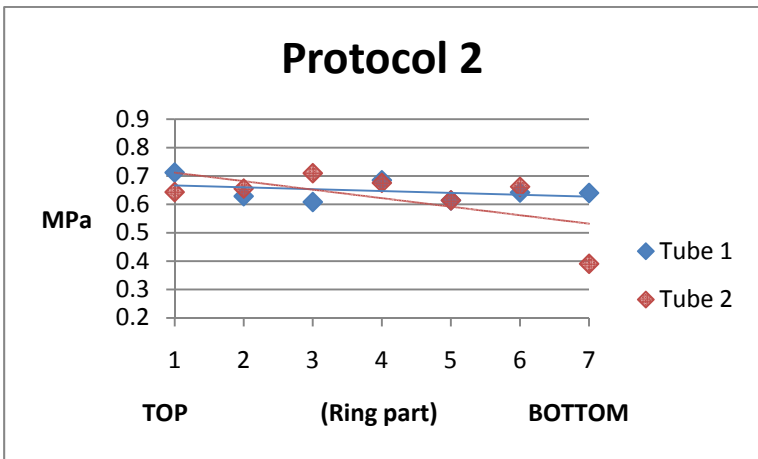

Chart 5. The chart shows all individual tensile tests performed on each ring, from the two tubes collected from protocol 2. Ring 1 represents the top and ring 7 the bottom of each tube. The chart indicates horizontal trends for all ring parts, except for an outlier on tube 2, ring 7.

Both the protocol 1 and protocol 2 experiments (charts 4 and 5) illustrate that the ring parts of a tube are similar in strength. This is given by the more or less horizontal data trend lines. There are exceptions of rings that are uncharacteristic but are classified as outliers in the box plot shown in chart 2, and if excluded the trend line would be even more horizontal. When comparing the reference tube with the other experiments, the charts from the protocol methods show no trend of decreasing strength or any significant unevenness, as the reference tube does. However, there are only a few produced tubes in which these methods have been performed. It indicates that these changes may very well be important for reproducibility and the possibility of creating a uniform BC tube production.

SEM pictures on cut-out tube segments were also obtained, with 100X magnification. This resulted in three pictures, which illustrate the top, middle and bottom segments of the tube.
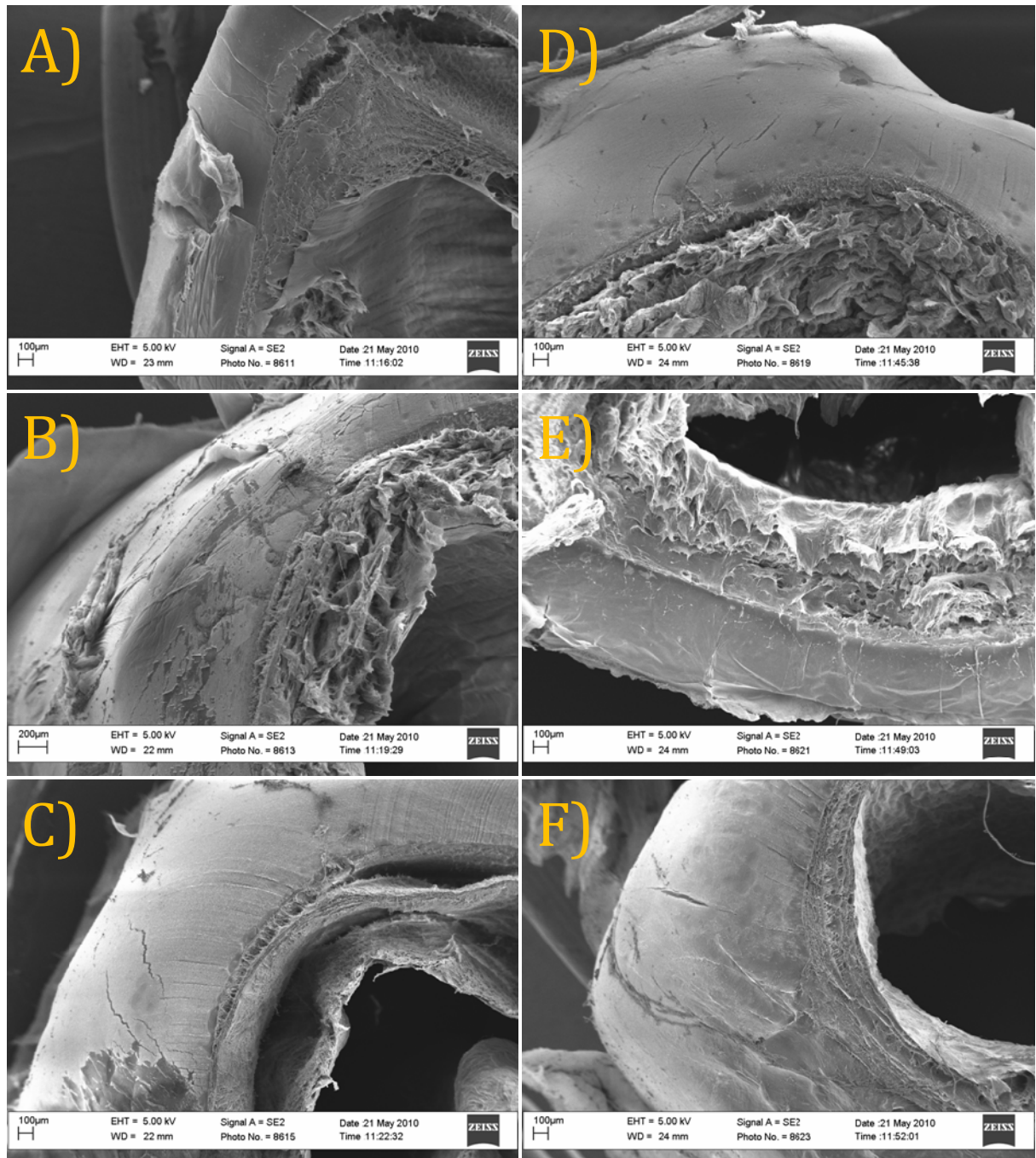
26

Figure 7. The six SEM pictures illustrated above (magnification = 100X) show three segments of two reference tube samples. Picture A is the top segment, B represents the middle segment and C is the bottom segment of reference tube 2 in chart 3A. It can be seen that the thicknesses of the BC rings differ a bit from each other, which was also suspected and now confirmed with pictures. Conversely, the top segment D, middle segment E and bottom segment F of reference tube 6, also seen in chart 3C, illustrate a more even wall thickness, which correlates to the more even results in chart 3C.

The pictures from the references differ in appearance. One difference, in which the tendency is that from top to bottom the thickness will decrease, illustrated in figure 7A-C, and was also confirmed by the mechanical testing results. However, some produced tubes were more even which is exemplified in figure 7D-F, and confirmed by mechanical testing results as well.
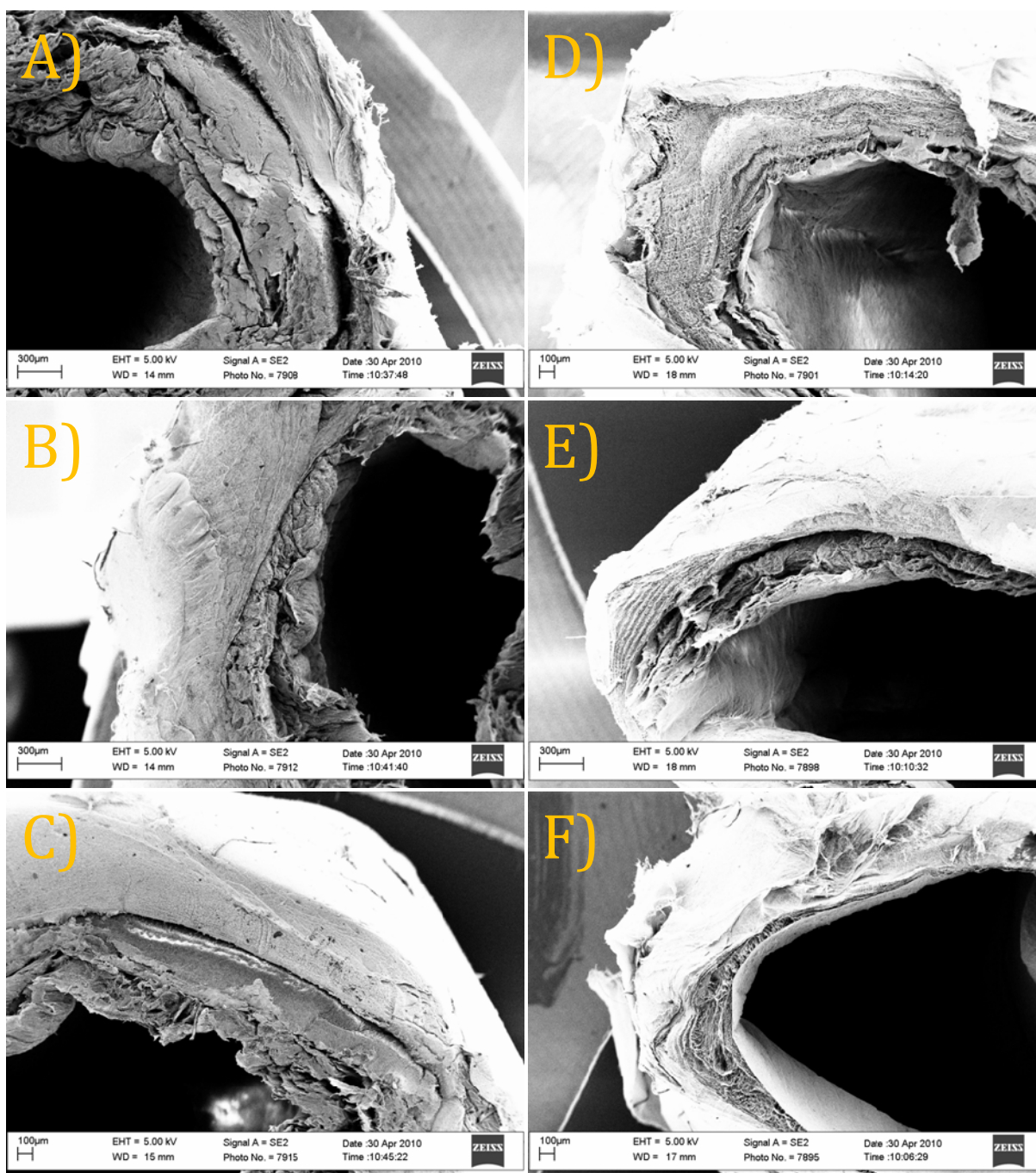
Figure 8. SEM pictures above show three parts of a tube from the protocol 1 experiments, and three parts of a tube from the protocol 2 experiments, 100X magnification. Pictures A, B and C illustrate the top, middle and bottom segments from the protocol 1 experiments. The D, E and F pictures show the protocol 2 experiments, also representing the top, the middle and the bottom segments. All pictures reveal a relatively similar tube thickness, thus confirming the even BC fermentation for each of the tubes.

The SEM pictures (figure 8) illustrate segments of one of the tubes from the protocol 1 changes. The A picture is from the top segment, B from the middle segment and C from the bottom segment. The wall thicknesses of the three pictures are similar. The D (top), E (middle) and F (bottom) pictures are taken from the protocol 2 experiments, in which the

three parts were also similar in thickness. The same magnification (100X) has been used for all pictures.

The SEM pictures must be studied with caution. This is due to the preparation steps of a specimen; e.g. rapid freezing and subsequent freeze-drying. A better methodology would be to use a wet chamber for the SEM. This would allow samples to be studied in their original state, thus avoiding any sample preparation.

PROTOCOL 3

The indication found from the protocol 1 and 2 experiments was followed up by the protocol 3 bioreactor set-up. Thus, the attempt of enhancing the bioreactor control and achieve a more homogeneous mass profile would be validated.

The radial tensile tests results are illustrated with data of tensile stress at break (MPa). The results are displayed as box plots, in a similar way as the previous experiments.
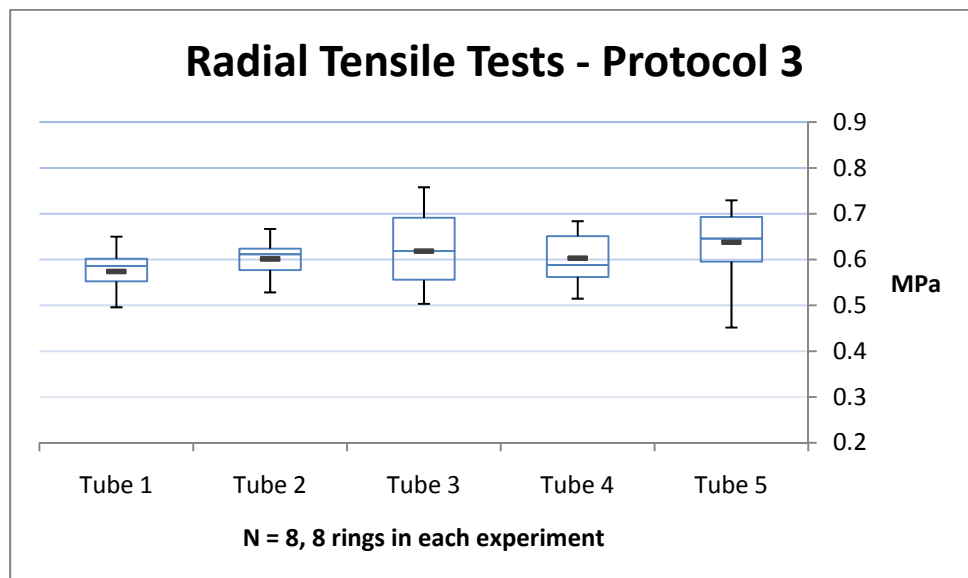
Chart 6. Radial tensile tests performed on the tubes fermented according to protocol 3 bioreactor set-up. The tensile test results are in MPa, including all eight ring parts from one tube in one box plot. The results give relatively small boxes, which are within a similar range of strength.

Chart 6 illustrates the radial tensile test results from the protocol 3 experiments. Compared to the small boxes identified from protocol 1 and 2 experiments, these boxes are somewhat larger. However, the mean values of these experiments are not as widespread, in comparison with the references. The reason for the larger boxes could be that the improvement from protocol 1 and 2 into protocol 3 was not sufficient enough or not properly incorporated.
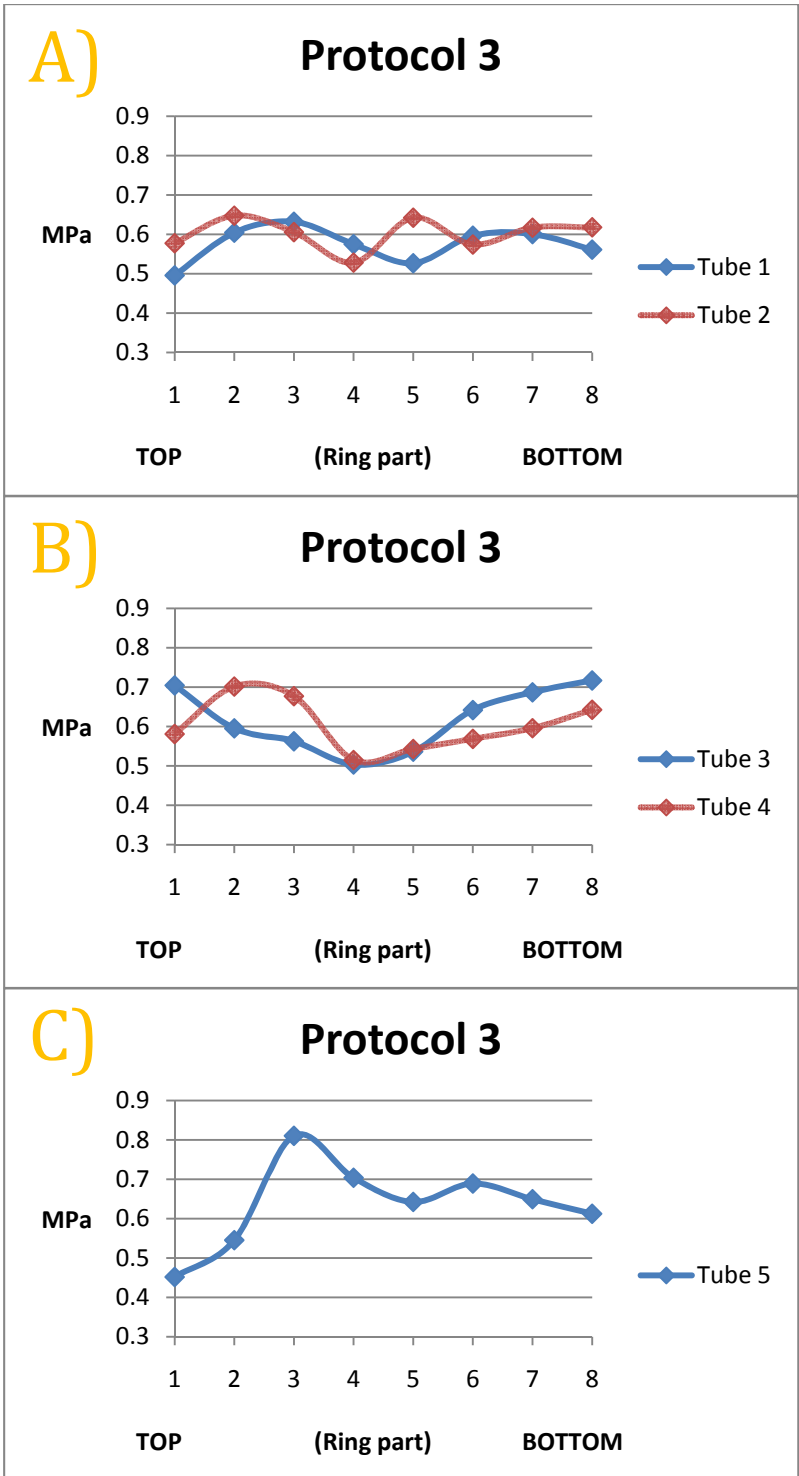
Chart 7. Tensile results of five different tubes produced according to protocol 3 set-up. Chart 7A illustrates tubes that exhibit more or less similar tensile test results from the top to bottom, analogous to the protocol 1 and 2 results. In B, the desired dip in the middle, with stronger ends at the top and the bottom, is illustrated. C is a result that is more similar to the reference tubes, i.e. random. Overall, the results show no particular trend, and the results are similar to the reference results.

Protocol 3 did not reveal any new information on how the BC tubes are produced. The results were more similar to the reference tubes; there were no real trends. The improvement from protocol 1 and 2 might have contributed to the random results.

CONCLUSIONS

Both protocol 1 and 2 were successful by generating a more reproducible mass profile. The obtained results indicate a good lead, as the reproducibility has improved. Compared to the old bioreactor set-up results, these results were tremendously improved and have provided increased knowledge on synthesis of BC tubes. Results from the radial tensile tests illustrated the improvement via box plots. Comparison of the reference results and the new protocol (1 and 2) results revealed that the variance in strength was much smaller. This was also confirmed by plotting of the tensile test results in a scatter plot, with ring segments next to each other. Similar mechanical strength was obtained for every ring, regardless of position in the experiment tubes. The produced reference tubes proved to exhibit more widespread results, which contributed to the larger box plots and the apparent uneven characteristics of the dot-charts. Thus, the concept of the desired homogeneous mass profile was proven. Supplemental SEM pictures were also obtained, which confirmed the biomechanical data

Due to the success of these experiments (protocol 1 and 2), the procedure was incorporated into the third protocol experiments. However, the hypothesis of obtainment of stronger ends at the gas inlets was not proven by the results. Instead, the results were similar to the results of the reference tubes. The reason could be due to insufficient incorporation of the improvement from previous protocols.

The low production of tubes resulted in a small amount of analyzed tubes. To further confirm the indications given by the results, more tubes need to be produced and analyzed. In the future, additional mechanical tests should be performed, e.g. suture, peel and burst strength tests. In addition compliance tests are important before *in vivo* trials. Preferably, a WET-SEM should be used in order to not deform the samples thus obtaining pictures of BC in its natural hydrogel form.

The effects of protocol 1 and/or 2 were found to be important, in order to achieve a homogeneous mass profile and a better reproducibility in the production. Further experiments have to be conducted to statistically confirm the indication. In the future, more experiments according to protocol 3 set-up should be conducted in which the incorporation is better affiliated with the new bioreactor. The new bioreactor set-up shows great potential, and with proper incorporation this might be the bioreactor set-up that will provide the solution to the reproducibility issue.

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