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Influence of carbon source complexity on porosity, water retention and extracellular matrix composition of *Neurospora discreta* biofilms

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Running Headline

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Abstract

Aims: To evaluate carbon source complexity as a process lever to impact the microstructure, chemical composition and water retention capacity of biofilms produced by *Neurospora discreta*.

Methods and Results: Biofilms were produced by non-pathogenic fungus *N. discreta*, using sucrose, cellulose or lignin as carbon source. Increase in complexity of carbon source from sucrose to lignin resulted in decreased water retention values (WRV) and wet weights of harvested biofilms. Confocal laser scanning microscopy (CLSM) was used to calculate porosity from bright field images, and relative stained areas of cells and carbohydrates from fluorescence imaging of samples stained with Trypan blue and Alexa Fluor 488. Porosity and relative quantity of cells increased with increase in carbon source complexity while the amount of carbohydrates decreased. Chemical analysis of the extracted extracellular matrix (ECM) showed that biofilms grown on more complex carbon sources had lower carbohydrate and protein content, which also explains the lower WRV trend, as carbohydrates are hydrophilic.

Conclusions: The nature of carbon source impacts the metabolic pathway of cells, thereby influencing the relative proportions of ECM and cells. This in turn impacts the microstructure, composition and water content of biofilms.

Significance and Impact of the Study: This work shows that carbon source can be used as process lever to control the properties of biofilms and presents a novel view of biofilms as potentially useful biomaterials.

Introduction

Worldwide public health and environmental concerns with synthetic polymers have led to a growing interest in biologically produced materials that are natural, renewable as well as biodegradable (Cole *et al.* 2011; Webb *et al.* 2013). This class of biomaterials include biocomposites based on wood and natural fibers such as cellulose, as well as microbially produced biopolymers. Microbial biopolymers produced by bacteria and fungi have an added advantage of process control and scalability as these can be produced in bioreactors: Examples include pullulan, bacterial cellulose, scleroglucan, xanthan, poly-3-hydroxybutyrate and exopolysaccharides (EPS) (Survase *et al*. 2007; Mohammadkazemi *et al*. 2015; Jiang *et al*. 2016; Majee *et al*. 2017; Girometta *et al*. 2019). Due to the diversity in their properties, these materials find application in various areas, being used as emulsifiers, stabilizers, gums, or for making biocomposites. A well-researched example is microbial cellulose produced by various bacterial species, which finds application in the food and medical industries as an additive, wound dressing material, packaging material, etc. (Raghunathan 2013; Mohammadkazemi *et al*. 2015). However, nearly all of these polymers are secreted by cells in soluble form, and require further downstream processing such as precipitation, purification and fabrication (Lee *et al*. 2009). A few recent studies have demonstrated the formation of solid fungal composites, which are formed by fungal mycelia grown on solid substrates such as cotton, straw, hemp, and sawdust (Elsacker *et al*. 2019; Girometta *et al*. 2019). Wood-decay fungi can penetrate these substrates, degrade the lignocellulosic components present, and eventually replace the substrate with fungal biomass resulting in mycelium-based bio-composites (Girometta *et al*. 2019). Appels *et al.* (2019) found that the fungal species as well as the substrate used, significantly impact the strength and stiffness of such bio-composites. More recently, hybrid composites made of wood, cellulose nanofibrils and fungal mycelia were reported (Sun *et al*. 2019,). Most of these fungal composites that form on solid substrates require further processing such as cold-pressing or curing at

high temperatures to achieve the mechanical strength required for their potential applications (Iordache *et al*. 2018; Girometta *et al*. 2019).

A relatively unexplored area in biomaterials research is the evaluation of 'biofilms' as useful materials. Biofilms are biological structures formed by microorganisms on various surfaces and interfaces, consisting of communities of cells embedded in a self-produced extracellular polymeric matrix (ECM) (Flemming and Wingender 2010). Owing to the detrimental effects of certain types of biofilms on medical and industrial equipment, research on biofilms has largely focused on their detection, prevention and removal (Francolini and Donelli 2010). On the other hand, biofilms formed in wastewater treatment plants are considered advantageous as they help in degradation and removal of contaminants, and provide a suitable habitat for the microorganisms involved in the process (Singh *et al*. 2006). In addition to immobilizing and housing cells, the purpose of biofilms is to enable cell-cell interactions, retention of extracellular enzymes, and transport of nutrients and waste products to and from the cells. This requires an intricate 3-dimensional structure, with varying pore sizes as well as an amphiphilic nature for selective transport. The result is a highly porous, viscoelastic material with the capacity to hold large amounts of water. Therefore, biofilms formed by non-pathogenic microorganisms can be viewed as renewable, biodegradable and sustainable porous biomaterials with potential applications as biological membranes. The main difference between biofilms and other microbial biopolymers is that biofilms are formed as intact materials that would not need further purification or downstream processing. Identification of parameters that can be used as process levers to tune the key properties of biofilms could therefore pave the way for a new area of biomaterials research.

Several physical factors such as pH, temperature and fluid shear stress influence the biofilm mode of life: We recently demonstrated that agitation of the growth reactor significantly impacts the microstructure and the mechanical properties of biofilms (Narayanan and Ahmed 2019). Among the

chemical factors that are critical to cell growth and biofilm formation are the nutrients available in the surrounding medium. Within the nutrient medium, the nature and quantity of carbon source have a profound influence on biofilm formation (Ellis *et al*. 2000; Wawrik *et al*. 2005; Ib and Chauhan 2015), and can be used as levers to control the structure and mechanical properties of biofilms, which would have a direct bearing on their applications.

This work pertains to biofilms formed by a locally isolated strain of non-pathogenic fungus, *Neurospora discreta*, on the air-liquid interface. When grown in liquid medium, *N. discreta* rapidly forms a biofilm on the surface of the medium, layer by layer, eventually forming a bio-composite of cells and ECM comprising carbohydrates, proteins, lipids and other polymeric substances. The intricate and interwoven matrix structure results in highly porous biofilms that hold >90% water by weight and the presence of the thick filaments of *N. discreta* reinforces the biofilms making them strong enough to be harvested and tested in an intact condition. The objective of the present work was to evaluate the effect of carbon source complexity on the properties of biofilms formed by *N. discreta*. Three carbon sources were tested in this study: (1) sucrose, a disaccharide of glucose and fructose, and a common carbon source in used in microbial growth media due to the ease of degradation, (2) cellulose, a natural plant-derived polymer and a polysaccharide of β-glucose units with 1,4-glycosidic linkages and (3) lignin, a bulky and recalcitrant phenolic polymer, consisting of several methoxylated derivatives of benzene (phenylpropanoid alcohols, also called monolignols). Lignin is a common by-product of biorefineries based on lignocellulosic biomass as well as paper and pulp industries and is metabolized by very few organisms, including white rot fungi such as *Phanaerochaete chrysosporium* (Boyle *et al.* 1992), as well as ascomycetes such as *N. discreta* (Pamidipati and Ahmed 2017). These carbon sources were selected as they represent three levels of relative complexity in structure, and differ in their molecular weights – all of which results in differences in how they are incorporated into the metabolic pathways of the

fungus. The biofilms formed from each of these carbon sources were analyzed in terms of their microstructure as well as chemical composition.

A look into the common metabolic pathways of microorganisms shows that simpler carbohydrates such as glucose or fructose are readily incorporated into the glycolytic pathway while a more complex polysaccharide needs to be broken down into simpler units before it can join one of the catabolic pathways. This results in differences in net ATP production, impacting the rate of cell growth as well as the nature and amounts of products released by cells. As the formation of biofilms is a direct function of cellular metabolism, we hypothesize that the complexity of the carbon source has a significant impact on biofilm composition, which in turn would influence other key properties of the material, such as porosity and the capacity to hold water. In the literature, the effect of carbon source on biofilm formation and ECM secretion has been reported (Xu *et al*. 2017; Turskaya *et al*. 2018), but its impact on physical, structural and chemical properties of biofilms has not been explored so far.

This work also introduces a new view of 'biofilms' as potentially useful biomaterials, in contrast to the vast literature on biofilms formed by pathogenic microorganisms responsible for diseases and industrial fouling. This is possible due to three reasons: first, *N. discreta* is a non-pathogenic and safe microorganism (Perkins and Davis 2000); second, the biofilms formed are strong enough to be harvested and handled as intact materials, possibly owing to the reinforcement provided by the long and thick hyphae of *N. discreta* as the biofilm grows; third, the biofilms have a membranous porous structure. Another unique feature of these biomaterials is that the formation on the air-liquid interface further contributes to ease of harvesting without damaging the structure.

Materials and Methods

Microorganism growth and maintenance

Neurospora discreta, isolated locally from the bark of a Subabul wood tree (Pamidipati and Ahmed 2017) was used for producing biofilms. *N. discreta* was maintained on potato dextrose agar plates (PDA) on which it grows as long filaments and produces abundant spores. The streaked plates were incubated for 3-4 days at 28-30^{o}C, after which the plates were refrigerated at 2-4^{o}C until further use.

Biofilm growth and harvest

Biofilms were grown in 250 ml Erlenmeyer flasks containing 100 ml Vogel's medium (Vogel 1964) with 2% total carbon source at an initial pH of 5.6 $\pm$ 0.2. For cellulose and lignin, this was added as 1.5% carbon source plus 0.5% sucrose: The latter was added to initiate cell growth. The flasks containing the culture medium were sterilized in an autoclave at 121^{o}C for 15 minutes. Once the medium cooled, 0.1% solution of filter sterilized biotin was added to each flask.

For inoculation of the flasks, cells of *N. discreta* grown on PDA were gently scraped and suspended into a few ml of sterile Vogel's minimal medium. The suspension was then filtered through a coarse muslin cloth to remove filaments and residual agar and the filtered spore suspension was used to inoculate the flasks and mixed thoroughly to obtain a spore count of ~0.2 million cells/ml in each flask. After the initial mixing, the flasks were incubated at 30^{o}C for 7 days without any agitation.

The first layer of the biofilm is formed about a day after inoculation after which it thickens by adding more layers. The biofilms were harvested by carefully removing them from the flasks using a long spatula and placing them on a filter paper for a few minutes to drain excess medium. The wet weights were then noted and the samples were sprayed with isopropyl alcohol to arrest further cell growth and then refrigerated in petri dishes at 2-4^{o}C until further analysis.

Water Retention Value (WRV) and Dry weights

A modified standard protocol from Tappi UM 256 (Cheng *et al*. 2010) was used to calculate the WRV of the biofilms. For this, a known amount of sample was soaked in water for an hour and then centrifuged at 1217 g for 15 minutes. Excess water was then removed and the sample was dried at 103.5 ^{o}C until constant weight was achieved. The dry weight was then noted and the ratio of the difference in wet and dry weights to the wet weight gave the WRV for each sample. Normalized dry weights were also calculated by dividing the dry weight by the wet weight of each sample.

Confocal Laser Scanning Microscopy

Porosity measurement

Biofilm samples were imaged using confocal laser scanning microscopy (CLSM) (Leica DMi8 inverted microscope) after drying at 30^{o}C for about 3 hours. The samples auto-fluoresce which was used to obtain bright field images with a 40X oil objective with z-step of 1 µm. From the bright field images porosity is calculated which is a measure of structural heterogeneity in biofilms: It is defined as the ratio of pore volume to biomass volume. The images were processed using ImageJ (Fiji) software. This entailed converting each image into a grey-scale binary image and segmenting the image by consistently applying a thresholding algorithm (Huang and Wang 1995)

The formula used for calculating porosity from confocal images is as follows:

$$\text{Porosity} = \frac{(\text{Field of view}) \times \sum(\text{Areal porosity} \times \text{Slice thickness})}{(\text{Volume of the sample})}$$

Where, $\text{Areal porosity} = \frac{\text{Number of void pixels}}{\text{Total number of pixels}}$

Sample staining:

Samples were stained for fluorescence imaging to quantify carbohydrates and cells. Trypan Blue was used to image the cells and Alexa Fluor 488 to image the carbohydrates (Zinchuk and Zinchuk 2008). For this purpose, a small strip of each sample was first washed in phosphate buffer saline (PBS) solution, after which 2000 µl of diluted Trypan blue stain (1:1500) was added to the sample and incubated for 30 minutes. After incubation, the sample was washed again with PBS and excess stain was removed using blotting paper. The film was then added to a well containing 200 µl of 15 µg/ml of Alexa Fluor 488 and incubated for 30 minutes. The concentrations of both stains were decided based on experiments that were designed to determine stain concentrations for which the stained areas were independent of sample thickness. Finally, the sample was washed again with PBS buffer and the excess stain was removed by using blotting paper. The film was then placed in a microscopic slide covered with a coverslip and dried at 35 ^{0}C before viewing in the fluorescence mode of the CLSM. The stained area for each stain was obtained using the software, and the stained volume was calculated using the number of slices. A feature of the fluorescent imaging protocol that we adopted is that information about carbohydrates and cells were obtained from the same area of the sample as the sample contained both the stains.

ECM extraction and analysis of composition

ECM was extracted from the samples and analysed for carbohydrate and protein content. For extraction we tried physical methods of centrifugation and ultrasonication. However, we report results only from the chemical extraction method that used a combination of formaldehyde and sodium chloride as it gave the highest yields (Jiao *et al.* 2010; Pan *et al.* 2010; Hemme 2015; Chiba *et al.* 2016).

The biofilms samples were soaked in 25 ml of 8.5% sodium chloride and 0.22% formaldehyde for 2 hours, then centrifuged and washed. The extracted EPS was then subjected to carbohydrate and protein estimation. Lowry's protocol was used to measure the protein content in the extracted EPS (Lowry *et al.* 1951). Carbohydrate analysis involved acid hydrolysis of the EPS using 98% sulfuric acid for 2 hours, followed by dilution to 4% and then autoclaving at 121^{o}C for 1 hour. The samples were then cooled, neutralized using 1N NaOH and the resulting sugars from the hydrolysis were measured using DNS reagent (Miller 1959).

Results

Biofilm growth, weights and water retention

In all flasks, biofilm growth started 18-24 hours after inoculation. Biofilms formed on the air-liquid interface and grew layer by layer each day for about 5 days after which there was no more visible increase in thickness. Biofilms grown on lignin were considerably thinner and more fragile compared to those grown on sucrose. Cellulose-grown biofilms were thicker than the lignin-grown ones but not as thick as the sucrose-grown biofilms. This was also reflected in the wet weights, as shown in table 1. As the carbon source complexity increased, the overall wet weight of the biofilm decreased. While this may appear to be an expected trend, owing to the ease of degradability of the carbon source, which would lead to greater cell growth in sucrose compared to lignin, the normalized dry weights (table 1) show the opposite trend with lignin-grown biofilm being the highest and sucrose-grown biofilm the least. These results indicate that the higher wet weight of sucrose-grown biofilms is due to the water held in the sample and not because of greater amounts of biomass. This can also be seen from the WRV, which is highest in sucrose-grown and lowest in lignin-grown biofilms. From these results it be concluded that water content is controlled by the carbon source, which has implications for the microstructure of the

biofilms formed. WRV is an important property of biomaterials as it provides insight into the hydrophilic nature and swelling capacity of the material (Cheng *et al*. 2010).

Porosity and stained areas of biofilms

Biofilms samples were dried and subjected to brightfield imaging using CLSM to determine the effect of carbon source on the microstructure and more specifically the porosity. Each biofilm layer consists of crisscrossing filamentous cells and ECM (Fig 1a) interspersed with pores (Fig 1b). The relative amounts of cells and carbohydrates in ECM as a function of carbon source can be seen qualitatively from the stained CLSM images in figure 2 and quantitatively from the plot of average stained areas in figure 3a. Analysis of the bright field images yielded average values of porosity and filament diameter of biofilms grown on each carbon source (Fig 3b). Sucrose-grown films contained the highest amount of carbohydrates and lowest amount of cells, while lignin-grown films displayed the opposite trend. This can also explain the trends in WRV (table 1), as carbohydrates are hydrophilic and therefore, higher the proportion of carbohydrates, greater the amount of water held by the biofilms. Therefore, sucrose-grown biofilms, which had relatively the highest carbohydrate content also had the highest WRV, and consequently the highest wet weights. Another quantity that we can deduce from the average stained areas is the amount of carbohydrates produced per cell which varied significantly across carbon sources. The ratios of carbohydrates to cells yields values of 1.3 for sucrose, 0.8 for cellulose and only 0.3 for lignin, indicating a difference in cellular metabolism in each case. When grown on sucrose, the metabolic energy seems to be diverted towards carbohydrate production rather than cell growth, whereas growth on lignin promotes cell growth much more significantly compared to carbohydrate production.

The porosity in sucrose-grown biofilms was the lowest while that in lignin-grown biofilms the highest. The space between crisscrossing network of the hyphae – filamentous cells (figure 1) define the

245 micropores. The ECM grows around the cells and consequently, extends into the space between the
246 cells. Therefore, for lignin-grown biofilms for which ratio of carbohydrates to cells is only 0.3, the
247 porosity is nearly 75% in contrast to sucrose-grown biofilms whose porosity is less than 50% for which
248 the ratio of carbohydrates to cells is 1.3. Sucrose-grown biofilms also have higher filament diameter
249 compared to lignin-grown biofilms (Fig. 3b), which also indicates basic differences in the metabolism. In
250 all cases, the values of the cellulose-grown biofilms fell between the two extremes of sucrose and lignin,
251 showing increasing and decreasing trends of parameters with complexity of the carbon source.

252

253 **Chemical analysis**

254 Further insight into the effect of carbon source on the composition of ECM was obtained by extracting
255 the ECM and determining its carbohydrate and protein content, as these are the two main constituents
256 of ECM. Among other functions, proteins promote enzymatic activity that is necessary for 'digestion of
257 exogenous macromolecules for nutrient acquisition' (Flemming and Wingender 2010). As seen from
258 figure 4, the relative amounts of carbohydrates and proteins in the ECM decreased with increasing
259 complexity of carbon source. This data corroborates the trends seen in the CLSM analysis, and shows
260 that the amount of carbohydrates in the ECM of sucrose-grown films was the highest and that in lignin-
261 grown films the lowest.

262

263 **Discussion**

264 There is contradicting evidence in the literature on whether ECM production is a growth-associated or
265 non-growth associated process, as some researchers report that microorganisms produce less ECM
266 during the active growth phase, while others report that there is a linear positive relationship between

cell growth and ECM production (Laspidou and Rittmann 2002). From the results of this study, it can be inferred that as the complexity of the carbon source increases from sucrose to lignin, the metabolism seems to favor cell growth over ECM production, resulting in biofilms with lower water-holding capacity. This can be explained by the differences in the manner in which carbon sources are metabolized by cells for growth as well as ECM production (figure 5). Metabolism of sucrose involves its hydrolysis to glucose and fructose. Glucose is phosphorylated to Glucose-1-phosphate and then to Glucose-6-phosophate, which subsequently gets converted to Pyruvate after a series steps as shown in figure 5. Pyruvate serves as a metabolic precursor to several other pathways and cycles including the TCA cycle for ATP generation. The fructose produced from the hydrolysis of sucrose also gets phosphorylated and eventually joins the glycolytic route. In a parallel pathway involving ECM production, Glucose-6-phosphate or fructose-6-phosphate are converted to Uridine diphosphate (UDP)-Glucose or UDP-galactose respectively, which eventually form repeating units that form the ECM (Ramos *et al*. 2001; Tang and Ya-jie 2004; Chai *et al*. 2012. The production of ECM follows a biosynthetic pathway involving the net consumption of ATP, rather than production of ATP. Therefore, when provided with an easily degradable carbon source such as sucrose, the cells have sufficient ATP for growth, and therefore can redirect the excess ATP to the anabolic steps involved in ECM production.

In case of cellulose, the polysaccharide is first broken down to cellodextrin, which then forms several units of cellobiose that finally break down to glucose, which can then follow the same path described above to form EPS or enter the glycolytic pathway. Therefore, the number of steps involved in breakdown of cellulose are greater than those required for breakdown of sucrose and the net ATP production would be lower as some would be consumed during breakdown of cellulose.

The degradation of lignin follows a completely different pathway compared to sucrose and cellulose, involving breakdown of lignin via multiple steps to bi-aryls, and then to mono-aryls which over several

more steps go through ring cleavage and eventually form pyruvate, which can enter the TCA cycle (Takayoshi 1986). Pyruvate can then either be utilized in the TCA cycle, or, under the right conditions enter the gluconeogenesis pathway, leading to the formation of glucose. Some evidence in the literature also exists on the presence of pyruvate itself in EPS. This means that the number of steps required for metabolizing lignin are even greater compared to those required for cellulose and the net ATP production and therefore the ATP available for producing ECM would be a lot lower compared to simpler carbon sources. Therefore, the amount of ECM produced per cell in the case of lignin-grown biofilms is a lot lower compared to that in sucrose-grown biofilms.

By impacting the composition of the biofilms, the complexity of the carbon source also influences the physical properties of the biofilms, such as the capacity to retain water. As the ECM is largely composed of carbohydrates, which are hydrophilic, the biofilms in which the relative amount of ECM is higher hold more water, as seen from the higher WRV. Biofilms grown on lignin on the other hand, have a higher proportion of cells that are less hydrophilic compared to ECM and therefore have lower WRV. The presence of greater numbers of cells in these biofilms also leads to increased porosity as the pores are formed by the crisscrossing of filamentous cells. Thus, complexity of carbon source could be an important process lever to grow fungal biofilms with customized mechanical properties.

This work shows that the carbon source used has a significant impact on the composition of biofilms, which in turn impacts the microstructure and the water-retention capacity. As the complexity of the carbon source increased, water retention decreased due to the decreased levels of carbohydrates and proteins. On the other hand, porosity increased with increasing complexity, owing to the presence of more cells and less ECM. Based on these results, carbon source can be used as a process lever to tune the porosity and composition of the biofilms, which would also have a direct bearing on the mechanical properties. These biofilms are natural, biodegradable and sustainable materials that can find

applications as membranes for wastewater treatment (Shukla *et al*. 2014; Quilès *et al*. 2016), and wound care materials (Smith *et al.* 2016; Suarato *et al*. 2018), which require flexible "housing" and a highly porous structures for exchange of fluids.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Table and Table Legend

Table 1. Listing of various parameters that characterizes the water content in the biofilms grown with different carbon sources

Carbon source	Wet weight (g)	WRV (g/g)	Normalized dry weight (g/g)
Sucrose	12.4 ± 0.5	98.9	0.011
Cellulose	11.1 ± 0.5	98.1	0.019
Lignin	6.9 ± 0.4	97.2	0.028

Figure Legends

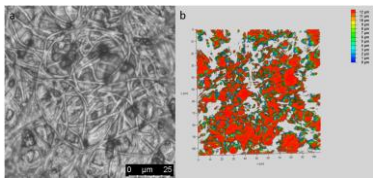
Fig. 1 (a) Representative bright field image of a biofilm sample which shows the crisscrossing filaments and resulting pores. **1(b)** Reconstructed 3-d image of a biofilm sample which shows the porous structure.

Fig. 2 CLSM images of biofilm samples grown on sucrose, cellulose and lignin. Samples were stained with Alexa Fluor 488 for carbohydrates (top) and trypan blue for cells (bottom).

Fig. 3 (a) Average stained areas for cells (shaded) and carbohydrates (unshaded) and obtained from CLSM images, **3(b)** Filament diameter (●) and porosity values (■) of biofilms grown on sucrose, cellulose and lignin.

Fig. 4 Carbohydrate and protein content in ECM of biofilms grown on sucrose, cellulose and lignin.

Fig. 5 Summary of the metabolic pathways involved in breaking down sucrose, cellulose and lignin and production of ECM. Adapted from multiple sources (Takayoshi 1986; Nelson *et al.* 2004; Fisher, B. and Fong, S. 2014). Dashed lines represent multiple steps.

456 

457 Figure 1

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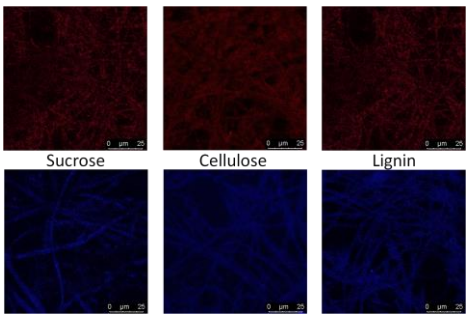
460 

461 Figure 2

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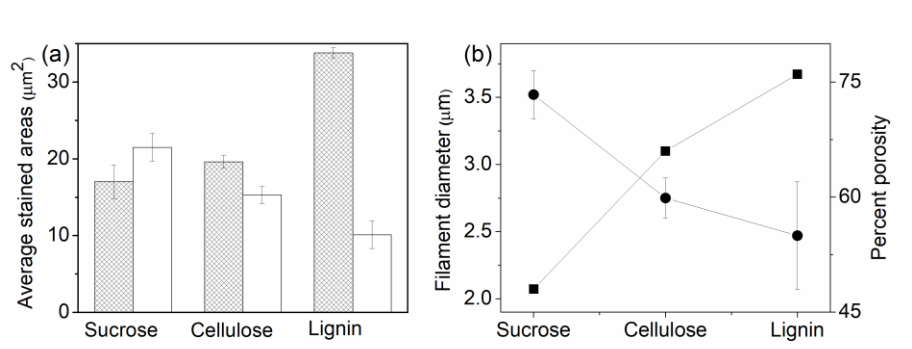

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464 Figure 3

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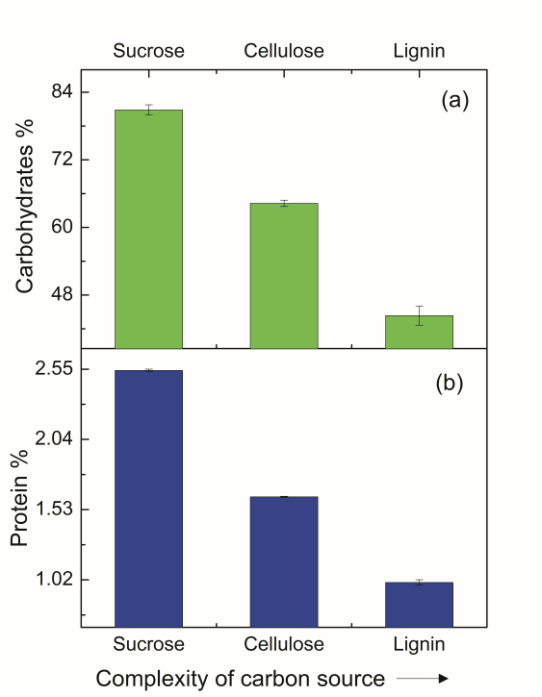

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468 Figure 4

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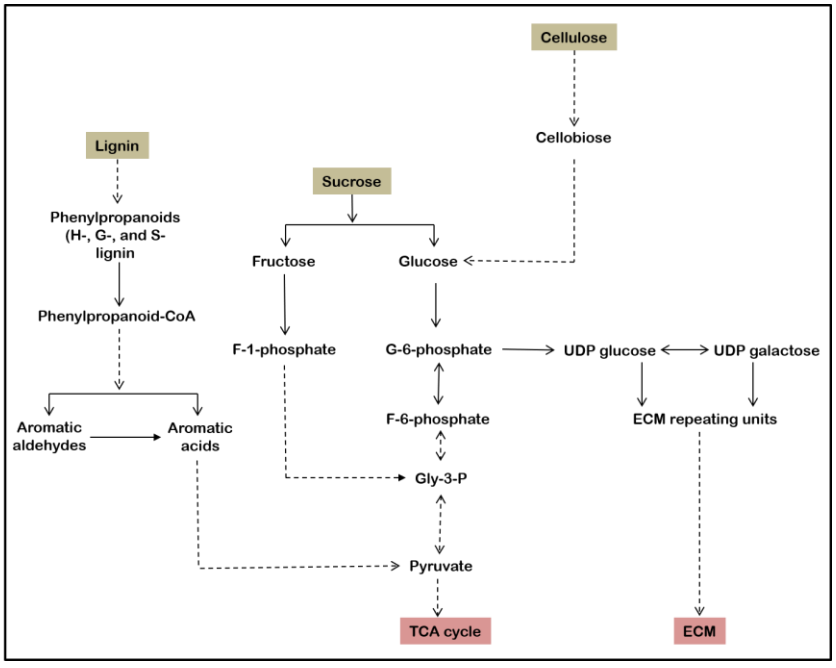

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471 Figure 5