Toxicity of Microcrystalline Cellulose Isolated Through Different Acidic Treatments

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Submitted by

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"In the name of Allah, the most gracious and the most merciful"

Declaration of Originality

I hereby declare that the work **"Toxicity of Microcrystalline Cellulose Isolated Through Different Acidic Treatments"** accomplished in this thesis is the result of my own research carried out under the supervision of **Dr. Muhammad Zia** and it is written and composed by me.

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CERTIFICATE

It is certified that the research work presented in this thesis titled "Toxicity of Microcrystalline Cellulose Isolated Through Different acidic Treatments" was conducted by **Mr. Muhammad Aslam Khan** under the supervision of **Dr. Muhammad Zia**. This thesis is submitted to the Department of Biotechnology in partial fulfillment of the requirements for the degree of Master of Philosophy (MPhil) in **Biotechnology.**

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Dedication

"I dedicate this work to my loving parents, dear siblings and to all those people-who despite the odds, struggle for the grandest good of humanity".

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All praises be to **Almighty Allah**, the source of all knowledge, wisdom within beyond my comprehension. All praises be to **Almighty Allah,** in which hands lies the will and strength of a man. Also unlimited Darood-o-salam on **Hazrat Muhammad (S.A.W.W)** who is the real teacher and guide to all.

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7th Feb, 2020

Muhammad Aslam Khan

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List of Abbreviations

BC	Bacterial cellulose
CAGR	Compound annual growth rate
CI	Crystallanity Index
EFSA	European food safety authority
DMSO	Dimethyl sulfoxide
FTIR	Fourier transform infrared spectroscopy
FMC	Food machinery corporation
GDP	Gross domestic product
HCL	Hydrochloric acid
HNO_3	Nitric Acid
H_2SO_4	Sulfuric acid
hRBCs	Human red blood cells
hWBCs	Human white blood cells
MCC	Microcrystalline cellulose
M	Molar
N	Normal
PLA	Poly lactic acid
SEM	Scanning electron microscope
XRD	X-ray diffractogram
ZOI	Zone of inhibition

ABSTRACT

Rising awareness about the global environment, government regulations, consumer and market demand has pushed for the development and use of renewable materials. Cellulose, a biodegradable, eco-friendly, sustainable and the most abundant biomaterial on earth has the potential to be substituted for many of the fossil based materials. In the present study, microcrystalline cellulose was successfully prepared from cotton wool fibers, by using different acidic treatments viz. hydrochloric acid, sulfuric acid and nitric acid. Isolated microcrystalline cellulose were characterized by X-ray diffraction (XRD), fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). Moreover, extraction yield, aqueous suspension stability, biocompatibility, cytotoxicity and antimicrobial potency were also evaluated and compared. The results revealed that type of the acid effect morphology, size, crystallanity and dispersion stability of microcrystalline cellulose. Sulfuric acid derived microcrystalline cellulose with average dimensions $40.46 \pm 28 \times 9.74 \pm 4.23$ μm, exhibited high crystallinity index (80.57%) and stable aqueous dispersions. Hydrochloric acid resulted in microcrystalline cellulose with high yield (73%) and average length and diameter 65.24 ± 30.35 and 13.25 ± 4.45 μm, respectively. High aspect ratio (6.9) microcrystals with mean dimensions $76 \pm 59 \times 11.5 \pm 4.1$ μm, were isolated with nitric acid. Despite having different physicochemical and morphological characteristics, isolated microcrystalline cellulose exhibited excellent compatibility against freshly isolated human red blood cells ($IC_{50} > 200$ μg/ml), macrophages ($IC_{50} > 200$ μg/ml) and brine shrimps ($LC_{50} > 200$ μg/ml). Furthermore, no growth inhibition effect was observed in any of the tested bacterial and fungal strains. The study thus concludes that cotton wool fibers is potential, economic source for manufacturing high valued, safe, microcrystalline cellulose that can be used for broad range of applications in biocomposites, pharmaceutical and food industries.

Chapter 01

Introduction

INTRODUCTION

Ever increasing concerns about the environment and consumer/market demand for sustainable products and services has pushed the governments and industries towards the development and use of renewable materials and products (Shatkin, 2014). Currently, there is a strong urge for transition towards bio-economy from contemporary fossil fuel economy that may rectify many of the challenges such as carbon footprint, plastic pollution, diminishing crude oil stocks, global warming and sustainability. (Toma *et al.*, 2017). Cellulose, the most abundantly available natural biopolymer on earth, is one potential alternative which can be used to propose rational solutions to overcome these issues.

Cellulose is ecofriendly, renewable, biodegradable and sustainable biomaterial with an estimated annual bio production of over 7.5×10^{12} metric tons (Trache, 2018). It is present in a wide variety of organisms including plants, some species of bacteria and several species fungi and amoeba (Moon, 2011). Naturally, cellulose exists in the form of linear chains which are synthesized by living cells and are then self-assembled into microfibrils. The microfibrils are compact bundles of linear cellulose chains and constitutes crystalline regions separated by weak amorphous domains (Fernandes *et al.*, 2011; Nishiyama, 2009).

Microcrystalline cellulose or cellulose microcrystals are partially depolymerized (micron sized) white, odorless powder prepared by treating refined α-cellulose with dilute mineral acids. The hydronium ions from the acid cleave the glycosidic bonds at weak, amorphous domains and thus results in rod shaped microcrystals (Azizi *et al.*, 2005). According to the figures, microcrystalline cellulose (MCC) market is estimated to increase up to 1.24 billion USD by 2023 at compound annual growth rate (CAGR) of 7.0 % (Markets and Markets, 2019). Currently, microcrystalline cellulose is manufactured by more than 10 major suppliers worldwide. Commercially microcrystalline cellulose is available as Avicel®, Microcel®, Noyagel®, Heweten®, and Nilyn® and used in various industries like pharmaceutical (tablet binder), food (rheology modifier), paper and in composites (Azizi *et al.*, 2005).

Excellent mechanical properties, low weight and stiffness combined with renewable, biodegradable and eco-friendly nature has drawn much attention towards microcrystalline cellulose isolation and their applications in bio-composites (Trache *et al.*, 2016).

Apart from the mineral acid hydrolysis, various other methodologies have been reported in literature for microcrystalline cellulose production. These technique includes steam explosion, extrusion, alkali hydrolysis and radiation enzymatic process (Khalili *et al.*, 2014; Delong 1987; Lamsal *et al.*, 2010). These methods are demonstrated as safe, ecofriendly, less dependent on acids but are associated with drawbacks such as difficulty in controlling yield, low crystallinity and may be too expensive to be viable at commercial scale (Adel *et al.*, 2011; Hanna *et al.*, 2001; Merci *et al.*, 2015).

The source for the extraction of cellulose microcrystals is also important as it defines the overall properties and performance and can be isolated from any lignocellulose source with high cellulose content. Various cellulose sources have been reported in literature for the isolation of MCC such as alfa fibers (Trache *et al.*, 2014), oil palm fronds (Owolabi *et al.*, 2017), Roselle fibers (Kian *et al., 2017)* filter papers (Ahmadi *et al.*, 2016), waste cotton fabrics (Chaiwutthinan *et al.*, 2015) and Groundnut Shell (Zango and Imam, 2018).

Cotton fibers are pure (~90%) and economic source of cellulose and the most abundant natural fibers. Global cotton production touched a high of 120.1 million bales in 2017 (Statista, 2017). Pakistan being the world's 5th largest cotton producing country, is a key player in global cotton market. The country produced 10.11million bales in 2018 (Dawn, 2019).

1.1 Objectives of the study

The aim of the current study was to prepare microcrystalline cellulose (MCC) with different acidic treatments from cotton wool fibers, as the most widely available native source of pure cellulose, their comparative physicochemical characterization and toxicological profiling. The goals of the study were

- Preparation of microcrystalline cellulose (MCC) from cotton wool fibers through hydrochloric acid (HCL), sulfuric acid (H_2SO_4) and nitric acid (HNO_3).
- Physicochemical, morphological and thermal characterization of the prepared MCC
- Biocompatibility of MCC against freshly isolated human red blood cells (hRBCs) and human white blood cells (hWBCs).
- Cytotoxicity against brine shrimps (*Artemia Salina*)
- Microbial toxicity against bacterial and fungal strains.

Chapter 02

Literature Review

LITERATURE REVIEW

2.1 Bio-Economy: A bio-based future

Currently, our world is facing a number of major environmental, economic and social challenges. To ensure, a healthy, safe and prosperous life for our future generations, these challenges need to be properly resolved. The transition from fossil fuel to bio-based economy, where agriculture not only ensure food security but also provide biomass, as a renewable raw materials for industries, is at the core of integrated bio-economy (Sasson and Malpica, 2018; Lokko *et al.,* 2018).

The term "bio-economy", also known as bio-based economy or green economy refers to knowledge driven exploitation, production and usage of bio-resources, state-of-the-art biological processes and principalities to deliver sustainable goods and services. It includes diverse products and key industrial sectors that include chemicals, bioenergy, bio-plastics, bio-materials, food ingredients, packaging materials, textiles and pharmaceuticals (Hetemäki *et al.,* 2017).

The global transition towards bio-based economy is accompanied by the ever increasing demand for renewable biomass. At the same time, rise in global biomass demand is also an opportunity for many agricultural-based, low income countries to diversify and improve their economies.

Pakistan, being a low income country relies heavenly on agriculture. It in fact, constitutes the largest sector of the country's economy and most of the population is dependent, directly or indirectly on the sector. The agriculture sector accounts for 24% of national GDP (gross domestic product) and is the largest source of foreign exchange reserves (Pakistan bureau of statistics, 2019).

Being, the world's 5th largest producer of cotton, Pakistan is a key player in global cotton market. Though the cultivation of cotton plants accounts for 5.5% of value-added in agriculture and about 1% of the GDP, Pakistan's economy is heavily reliant on cotton and its derivatives (Ashraf *et al.,* 2018). According to the recent figures, cotton production in the year 2018, stood at 10.273m bales (Dawn, 2019).

Beside, widely used in textile industry cotton is a pure source of cellulose (~90%), the most abundantly available biomaterial on earth.

2.2 Cellulose: The most abundant biomaterial

Cellulose, the most abundantly available, renewable biopolymer on the earth occupies a central place in the annals of polymers. About 10^{11}-10^{12} tons of cellulose is biosynthesized in the biosphere annually. For centuries, it has served mankind with diverse purposes: either in the form of construction material (intact wood, textile fibers) or paper and board (Klemm *et al.*, 2005)

Since the Egyptian papyri, cellulosic raw materials have played a significant role in human civilizations However, advances in the scientific knowledge about cellulose is correlated with the invention of different characterization techniques.

Henri Braconnot, in the early 19th century, was the first scientist who work one the acid hydrolysis of substances derived from the cell walls of plants (Braconnot, 1819). Anselme Payen, however first time discovered that all plant cell walls have fibrous component which has a distinctive chemical structure. He found that a fibrous material was formed when plant tissues are refined with an acid-ammonia treatment, and then followed by water extraction and named it cellulose (Payen, 1838). After a year, he successfully transformed cellulose into dextrose by using concentrated sulfuric acid and thus found that sugar, starch and cellulose could have the identical chemical composition. After more than 75 more years, Willstätter and Zechmeister established the basic cellulose formula $(C_6H_{10}O_5)_n$ (Willstätter and Zechmeister,1913).

2.2.1 Structure and Chemistry

The structural investigation of cellulose is underway since the development and advancement in polymer science. Cellulose, a polysaccharide is aggregate of linear polymers of D-glucopyranosyl residues. These residues are linked together through β-(1→4) configuration to form linear chains. The adjacent D-glucopyranose rings are organized in such a way that glucosidic oxygens point in opposite directions and the repeating unit in a cellulose chain consists of two β-D-glucopyranose to form a cellobiose unit as shown in figure 1.1 (Wüstenberg, 2014).

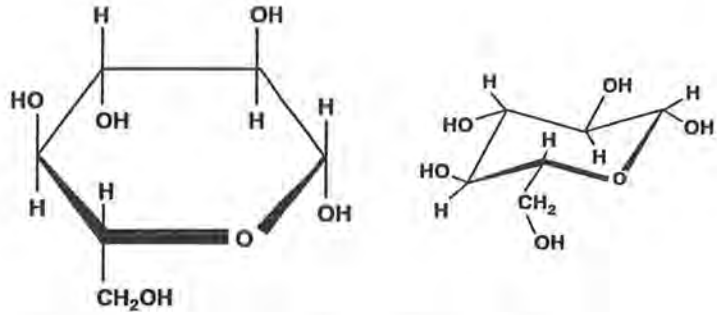

Figure 2.1 β-D-glucose ring: smallest building block of cellulose (adapted from Wüstenberg 2014)

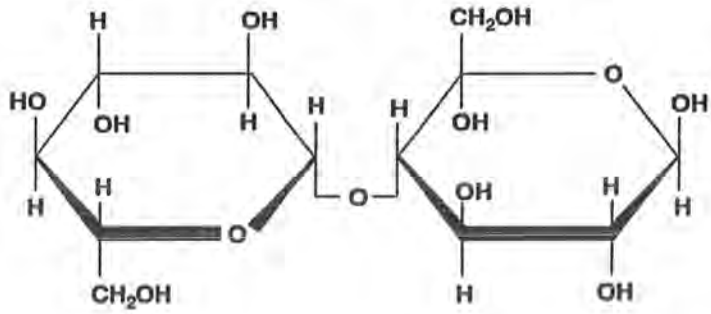

Figure 2.2 Cellobiose unit: secondary building block of cellulose (adapted from Wüstenberg 2014)

Figure 2.3 Twofold symmetry of cellulose linear chain (adapted from Wüstenberg 2014)

$(C_6H_{10}O_5)n$ or $(C_6H_{10}O_5)p$, is basic chemical formula of cellulose in which n is the number of basic glucose units and p refers to the degree of polymerization of the cellulose chain. Payen in 1842, found that elementally cellulose consists of carbon (C), oxygen (O) and hydrogen (H) with 44.0%, 48.5–50.0% and 6.0-6.5 % by weight respectively. Thus molecular mass of cellulose molecule is 162 $g.mol^{-1}$ (Wüstenberg, 2014).

Walter Norman Haworth, a British chemist was the first to find out the existence of H-bonding within and between the glucose monomers in cellulose molecule

Cellulose, derived from plant commonly exist in natural composite with, hemicellulose, lignin, pectin and other substances, whereas bacterial cellulose (BC) is considered the most pure form, with high water content and improved mechanical properties (Klemm *et al.*, 2011)

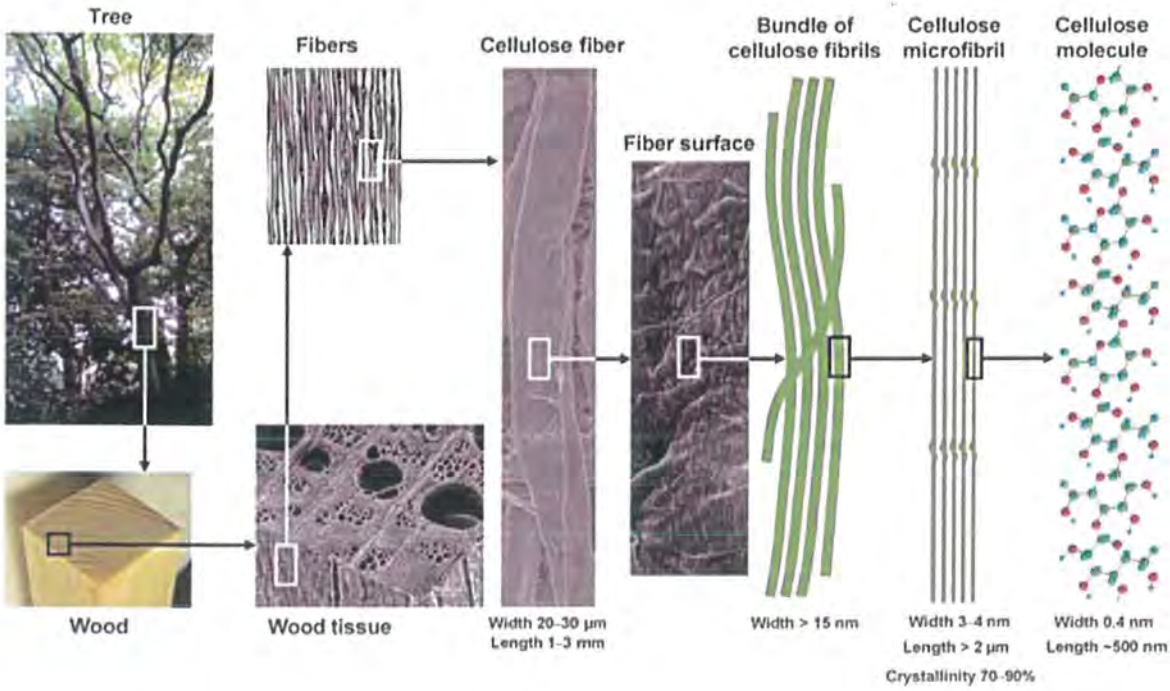

Figure 2.4 Hierarchical structure of plant cellulose (adapted from Isogai *et al.*, 2011).

Cellulose is known as hygroscopic substance, but it is considered inert as it shows minimal swelling in water. It is non-soluble in water, majority of organic solvents and dilute acids. However, strong acids dissolution can be accomplished but that too at the cost of degradation. It has been found that alkaline solution leads to substantial swelling (Svagan *et al.*, 2011).

2.2.2 Sources

Cellulose one of the most widely available natural polymer, is distributed throughout the nature. It can be extracted from wide variety of microbes, plants and some animals (Figure 2.5)

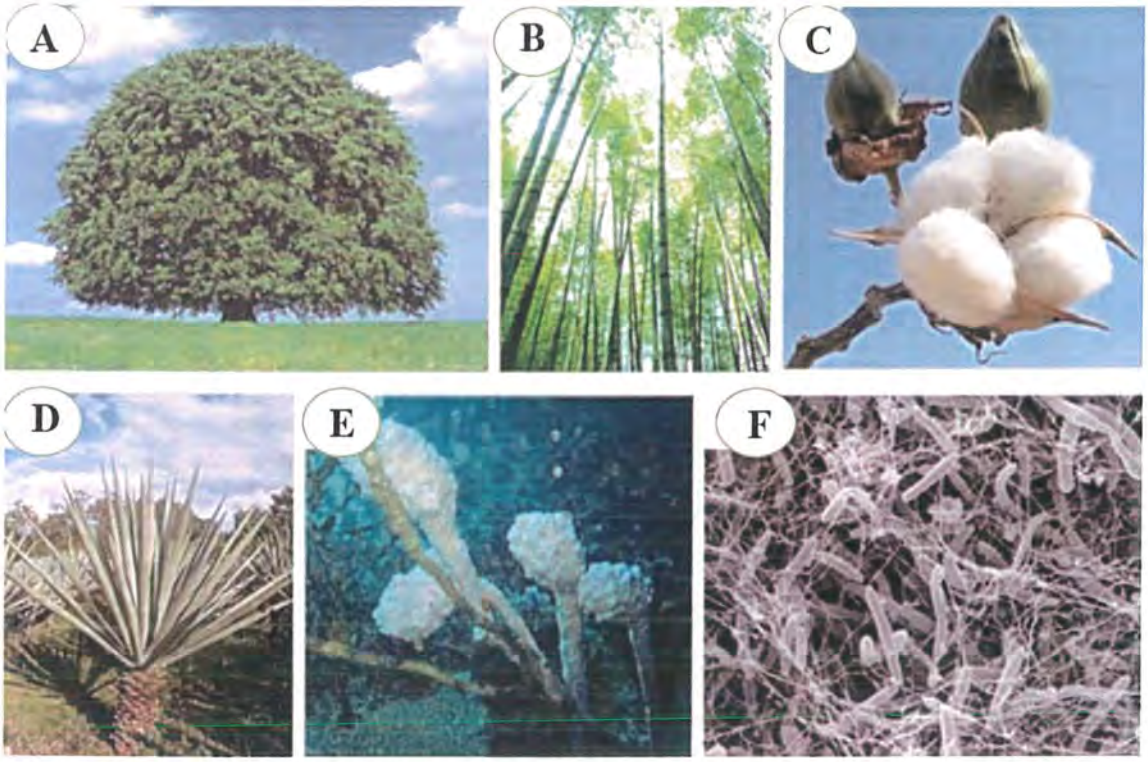

Figure 2.5 Pictorial presentation of important cellulose sources: **A)** beach tree, **B)** bamboo, **C)** cotton, **D)** sisal, **E)** tunicin, **F)** *Gluconacetobacter xylinum* (adapted from Ciolacu, 2011).

2.2.2.1 Plants

The source is of key significance as it effects the size and features of the extracted cellulose. Among all, plant fibers are widely employed sources for cellulose extraction. In fact, natural cellulose yields about 40% to carbon fraction in plant cells. Moreover, cellulose also serve as building units within the multifaceted structure of cell walls. In plants, it usually occurs in a natural composite, in combination with other natural polysaccharides such as hemicellulose, lignin and small amounts of extractives. The overall amount of cellulose varies from plant species, for example woody portion of the plant contains 40–50 wt% of cellulose, straw (40–50 wt%) sugarcane bagasse and bamboo (35–45 %), bamboo (40–55 %) respectively. Some plants even contains higher amounts of cellulose such as flax. Hemp, jute and ramie where cellulose exist by 70–80 %, 75–80 w%, 60–65%, and ramie 70–75% respectively. Cotton is the one of the most abundant and economic source that contain more than 90% cellulose (Kargarzadeh *et al.,* 2006).

2.2.2.2 Bacteria

Apart from plants, certain types of bacteria such as *Glucanacetobacter xylinus* produce cellulose as by product of primary metabolism. When the optimal condition are maintain, certain bacteria yield a thick gel that contains cellulose microfibrils (CMFs) and water (97%-99%). The degree of polymerization (DP) of bacterial cellulose depends upon the algal species but is usually between 2000 and 6000. One of the key features of BC is its high purity as compared to plant cellulose which exist in combination with other polysaccharides such as hemicelluloses and lignin (Ashjaran *et al.*, 2013).

2.2.2.3 Algae

Various species of red, green, brown and gray algae have also been reported as cellulose source. Species such as, *Valonia*, *Micrasterias rotate*, *Micrasterias denticulate Coldophora* and *Boerogesenia* are widely reported in literature as cellulose source (Hua, 2015).

2.2.2.4 Tunicates

Tunicates are the members of the subphylum Tunicata, the marine invertebrate animals though most of research has focused on class *Ascidiacea*. *Ascidiacea* commonly known as sea squirts, consist of over 2300 species. Among those species, *Halocynthia roretzi* and *Halocynthia papillosa* are the most widely used species for cellulose extraction (Iwamoto *et al.*, 2011). Cellulose is produced in tunic,outer tissue, from cellulose fraction known as tunicin is further purified. Tunicin, has high crystallinity index, high specific surface area (150–170 $m^2 g^{-1}$) and a very large aspect ratio (60–70) (Zhao *et al.*, 2014).

2.3 Microcrystalline Cellulose

Microcrystalline cellulose (MCC) also known as cellulose microcrystals are micron-sized odorless, biodegradable, white powdered material, which are derived from cellulose. Microcrystalline cellulose are used for diverse applications in food, pharmaceuticals and cosmetic industries (Sakhawy and Hassan, 2007; Uthai and Charuchinda, 2010).

MCC was first discovered by Battista and his co-worker Smith in 1955 and in 1962 was first commercialized by FMC under the brand name Avicel. In 1965, FMC (Food machinery and chemicals) introduced Avicel PH to the pharmaceutical industry (ingredient for direct compression tableting) and was first registered in the supplement in 1966 (Suzuki and Nakagami, 1999). Currently, microcrystalline cellulose is manufactured by more than 10 major suppliers worldwide (Thoorens *et al.*, 2014).

2.3.1 Commercialization

According to markets and markets report, microcrystalline cellulose (MCC) market is estimated to increase from 885.1m USD to 1.24b USD by 2023 at CAGR (compound annual growth rate) of 7.0|%. The growth in the market of MCC is due to increasing demand in pharmaceutical, cosmetic and food industries. The wood derived MCC remained the major source in commercial manufacturing. Moreover, Europe was the largest market for MCC in 2018 (Markets and markets, 2019).

Globally, the major manufacturers of MCC include DowDuPont (US), Asahi Kasei Chemicals Corporation (Japan), Roquette (France), Rayonier Advanced Materials (US), and DFE Pharma GmbH & Co.KG (Germany) (Global Markets Insight, 2019).

2.3.2 Sources

Microcrystalline cellulose is isolated from different lignocellulose sources. Cellulose source, extraction methods and conditions affects the molecular weight, crystallinity, particle size, surface area, moisture content and porous structure of the prepared cellulose microcrystals. The source for the extraction of cellulose microcrystals is important since it dictates the overall properties and performance of the particles. Wood and cotton are the main industrial sources of cellulosic fibers, and are thus the most important feedstock employed in the manufacturing of MCC. Other than wood pulp and cotton, sources such as waste paper (Okwonna, 2013), sugar beet pulp, oil palm fronds (Owolabi *et al.*, 2017), roselle fibers (Kian *et al.*, 2017), pomelo peel (Liu *et al.*, 2018), rice husk (Collazo-Bigliardi *et al.*, 2018), Groundnut Shell (Zango and Imam, 2018), filter papers (Ahmadi *et al.*, 2015) and waste cotton fabrics (Chaiwutthinan *et al.*, 2015) have also been reported in the literature.

2.3.3 Preparation

Mineral acid hydrolysis is the most common method used for the preparation of cellulose microcrystals, however other methods like alkali hydrolysis, steam explosion and extrusion technology have also been employed. Before using any of the methods, cellulose is needed to be isolated from a lignocellulose source. In plants cellulose exist in the form of natural composite i.e. it coexist with hemicellulose, pectin, lignin and small amount of extractives. Numerous physical, chemical, enzymatic and combined methods for the isolation and purification of cellulose are reported in the literature (Trache *et al.,* 2016). The typical steps involved in the isolation of microcrystalline cellulose through mineral acid hydrolysis are illustrated in figure 2.6.

Figure 2.6 Typical procedure for the isolation of MCC from purified cellulose

2.3.3.1 Alkali hydrolysis

Alkali treatment is the commonly employed method for delignification of lignocellulose biomaterials. The process separate lignin from lignocellulose by disrupting the structural linkage between carbohydrate and lignin. To overcome the disadvantages associated with mineral acid hydrolysis some researchers reported successful isolation of microcrystalline cellulose with alkali treatment (Khalil *et al.,* 2014). For instance, Trusovs, prepared cellulose microcrystals by treating cellulose with an alkaline solution, followed by treatment with hydrogen peroxide to depolymerize

the cellulose. The depolymerized cellulose was then filtered and later made neutral, washed and dried. The method is safe, economic and eco-friendly but very difficult to control to avoid the undesirable degradation of cellulose to obtain intact microcrystals (Trusovs, 2002; Nguyen, 2006).

2.3.3.2 Steam Explosion Technology

Steam explosion is promising technique for mechanical pulping and was first utilized by De Long for the production of microcrystalline cellulose. He treated lignocellulose material with steam in two steps. In the first stem, cellulose with low degree of polymerization (DP) was archived. The obtained cellulose was then treated with second steam in the presence of a strong acid (Jackquet *et al.*, 2012; Delong 1987). However, Ha and Landi, successfully derived cellulose microcrystals by employing steam explosion without assistance of any acid in just single step. During the procedure, the lignocellulose source was introduced into a pressurized reactor and under controlled conditions mcc were prepared by steam explosion. It is thought that shearing forces, steam heat and production of an organic acid during the process results in breakdown of glycosidic bonds in amorphous domains of cellulose chains that yield to microcrystalline cellulose (Ha and Landi 1997).

2.3.3.3 Extrusion Technology

Extrusion technology is brief, and high temperature hydrolytic method with no effluents and provides high flexibility. The technique provides an environment of a continuous reactor and combinatorial working of chemical and thermo-mechanical system for microcrystalline preparation (Lamsal *et al.*, 2010; Merci *et al.*, 2015). Hanna reported isolation of microcrystalline cellulose with rod shape particle and high crystallinity index (70%) from soybean hulls by employing a two-step extrusion process. The process is highly eco-friendly but rarely reported in literature (Hanna *et al.*, 2001).

2.3.3.4 Radiation-Enzymatic Technique

Radiation enzymatic, a rarely explored process is another potential method of microcrystalline production. In a study, mcc was isolated from bleached pulp of mountain spruce using a two-step radiation enzymatic technique. In the first step, the cellulose was irradiated by an electron beam followed by swelling in a chemical solution and washing. The washed sample was then subjected to enzymatic action. At the end the sample was washed and dried to produce mcc. The

microcrystalline cellulose obtained had crystallinity index 64% with degree of polymerization (150). The main drawbacks associated with this methods include low crystallinity of the mcc and too expensive to be viableat at commercial scale (Adel *et al., 2011*).

2.3.3.5 Acid Hydrolysis

Mineral acid hydrolysis is the most commonly employed method for the isolation of cellulose microcrystals. Structural investigation shows that cellulose fibers are composed of millions of micro-fibrils while each micro-fibril consists of two major domains (regions). A crystalline region, which consists of tightly bound chains having linear arrangement and a para-crystalline region, which is disordered, flexible mass of cellulose chains. Thus native cellulose has both crystalline and amorphous regions. Throughout the entire length, cellulose fibers has both alkali and acid sensitive bonds in both the regions. However, these sensitive bonds are not easily accessible in the crystalline domains. During the hydrolysis, the hydronium ion from the acid cleave the bonds in the disordered, amorphous region and thus results in microcrystalline cellulose as illustrated in figure 2.7 (Trache *et al.*, 2016).

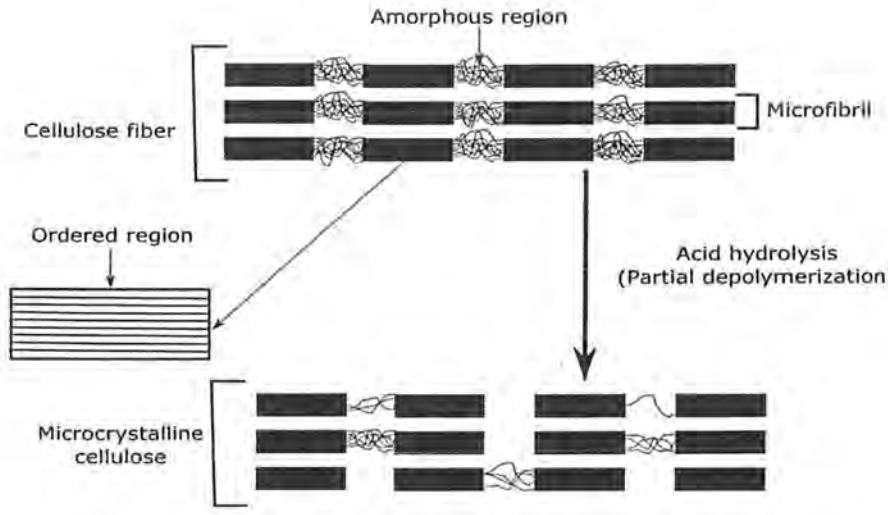

Figure 2.7 Schematic presentation of MCC isolation during acid hydrolysis of cellulose

After completion of the reaction, the obtained MCC paste is washed, neutralized and re-washed to make it free from impurities, followed by drying to obtain the powder (Thoorens *et al.*, 2015). Different researches have investigated the influence of cellulose source, type of acids and processing conditions on the physicochemical and thermo-mechanical properties. The effect of different acidic treatments and processing condition on various cellulose sources is tabulated in the table

Table 2.1 Preparation of MCC from different cellulose sources through acid hydrolysis

Source	Acid	Temp (°C)	Time (h)	Length (μm)	Diameter (μm)	Reference
Alfa fibers	HCL	85	3	20-200	10-20	Trache *et al*, 2014
Jute	CH_2O_2	100	3	--	8.1	Jahan *et al.*, 2011
Rice straw	H_2SO_4	--	.45	3-6	--	Sakhawy *et al.*, 2005
Wheat bran	HCL	80	.5	--	--	Wang *et al.*, 2013
Sago seed shells	HCL	--	12	--	--	Naduparambath 2016
Bean hulls	HCL	100	3	125	86	Adel *et al.*, 2010
Bacterial cellulose	H_2S0_4	--	3	90	30	Oliveira *et al.*, 2011

Study shows that source of the cellulose, type of the acid, reaction conditions including acid to cellulose ration, reaction temperature and reaction time greatly influence the physico-mechanical and thermal properties of microcrystalline cellulose. The most typical acid used for hydrolysis is hydrochloric acid (Wang *et al.*, 2013; Naduparambath, 2016). For instance, in a study by Trache and his colleagues, microcrystalline cellulose with length 20-200 μm and diameter 10-20 μm were prepared using hydrochloric acid at 85 °C with reation time of 2 hours. Apart from typical used hydrochloric acid, few studies have also reported the use of sulfuric acid and formic acid (Oliveira *et al.*, 2011; Jahan *et al.*, 2011). In a study by Sakhawy and Hassan, the effect of sulfuric acid and hydrochloric acid was evaluated on cellulose derived from bagasse and rice straw. The studied concluded that both hydrochloric acid and sulfuric acid can be used to isolate microcrystalline cellulose from the agricultural residues, however, type of the acid effect particle size, thermal stability, cohesiveness and tensile strength (Sakhawy *et al.*, 2005).

Beside simple hydrolysis method, other methods that use hydrolysis in combination with other processes were also explored to produce cellulose microcrystals. For example, MCC was prepared by a single step acid hydrolysis in combination with bleaching process. In this method, Schaible and Sherwood hydrolyzed the pulp from the southern pipe by 2N HCl +H_2SO_4 and 0.2 M ozone for 60 minutes at boiling temperature. After cooling, the product was filtered, washed and then finally lyophilized. The authors believed that eliminate the multiple steps of bleaching and hydrolysis and combined both the process in a single step to achieve high grade cellulose microcrystals (Schaible and Sherwood, 2003).

Though the preparation of microcrystalline cellulose from various lignocellulose by acid hydrolysis is still in the progress of perfection yet it has some pitfalls. The main drawbacks associated with the process include high cost, high reagents consumption, corrosiveness, hazards, and treatment of effluents is needed to avoid the water pollution (Trache *at al.*, 2016).

2.3.4 Applications

The three major areas where microcrystalline cellulose is used for diverse applications including bio-composites, biomedical and in food industries (figure 2.8)

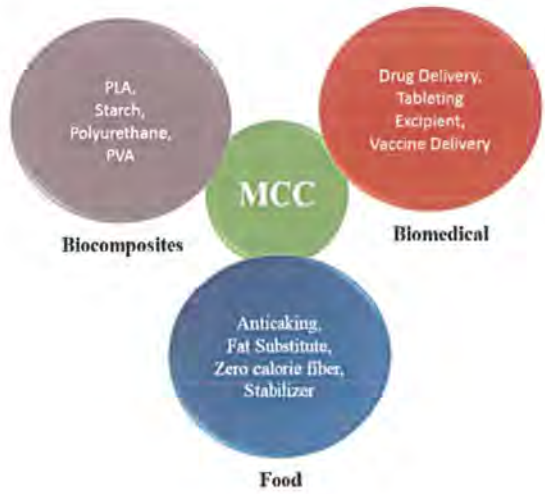

Figure 2.8 Graphical illustration of microcrystalline cellulose applications

2.3.4.1 Bio-composites

The potential of microcrystalline cellulose as a reinforcing agent in composite materials is promising sectors. Such bio composites are utilized in automotive, construction, building and household products. Such an approach of using renewable and sustainable materials will overcome many of the environmental concerns. High surface area, crystallinity, light weight in combination with excellent mechanical properties, thermal stability and biodegradability make cellulose microcrystals as attractive reinforcement agent. Microcrystalline cellulose is used as filler in different composite with poly lactic acid (PLA), polypropylene, polyurethane, rubber, nylon, poly(D,L-lactide) and starch (Mathew *et al.*, 2005; Haafiz *et al.*, 2013; Spoljaric *et al.*, 2009; Wu *et al.*, 2007; Bai and Le 2009; Kiziltas *et al.*, 2011; Rico *et al.*, 2016; Area *et al.*, 2019).

Biodegradable composites were prepared by using polylactic acid (PLA) as matrix and microcrystalline cellulose isolated from acid hydrolyzed wood pulp. Thermal analysis revealed that the composite showed improved storage modulus (Mathew *et al.*, 2005). Similarly in another study, MCC/PLA composites were prepared with microcrystals derived from oil palm biomass. The authors reported improved thermal stability and mechanical properties of the composite (Haafiz *et al.*, 2013).

In another study, high strength composites of microcrystalline cellulose were successfully prepared in a polyurethane matrix. The composite exhibited enhanced strength and strain as compared to normal polyurethane (Wu *et al.*, 2007).

MCC has the potential to significantly reduce the energy requirement for dispersion during composite preparation. Bai and Li reported that by replacing silica with cellulose microcrystals dispersion energy for rubber composites can be reduced many fold. The found that partial replacement of silica up to 18% not only reduce energy but also improves thermal stability and keeping the mechanical properties of rubber composites intact (Bai and Li, 2009).

2.3.4.2 Biomedical Applications

After discovery by Battista and Smith in 1955, FMC Corporation for the first time commercialized microcrystalline cellulose with brand name Avicel™ and in 1964 FMC introduced Avicel PH™ as

material for direct compression tableting for pharmaceutical industries. Beside ingredient for tableting, microcrystalline cellulose has been studied for controlled drug release, carrier for vaccines and as antibacterial agent.

In a study by Jeon and his colleagues, microcrystalline cellulose (Avicel PH-101) was used as delivery vehicle for recombinant protein against Erysipelas. The cellulose binding domain-surface protecting antigen (CBD-SpaA) was bound to the surface of microcrystals. The adsorption was then evaluated for immunogenicity by enzyme linked immunosorbent assay (ELISA) (Jeon *et al.*, 2018).

In another study, microcrystalline cellulose was evaluated for the adsorption of various drugs. The results concluded that microcrystals exhibited less adsorption capacity for drugs like chlorpheniramine, diphenhydramine, isoniazid and paminobenzoic. However, adsorption capacity for phenothiazine derivatives like acrinol was quite large. High affinity of those drugs was attributed majorly to ion-exhange mechanaism and appreciable non-electrostatic forces between the drugs and microcrystals (Okada *et al.*, 1986).

A novel form of microcrystalline cellulose AaltoCell™, was investigated for drug release and enzyme immobilization. The well characterized gel-like matrix constituting AaltoCell™ remained stable under various physiological conditions and shown the potential for controlled release of metronidazole and lysozyme (Dong *et al.*, 2018).

Moreover, surface modification of microcrystalline cellulose may improve its biological properties. For instance, physicochemical properties, antibacterial and antioxidant capacity were shown to improve by surface modification of cellulose microcrystals by sodium periodate ($NaIO_4$) (Zhang *et al.*, 2017).

2.3.4.3 Food Industry

Microcrystalline cellulose with size up to 30 μm, is used in food industry in both powdered and colloidal form. Powdered grade microcrystalline cellulose is used as zero calorie bulking agent and high quality fiber source in food products. Being flavorless and without any flavor masking properties, cellulose microcrystals are employed in dietetic drinks as fibers source. Recently,

colloidal microcrystalline cellulose was studied as fat substitute and stabilizer material in emulsified products (pate) and cooked meats (sausages). Colloidal MCC are also utilized breadings and batters, chocolates bars, low fat sour cream, frozen desserts and ice-creams because of various benefits that it offers. The advantages include reduction of drying time, low fat absorption during frying, clinging improvement, emulsion stabilization, improves stability and leads smaller ice crystals. On the other hand powdered grade microcrystalline cellulose finds its applications in puffed snacks, confectionaries and high fiber drinks because of numerous benefits like improve homogeneity, source of dietary fibers and non-nutritive bulk filler (Imeson 2011; Wuestenberg 2014).

2.3.5 Toxicity

Toxicity is the extent to which a substance/material harm a living organism. A substance may effect an organism at different levels i.e. at cell (cytotoxicity), organs (e.g. hepatoxicity) and organism as a whole. Generally, methods for evaluation of cytotoxicity are grouped in three categories including 1) methods assessing cell growth 2) methods that assess cell damage 3) methods that asses metabolic activities (Roman, 2015).

Toxicity of microcrystalline has been rarely reported in the literature. Most of the data that exists in the literature refers to Avicel and its derivative series, from wood pulp as a primary source. The studies showed that Avicel exhibited no adverse effects both *invitro* and *invivo* (Kotkoskie *et al.*, 1996; Murli 1994; Mckeon, 1992; Younes *et al.*, 2018).

For instance, a subchronic toxicity was performed by Kotkoskie to evaluate the possible toxic potential of microcrystalline cellulose. Microcrystalline cellulose manufactured by FMC with micron size 6μm were fed to Sprague-Dawley rats for 90 days (up to 5000mg/kg/day). The study concluded no observed adverse effect level (NOAEL) for the highest concentration tested.

A couple of study conducted in the 90s also demonstrated that the microcrystals has no genotoxic potential. An invivo mammalian micronucleus assay on bone marrow polychromatic erythrocytes of mice revealed that Avicel (FMC) did not possess any genotoxic potential (Murli 1994).

Moreover, recently, a detailed study was conducted by Younes and his colleagues for the reevaluation of biosafety of microcrystalline cellulose on the request of European Commission, the EFSA Panel on Food Additives and Nutrient sources added to food (ANS). The pilot study did not found any potential harmful effects of microcrystalline cellulose added to human diet and in various food products (Younes *et al.,* 2018).

However, majority of the toxicological study conducted on microcrystalline cellulose are FMC supported and financed. Moreover, the effect of source and the type of acid has not been evaluated since the source and acid highly effect the physico-chemical, mechanical, morphological and thermal properties of the prepared microcrystals.

Chapter 03

Materials and Methods

MATERIALS AND METHODS

The present study was accomplished under the supervision of Dr. Muhammad Zia. Practical work was performed in Department of Biotechnology and Department of Pharmacy (Quaid-i-Azam University, Islamabad). Characterization of the material was carried out in the Department of Chemistry (Quaid-i-Azam University, Islamabad), Department of Physics (Allama Iqbal Open University, Islamabad) and Institute of Space Technology (IST), Islamabad.

3.1 Materials

The materials used in the current study broadly includes chemicals and reagents, apparatus and equipment, microbial strains, freshly isolated human red blood cells (hRBCs), human white blood cells (hWBCs) and brine shrimps.

3.1.1 Chemicals and reagents

Cotton wool was bought from local market (Kohinoor Textile Mills Limited). Sulfuric Acid (H_2SO_4), Hydrochloric Acid (HCL) and Nitric Acid (HNO_3) were bought from Sigma Aldrich (Sigma-Aldrich Germany). DMSO were purchased from Sigma (Sigma-Aldrich, Germany); Distilled water used in the process was prepared freshly; dried yeast and phosphate buffer saline (PBS) were acquired from Riedel-de-Haen (Germany), nutrient agar, standard antibacterial drug (Cefixime, Roxithromycin), standard antifungal drugs (Clotrimazole) and Doxorubicin were also purchased from Sigma (Sigma-Aldrich).

3.1.2 Apparatus and equipment

Beakers, Erlenmeyer flasks, Funnels, Sonicator (Sweep Zone technology, USA), Micropipette (Sartorius France) and Pasteur pipette, Centrifuge (B. Bran, Germany); Freezer 9170 WB M (Dawlance, Pakistan), Incubator IC83 (Yomato, Japan); CO_2 incubator MCO-17AIC (Sanyo, Japan); Microplate reader ELX 800 (Biotek, USA); 96 well plate (SPL life science, Korea); Vernier caliper; X-ray diffractometer (Brucker, USA); Scanning electron microscope (Teskon, Czech Republic); Fourier transform infrared spectroscope (Brucker, USA).

3.1.3 Microbial strains

Bacterial strains:

Gram negative strains: The gram negative bacteria used in the experiment included *Escherichia coli* (ATCC25992) and *Klebsiella pneumoniae* (ATCC# 4617).

Gram positive strains: The gram positive bacteria used were *Staphylococcus aureus* (ATCC-6538), *Pseudomonas aeruginosa* (ATCC-15442), *Bacillus subtilis* (ATCC-6633).

The bacterial test strains were cultured and maintained on nutrient agar slants followed by incubation at 37°C while the stock cultures were maintained at 4°C.

Fungal strains:

The fungal strain used in the experiment included *Aspergillus flavus* (FCBP 0064), *Aspergillus fumigatus* (FCBP 66), *Aspergillus niger* (FCBP 0198), *Fusarium solani* (FCBP 0291) and *Mucor* species (FCBP 0300).

The fungal test strains were cultured and later on maintained on Sabourad dextrose agar (SDA) followed by incubation at 28-30°C while stock cultures were maintained at 4°C.

3.1.4 Brine shrimps

Brine shrimp eggs were purchased from Ocean star Int., USA and were stored at freezing temperature.

3.1.5 Human red blood cells (hRBCs) and white blood cells (hWBCs)

Red blood cells and white blood cells (macrophages) were isolated from freshly drawn blood of a healthy volunteer with an informed consent.

3.2 Methods

The research method outlined in the current study can be simply be categorized into three steps i) preparation of microcrystalline cellulose ii) characterization of the prepared microcrystals and iii) Toxicological profiling.

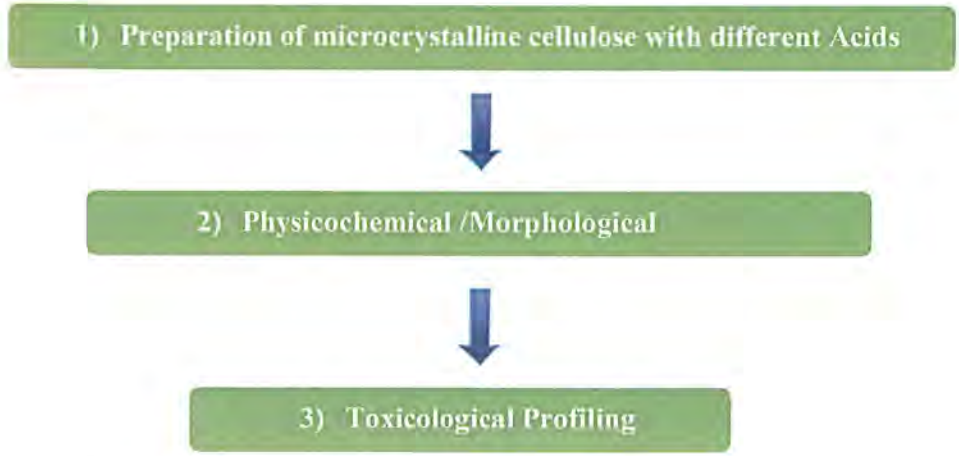

Figure. 3.1 Graphical representation of the generalized steps followed in the study

3.2.1 Preparation of microcrystalline cellulose

H_2SO_4-MCC were prepared by treating cotton wool with 5 M H_2SO_4 for 20 hours at 45 °C, with moderate agitation on magnetic stirrer. Hydrolysis reaction was terminated by addition of 10 fold ice cold water. The resultant suspension was centrifuged at 10,000 rpm for 10 min followed by subsequent centrifugations with distilled water. The resultant acid free sediment was collected and neutralized by NaOH solution and re-washed thrice with distilled water. The acid-free, washed suspension was then subjected to ultra-sonication for 10 minutes followed by oven drying at 105 °C. The dried material was then gently grind in pastor and mortar into fine powder. HCL-MCC and HNO_3-MCC were prepared following similar procedure instead the reaction temperature was maintained at 55 °C.

3.2.2 Characterization of microcrystalline cellulose

The fine powder obtained was used for physico-chemical characterization, biocompatibility and microbial toxicity evaluation.

3.2.2.1 Yield

The percent yield of the prepared cellulose microcrystals was calculated by placing the acid free, neutralized and washed suspension of respective mcc in a weighing bottle in a drying oven. The sample was dried at 105 °C until a constant weight was achieved. After cooling at room temperature, weight of the samples were measured in analytical balance to calculate the % yield.

$$\text{Yield (\%)} = \frac{m}{M} \times 100$$

Where m is the weight of the mcc obtained and M is weight of the cotton wool before hydrolysis reaction. Large clumps and non-hydrolyzed fibers, before and after drying were carefully removed before weighing the mcc samples.

3.2.2.2 Dispersion studies

The dispersions of the prepared microcrystals were probed visually by observing the stability of dispersions prepared in dH_20 with varying pH: 2, 7, 9, 12 as function of storage time. Briefly, 10mg/ml of microcrystals in a corresponding solvent were suspended in a glass vial (each 7ml) after brief sonication. Pictures were taken immediately after sonication and subsequently after 1 h and 24 h.

3.2.2.3 X-ray diffraction (XRD)

X-ray diffraction patterns of the prepared samples were measured using an X-ray diffractometer (PANalytical Xpert Pro MRD Diffractometer, Amsterdam, Netherlands). The samples were scanned over the range of diffraction angle of $2\theta = 10°–80°$ with a scanning rate of 0.4°/min at room temperature. The crystallinity index (CI) was calculated according to the Segal empirical method (Segal *et al.*, 1959).

$$\text{Crystallinity index } \% = 100 \times \frac{I200 - Iam}{I200}$$

where I_{200} is the intensity of the 200 peak (at $2\theta = 22°$) and I_{am} is the intensity corresponds to the amorphous structure which showed the lowest intensity value between 22° and 16° ($2\theta = 18°$).This method is easily implemented with data from a powder diffractometer and is still widely used as it constitutes a fast and easy tool to analyze and compare qualitatively different cellulose structure..

3.2.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a fascinating technique to evaluate structural variations on samples due to chemical treatments. The functional groups on the cotton fibers and microcrystalline cellulose were investigated by FTIR spectroscopy using a Nexus Thermo FTIR (ThermoNicolet, USA) spectrophotometer. The well dried samples mixed with KBr in a ratio of 1:200 (w/w) and pressed under vacuum to form pellets. The FTIR spectrum of the samples was recorded in the transmittance mode in range of 4000-500 cm-1.

3.2.2.5 Scanning Electron Microscopy (SEM)

The surface morphology and dimensions of the samples were examined using scanning electron microscope (SEM) (Tescan, Czech Republic) with an accelerating voltage of 15 –20 kV. Fine layer of gold was sprayed on samples by an ion sputter coater with a low deposition rate before investigating the samples. Images showing surface morphologies and dimensions of the samples were taken at various magnifications.

3.2.3 Toxicological profiling

The prepared microcrystalline cellulose was evaluated for compatibility against freshly isolated human red blood cells and macrophages. Cytotoxicity was probed against brine shrimps *(Artemia Salina)*. Moreover, growth inhibition potency was evaluated against bacterial and fungal strains.

3.2.3.1 Biocompatibility against human RBCs (hRBCs)

To determine the toxic nature of MCCs against isolated human red blood cells (hRBCs), a hemolytic assay previously established (Malagoli, 2007) was performed. Briefly, fresh blood

(about 5mL) was collected from a healthy individual and dispensed into a sterile EDTA tube. 1 mL blood was centrifugation (13000 rpm for 10 min) for the separation of RBCs. For the preparation of erythrocytes suspension in phosphate buffered saline (PBS), 200 µL of the pelleted erythrocyte were added to 9.8 mL of (PBS, pH: 7.4) followed by gentle shaking. In separate eppendorf tubes, erythrocyte suspension (100 µL) and test sample (100 µL) of different concentrations were gently mixed, and incubated for one hour at 35 ^{0}C. The mixture was further centrifuged at 12,000 rpm for 10 min and supernatant was collected. The hemoglobin release was monitored using micro-plate reader (BIOTEK) at 540 nm after dispensing the supernatant in the 96 well plate. Triton X-100 and distilled water were used as positive and negative controls respectively. Results were calculated as percentage hemolysis induced by cellulose microcrystals dilutions calculated through the following equation.

$$\% \text{ Hemolysis} = \left[\frac{\text{ABs} - \text{ABnc}}{\text{ABpc} - \text{ABnc}}\right] \times 100$$

Where ABs is the absorbance of the tested sample and ABnc and ABpc depicts absorbance of negative and positive control respectively.

3.2.3.2 Biocompatibility against human WBCs (hWBCs)

For further assessment of biocompatibility of MCCs against isolated human white blood cells (hWBCs), Ficoll–Gastrografin (sodium diatrizoate) method was followed. The cytotoxicity was inspected against human macrophages isolated from peripheral human blood. This isolation protocol is based on ficoll-gastrografin density gradient (density=1.070 g/mL) (De Almeida *et al.*, 2000; Fatima *et al.*, 2015). Briefly, in the mixture of 95 mL of deionized water and 5 mL of gastrografin, ficoll (5.7 g) was gradually dissolved. Hank's buffer salt solution (HBSS) was used for the dilution of blood which was layered gently on the ficollgastrografin. The solution was centrifuged for 30 min at 400g followed by purification with percoll gradient (density 1.064 g/mL) adjusted with sterilized deionized water. The suspension of isolated cells was made in RPMI medium supplemented with Hepes (25 mM), fetal bovine serum (10 %) and antibiotics (penicillin:100 U/mL; Streptomycin: 0.1 mg/mL). The isolated macrophages were cultured to the

density of 1 × 105 cells/well in humidified incubator with 5 % CO_2. Percentage inhibition was calculated using the formula;

$$\% \text{ inhibtion} = \left[1 - \left\{\frac{\text{ABs}}{\text{ABc}}\right\}\right] \times 100$$

Where ABs is the absorbance of test sample and ABc is the absorbance of control.

3.2.3.3 Brine shrimp *(Artemia Salina)* lethality assay

A 24 h lethality test, previously reported protocol was followed for assessing the cytotoxic potential of cellulose microcrystals against brine shrimp (*Artemia* salina) larvae (Bibi *et al.* 2011). In brief, a specially designed, perforated, bi-compartment plastic tank was filled with simulated sterile seawater (34g sea salt per liter supplemented with dried yeast 6mg/L). *Artemia salina* eggs (Ocean Star International) were poured in sufficient amount in one of the compartments covered with aluminum foil. The eggs were allowed to hatch at 30°C for 24 h. After incubation period, the hatched shrimps (nauplii) move towards the light source placed over the uncovered compartment of the tank. The mature phototropic nauplii were collected with Pasteur pipette and were transferred to a beaker having artificial sea water from where motile naupli were to be transferred to each well of the plate. Each well to be used in the assay received 150 µl of sea water and 10 mature nauplii. Different dilutions of test samples were then introduced to the corresponding wells to have respective concentrations (200, 100, 50 and 25µg/mL). Lastly, sea water was used to make the final volume of each well up to 300 µl. In the experiment, DMSO alone was used as a negative control while Doxorubicin served as a positive control. After incubation period of 24 h at 37°C, the number of dead nauplii were counted in each of the wells. Percent mortality was calculated using the following formula:

$$\% \text{ mortality} = \frac{\text{number of dead shrimps}}{\text{number of alive shrimps}} \times 100$$

3.2.3.4 Antibacterial assay

Agar well diffusion method as documented previously was used with some modifications for the assessment of in vitro antibacterial potential of microcrystals against different bacterial strains (valgas *et al.*, 2007) According to the test specification (1×106 CFU/ml) seeding density of the bacterial culture was adjusted. To prepare lawn on the nutrient agar plates 50 µl of refreshed

cultures in nutrient broth were used. Each well was carefully filled with (10 µl) of test sample. Accordingly, the seeded plates were also labeled. Cefixime and Roxithromycin (standard antibiotics) acted as positive controls while DMSO served as negative control. Incubation was done at 37°C for 24 hours. After incubation each well loaded with tested samples and controls were checked for the appearance of ZOI (zone of inhibition). The diameter of zone of inhibition was measured with a vernier caliper to the nearest mm.

3.2.3.5 Antifungal assay

Antifungal potency of microcrystalline cellulose was evaluated by disc diffusion method some modifications (Magaldi *et al.*, 2004). 100 µl of spore suspension was taken for each fungal strain from Tween 20 solution (0.02 % v/v) and swabbed on separate petri plates containing sterile SDA media. Each tested sample (10 µl) were applied to the sterilized filter paper discs which were then retained on the seeded agar plates. The seeded plates were also labeled accordingly. Clotrimazole was used as positive control while DMSO acted as negative control. For fungal growth, the plates were then incubated at 28°C for 24-48 hours. After incubation, each loaded disc with tested samples and controls were checked for the appearance of ZOI (zone of inhibition). The diameter of ZOI was measured by using a vernier caliper to the nearest mm.

Chapter 04

Results

RESULTS

4.1 Preparation of microcrystalline cellulose

The hydrolysis of the cotton wool fibers with H_2SO_4, HCL, and HNO_3, at high temperature and vigorous shaking resulted in white suspension of microcrystalline cellulose. The subsequent steps of centrifugation, neutralization and drying resulted in an acid free, dried white powder of cellulose microcrystals with pH 7 ±1 (Figure 3.1).

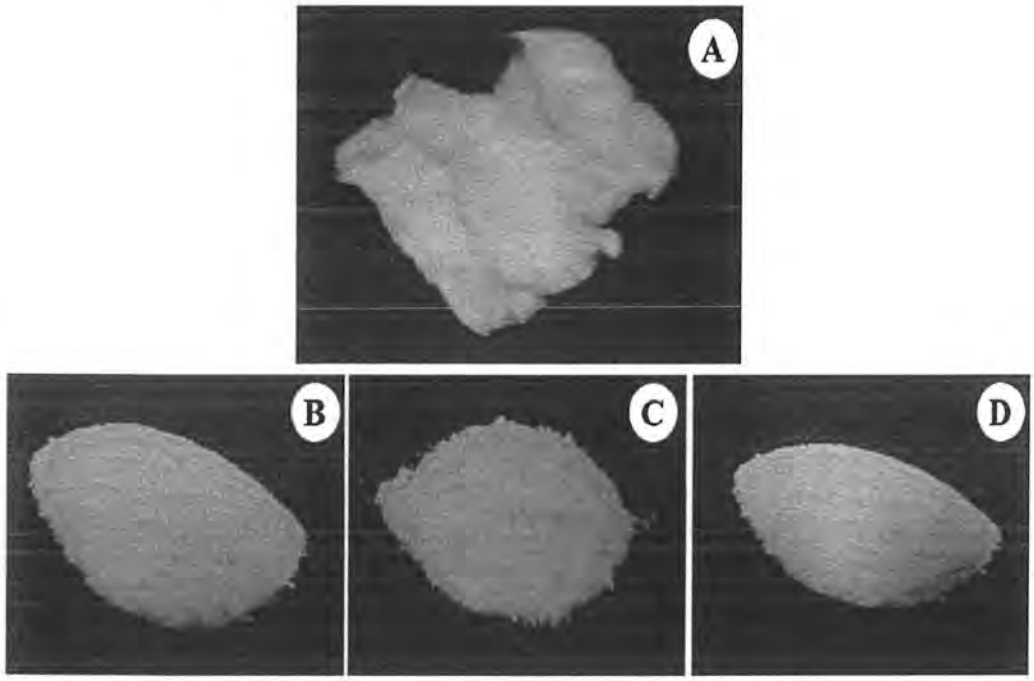

Figure 4.1 Pictorial presentation of cellulose source and prepared MCC; **A)** Untreated cotton wool (CW) fibers **B)** H_2SO_4-MCC **C)** HCL-MCC **D)** HNO_3-MCC

Large clumps and non-hydrolyzed long fibers were carefully removed before and after drying the samples. The obtained powder was stored in air-tight glass vials and were labelled as H_2SO_4-MCC, HCL-MCC, and HNO_3-MCC. Finally the vials were stored at room temperature for further use.

4.2 Yield calculation

Yield of the MCC obtained from any cellulose source is greatly influenced by origin of the source, type of the acid used and preparation conditions (acid to cellulose ratio, temperature and time). The yield (%) of the well dried MCC obtained from cotton wool through various acidic treatments is shown in table 4.1.

Table 4.1. % Yield of MCC obtained after different acid treatments

Cotton wool	Acid	Cotton: Acid	Temp	Time	MCC	% Yield
1.0 g	H_2SO_4 (5 M)	1:300	45 °C	20 h	0.64 g	64
1.0 g	HCL (5M)	1:300	55 °C	20 h	0.73 g	73
1.0 g	HNO_3 (5M)	1:300	55 °C	20 h	0.58 g	58

Note: M = Molar, cotton: acid represents cotton to acid ratio (g:ml), Temp simplifies temperature.

The table shows that under the experimented conditions, Hydrochloric acid (HCL) hydrolysis resulted in maximum yield (73 %) of MCC. While H_2S0_4 and HNO_3 yielded 64% and 58 % MCC respectively. The experimental conditions were retained same for different acidic treatments except the reaction temperature for H_2SO_4 hydrolysis. It was observed that beyond 45 °C, H_2S0_4 start to degrade cotton fibers as could be seen from the color change (dark brown). Overall, the yield of the prepared microcrystals decreased with the following trend: **HCL-MCC > H_2SO_4-MCC > HNO_3–MCC.**

4.3 Dispersion stability

The dispersion stability of microcrystalline cellulose is an important parameter in the preparation of green composites. The dispersion of the crystals highly depends on aspect ratio, surface chemistry of the particles and the ability of the solvent and surface groups to counterbalance the attractive hydrogen-bond interactions exerted by abundant hydroxyl groups. Figure 4.2 shows the photographs, illustrating aqueous suspension stability cellulose microcrystals with varied pH.

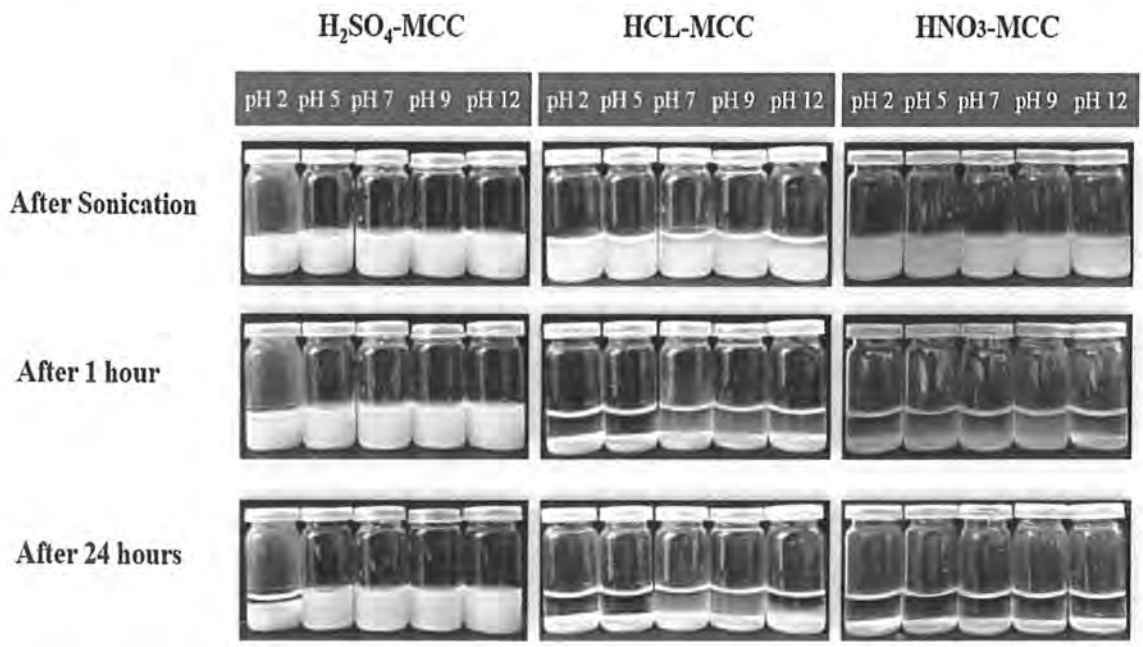

Figure 4.2 Pictorial presentation of aqueous dispersion studies; illustrating H_2SO_4-MCC, HCL-MCC and HNO_3-MCC dispersions in deionized water at various pH values, as function of time storage.

As can be seen in figure 3.2, that H_2SO_4-MCC exhibited high dispersion stability in aqueous medium with varied pH as compared to HCL-MCC and HNO_3-MCC. Even after 24 hours of sonication, the sulfuric acid isolated microcrystals maintained excellent suspension stability except at pH 2, where little phase separation can be seen. The dispersion stability is attributed to the negative charged sulfate groups on the surface of microcrystals which are conferred upon during hydrolysis. Contrary to H_2SO_4-MCC, HCL-MCC and HNO_3-MCC demonstrates very weak or no dispersibilty at any of the pH employed. Contrary to H_2SO_4-MCC, HCL-MCC and HNO_3-MCC started to flocculate after sonication and gradually settled at the bottom of the glass vials. The sedimentation was more prominent in case of HNO_3-MCC whereas HCL-MCC shown very weak dispersion ability after 24 hours at neutral pH. Overall, the aqueous dispersion stability of the microcrystalline cellulose follows the as mentioned trend: **H_2SO_4-MCC > HCL-MCC > HNO_3 – MCC.**

4.4 Morphological study

Scanning electron microscope (SEM) was used to study the morphology and dimensions of the obtained microcrystalline cellulose.

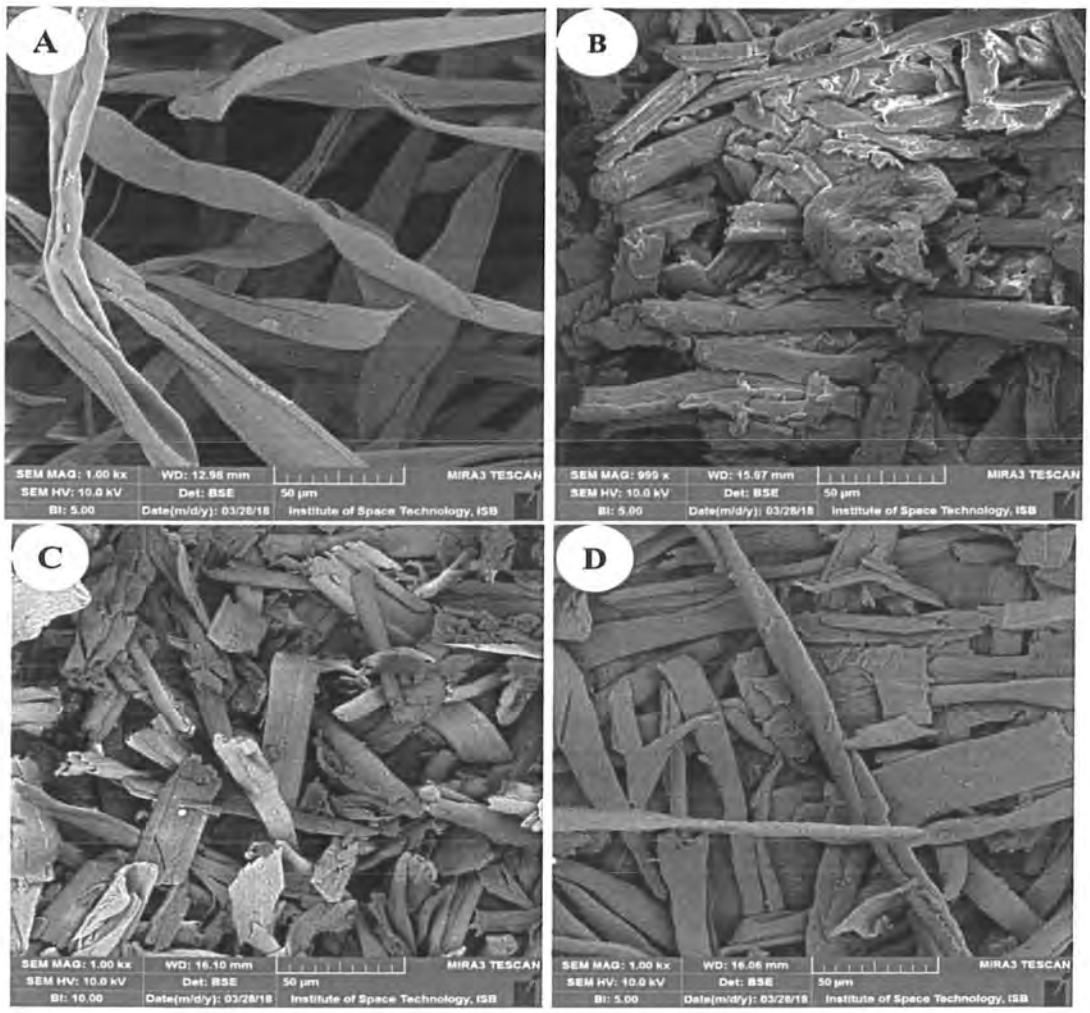

Figure 4.3 Typical scanning electron micrographs of cellulose source and prepared MCC; **A)** cotton wool fibers **B)** H_2SO_4-MCC **C)** HCL-MCC **D)** HNO_3-MCC

Fig 3.3 illustrates successful disintegration of cotton wool fibers into microcrystals after different acidic treatments. Fig 3A shows morphology of the original wool fibers, each having a smooth, curled and compact structure, typical of a lignocellulose fiber. The curliness increases the surface

area and makes the fibers more reactive (Morais *et al.*, 2013). To produce microcrystals, cellulose has to be treated with an acid. The micrographs (Fig. 3B, Fig. 3C and 3D) shows the level of morphological and dimensional changes after hydrolysis with H_2SO_4, HCL and HNO_3. Generally, the isolated microcrystals displayed rod like shaped particles with swelled and rough morphology. Similar results were also reported by other authors (Elanthikkal *et al.*, 2013; Adel *et al.*, 2011). The microcrystals are the result of preferential cleavage of cellulose fibers at amorphous domains during hydrolysis reaction.

Table 4.2 Morphology and dimensions of the prepared MCC

S No	Samples	Length *(l)* (μm)	Diameter*(d)* (μm)	*(l/d)*
1	H_2SO_4-MCC	40.46±28	9.74±4.23	4.1
2	HCL-MCC	65.25±30.35	13.25±4.45	4.9
3	HNO_3-MCC	76.00±59	10.20±4.1	6.9

Note: The values represented here are mean of more than 50 particles calculated with ImageJ (software) in each SEM micrograph. *(l/d)* refers to aspect ratio.

The average length and width of H_2SO_4-MCC were 40.46 μm (ranging from 10 to 160) and 9.74 μm (ranging from 4 to 15) with mean aspect ratio (l/d) 4.1. In case of HCl hydrolysis, obtained microcrystals had average length 65.25 μm (ranging from 21 to 140 μm) and width 13.25 μm (ranging from 7 to 20) with average aspect ratio of 4.9. HNO_3 being weak acid, yielded much longer cellulose microcrystals with average length 76 μm (ranging from 21 to 250 μm) and width 10.2 (ranging from 5 to 19 μm) with aspect ratio 6.9. The aspect ratio of the prepared microcrystals, decreased with the following trend: **HNO_3-MCC > HCL-MCC > H_2SO_4-MCC.**

4.5 Crystallinity

The effect of different acidic treatments on the crystallinity of the prepared microcrystals was investigated by X-ray diffraction patterns. The X-ray diffraction spectra of the samples are shown in fig 3.4.

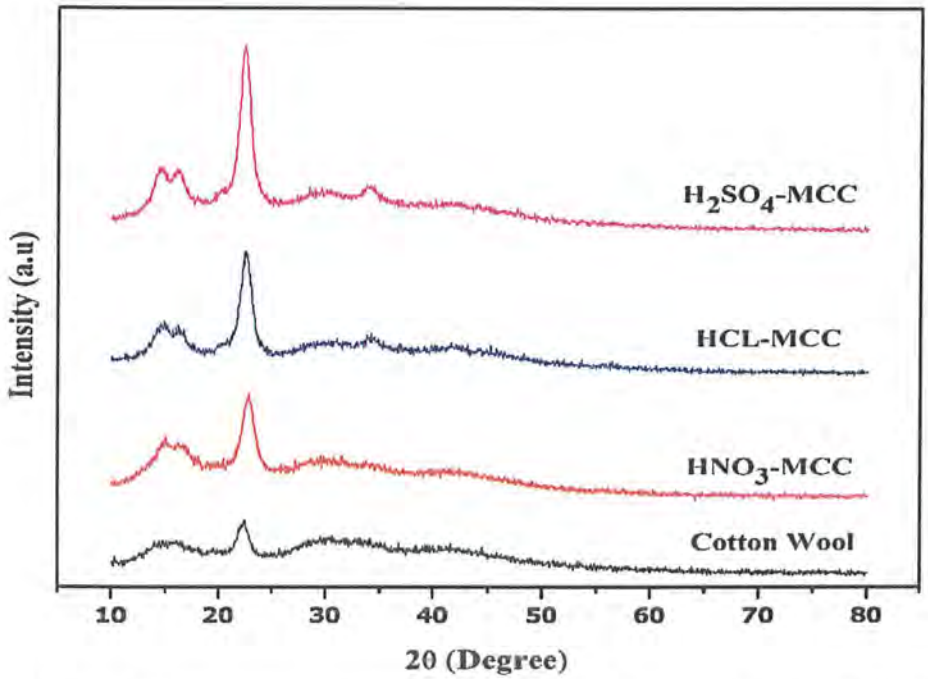

Figure 4.4 X-ray diffractograms of Cotton wool, HNO_3-MCC, HCL-MCC, H_2SO_4-MCC

As can be seen from the diffractograms, all the samples display characteristic peaks at 16° and 22°, representing typical cellulose 1 structure (Pachuau *et al.*, 2019; Naduparambath and Purushothaman 2016; Chandra *et al.*, 2016; Haafiz *et al.*, 2013). The crystalinity index (CI) of the engineered microcrystals calculated by segal equatiom are given in table 3.4.

Table 4.3 Crystallinity Index (CI) of the prepared MCCs from cotton wool

S. No	Samples	Crystallinity Index (%)
1	Cotton wool	49.80
2	H_2SO_4-MCC	80.57
3	HCL-MCC	75.55
4	HNO_3-MCC	61.40

It is well known that cellulose fibers contain both crystalline and amorphous regions. During acid hydrolysis, the hydronium ion from the acid penetrates into weak, disordered and amorphous regions of the fibers and encourages hydrolytic cleavage of the glycosidic bonds. As can be seen

from the table, the type of acid affects the crystallinity of microcrystalline cellulose. Sulfuric acid resulted in highly crystalline (80.57 %) cellulose microcrystals as compared to the cotton wool (49.8). Between HCL and HNO_3, high crystalline particles were obtained by HCL while HNO_3-MCC had 61.4 % crystallinity. Overall, crystallinity of the materials decreased in the following order: **H_2SO_4-MCC > HCL-MCC > HNO_3-MCC > Cotton wool.**

4.6 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) was used to investigate chemical changes due to different acidic treatments on cotton wool fibers. FTIR spectra of the raw, H_2SO_4, HCL and HNO_3 treated cotton wool are presented in figure 3.

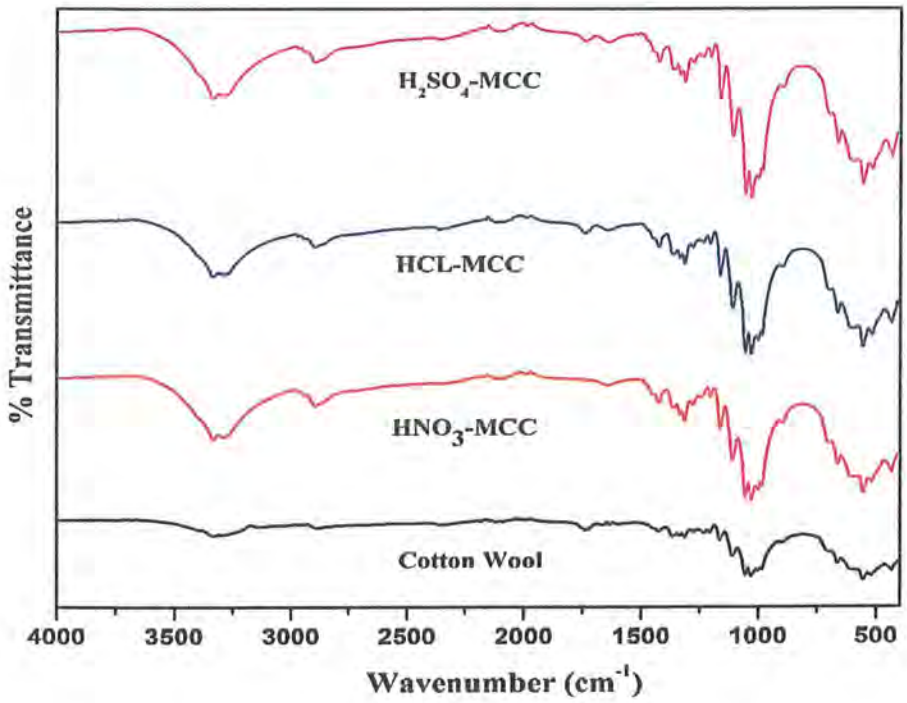

Figure 4.5 FTIR spectra of cotton wool, HNO_3-MCC, HCL-MCC and H_2SO_4-MCC

All the samples shown two main absorbance regions. One that ranges from 600 from 1200 cm^{-1} and the second region that ranges from 2700-3700 cm^{-1}. The absorption bands in the range 3319-3402 cm^{-1} and 2956-2990 cm^{-1} found in the spectra of all the samples represents O-H stretching vibrations of the hydroxyl groups and C-H groups of cellulose (Avolia *et al.*, 2012; Chandra *et al.*,

2016). The peak appearing at ~1108 cm^{-1} is due to C-O-C stretching vibrations of the β (1,4) - glycosidic bonds between glucose units in the cellulose chain. The peak intensity increased with respective acidic treatments (Marchessault, 1962). The absorption at 1644 cm^{-1} to 1634 cm^{-1} present in all the spectra is related to vibrations of O-H groups of waters adsorbed by the polymer. In the CW spectra, a peak at 1161 cm^{-1} was observed which is related to the C-C bond vibration in the carbon skeleton. The peak present at 1730–1740 cm^{-1} in the in the raw fibers could be due to the presence of C-O linkage, which is a characteristic group of lignin and hemicellulose, at 1765–1715 cm-1. (Abraham *et al.*, 2011). Additionally, all the acidic treated spectra shown peak intensity at 1737 cm^{-1}, which is attributed to the vibration of C=O group of ester that was formed after hydrolysis process. The peaks observed at 1427cm^{-1} in all the spectra corresponds to symmetric bending of CH_2 and in the range 1334^{-1} 1360 cm-of cotton wool (CW) spectra were attributed to the bending vibrations of the C–H and C–O groups in the aromatic rings of cellulose (Janoobi *et al.*, 2011). Finally, the absorbance peaks observed in the 1108–1161 cm-1 range were attributed to C–O stretching and C–H rocking vibrations of the pyranose ring skeleton (Alemdar and Sain, 2008). Moreover, the absence of the 1,245 cm-1 peak in acid derived samples, suggests the effective removal of hemicelluloses in the treated fibres The differences between the FTIR spectrum of cotton wool fibers and microcrystalline cellulose indicates the successfully isolation of MCCs from cotton wool by acid hydrolysis treatment.

4.7 Biocompatibility against human red blood cells (hRBCs)

Cellulose is considered as non-toxic and biocompatible material (Thakur *et al.*, 2013; Credou and Berthelot, 2014). However, physicochemical and morphological characteristics of micro and nano-materials are different from their bulk materials (Khan *et al.*, 2017). To evaluate the possible toxicological potential, engineered MCCs were tested for biocompatibility with human red blood cells (hRBCs), freshly isolated human macrophages. Red blood cells hemolytic assay was performed to test the cytocompatibility of the prepared samples. In hemolytic assay, human red blood cells (hRBCs) and prepared samples were co-incubated in the buffer that mimic extracellular environment (Evans *et al.*, 2013). The assay is based on the release of hemoglobin from RBCs which can be induced by the test sample if it has capability to rupture RBCs. The RBCs lysis is then quantified by measuring the hemoglobin release in the medium spectrophotometrically

(405nm). The assay was performed at different concentrations i.e. 400 µg/ml, 200 µg/ml, 100µg/ml, 50 µg/ml of the test samples.

Table 4.4 RBCs hemolytic profile of the prepared MCC

	% Hemolysis (concentration:µg/ml)				
Samples	**400**	**200**	**100**	**50**	**LC_{50}**
H_2SO_4-MCC	3.40±02	1.4±0.2	0.6±0.1	0.2±0.01	>400
HCL-MCC	3.33±0.1	1.25±0.2	0.8±0.2	0.3±0.02	>400
HNO_3-MCC	3.21±0.2	1.34±0.2	0.6±0.2	0.2±0.01	>400

Generally, any hemolysis >25% is considered hemolytic while hemolysis <10 % is considered non-hemolytic (Amin and Dannenfelser, 2006). As can be seen from the table, that the prepared microcrystals are highly compatible as even at the highest concentration of 400 ug/ml a slight hemolytic activity was shown while at all other concentrations, MCCs are non-hemolytic. This confirms the general biocompatible nature of microcrystalline cellulose irrespective of the acid used in the preparation process. The toxicity of the sample generally decreased with following trend, with very little difference: **H_2SO_4-MCC > HCL-MCC ≥ HNO3-MCC**.

4.8 Biocompatibility against human white blood cells (hWBCs)

To further asses the bio-safe nature, toxicity testing against the freshly isolated macrophages, was carried out through Ficoll–Gastrografin (sodium diatrizoate) method. Macrophages are essential components of the immune system and are involved in immune response against the foreign particles when enters the blood stream. The results obtained summarized in table 4.5

Table 4.5 % inhibition of the isolated macrophages by MCC

	% Inhibition (concentration:µg/ml)				
Samples	**400**	**200**	**100**	**50**	**IC_{50}**
H_2SO_4-MCC	14±3	4±0.12	2±0.43	0.3±0.01	>400
HCL-MCC	12±1	3±0.24	3.4±0.22	0.2±0.02	>400
HNO_3-MCC	13±2	3±0.21	3±0.14	0.2±0.01	>400
Vincristine	93.34±1	59±1	47±0.78	31±0.2	6.66±0.5
DMSO	--	--	--	--	--

Results illustrates, the response of the isolated macrophages was dose dependent. The % inhibition increased with increase in concentration and vice versa. Overall, the assay revealed a non-toxic nature of the prepared MCCs as the $1C_{50}$ of all the tested samples were >200 µg/ml as compared to the positive control Vincristine (IC_{50} 6.66±0.5). This confirms the hemo-compatibility nature of the engineered microcrystals. In general the toxicity of the samples decreased by the following trend with little difference: **H_2SO_4-MCC > HCL-MCC ≥ HNO3-MCC**.

4.9 Brine shrimps (*Artemia salina*) lethality assay

The possible cytotoxic nature of engineered microcrystals was also determined against brine shrimps (*Artemia salina*). Brine shrimp lethality bioassay is a simple, high throughput screening that is widely used for preliminary toxicity profiling of materials, pesticides, medicines, synthetic compounds and plant extracts (Wu, 2014). The results are summarized in the table 4.6

Table 4.6 Brine shrimp lethality profile of the isolated MCC

Sr No	Samples	% Mortality (concentration:µg/ml) 200	100	50	25	LC_{50}
1	H_2SO_4-MCC	20±20	10±10	10±10	10±5	>200
2	HCL-MCC	10±10	10±10	10±10	10±5	>200
3	HNO_3-MCC	10±10	10±10	10±10	10±5	>200
4	Doxurubicin	100	80±10	70±10	70±5	5.93±2
5	DMSO	--	--	--	--	--

The values are mean of triplicate with ± standard deviation (SD). Doxurubicin was used as positive control. DMSO was used as negative control. "–"shows no activity of the sample.

The samples showed excellent biocompatibility against *Artemia salina* larvae at high concentrations. All the samples exhibited LC50 > 200 ug/ml as calculated by the TableCurve 2D software and were categorized as non-toxic. The toxicity of the microcrystals decreased with the following trend with very little difference: **H_2SO_4-MCC, HCL-MCC, HNO_3-MCC.**

4.10 Antibacterial Assay

The antibacterial activity of the samples was probed against five different bacterial strains (table 4.7) for their growth inhibition effect but none of the samples revealed any activity against any of the tested strains at highest concentration of 200 µg/well.

Table 4.7 Antibacterial activity of MCC at 200 µg/ well

Sr. No	Samples	Zones of inhibition (mm) *P.aeruginosa*	*E.coli*	*B.subtilis*	*K.pneumonae*	*S. aureus*
1	H_2SO_4-MCC	--	--	--	--	--
2	HCL-MCC	--	--	--	--	--
3	HNO_3-MCC	--	--	--	--	--
4	+ve	35	20	35	22	9.8
5	-ve	--	--	--	--	--

Values presented here are mean of triplicate ± standard deviation (SD). Roxitromycin and Cefixime were used as positive control. DMSO was used as negative control. "–"shows no activity of the sample.

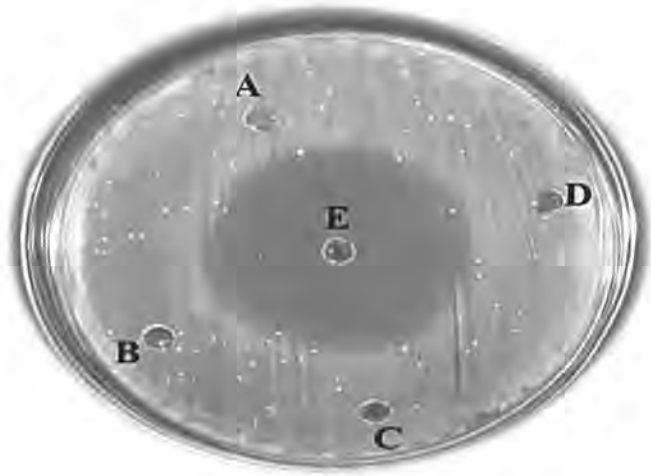

Figure 4.6 Pictorial presentation of antibacterial activity by agar well plate; **A)** H_2SO_4-MCC **B)** HCL-MCC, **C)** HNO_3-MCC **D)** DMSO **E)** Amphotericin B against *Pseudomonas* aeruginosa by agar well diffusion method

4.11 Antifungal assay

The growth inhibition effect of microcrystalline cellulose was also evaluated against five fungal strains including *Aspergillus flavus, Aspergillus fumigatos, Aspergillus niger, Solani and Mucor. The results are* as shown in table 3.5. Like bacterial strains, MCC did not induce growth inhibitory response in any of the tested fungal strains.

Table 4.8 Antifungal activity of MCC at 200 ug/ disc

Sr. No	Samples	Zone of inhibition (mm)				
		A. *flavos*	A.*fumigatos*	A.*Niger*	*Solani*	*Mucor*
1	H_2SO_4-MCC	--	--	--	--	--
2	HCL-MCC	--	--	--	--	--
3	HNO_3-MCC	--	--	--	--	--
4	Clotrimazole	35±2	20±3	35±2	22±1	9.8±3
5	DMSO	--	--	--	--	--

Values presented here are mean of triplicate ± standard deviation (SD). Clotrimazole was used as positive control. DMSO was used as negative control.

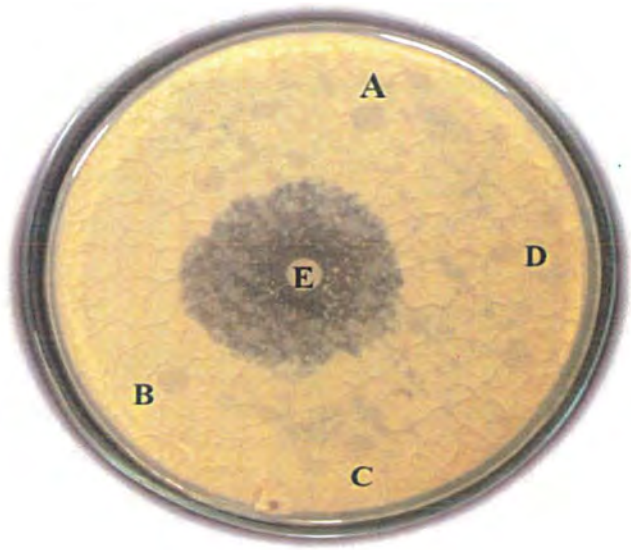

Figure 4.7 Pictorial presentation of antifungal activity by disc diffusion method of **A)** H_2S0_4-MCC **B)** HCL-MCC **C)** HNO_3- MCC **D)** DMSO **E)** Clotrimazole against *Solani*

Chapter 05

Discussion

DISCUSION

Ever increasing concerns about the global environment, government regulations and consumer demands has pushed for the development of ecofriendly, sustainable and renewable materials and minimization of fossils derived materials. This transition from fossil fuel economy towards bio-based or bio-economy is co-related with an increasing demand for renewable biomass. At the same time, an increasing demand of the biomass is an opportunity for low income, agricultural countries to further diversify and improve their economies (Lokko *et al.*, 2018).

Pakistan is the world's fifth largest producer of cotton and a key player in global cotton market. In fact, cotton constitute 1% of the country's GDP and the economy is heavily reliant on cotton and its derivatives. Beside used in various industries like textile, cotton is also a pure source of cellulose (~90%), the most abundant natural polymer on earth (Ashraf *et al.*, 2018; Dawn 2019).

Cellulose microcrystals or microcrystalline cellulose (MCC) is partially depolymerized, white, odorless powder. It is prepared by treating cellulose with dilute mineral acids and has broad range of applications in food, cosmetics, bio-composites and pharmaceutical industries (Azizi *et al.*, 2005).

The present study was aimed at the isolation and evaluation of microcrystals from cotton, as the most economic and abundantly available, native source of cellulose. The MCCs were prepared by treating cotton wool fibers with different mineral acids including sulfuric acid, hydrochloric acid and nitric acid. The microcrystalline cellulose derived through sulfuric acid, hydrochloric acid and nitric acid (H_2SO_4-MCC, HCL-MCC and HNO_3-MCC) were investigated for comparative physicochemical characterization and potential toxicity. Yield and suspension stability of the prepared microcrystals at varied pH was determined. Morphology and dimensions were studied by scanning electron microscope (SEM). The chemical changes and crystallinity of MCCs as a result of the different acidic treatments was probed by fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). To evaluate the bio-safe nature, toxicity of the engineered microcrystals was evaluated against isolated human red blood cells (hRBCs), macrophages (hWBCs) and brine shrimps (*Artemia Salina*). Moreover, cellulose microcrystals were also tested for their growth inhibitory effects on bacterial and fungal strains.

Yield of microcrystalline cellulose highly depends on the source, type of the acid used and processing conditions like acid to cellulose ratio, temperature of the reaction and reaction time. In the study, the hydrolysis conditions were retained same for the acids used except reaction temperature for sulfuric acid hydrolysis. It was observed that increasing temperature above 45 °C, cellulose starts to degrade, as visualized by the color change from white to dark brown. Among the acids used, hydrochloric acid resulted in highest yield of MCC which was 73%. On the other hand, sulfuric acid and nitric acid yielded 64% and 58% MCC respectively. The yield is comparable to a study performed by Liu who obtained 78.6% MCC from hydrochloric acid hydrolysis of cotton linter (Liu *et al.*, 2011). However, the yield is far more than those reported in other studies. For instance, in a study 53.6 % yield of MCC was obtained by hydrochloric acid hydrolysis of α-cellulose obtained from bamboo. In another study by Jahan, 59.8% yield was obtained from bamboo cellulose through formic acid/peroxyformic acids hydrolysis (Kharismi *et al.*, 2018; Jahan *et al.*, 2011).

It is well known that cellulose fiber consist of both crystalline and amorphous domains. During the acid hydrolysis process, hydronium ion from the acid penetrates the weak, disordered and amorphous domains of the cellulose fibers and catalyze the breakdown of glycosidic bonds. This preferential cleavage of cellulose fibers at amorphous regions results in microcrystals of varied dimensions. Scanning electron microscope (SEM) micrographs shown successful disintegration of cotton wool fibers into microcrystals by acidic treatments. The microcrystals resulted, had rod shaped like structure with rough, swelled morphology as compared to cotton wool fibers with compact, smooth and spiral long threads. Similar, morphological patterns were also reported in other studies (Trache *et al.*, 2014; Elanthikkal *et al.*, 2013; Adel *et al.*, 2011; Uesu *et al.*, 2000). In the current study, cellulose microcrystals of different dimensions were obtained with different acidic treatments. For instance, the average length and diameter of H_2SO_4-MCC was found to be 40.46 μm and 9.74 μm with an aspect ratio 4.1. Contrary to sulfuric acid, hydrochloric acid and nitric acid yielded microcrystals with increased length and diameter and high aspect ratio. The average length of HCL-MCC and HNO_3-MCC was measured as 65.25 μm and 76 μm with diameter 13.25 μm and 10.2 μm respectively. While the aspect ratio was calculated as 4.9 and 6.9 for HCL-MCC and HNO_3-MCC respectively.

Cellulose is semi crystalline biopolymer as it consist of crystalline regions separated by amorphous domains. The harsh conditions during hydrolysis not only alter morphology of the cellulose fibers but also effects crystallinty of the isolated microcrystals. Our results affirms that crystallinity of the microcrystalline cellulose depends on the type cellulose source, acid and hydrolysis conditions. The X-ray diffractograms revealed that H_2SO_4-MCC exhibited high crystallanity index (80.57 %) as compared to cotton wool fibers which was 49.80%. Thus, 30.7% increase in crystallinity was achieved with sulfuric acid hydrolysis. Unlike sulfuric acid, hydrochloric acid and nitric acid resulted in microcrystals with 75.55% and 61.40%. A correlation was observed in our study between aspect ratios and crystalinity Index of the MCCs. The crystallinity index was found to increase with decrease in aspect ratios of the engineered microcrystals and vice versa.

Fourier transform infrared spectroscopy (FTIR) revealed that no major chemical changes occurred on the cellulose structure during successful disintegration of wool fibers into respective microcrystalline cellulose as a result of hydrolytic reactions. The major peaks observed were in the range 3,319- 3,402 cm^{-1}, 2,956-2,990 cm^{-1} and 1,108 which represents O-H stretching vibrations of the hydroxyl groups, C-H groups of cellulose and C-O-C stretching vibrations of β (1,4) -glycosidic bonds in the cellulose chains (Avolia *et al.*, 2012; Chandra *et al.*, 2016; Marchessault, 1962). Absorption peaks in the range 1644 cm^{-1} to 1634 cm^{-1} founded in all the spectra is due to vibrations of O-H groups of water molecules adsorbed by the polymer. Such peak were also reported in different studies previously (Abraham *et al.*, 2011; Janoobi *et al.*, 2011). Moreover, the absence of 1,245 cm^{-1} peak and presence of peak intensity at 1737 cm^{-1} (C=O group of ester) in only acid isolated microcrystals indicated successfully isolation of MCCs from cotton wool fibers.

The dispersion stability of cellulose microcrystals is one of the important parameters worth considering particularly in engineering bio-composites. The key factors that contribute to suspension stability are aspect ratio, surface chemistry and ability of the solvent and functional groups on microcrystals to counterbalance the strong H-bonds interactions (Espinosa *et al.*, 2013). In our study, H_2SO_4-MCC shown good aqueous dispersion capability at varied pH. The dispersion capability may be attributed to negatively charged sulfate groups on H_2SO_4-MCC and

low aspect ratio. Unlike H_2SO_4-MCC, HCL-MCC and HNO_3-MCC displayed very weak or no aqueous dispersion stability on any of the pH probed.

Cellulose is considered as non-toxic and biocompatible material (Thakur *et al.,* 2013; Credou and Berthelot, 2014). However, physicochemical and morphological characteristics of micro and nano-materials are different from their bulk materials (Khan *et al.,* 2017). To evaluate the possible toxicological potential, engineered MCCs were tested for biocompatibility with human red blood cells (hRBCs), freshly isolated human macrophages (hWBCs) and brine shrimps (*Artemia Salina*)..

Irrespective of the type of acid used, all the engineered microcrystals revealed excellent compatibility against hRBCs and IC_{50} was found to be > 200 µg/mL. To further asses the hemocompatibility nature, materials were tested against freshly isolated macrophages. Macrophages are differentiated monocytes with large cells and plays a significant role in immunity and immune response (Tao *et al.,* 2016; Mosser and Edwards, 2008). Our results indicated a dose dependent interaction between MCCs and isolated macrophages and IC_{50} for all the derived microcrystals was found to be >200 µg/mL as compared to positive control, Vincristine (IC_{50} = 6.65± 0.5). The assays affirm the generally accepted low toxic and biocompatible nature of microcrystalline cellulose as even at the highest concentrations a very slight toxicity was observed

Brine shrimps lethality profiling also augmented to the bio-safe in-vitro nature of the isolated microcrystals. Brine shrimp lethality bioassay is a simple, high throughput screening that is widely used for preliminary toxicity profiling of different materials, medicines, synthetic compounds and plant extracts (Wu, 2014). In our study, all the tested samples exhibited LC_{50} > 200 µg/mL as calculated by the TableCurve 2D software and were categorized as non-toxic.

Last but not the least, antibacterial and antifungal activity also depicted no growth inhibitory potency of the prepared microcrystalline cellulose. Gram positive bacteria (*Staphylococcus aureus Pseudomonas aeruginosa* and *Bacillus subtilis*) and gram negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) were exposed to 200µg/mL of the tested samples by agar well plate method. After 24 hours incubation, no growth inhibitory effect was observed for any of the

sample against any strains. Likewise, no antifungal activity was visualized against various fungal strains (*Aspergillus flavus, Aspergillus fumigatos, Aspergillus niger, Solani and Mucor).*

Our study thus concludes that choice of the acid highly effect the physicochemical and morphological properties of microcrystalline cellulose from cotton wool fibers. Type of the acid yielded microcrystals with different surface morphology, physical dimensions, aspect ratios, surface chemistry, crystallinity and suspension stability. However, despite such diverse characteristics, the interaction of the engineered microcrystals remained less or nontoxic with human blood cells, invertebrate model (*Artemia Salina*), and microbes (bacterial and fungal strains).

Cotton wool fibers are thus potential economic source for engineering tailored and bio-safe cellulose microcrystals for particular applications. However, the process of production is further needed to be made efficient, less time consuming and scaled up to harness the economic value

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