STUDIES ON BACTERIAL CELLULOSE PRODUCTION USING INDUSTRIAL WASTEWATER

Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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D E C L A R A T I O N

I, G.Gayathri hereby *declare* that the Research Thesis entitled "**STUDIES ON BACTERIAL CELLULOSE PRODUCTION USING INDUSTRIAL WASTEWATER"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in Department of Chemical Engineering is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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C E R T I F I C A T E

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Life to me is a journey - you never know what may be your next destination – David Russell

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ABSTRACT

Industrial wastewater management remains a critical environmental concern worldwide. Several new and modified methods have been implemented to manage the effluent discharge from the industries. These wastewaters predominantly contains huge amount of organic loads; which can be utilised as a nutrient source for cultivating microorganisms to obtain valuable products from the fermentation process. Bacterial Cellulose is one such useful biopolymer produced by certain class of bacterial strains. These have been extensively studied for their distinctive properties and applications. However, production of Bacterial Cellulose from wastewaters using a potential bacterial strain is still limited. *Komagataeibacter saccharivorans* BC1, a novel cellulose producing strain was isolated from rotten green grapes and studied for the production of Bacterial Cellulose. The strain was initially grown and optimised on standard medium for cellulose production. Later, the strain was evaluated for the production of BC in crude distillery effluent medium. 23.6% reduction in COD and 11.9% reduction in BOD value along with the production of 1.24g/L of BC were recorded. Scanning electron microscopy analysis revealed thin microfibrils with good porosity. Fourier Transform Infrared Spectroscopy studies indicated similar functional groups as that of cellulose derived from standard medium. XRD analysis revealed crystallinity index of 80.2% and crystallite size of 8.36 nm. Solid state ^{13}C NMR analysis helped to study the structural framework of the synthesised cellulose. Further, the Bacterial Cellulose films were used to study *in vitro* drug release. The study demonstrated the absorption and release of the model drug for over 8 hours. The films were also assessed for their cytotoxicity activity using A549 cells and showed an IC50 value of 210µg/mL. Thus, production of a useful biopolymer from wastewater as a nutrient medium proves a sustainable approach to reuse the waste to produce a value-added product which could benefit both the environment and humanity.

Keywords: Bacterial Cellulose; biopolymer; wastewater; characterisation; drug release

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ABBREVIATIONS

BC	Bacterial Cellulose
HS	Hestrin Schramm
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
XRD	X-Ray Diffraction
TLC	Thin Layer Chromatography
TGA	Thermogravimetric Analysis
^{13}C-NMR	Carbon Nuclear Magnetic Resonance
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen demand
TOC	Total Organic Carbon
TDS	Total Dissolved Solids
TSS	Total Suspended Solids
CPCB	Central Pollution Control Board
CS	Cellulose synthase
UDPG	Uridine Diphosphate Glucose
DP	Degree of polymerisation
CI	Crystallinity Index

CrS	Crystallite Size
WHC	Water Holding Capacity
FDA	Food and Drug Administration
FAO	Food and Agriculture Organization
APHA	American Public Health Association
IbuNa	Ibuprofen Sodium Salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
EE	Encapsulation Efficiency
LC	Loading capacity
CFU	Colony Forming Units
DMSO	Dimethyl Sulphoxide
DNS	Dinitro Salicylic Acid
ANOVA	Analysis of variance
SD	Standard Deviation

NOMENCLATURE & SYMBOLS

°C	Degree Celsius
%	Percent
g	Gram
mg	Milligram
mL	Milliliter
mg/L	Milligram per Litre
g/mL	Gram per Milliliter
g/L	Gram per Litre
min	Minute
hrs	Hours
nm	Nanometer
mm	Millimeter
cm	Centimeter
θ	Theta
α	Alpha
β	Beta
Å	Angstrom
µl	Microlitre
µm	Micrometre

µg	Microgram
M	Molar
kV	Kilovolt
A	Ampère
mM	Milimolar
kHz	Kilo hertz
s	Seconds
ppm	Parts per million
W	Weight

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

The treatment and disposal of industrial wastewater is an environmental problem facing all over the world. In the late 1980s, "Zero discharge" concept in industries started gaining importance, with environmental awareness. The concept of zero discharge effluent focuses on avoiding the waste discharges from industries directly into the environment, instead, recycle and reuse all the types of treated wastes (solid and liquid), and cut down on air emissions to the lower levels along with the reduction of toxicity levels. There are various industries such as textiles industries, cellulose and paper pulp industries, tannery and leather industries, brewery and fermentation industries, metal processing factories, chemicals manufacturing industries etc. which discharges heavy organic loaded pollutants daily. Generally, biological wastewater treatments (aerobic and anaerobic) are employed to breakdown the organic compounds and later remove solids from the treated wastewater. However, these require high energy and use of chemicals, leading to high costs for the overall treatment of the wastewater. Sustainable development is an essential need of the hour to ensure overall economical growth and well-being. Hence, wastewater treatment requires an efficient method by which these waste substances can be converted to useful products. Lately, various strategies and technologies have been developed to reuse this industrial discharge such that the treatment process can be reduced and the effluent can be utilised for producing value-added products (Pagliano et al. 2017).

One of them is to utilise the effluent as a nutrient medium such that microorganisms can utilise the organic carbon in the effluent and grow on it. This can meet two demands/ objectives, namely, one to reduce the amount of organic load required for further treatments and the second is to grow suitable microorganisms which can release value-added products such as biopolymers. Keeping this in mind, the current study aims to isolate a suitable bacterial strain which is capable of producing Bacterial Cellulose (biopolymer) from the wastewaters. Although several studies have been carried out work on Bacterial Cellulose (BC) production, certain limitations such as

low yield of BC, identification and charcterisation methods, suitable medium to cultivate BC still exists. The commercial scaling up of the process using synthetic media leads to an increase in overall production cost. Therefore, there is a need for identifying effluent medium with a high organic content and possible microorganisms which can thrive on this medium and produce BC.

Macromolecules which are made up of repeating structural units of monomers are called polymers. These polymers are broadly classified into two main classes, namely: natural or synthetic. The class of natural polymers comprises cellulose, silk, collagen, etc., whereas synthetic polymers are those of plastics, nylon, teflon etc. These materials play a vital and essential role in our everyday life. Some of them include conventional plastics to natural biopolymers, such as carbohydrates and proteins (Ghosh 2006). The natural polymers which are obtained from the living organisms are called biopolymers. These naturally derived polymers exhibit unique properties, making them suitable for several applications.

In this respect, Cellulose is one such biopolymer which is available in abundance on the earth. This polymer forms the basic building blocks of the plant cell wall. Chemically, cellulose can be defined as complex carbohydrate $(C_6H_{10}O_5)_n$, which is made up of several glucose units arranged linearly with β-1,4 glycosidic linkage (Klemm et al. 2005) (Figure 1.1). Cellulose constitutes 33% of whole plant matter. In plants, such as angiosperms, gymnosperms and also in ferns and mosses, cellulose is present in the cell wall in combination with other polymers such as lignin, hemicellulose (Brown 2004). Also, other members of the plant kingdom, such as red algae *Gelidium,* green algae *Cladophora* spp., *Valonia* have known to produce cellulose (Mihranyan 2011; Chen et al. 2016).

Other than plants, certain strains of bacteria have also been reported to produce this polymer extracellularly (Jonas and Farah 1998). Mostly gram-negative strains such as *Acetobacter, Agrobacterium, Escherichia, Enterobacter, Gluconoacetobacter (formerly Acetobacter), Komagataeibacter (formerly Gluconoacetobacter), Pseudomonas, Rhizobium, Salmonella* and *Sarcina* are some of the bacterial genera reported to produce cellulose as an extracellular film (Chawla et al. 2009; Cannon and

Anderson 1991; Ross et al. 1991; Bielecki et al. 2005; Klemm et al. 2005; Sunagawa et al. 2012; Römling and Galperin 2015). Such a type of cellulose is called Microbial cellulose or Bacterial Cellulose (since bacteria is the source of origin).The cellulose synthesized by the bacteria aids in adhesion and aggregate to a substratum (biofilm formation), protection of bacterial cell from harmful UV radiation, resistance to adverse environmental conditions, quorum sensing, and provides an aerobic environment for the bacteria (Bandyopadhyay et al. 2018; Augimeri et al. 2015).

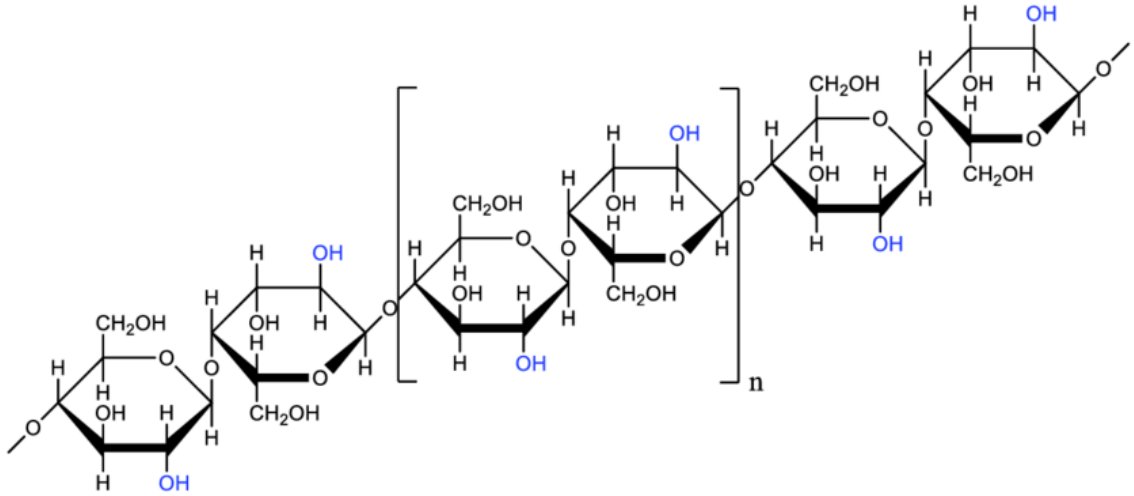


Figure 1.1 Structure of Bacterial Cellulose (Wang et al. 2018)

Past few decades, researchers have been exploring the potential of bacteria to produce biopolymers as the process is eco-friendly and cost-effective over obtaining the resources from plants/woods. The cellulose required for several commercial applications is extracted from the trees, this account to around 50% to 90% (approx.) dry weight of cellulose obtained from woods and cotton respectively.

1.1 The Need for Bacterial Cellulose (BC)

The rising industrial demands on cellulose have put tremendous pressure on plant biomass resources. In the present time, sustainable development of natural resources is gaining importance, and people are continually looking for alternatives to ease the burden on the exploitation of natural resources. A better substitute for plant-derived cellulose is Bacterial Cellulose. BC serves as a better alternative due to the following reasons:

- The pulping and bleaching process to obtain the cellulose fibres from woods and the extraction of pure cellulose from lignin and hemicellulose is energy and chemical-intensive processes; dumping of bleached effluents contributes to environmental pollution. Also, these tend to weaken the basic structure of the polymer. In contrast, the BC is the purest form of the cellulose polymer, as it is devoid of lignin and hemicellulose but still having the same chemical structure as that of wood/plant-derived cellulose. Hence production and purification of BC are easier compared to the former.

- BC has better properties over plant-derived cellulose such as high crystallinity, high water holding capacity, biocompatibility owing to its high purity, good mechanical properties.

Although it cannot completely replace plant cellulose, it can still serve as a good option for the medical/industrial applications which require greater levels of chemical purity. The ability of high water retention, moulding into any desirable shape and nanofibrous network makes it highly porous, thereby making BC a potential precursor material for designing several industrial products (Dubey et al. 2017).

In recent times, several studies have been aimed at converting organic wastes into bioenergy and essential biomaterials with the help of microbial biotechnologies. Wastewaters derived from agriculture, food processing factories, and other municipal organic waste which have large amount organic waste content have been effectively reused to produce biopolymers such as extracellular polysaccharides (EPS), polyhydroxyalkanoates (PHA), bioplastics, and other forms of energy such as biohydrogen and biogas. The usage of by-products and wastewater generated from industries as substrates could reasonably reduce raw material costs (Gumel et al. 2012). Hence, these approaches in waste management, in addition to being eco-friendly and reuse of the waste to value-added products also prove beneficial both to the industries and the environment. Accordingly, the present work aims to evaluate the potential of a bacterial isolate in production of Bacterial Cellulose using wastewater (crude distillery effluent) as the fermentation medium and study the properties of BC synthesised.

1.2 Objectives

The objectives have been described as follows:

1. Isolation and screening of bacteria which are capable of producing Bacterial Cellulose from rotten fruit sources.
2. Production and optimisation of BC in the standard medium by one variable at a time approach.
3. Evaluation of BC production in an alternative medium such as crude distillery effluent.
4. Study various physiochemical properties of the synthesized BC.
5. Application of the synthesised BC in studying *in vitro* drug release and absorption potential using anti-inflammatory model drug Ibuprofen.

1.3 Organisation of the thesis

The organisation of the thesis is in five chapters. **Chapter 1** provides the justification and background to the present work. **Chapter 2** contains a detailed literature review on various aspects related to the current work. **Chapter 3** includes all the materials and the methodologies involved in the work. **Chapter 4** provides a detailed interpretation of the results and the observations made during the research work along with the comparison of results to the previous available literature. **Chapter 5** gives the summary and conclusions of the current work. It also highlights the significant findings made and the scope for future work.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Until the late 18th century water pollution was mainly limited to a minimal area. The beginning of the industrialisation era led to the setting up various large and small scale factories. These industries used a large amount of water as a raw material for multiple purposes and as a result, generated huge quantities of wastewater which were later discharged with/without appropriate treatments into the environment. This phenomenon gradually led to the pollution of water bodies, which further raised several environmental concerns. The industrial wastewaters can be broadly classified into two types: inorganic and organic wastewaters. The inorganic wastewaters are the wastewaters generated out of coal, steel, and other non-metallic industries. These waters mostly consist of suspended solid matters which can be easily removed using the methods such as sedimentation or by chemical flocculation. These waters also contain extremely harmful solutes such as cyanides, chromates, which needed to be treated before further discharge. The other type is organic wastewaters which include the waste effluents from other industries such as pharmaceutical, pesticides and insecticides, soaps and detergents, textile and dyes, tannery and leather, fermentation and brewery, paper and pulp, sewage, oil refining and metal processing industries. These industrial generated effluent high amounts of BOD, COD and a large amount of organic loads. These require anaerobic and aerobic methods of wastewater treatments to get rid of harmful impurities before discharging into the surroundings (Shi 1998; Ranade and Bhandari 2014).

The wastewaters are characterised by three major types, namely, physical, chemical and biological (Henze and Comeau 2008).

- The physical characteristics are based on odour, temperature, density, turbidity and colour.
- The chemical characteristics are based on measurements of pH, gases, organic and inorganic matter present in the water (Table 2.1), i.e., BOD, COD, TOC,

TDS, TSS etc. and the chlorides, nitrogen, phosphorus, sulphur, heavy metals respectively.

- The biological characterisation of wastewater is by the types of microorganisms constituting the effluents, their amount, the pathogenicity and toxicity levels of the effluents.

Table 2.1 General domestic wastewater composition (FAO, 2016)

PARAMETERS	UNITS	WEAK	MEDIUM	STRONG
TSS	mg/L	350	720	1200
TDS	mg/L	250	500	850
Suspended solids	mg/L	100	220	350
BOD	mg/L	110	220	400
COD	mg/L	250	500	1000
TOC	mg/L	80	160	290
Nitrogen	mg/L	20	40	85
Phosphorus	mg/L	4	8	15
Chloride	mg/L	30	50	100
Alkalinity($CaCO_3$)	mg/L	50	100	200
Oil and Grease	mg/L	50	100	150
Free ammonia	mg/L	12	25	50
Nitrates	mg/L	50	100	200
Phosphates	mg/L	1	3	5
Lead	mg/L	1	2	5
Cadmium	mg/L	0.03	0.5	1
Mercury	mg/L	0.0000002		
Total coliform bacteria	no/100 mL	10^5- 10^7	10^7-10^8	10^8-10^9
Temperature	35-40°C			
pH	5.5-9			

2.1 Wastewater treatment methods

2.1.1 Conventional wastewater treatment methods (Benford 2003; Mackenzie Davis 2010).

- Preliminary treatment
- Primary treatment
- Secondary treatment
- Tertiary treatment
- Disinfection

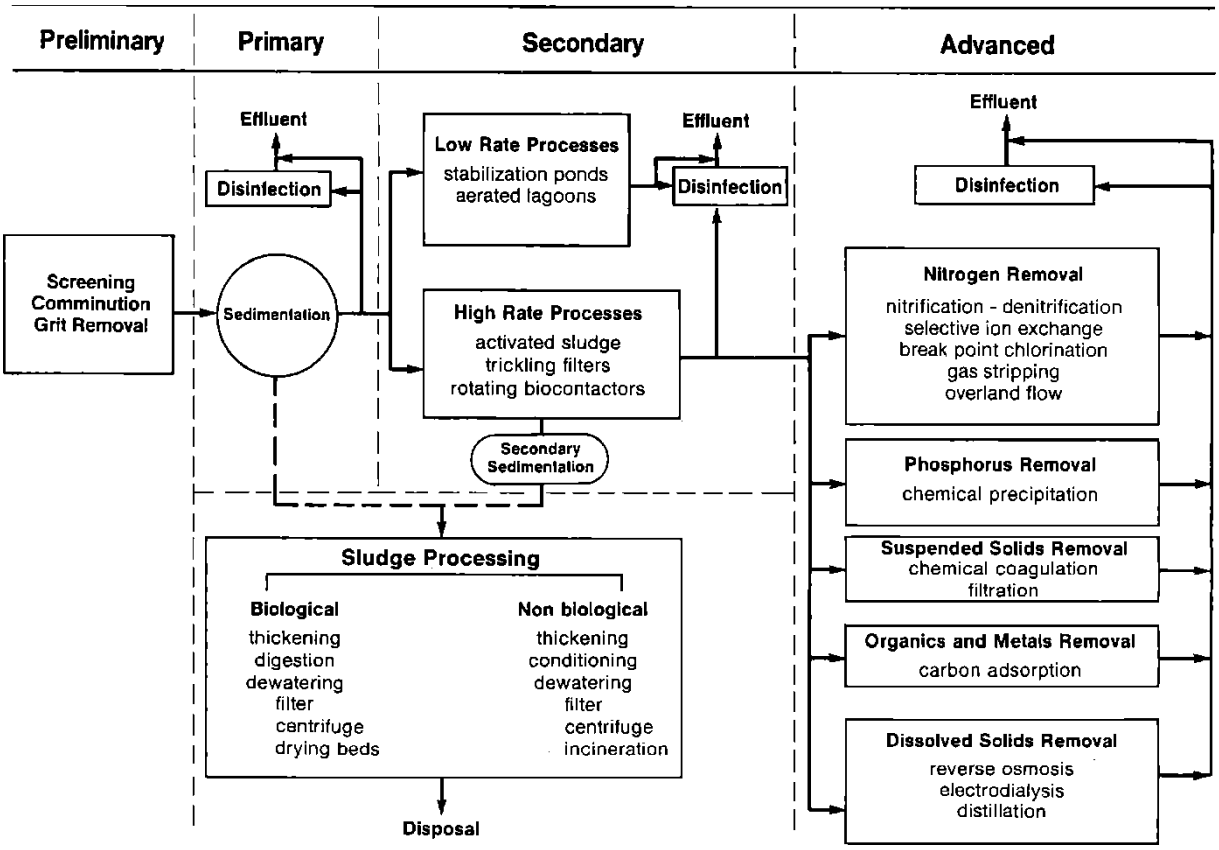


Figure 2.1 General flowchart of conventional wastewater treatment (Pettygrove et al. 2019)

Preliminary treatment: involves removal of debris and heavy inorganic solids using grit chambers, removing coarse solids and large particle aids in better treatment in the subsequent treatment steps.

Primary treatment: The primary treatment involves removal of settleable organic and inorganic solids by sedimentation, and the removal of floating materials by the process of skimming.

Secondary treatment: The secondary treatment mainly involves the removal of the residual organics and suspended solids left out after primary treatment. The removal of biodegradable dissolved and colloidal organic matter using aerobic biological treatment processes. This step is followed by anaerobic digestion, wherein the primary sludge is subjected to breakdown by anaerobic and facultative bacteria in digesters.

Tertiary treatment: Tertiary and advanced wastewater treatment is performed to remove specific wastewater constituents such as nitrogen, phosphorus, additional suspended solids, refractory organics, and heavy metals (Mackenzie Davis 2010).

The CPCB has listed out following wastewater treatments processes which are being employed by various industries (CPCB 2005).

Aeration method: the process where oxygen or other gases mix with the wastewaters by:

a. aiding in evaporation of volatile molecules present in the water and

b. dissolution of essential gases to the wastewater.

The volatile molecules may be organic or aromatic in nature. O_2 and CO_2 are the most common gases which mix into the wastewaters. Aerators are used for this process. Spray and mechanical aerators are predominantly used ones.

Coagulation method: involves a mixing a coagulant to the crude wastewaters that results in neutralisation of substances based on charge present on them. The substance used for coagulating can be either organic or inorganic (Generally, 1-100 mg/l coagulant is added to the wastewater to bring about coagulation) (Bratby 2016).

Flocculation method: The step which is followed after coagulation of molecules is flocculation. This involves mild agitation or stirring process so as to make the coagulated particles to settle down, also to collect these substances based on density or size so as to be removed by further treatment methods (Bratby 2016).

a) Sedimentation and filtration methods

The wastewater which is treated by flocculation sent to sedimentation tanks/clarifiers for the removal of accumulated particles and later filters are employed to reduce/remove the turbidity.

b) Backwashing step

This step involves removal or cleaning up of solids which are held back in the filter such that to clear filter pores are available for better filtration along with effective reuse of same filters for further treatments.

Disinfection step

Disinfection step is carried out so as to effectively obtain pure and clean water devoid of any harmful substances or infectious microorganisms. Chlorine is the most commonly used substance for this step.

Limitations of conventional methods of water treatment (Rajasulochana and Preethy 2016).

- Coagulation-flocculation: Sludge production and sludge disposal process.
- Aeration: expensive, process needs to be carried out at frequent intervals and requires more energy.
- Filtration methods (Ultrafiltration, Nano filtration, RO): Expensive and membranes requires frequent maintenance.
- Aerobic methods of treatments: Aerobic digestion requires aeration, which uses a large amount of electrical energy. This is usually consumed for the burning of fossil fuels. Aerobic digestion also results in large amounts of organic solids and sludge which require disposal. Inappropriate release of the nutrient-rich sludge into rivers or ponds causes eutrophication, which affects aquatic flora and fauna. Although biological wastewater treatment is efficient

in removing most organic contaminants, studies indicate that some chemicals, such as pharmaceuticals, cosmetics and industrial compounds, remain in the wastewater.

- Although anaerobic digestion of contaminants in wastewater is less efficient than aerobic digestion. Anaerobic microbes attack a smaller range of pollutants. Application of anaerobic treatments requires large reactors, which increases the treatment costs. Low reaction rates, long start-up periods and difficulty in recovery are some of the limitations of anaerobic digestion

CPCB in 2008 reported that biological wastewater treatment methods were cheaper and viable over the conventionally used treatment methods and proposed some methods such as duck weed-based water treatment, enzymatic treatment, biofilters etc.

Zero Liquid Discharge System: Currently, this method has gained popularity in wastewater treatments. This method involves the usage of thermal technologies with the help of evaporators, distillation units, mechanical vapour compressors, crystallizers, and recovery of the condensate. These plants aim to produce solid dry wastes and recycle or reuse the liquids back to industries (Tong and Elimelech 2016).

Recycle and Reuse via membranes: In this method, wastewater is recycled using the membrane-based system. Membrane bioreactors use the simple science of ultrafiltration with a bioreactor to treat wastewater. This is a commonly used method in industrial and municipal wastewater management units. Treated water is recycled for various purposes, such as irrigation, flush tanks in toilets and in some cases as a cooling agent and other uses which do not require potable quality of water (Melin et al. 2006).

The use of wastewater or greywater for a variety of purposes is gaining importance as a means of preservation of scarce water resources. Economic and environmental concerns have led to the development of methods for recycling and reusing of the wastewater, which includes irrigation of food and non-food crops, expansion of green habitats, recovering arid land, industrial cooling and processing, sanitation. The beneficial use of wastewater decreases the impact on the environment of disposal of

sewage or industrial effluent. The end use of wastewater determines the required quality of the water and management procedures required to ensure safety (Drechsel et al. 2015). Effluents have been successfully used as cooling water or boiler feed water in industries. The major determining factors for effluent reuse includes (i) availability of natural water, (ii) quality and quantity of effluent generated (iii) transportation cost of effluent, and (iv) quantity of industrial water disposed into the environment. Industrial generated effluents have been directly reused for industrial washings and processing, fire protection, organic content loaded wastewater for large scale biogas production and aquaculture of flora and fauna (Visvanathan and Asano 2009). The use of industrial effluents and municipal sewage for irrigation has emerged as an economical way of the utilisation of wastewater due to the presence of considerable quantities of nitrogen and phosphorus and assimilation of certain pollutants by the plants to some extent. Nevertheless, the usage of effluents for agriculture is impaired by several constraints such as soil salinity, the interaction of a harmful chemical with the uptake of nutrients by the plant species and changes in soil ecology and associated microbes (Noel and Rajan 2015). The unfit industrial recycled water after appropriate treatments is disposed of into the nearby water bodies.

The other way to reuse of these effluents is by cultivating potential microorganisms which not only break down and assimilate the carbon source but also synthesise a valuable product from waste raw material as they are rich in organic carbon and other nutrients. In this way, we meet two main goals one the reduction organic content of the wastewater, thereby making it easier for further treatments and two to obtain a valuable product which can be beneficial for our applications.

2.2 Bacterial Cellulose and its importance

Biopolymer Cellulose is one of such a useful product that has influenced the day to day lifestyle of humans. Although commercialisation of this product is still underway, in due course of time, it is believed that Bacterial Cellulose will be able to replace natural cellulose in various aspects. This chapter discusses briefly on Bacterial Cellulose: on its history of origin and synthesis, strains used for isolation and

production of microbial cellulose, nutritional and physiological factors during fermentation, production and harvesting of polymer followed by characterisation techniques used to physicochemical properties and several applications of this polymer.

Generally, cellulose is obtained from the in trees/woods and plants that constitute about 40-50% cellulose along with other polymers, namely lignin and hemicelluloses and waxes (Suresh 2018). Cotton and wood are the significant contributors of cellulose are; which forms the primary raw material for the production of paper, cardboard, textiles, chemicals and various derivatives of cellulose (Keshk 2014). Other important applications include optical films, like paints and coating additives, in designing electronic equipment and products, foods and packaging materials, cosmetics, biomedical devices, and for manufacturing of biofuels (Suresh 2018; Davenport 2010).

To obtain the cellulose in its purest form, chemical and energy-intensive processes are used. These processes raise several environmental issues such as large scale deforestation, discharge of toxic waste from industries resulting in pollution of natural resources; and fuels used in these industries are non-renewable forms of energy. Therefore, to reduce these environmental concerns as well as to cut down on chemical, energy expenses, biological alternatives are sought after. One of them is employing enzyme application; several enzymes such as cellulase, laccase, lipase, xylanase, esterase etc. are used in several steps for softening the plant/wood fibres, bleaching the pulp, pitch control and removing sticky materials during the cellulose production process (Jegannathan and Nielsen 2013). However, this method as well has few drawbacks such availability and cost of enzymes; lack of in-depth knowledge on functioning mechanism and stability of these enzymes. Another approach was to employ microorganisms or genetically modified organisms to produce this biopolymer. Over the past few years, several types of research have focused on bacterial production cellulose (BC). The studies are increasing significantly due to the two main reasons, i.e., unique and distinguished properties of BC and a varied range of applications (Dubey et al. 2017).

What makes Bacterial cellulose (BC) unique?

The cellulose which is produced by the microorganisms, specifically the bacteria are called Bacterial Cellulose. The table below brings out the main differences between the plant and bacterial synthesised cellulose (Chawla 2009; Suresh 2018) (Table 2.2). Therefore, BC has several distinguishable features and properties over cotton/wood-derived cellulose such as high purity, good WHC (water holding capacity), good mechanical properties, good DP and CI values, good flexibility to take desired shape, environmentally biodegradable, *in vivo* biocompatibility (Suresh 2018) (Figure 2.2). Superior properties compared to the counterpart, making it a versatile polymer for several biotechnological applications such as raw material for tissue scaffolds, as food, as filtration membranes, as a cosmetic face mask etc. Kudlicka and Brown (1996) had stated that 1-hectare area could yield around ten thousand kg of microbial cellulose per year, whereas only 600 kilograms of cotton could be harvested from the field of the same area. Thus, the production of BC has significant importance over the coming years in terms of economic aspect as well.

Figure 2.2 Various properties of BC (Hussain et al. 2019)

Table 2.2 Cellulose comparison based on the source of origin (Suresh 2018)

Plant Cellulose	Bacterial Cellulose
A heterogeneous mixture of cellulose blended between lignin, hemicellulose, pectin, waxes.	Pure cellulose devoid other constituents
The dry weight of Cellulose ranges approximately around ~40–50%	About 80-90% dry weight
The amorphous region surrounds the crystalline core. Crystallinity ranges around 60-70% in wood fibres	Mostly crystalline.
The Iβ form of cellulose is present in wood and plant fibres	Cellulose derived from algae and bacteria are abundant in Iα form.
Present in plant cell walls and serves as their backbone providing mechanical strength	Produced as extracellular biofilms which helps in the defense mechanism, adherence to any substrate.
Essential polymer for overall plant growth and development.	Non-essential to the microbe, but gives extra benefits to the host microorganism.
Synthesis independent of environmental parameters	Production depends on physiochemical and nature of growth medium used.

2.3 Bacterial Cellulose: Structure and features

The structure of Bacterial Cellulose ($C_6H_{10}O_5$) is made up pile of glucan chains. These chains are linear, made up of which are glucopyranose rings which are held together by β-D-1, 4 linkages (between the OH situated between C1 and C4 carbon). Due to this design of the structural arrangement, it gives both hydrophilicity and hydrophobicity nature to the polymer. Water-insoluble property can be attributed to the presence of more than six-eight glucose units (Gray et al. 2003). The number of glucose molecules also ascertains the degree of polymerisation (DP) of the cellulose polymer. This number could vary depending on the source of their origin or the synthesis conditions in the lab (Brown Jr 1996). For example, in vascular plants (wood) the cellulose chain has a DP of 8,000-12,000 (Somerville 2006), whereas, in

green algae, there are 25,000 or more glucose molecules in the single-chain (Shibazaki et al. 1998).

The cellulose has parallelly arranged glucan chain is made up of a nonreducing end and reducing ends. The nonreducing end has free unmodified C4 hydroxyl group, and the reducing end has free C1 hydroxyl group (Figure 2.3). The C4 hydroxyl groups are chemically unmodified, as mentioned before, in contrast to the C1 hydroxyl group, which can be altered chemically. Chain polymerisation occurs by the linking of new glucose units to the nonreducing ends of the monomers within a cell (Brown and Saxena 2000).

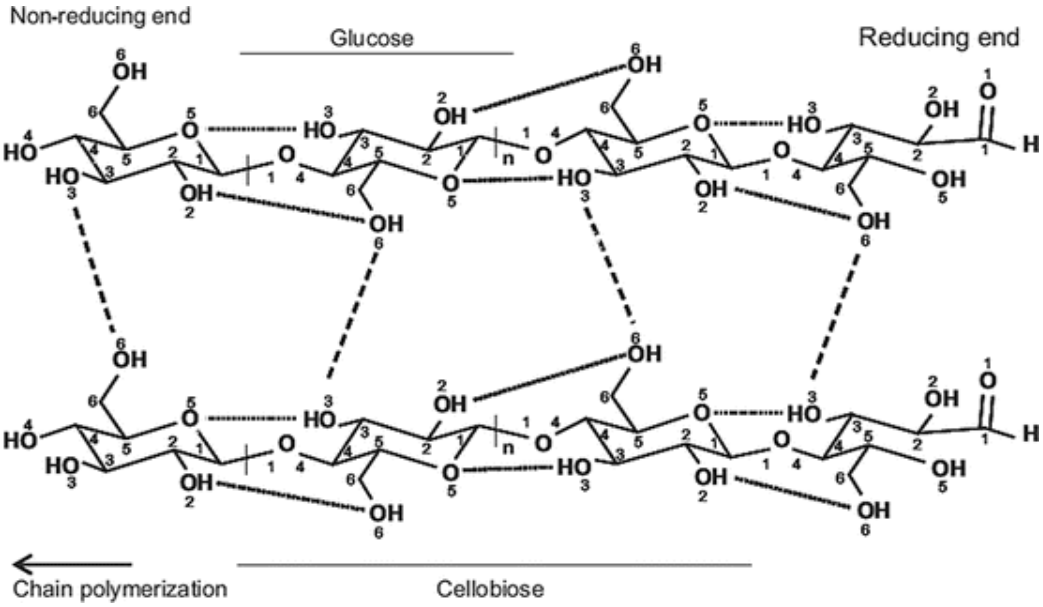


Figure 2.3 Structure of Bacterial Cellulose (BC) displaying hydrogen bonding within the chain (Festucci-Buselli et al. 2007).

The structural arrangement of the polysaccharide begins with the assembling of several linear glucan chains parallel to one another resulting in the structure called protofibril. The diameters of these protofibrils range from 2 to 20 nm (varying according to the source of origin). Several protofibrils align together to form a bulkier structure called microfibrils. The microfibrils are stabilised through Van der Waal's interactions, H-bonding (intermolecular and intramolecular). The arrangements of these microfibrils are responsible for determining the mechanical properties and crystallinity of the cellulose molecule. This entire process of chain synthesis and

polymerisation occur within the cell. On the broader view, the clusters of microfibrils appear as ribbon-shaped. Cellulose exists in six different polymorphic forms, namely cellulose I, II, III_I, III_{II}, IV_I, and IV_{II}. Cellulose I and cellulose II are naturally occurring forms of cellulose. Cellulose I is the most abundant type present in plants and woods. Cellulose II type is found in some algae and bacteria. They differ based on the type of hydrogen bonding within the chains and type of chain alignment (Kuga and Brown Jr 1987; O'Sullivan 1997).

The glucan chains of Cellulose I are arranged in parallel fashion in contrast to Cellulose II, wherein the antiparallel arrangement is observed. Second, the type of H-bonding varies slightly between the two polymorphs, i.e., Cellulose I shows O3--H--O6 intermolecular H bonding whereas Cellulose II shows O2--H--O6 intermolecular bonding. Apart from this, both show O3--H--O5 intramolecular hydrogen bonding. Employing any one of the two chemical methods also can produce Cellulose II type (i) Regeneration by dissolving of cellulose I in a suitable solvent followed by precipitation (ii) Mercerization: Native cellulose is treated with concentrated alkali (sodium hydroxide).

Cellulose III_I and III_{II} are obtained from dissolving cellulose I with liquid ammonia and cellulose II with diamines, respectively (O'Sullivan 1997). Similarly, Cellulose IV_I and IV_{II} can be prepared from cellulose III_I and III_{II} by heat treatment in glycerol at 206 °C, respectively (O'Sullivan 1997). The native form of Cellulose I exhibit two sub allomorphs based on the type of microfibrillar arrangement, namely Cellulose I_α and Cellulose I_β. Cellulose I_α shows the triclinic arrangement of lattice, cellulose I_β monoclinic type. Vascular plants/wood cellulose contains a large amount of cellulose I_β while bacterial and algal cellulose constitutes I_α type. These allomorphs Cellulose I_α and I_β always coexist with together in nature often within the same chain.

2.4 Brief history on discovery of Bacterial Cellulose

The earliest report of the production of microbial cellulose dates back to the 19^{th} century in the year 1886 when Brown observed a gelatinous mat or pellicle floating at the air-liquid interface of vinegar fermented broth. Further through chemical analysis, it was revealed that the pellicle resembled cellulose molecule. The pellicle floating on

the surface of the culture medium was called "*Vinegar plant*", and the microorganism responsible was called Bacterium aceti (Brown 1886).

Later in the year 1947, a study reported that bacterial cells of *Acetobacter xylinum* in the presence of a carbon source (glucose) and oxygen were able to synthesise cellulose (Hestrin et al. 1947). Since then, extensive research began in this field on several aspects from its production parameters to applications. (Brown 2004; Chawla et al. 2009).

Mühlethaler (1949) carried out the very first electronic microscopic observation of the cellulose polymer synthesised by *Acetobacterium xylinum*, stating that the Bacterial Cellulose exhibited fibrillar arrangement as that of plants. Hestrin et al. (1947) reported that *A. xylinum* produced a floating cellulose pellicle at the air-liquid interface after a period of static incubation. Further, Schramm and Hestrin (1954) studied upon several parameters that affected BC production and observed bubbles formation beneath the pellicle in the submerged fermentation medium. They concluded that the pellicle/mat formation at the surface occurred due to the formation of gas bubbles, which aided in floating of the pellicle. Bernardo et al. (1998) reported BC as a commercially edible product. Kuga and Brown (1987), investigated on structural morphology of glucan chain giving insights on the width of Bacterial Cellulose of about 10–25 nm and on clustering of the fibrils to form individual crystallite structure. Bureau and Brown (2006) reported that the plasma membrane or the cell membrane of the bacterium was the region where enzyme cellulose synthase was located, the enzyme responsible for the production of cellulose by bacteria.

2.4.1 Significance of Bacterial Cellulose production

Cellulose, which forms the major component backbone of the plant cell wall, essential for the protection of the living forms to survive in harsh climatic conditions (Brown 2004). The biopolymer is synthesised using photosynthesis in plants, cyanobacteria and with the help of different carbon sources in other organisms. Unlike plants, the synthesis of BC is not mandatory for the survival of the organisms, but they do confer certain benefits/ properties to the bacteria producing them (Shoda and Sugano 2005; Chawla et al. 2009) as mentioned below

- **Enhances aerobic nature in the medium:**

Bacterial cells adhere to the pellicle or the polymeric surface, this helps in the better exchange of gases at the air-liquid interface; in turn, helping them for better growth (Cannon 2000; Jonas and Farah 1998).

- **Provides colonisation:**

The mat surface provides adhesion of the bacterial cells to the pellicle, thereby aiding in substrate biofilm growth, proper nutrient supply and cell to cell interactions (Ross et al. 1991). Also, the moisture retention capacity facilitates dry out of the substrate and hence, helps in the colonisation of bacterium (Jonas and Farah 1998).

- **Protection against harmful environmental conditions:**

The hydrophilic and viscous nature of BC enhances the bacterial cells colonising the pellicle against harsh or harmful environments (variation in pH, temperatures). Also, they protect from harmful UV radiations (Klemm et al. 2005). A study by Ross et al. 1991 revealed that around 23.0 % of the bacterial cells trapped beneath the BC survived UV irradiation up to 1-hour treatment, the viability of the cells reduced with the removal of BC pellicle (Ross et al. 1991).

2.5 Microbial Cellulose Producers

Different strains of Gram-negative bacteria such as *Acetobacter, Agrobacterium, Alcaligenes, Achromobacter, Azotobacter, Aerobacter, Enterobacter, Escherichia, Gluconoacetobacter (formerly Acetobacter), Komagataeibacter (formerly Gluconoacetobacter), Pseudomonas, Rhizobium, Salmonella*, and *Sarcina* and few Gram-positive are reported so far to synthesize BC (Deinema and Zevenhui 1971; Matthysse et al. 1986; Cannon and Anderson 1991; Ross et al. 1991; Park et al. 2003; Bielecki et al. 2005; Klemm et al. 2005; Römling and Galperin 2015; Brown 1985; Geyer et al. 1994). Various strains capable of producing cellulose are tabulated below (Table 2.3)

Table 2.3 Different bacterial strains producers of Bacterial Cellulose

Microorganisms	Morphological nature of cellulose	Function/role	References
Acetobacter	Cellulose ribbon	Provide aerobic environment	Cannon and Anderson (1991); Fiedler et al.(1989)
Achromobacter	Cellulose fibrils	Flocculation	Fiedler et al. (1989)
Aerobacter	Cellulose fibrils	Flocculation	Deinema and Zevenhuizen (1971)
Agrobacterium	Short fibril	Attach to plant tissue/pathogen	Matthyse (1983)
Alcaligenes	Cellulose fibril	Flocculation	Fiedler et al. (1989)
Pseudomonas	No distinct fibril	Flocculation	Cannon and Anderson (1991)
Rhizobium	Short fibril	Attached to root nodules	Cannon and Anderson (1991)
Sarcina	Amorphous cellulose	Unknown	Canale and Wolfe (1964)
Zoogloea	Undefined	Flocculation	Matthyse (1983)

Amongst all the bacteria strains, *Acetobacter* species have been reported and extensively studied on its potential to produce BC extracellularly (Brown 2004). *Acetobacter* sp. is rod-shaped; Gram-negative, aerobic and nonpathogenic bacteria, which are synthesising cellulose extracellularly in the fermentation medium (Jonas and Farah 1998; Klemm et al. 2001). Puri (1984) reported that the bacteria were capable of growing on a varied range of substrates and exhibited no cellulase activity. The bacteria belonging to the class of acetic acid family of bacteria were previously categorised mainly into 2 genera namely *Acetobacter* and *Gluconobacter*. As the classification and nomenclature systems evolved a new genus known as *Komagataeibacter* was found. Subsequently, *Acetobacter xylinum* was categorised under new genus called *Gluconacetobacter* and renamed as *Gluconacetobacter xylinus,* which is grouped along with other together like *G. hansenii, G. oboediens, G. intermedius* etc. (Yamada et al. 2000).

2.6 Biochemical Pathway for Cellulose Synthesis

The biopolymer synthesis is regulated by the multi-step enzyme-mediated process. The systematic regulation of BC synthesis within a bacterial cell has been depicted in Figure 2.4. The synthesis of cellulose occurs either via citric acid cycle or pentose phosphate cycle along with gluconeogenesis pathways. The main enzymes which participate in the synthesis of cellulose are glucokinase (EC 2.7.1.2), phosphoglucomutase (EC 5.4.2.2), glucose-1-phosphate uridyltransferase / uridine 5′-diphosphoglucose pyrophosphorylase (EC 2.7.7.9) and cellulose synthase (EC 2.4.1.12) (Klemn et al. 2001).

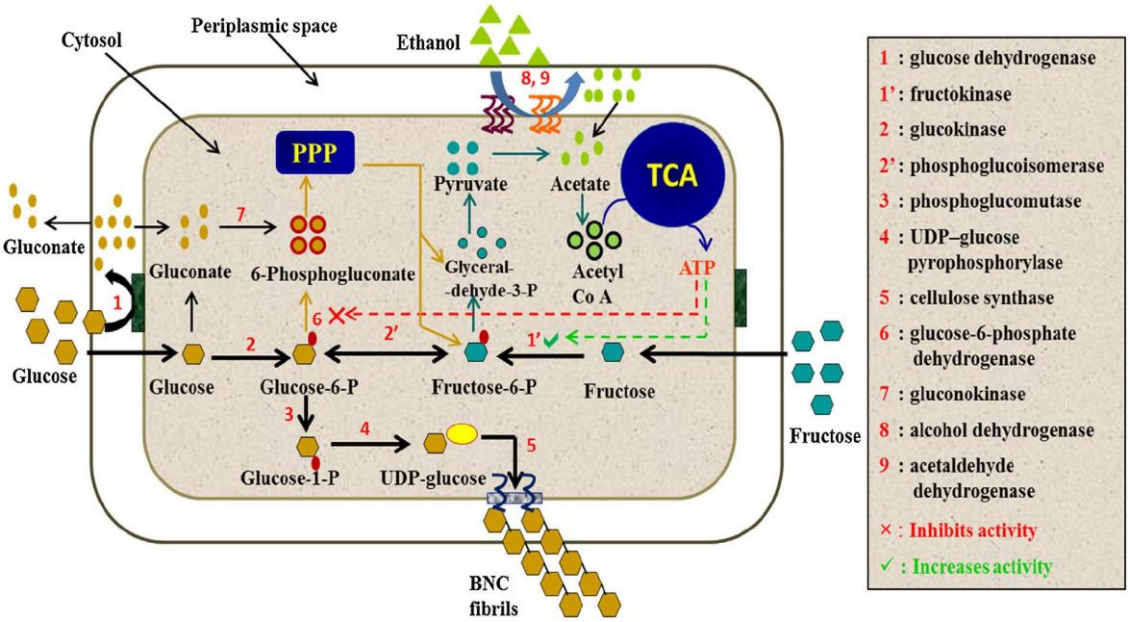


Figure 2.4 Schematic representation of Bacterial Cellulose synthesis within the bacterial cell (Dubey et al. 2017)

Enzyme Glucokinase brings about phosphorylation of the glucose molecule; thereby imparting a net negative charge to the molecule. This is followed by enzyme phosphoglucomutase that is responsible for isomerising of glucose-6-phosphate to glucose-1-phosphate. Next step is production of uridine 5-diphosphoglucose (UDPG) from UTP and glucose-1-phosphate catalysed by glucose-1-phosphate uridyltransferase / uridine 5′-diphosphoglucose pyrophosphorylase. This enzyme is precursor for cellulose production step and UDPG molecule is most essential molecule for the BC synthesis. The final step involves the cellulose synthase, a transmembrane enzyme which catalyses the conversion of UDPG into a final product

called cellulose. This step requires chain polymerisation β-1, 4 glucan chains, assembly of ribbon-shaped microfibrils and finally translocation of cellulose to the extracellular space. This transmembrane enzyme complex often referred to as terminal complexes consist of catalytic, regulatory, and accessory units which are primarily responsible for the biosynthesis and transportation of cellulose. Ross et al. (1991) reported that a single cell of *Komagataeibacter xylinus* showed chain polymerisation of approximately two lakh glucose molecules/second. Cellulose synthase (CS) or 1, 4 β-glucan β-glycosyltransferase (EC 2.4.1.12) is a common enzyme present both in plants and bacteria responsible for the synthesis of cellulose. Terminal complexes (TCs) are sites where functions such as glucan chain polymerisation, assembly, organisation and translocation take place. Earlier studies have shown TCs played not only an essential role in the synthesis of cellulose but also in determining the type of cellulose fibrils, shape and ratio of crystallinity (Nishiyama et al. 2003; Ross et al. 2001; Saxena and Brown 2005).

2.6.1 Mechanism of Biosynthesis

The site for Bacterial Cellulose synthesis is located in the transmembrane situated in between the plasma membrane and outer membrane. Cellulose synthase is a protein present in the cytoplasm, a globular region of this protein conserves the amino acid sequence which is responsible for the polymerisation of the chain. The synthesis of cellulose mainly by two steps

1. Glucan chain formation with polymerisation
2. Assembly and crystallisation of cellulose chain

1. Glucan chain formation with polymerisation

The conversion of glucose monomers into polymeric chain involves UDP-α-glucose as the substrate, catalysed by cellulose synthase. This is a single step process wherein the glycosyl group is transferred by inversion of structural configuration to the anomeric carbon. Also, a single cellulose synthase molecule can carry out the initiation, elongation and termination of the polymerisation process. Hence, it

implies that substrate UDP-glucose is directly converted to the product by cellulose synthase without any intermediate molecule (Saxena and Brown 2005).

Two hypotheses have been proposed to explain the mechanism of 1, 4-β glucan chains polymerisation.

As per the first hypothesis, the glucose unit was converted into a lipid intermediate with catalysed by an enzyme glycosyltransferase. This lipid intermediate is said to further take part in biosynthesis by addition of glucose residues to obtain the cellulose polysaccharide. Also, some literature studies have proved synthesis of acetan polymer (a soluble polysaccharide associated during cellulose synthesis in *A.xylinum* species) is linked to the production of lipid intermediate. However, if the lipid intermediate mediates, the process remains unknown (Iannino et al. 1988).

Concerning the second hypothesis, it stated that the non-reducing end of glucan chains is the site where addition of the glucopyranose residues (derived from UDPG) simultaneously occurred during the polymerisation process. Here, an assumption was made that the lipid intermediate was not required to transfer monomers of glucose for the formation nascent glucan chains during polymerisation process (Brown 1996; Brown and Saxena 2000; Koyama et al. 1997). The cellulose synthesised by the bacteria is a bunch of microfibrils with an approximate number of forty-six microfibrils at the rate of two micrometres per minute.

A study by Benziman et al. (1980) showed that polymerisation and crystallisation were an independent but continuous process; the team established the relationship between the two processes by the addition of Calcofluor white stain which binds to cellulose fibrils. The study showed that binding of the dye showed disruption in crystallinity but accelerated polymerisation; on the removal of the stain revealed both the steps to occur at normal rates. The microfibrils of cellulose range about 3nm in width made up of microfibrils parallelly arranged glucan chains; the thickness could increase up 20 nm in some algal forms (Jarvis 2003). A single cell of *Acetobacter xylinum* polymerise up to approximately 2, 00,000 glucose molecules per second, which are later extruded from the Terminal Complexes (TCs) and translocate outside the bacterial cell into the medium (Schramm and Hestrin 1954).

2. Process of Assembly and crystallization of glucan chains

The assembly of nascent glucan chain into protofibrils and these protofibrils accumulate together in parallel arrangement to form microfibrils. The next structure in line after microfibrils, nearly 100 to 1000 β-1, 4 glucan chain that is bound loosely (Iguchi et al. 2000). The final assembly and crystallisation take place in the extracellular environment of the cell (Ross et al. 1991). Although the process of assembly and crystallisation of cellulose occurs extracellularly, it was depicted as cell-directed process due to the self-aggregation of microfibrils resulting in formation of glucan chains which appeared as ribbon like structures (Ross et al. 1991).

2.7 Isolation of Bacterial Cellulose Producing Strains

One of important factor that determines the BC production is the strain or the type of bacterium (Lima et al. 2017). The yield and other mechanical properties of the Bacterial Cellulose are dependent on the bacterial strain. Although wide ranges of bacterial species are found in nature, acetic acid producing bacteria are the dominant class of bacteria which are able to produce the cellulose. Acetic acid bacteria derive energy from the oxidation of alcohol to produce acetic acid. They belong to the class of aerobic, rod-shaped bacteria and Gram-negative bacteria. *Acetobacter xylinus / Gluconacetobacter xylinus* strain known for the highest BC production (Oikawa et al. 1995; Pecoraro et al. 2007).

Several studies have been carried out on isolation of BC producers from various sources such as rotten fruits and vegetables, fruit and vinegar waste, apple wine, fermented foods and wastes, coffee husk, malt extract, soil, cream, dairy products and temple wash waters etc. The various BC producers and their sources have been tabulated, as shown in Table 2.4.

Table 2.4 Cellulose producing bacterial strains

Bacterial strain	Sources of isolation	Strain number	Reference/s
Acetobacter sp.	Flowers, fruits, nuts	BPR 2001	Toyosaki (1995)
Gluconacetobacter xylinus	Grapes	BPR 2002	Kojima et al. (1998)
Gluconacetobacter xylinus	Melon fruit	BPR 2003	Kojima et al. (1998)
Acetobacter xylinum	Rotten grapes	KJ-1	Son et al. (2002)
Gluconacetobacter hansenii	Rotten fruits and vinegar wastes	P2A	Park et al. (2003)
Gluconacetobacter oxydans	Malt extract	TQ-B2	Jia et al. (2004)
Gluconacetobacter rhaeticus, Gluconacetobacter swingsii	Apple juice	DST GL02T DST GL01T	Dellaglio et al. (2005)
Gluconacetobacter hansenii	Vinegar fermentation wastes	AS6, AS14	Aydin and Aksoy (2009)
Enterobacter amnigenus	Rotten apple	GH-1	Hungund and Gupta (2010a)
Komagataeibacter Persimmonis	Nata	GH-2	Hungund and Gupta (2010b)
Gluconacetobacter hansenii	Grape wine	UAC09	Rani et al. (2011)
Gluconacetobacter sp.	Persimmon vinegar	RKY5	Young-Jung Wee (2011)
Gluconacetobacter sp.	Rotten tropical fruits	PAP1	Suwanposri et al. (2013)
Acetobacter sp.	Wastewater	NU4	Premjet Siripong (2012)
Pseudomonas spp.	Rice, tomato, cheese, bread, fruits	F3	Kazim (2015)
Gluconacetobacter sp.	Citrus fruit juice	SEA623-2	Kim et al. (2017)
Komagataeibacter europaeus	Rotten black grapes	SGP37	Dubey et al. (2017)

2.8 Bacterial Cellulose Production

2.8.1 Static fermentation

The synthesis of cellulose from bacteria can be performed by either static or submerged conditions (Hornung et al. 2006; Chawla et al. 2009). There are several advantages and disadvantages to both methods. Static fermentation is the most preferred method of Bacterial Cellulose production. In the static mode of fermentation, flasks are inoculated with the bacteria cultures and incubated at static condition till the formation of the pellicle of the top surface of the growth medium (Son et al. 2001; Lin et al. 2014). The BC synthesis is directly proportional to surface area and volume on the area of air (Okiyama et al. 1992). The cellulose pellicle thus formed is an air-permeable membrane which facilitates adequate aeration in the medium.

In the static medium, there is an accumulation of acids which tends to decrease the pH of the medium, in turn affecting the growth. In static fermentation, a thick pellicle takes a long time to be produced, which makes static method unsuitable on commercial-scale production. (Chawla et al. 2009; Huang et al. 2015; Son et al. 2001). Yan et al. (2008) investigated the static fermentation of BC and found it more suitable for various applications due to uniformity of cellulose matrix static in contrast to the agitated mode wherein they are produced as spheres or irregular pellets. The disadvantage in static mode is high production cost and longer periods of cultivation (Çakar et al. 2014; Lee et al. 2014; Tyagi and Suresh 2016).

2.8.2 Agitated Fermentation

The agitated fermentation process is more effective for industrial-scale production of BC. This method requires proper aeration and agitation to maintain homogeneity of the medium and effective mass transfer (Watanabe et al. 1998). Several literature reports have highlighted the differences between the static and agitated method of production of BC (Czaja et al. 2004; Jung et al. 2005). Due to increased viscosity of the culture medium, oxygen distribution is disturbed in the broth, which affects the BC production. The two main drawbacks of this mode of fermentation are (i) non-

uniform bacterial sizes and shapes, (ii) inconsistency in oxygen supply and development of cellulose deficient mutants (El-Saied et al. 2004).

A study by Son et al. (2001) reported that the ethyl alcohol when added to the medium prevented the production of cellulose deficient mutant strains. Strains like *Acetobacter xylinum* NUST4.1, *Glucoacetobacter hansenii* KCTC, *Acetobacter xylinum* BPR etc. were some of the strains suitable for Bacterial Cellulose production on the agitated mode of fermentation (Toyosaki et al. 1995; Naritomi et al. 2002). BC was produced in the form of pellets. Another disadvantage of this process is several structural and morphological defects observed in BC synthesised due to the shear and stress (Zuo et al. 2006; Zhou et al. 2007). Lu and Jiang (2014) found that the crystallinity index of Bacterial Cellulose produced from the static medium was higher than that of agitated fermentation. The microbial cellulose produced by the airlift reactor formed a unique ellipse pellet different from the fibrous form that was produced in an agitated stirred tank fermentor (Chao et al. 2000).The major limitations concerning agitated culture are the non-newtonian behaviour leading to instability of the bacteria, and the high shear force. Moreover, BC produced by this mode of fermentation lower DP value, lower crystallinity index, and lower mechanical properties (Kouda et al. 1997). A BC membrane is usually formed at the top surface of broth media under static mode, whereas in the agitated method, BC is produced from the centre of the medium and then grows outwards. Hence, a layered formation can be observed in the sphere-like structured BC with hollow interiors. Hu and Catchmark (2010) reported denser fibres of BC within the layered structure of the sphere. Despite these limitations, some researchers have suggested that the agitated culture could be economical for commercial scaling up of BC.

Another limitation concerning BC production on agitated fermentation is that there is a high probability of production cellulose-negative mutants due to increased turbulence and shear stress (Kim et al. 2007). Thus, different reactors have been designed to enhance cellulose productivity while taking care to prevent the formation of cellulose-negative bacterial strains (Ul-Islam et al. 2017). Studies have shown the use of Stirred-tank bioreactors for the production of BC. However, the crystallinity index, elasticity and tensile strength, and DP of the synthesised BC were lower than

that of static fermented BC (Watanabe et al. 1998). Shoda and Sugano (2005) reported that the BC suspension in stirred tank bioreactors produced high viscosity, which in turn limited the oxygen transfer rate and required a lot of energy for the continuous agitation. Another reactor which has been used is an airlift bioreactor. This is advantageous over the stirred tank reactor as this involves less shear stress. In this oxygen, the transfer occurs from the bottom to top of the fermentation medium. Chao et al. (1997) used an airlift bioreactor for the first time to study BC production. Wu and Li (2015) used a modified airlift bioreactor which was made of series of discs placed one below another to synthesise BC in the form of a mat. The resulting membranes demonstrated higher WHC (water holding capacity) than BC obtained from static fermentation, and the elastic modulus was adjusted by either decreasing or increasing the number of discs.

2.9 Process Parameters

As we know that various physiological and nutritional factors play an important role in the synthesis of BC. The overall BC production process could be improved by the optimisation of these parameters. Several researchers have focused on various aspects such as temperature, pH, incubation period and nutritional factors such as carbon and nitrogen sources (organic and inorganic) for better yield and production of BC.

2.9.1 Effect of Temperature

The temperature is one of the essential parameters to determine cellulose production and yield (Chawla et al. 2009). Literature studies have revealed the temperature for Bacterial Cellulose production ranges from 25 to 30°C (Bielecki et al. 2005; Hestrin and Schramm 1954; Geyer et al. 1994; Jonas and Farah 1998; Son et al. 2001). Panesar et al. (2009) reported the highest BC yield of 1.6 g/L at 28°C using *Acetobacter* strain variant. Pourramezan et al. (2009) studied the effect of temperature and examined the highest BC yield at 30°C and lowest at 45°C. Mohite et al. (2013) reported the cellulose yield (3.1g/L) at 25ºC. A study by Kalifawi and Hassan (2014) showed that temperature acted as a vital parameter in the production of BC, and the production stopped above 50°C. However, many researchers showed the maximum

yield at 30°C (Hugund and Gupta 2010a; Rani et al. 2011; Raghunathan 2013; Atwa et al. 2015; Kim et al. 2017).

2.9.2 Effect of pH

The pH is another critical parameter that acts one of the factors to influence the process of bacterial growth and fermentation. Hence, it is an essential physiological factor that determines the physiology, enzyme activity and chemical environment of the bacteria. Thus, the pH of the growth medium has to be monitored during the BC production. Several studies have been carried to evaluate the effect of pH on BC production. The optimum pH for BC production has been shown to range from 4 to 6 (Son et al. 2001; Bae and Shoda 2005). The pH of the fermentation medium depends on the type of carbon source used as the nutrient source (Shirai et al. 1994; Keshk and Sameshina 2005).

Sugars such as glucose metabolise to acids, as a result pH value drops and BC synthesis is affected (Kongruang 2008). Masaoka et al. (1993) found that the yield of cellulose decreases below pH 4. A study by Embuscado et al. (1994) found that the strain *Acetobacter xylinum* showed maximum yield at pH of 4.5 and absolutely no yield at lower pH of 3.5. Jonas and Farah (1998) revealed the Bacterial Cellulose production was observed from of pH is 4.0 - 4.5. In a study by Erbas et al. (2015) reported maximum BC yield (0.15g/L) at pH of 8 and a temperature of 28°C. Jung et al. (2010) found that the pH of 6.5 was optimum for Bacterial Cellulose production.

In another study, Hugund and Gupta (2010b) reported the highest cellulose yield (5.14 g/L) at pH 5.5 using strain *Gluconacetobacter persimmonis* GH-2. Jagannath et al. (2008) obtained the maximum yield at pH 4.0 with 10% sucrose as carbon source, whereas Kim et al. (2017) reported maximum BC production at a lower pH of 3.5 using by *Gluconacetobacter sp.* gel SEA_623-2 strain and citrus juice as the fermentation medium. Raghunathan (2013) obtained Bacterial Cellulose yield (0.81 g/L) optimal pH 7 by *Acetobacter xylinum* DR1 strain fermenting wastewaters. Therefore, it is crucial to optimise the pH of BC fermentation medium according to strain employed and regularly monitored to ensure good BC yield and growth conditions.

2.9.3 Dissolved Oxygen

The dissolved oxygen (DO) is an important parameter to ensure proper cellular growth of the bacteria along with proper cellulose synthesis. Kouda et al. (1997) investigated the role of DO during BC production. The study proved that BC yield increased with increase in oxygen transfer; however, carbon dioxide produced by the cells affected the cellulose production. A study by Tantratian et al. (2005) reported that the higher value of DO in the fermentation medium caused the increase in the gluconic acid, which in turn slowed down BC production. Chao et al. (2000) found that the volumetric oxygen transfer coefficient (kLa) played an essential role in determining BC yield produced using fermenter. Bae and Shoda (2005) reported that the highest BC yield was obtained using DO of 30% in ten litre fermenter.

2.10 Effect of Nutritional sources

The essential components of any fermentation broth are carbon, nitrogen and some micronutrients which are vital for bacterial growth. Therefore, it is necessary to provide the optimal concentrations of the carbon, nitrogen sources, along with additives for maximum product production. The effects of different media components on BC production are described below:

2.10.1 Effect of Carbon Sources

The carbon source is one of the major nutrients which are essential for bringing proper cell development and product formation. The standard medium for BC is Hestrin and Schramm (HS) medium composed 20 g/L; bacteriological yeast extract, 5 g/L; bacteriological peptone, 5 g/L; sodium hydrogen phosphate, 2.7 g/L; citric acid, 1.15 g/L. Although the standardised medium has been used to cultivate BC, several studies have been carried out using various other carbon sources other than glucose such as fructose, sucrose, starch, mannitol, glycerol etc. A study by Matsuoka et al. (1996) found that 4% lactate (w/v) was found to increase cellulose yield. Ramana et al. (2000) used glucose, mannitol and sucrose as carbon sources for BC production by *A. xylinum* NCIM 2526 strain and found sucrose to be the optimum carbon source. Similarly, a study by Son et al. (2001) showed that ethanol added at 1.4% (v/v) to the

production medium produced four times higher yield of BC (15.2 g/L) by employing *Acetobacter* sp. A9 strain. A study by Ishihara et al. (2002) reported wood sugar to give optimum BC yield (3.0 g/L) by *A. xylinum* IFO 15606 strain. Krystynowicz et al. (2002) used sucrose (4%) and found the maximum cellulose yield. *Gluconacetobacter hansenii* PJK (KCTC 10505 BP) strain gave the highest yield (1.72 g/L) using glucose as the sole carbon nutrient source (Park et al. 2003). Park et al. (2003) also evaluated the effect of ethanol as another carbon source and observed that the BC production increased from 1.3 to 2.31 g/L along with effective degeneration of non-cellulose producing mutant bacterial cells of *Gluconacetobacter hansenii* strain. Keshk and Sameshima (2005) studied using several various carbon sources, of which glycerol gave the highest BC yield of 5.4 g/L. Similarly, Kim et al. (2006) obtained yield carbon and nitrogen sources for *Acetobacter xylinum* strain, and maximum cellulose production was found using mannitol and glucose. Mikkelson et al. (2009) optimised the fermentation medium with carbon sources galactose, sucrose, fructose, glycerol and mannitol, and obtained the maximum cellulose production 3.83 g/L using sucrose.

Mohammadkazemi et al. (2015) studied the effects of different carbon sources using various media formulations on *Gluconacetobacter xylinus* strain PTCC 1734 and found that mannitol followed by sucrose, which gave the highest BC yield. Molina-Ramírez et al. (2017) evaluated *Komagataeibacter medellinensis* strain using sucrose, fructose and glucose and found that glucose (2%) gave the highest yield of 2.8 g/L. Singhsa et al. (2018) evaluated five different strains of *Komagataeibacter xylinus* with glucose, fructose, lactose, maltitol, sucralose, and xylitol and found that maximum cellulose yield of 1.84 g/L using glucose as carbon source. Wang et al. (2018) investigated the effects of several carbon sources and found that fructose gave the highest yield of 1.59 g/L. Pourramezan et al. (2009) evaluated the BC yield by *Acetobacter* sp. 4B-2 in an optimised medium using various carbon sources such as monosaccharide and disaccharides and found that sucrose gave the maximum yield (11.98 g/L), followed by other carbon sources. In another study, 3% fructose gave a yield of 6.29 g/L BC by the strain *Enterobacter amnigenus* GH-1 (Hugund and Gupta 2010a). Maximum yield of 5.0 g/L was obtained using 2% sucrose as carbon source by the strain *Gluconacetobacter hansenii* NCIM 2529 (Mohite et al. 2013).

Apart from ethanol, some organic acids have also been known to increase the yield of BC, i.e., acetic acid (20g/L) added to the medium increased the BC yield from 0.6 to 3.8 g/L of (Keshk and Sameshima 2006). Another study by Keshk and Sameshima (2005) obtained the highest yield of BC from *Gluconacetobacter xylinus* ATCC 10245 using glycerol as carbon source over glucose which showed higher consumption efficiency over glycerol, lesser productivity compared to glycerol. Dubey et al. (2017) obtained maximum cellulose yield (6.49 g/L) from strain *Komagataeibacter europaeus* SGP37 using fructose as a carbon source.

2.10.2 Effect of Nitrogen Sources

Nitrogen plays an important role in building protein and nucleic acids of the bacterial cell. Many researchers have investigated on different types (organic and inorganic) types of nitrogen sources. The standard media (Hestrin and Schramm) has been modified by replacing with various and evaluated for BC production using different strains of bacteria. The HS medium has peptone and yeast extract as nitrogen sources; several studies have been carried out using varied concentration and replacing the original nitrogen in the medium. Beef extract, yeast extract and peptone are the most common nitrogen sources used for BC production.

Yang et al. (1998) obtained a yield of 6.7 g/L cellulose with the optimised carbon and nitrogen (yeast extract) were 20 g/L and 40 g/L, respectively. The nitrogen concentration used for the study was 4% (w/v). A Study by Kurosumi et al. (2009) found that fermentation using strain *Gluconacetobacter xylinus* NBRC13693 when the medium was supplemented with fruit juice as the nitrogen source along with 2.0% peptone, 0.5% yeast extract, increased BC production up to 7%. Pourramezan et al. 2009 reported 11.65 g/L cellulose was produced from (7 g/L yeast extract and 9 g/L peptone) along with carbon source; it was proven that both the nitrogen sources were essential. Hungund and Gupta (2010b) investigated the effect of different nitrogen sources on BC production by the strain *Gluconacetobacter persimmonis* GH-2 and found that beef extract [0.5% (w/v)] gave the maximum yield of 6.25 g/L at followed by casein hydrolysate that gave an yield of 5.25 g/L. A study by Panesar et al. (2009) reported that sodium nitrate at 1% (w/v) was the best nitrogen source to produce BC

yield of 1.6 g/L using *Acetobacter aceti* MTCC 2623 strain. Çoban and Biyik (2011) carried out a study to evaluate the effects of nitrogen source on BC. They found that yeast extract gave the highest yield over ammonium sulphate, casein hydrolysate and peptone.

Similarly, a study by Biyik and Coban (2017) found that 5 g/L casein hydrolysate as an ideal nitrogen source. Another study by Santos et al. (2013) showed that yeast extract-corn steep liquor (both at 5 g/L), along with fructose was an optimum nutrient medium for the production of BC by *G. sucrofermentans* CECT 7291. Costa et al. (2017) reported that corn steep liquor (10g/L) was ideal nitrogen source instead of yeast extract and peptone with a BC yield of 5.8 g/L using strain a strain of *Gluconacetobacter hansenii* UCP1619. Ramana et al. (2000) obtained maximum cellulose yield using casein hydrolysate in sucrose driven fermentation medium using strain *Acetobacter xylinum*.

2.10.3 Effect of supplements/additives

Studies have been performed by researchers to enhance the yield of BC by addition of additives which can act as a precursor or stimulators for cellulose synthesis. The mechanism by which additives function in fermentation is by shear force reduction, increased viscosity etc. (Bielecki et al. 2002). The BC production reportedly increased up to 6% with the addition of components such as carboxymethyl cellulose, organic acids, sodium alginate to the production medium (Zhou et al. 2007). A study by Naritomi et al. (1998) showed that ethanol and lactate increase BC productivity; although ethanol gave a higher yield over lactate. Similarly, Krystynowicz et al. 2002 reported the increase in the BC yield with the addition of ethanol. Lu et al. (2011) observed step up in the yield after addition of sugar alcohol such as mannitol and butanol to the production medium. Dayal et al. (2016) reported that addition of supplements like corn steep liquor, gelatin, cornstarch added in different concentrations to the medium had improved the yield of Bacterial Cellulose along with enhancing physical properties of the polymer.

2.11 Bacterial Cellulose production from various industrial wastes

2.11.1 Agricultural industries and by-products

Generally, overall BC production is a costly affair as it is usually synthesised from commercially available media components that include carbon, nitrogen sources and other essential components. This, on the whole, affects the commercialisation of cellulose on a large scale. Therefore, several studies have shifted their focus on finding alternatives and utilising waste products to reduce production cost. Several materials have been studied such as agricultural wastes and by-products of fermentation industries, cheese whey, lipid fermentation waste, coffee husk, crude glycerol, corn steep liquor, molasses, thin stillage, fermentation wastewaters, hot water extracts of wood, and rice wastewater (Shamolina 1997). Thus synthesising BC from the naturally derived raw materials, not only benefits in lowering the production costs of polymer but also makes the process environment-friendly.

Thompson and Hamilton (2001) carried out BC fermentation process using cheese whey, potato effluents and beet sugar by using two strains namely *Acetobacter xylinum* ATCC 10821 and 23770 and found yield was 17 % higher than that by glucose as carbon source. A study by Bae and Shoda (2004) reported that the sulphuric acid-treated molasses gave 76% higher yield over untreated molasses using *Gluconacetobacter xylinus* BPR2001 by 7.82 g/L by fed-batch fermentation and 6.3 g/L by continuous fermentation method. In another study, Bae and Shoda (2005) used evaluated cellulose production using molasses as nutrient source. Keshk et al. (2006) studied the ability of strain *Gluconacetobacter xylinus* ATCC 10245 to synthesise BC using beet molasses as production medium and obtained an increased yield of 7g/L when compared to 5.36 g/L when glucose was the carbon source. A study by Keshk and Sameshima (2006) reported higher BC production when glucose of the standard Hestrin-Schramm medium (HS medium) was replaced with molasses. The study was also carried out by coupling molasses with lignosulfonate and it was found to increase the yield of Bacterial Cellulose along with reduced acid production (gluconic acid). They also reported that polyphenolic and antioxidant contents present in lignosulfonate were responsible for reduced acid production.

Premjet et al. (2007) studied the effect of sugar cane molasses on BC production by *Gluconacetobacter xylinus* ATCC 10245 which included amino acids, vitamins, carbohydrates, nucleic acids and black colour substances (derived from sugar cane molasses) along with fructose and sucrose as carbon source. Amongst all, it was the black coloured substance increase in the BC yield to 255%. Kongruang (2008) carried out the study using juices extracted from pineapple and coconut waste as primary carbon nutrient source for cellulose synthesis by the strain *Acetobacter xylinum* TISTR 998 and *Acetobacter xylinum* TISTR 893. Of the two, *Acetobacter xylinum* TISTR 998 strain produced the highest yield of cellulose with coconut juice as the substrate. Dahman et al. (2010) studied the effect of agriculture wastes such as corn fibres and wheat straw along with single or mixed carbon substrates. The maximum Bacterial Cellulose yield (5.65 g/L) was obtained with fructose (40g/L) coupled with corn fibres followed by glucose coupled with wheat straw. Jung et al. (2010) found that synthesis of BC increased by two folds when molasses was supplemented to the medium as the carbon source and corn steep liquor as nitrogen source.

Kumalaningsih et al. (2012) investigated the effect of pineapple concentrate for BC production by *Acetobacter xylinum*. The study was performed along with carbon and nitrogen source at different concentrations, and it was observed that sucrose (5%) and ammonium sulphate (0.7%) gave the BC of 5.67mm thick and weight of 217g. Rani and Appaiah (2011) supplemented agricultural by-products to the production medium and found three folds increase in the BC yield (5.6-8.2 g/L) using the strain *Gluconacetobacter hansenii* UAC09. The supplements were used as carbon and nitrogen sources for the medium such as acetic acid, ethyl alcohol, coffee cherry husk, corn steep and urea were used at various concentrations. Coffee cherry husk extract 1:1 (w/v), corn steep liquor 8% (v/v), acetic acid 1.0% (v/v), urea 0.8% (w/v), ethyl alcohol 1.5% (v/v).

Zeng and coworkers (2011) used maple syrup as a supplement to the BC production medium by the strain *Gluconactobacter xylinus* BPR2001. However, the yield obtained (1.51 g/L) was slightly lower than that of fructose only medium (1.6 g/L). Carreira et al. (2011) used various agroindustry derived products such as cheese whey, sulfite pulping liquor, grape skins extracts and crude glycerol for BC

production as an alternative to normal nutrient (C&N) sources in the medium. It was found that the grape skins aqueous extract gave the maximum yield of 0.6g/L Bacterial Cellulose after 6 days of the fermentation period. A study by Hungund et al. (2013) evaluated different types of fruit juices of pineapple, pomegranate, muskmelon, orange, tomato and watermelon. Other sources included coconut water, coconut milk, molasses, starch hydrolysate and sugarcane juice for cellulose production from *Gluconacetobacter persimmonis*. 8.08 g/L of cellulose was the maximum yield obtained from muskmelon. Another study by Lin et al. (2014) investigated the waste beer yeast from breweries (WBY) as a nutrient source for *Gluconacetobacter hansenii* CGMCC 3917 strain to synthesise BC. The waste beer yeast was hydrolysed with strong alkali NaOH. This was used as a production medium for cellulose. It was found that the yield (7.02 g/L) was six times higher than untreated one (1.21 g/L).

Suwanposri et al. (2014) used soya bean whey to optimise BC production medium. Soya bean whey is generated as a by-product during soy milk processing. They found that *Komagataeibacter* sp. PAP1was capable of producing BC 3.6 times higher than that using HS medium. Another study by Afreen and Lokeshappa (2014) used waste juices of papaya and muskmelon and coconut water. The study revealed that *Acetobacter xylinum* (NCIM 2526) was able to ferment the juice and produced increased yield of BC with papaya juice with the maximum of 4.52g/100mL, followed by coconut water giving 2.43 g/100mL and muskmelon giving 1.68g/100mL of BC.

Similar to this study, Lestari et al. (2014) also used the waste coconut water and pineapple juice as a nutrient source for the fermentation. The process was carried out in two different phases, i.e., shaking culture and static culture with the help of *Acetobacter xylinum*. In both cases, it was observed that the conversion of glucose into cellulose biomass was higher when coconut water was used and concluded that it was more suitable and efficient substrate over pineapple juice. Some of the agro-industrial by-products used for Bacterial Cellulose production are listed in Table 2.5. Li et al. (2015) used wastewater obtained from candied jujube-processing industry to produce BC using the strain *Gluconacetobacter xylinum CGMCC No.2955* and found that 1.5 g/L Bacterial Cellulose was obtained after two-fold dilution of the

wastewater. Abdelhady et al. (2015) used a modified HS medium with sugar cane molasses to produce Bacterial Cellulose from a novel isolated strain *Komagataeibacter saccharivorans* PE 5. The study reported an increase in the yield of BC to about 1.77 times.

Similar to the previous study, Farag et al. (2016) used H_2SO_4 treated molasses was treated for the production of Bacterial Cellulose using the isolated strain *Achromobacter* sp. and obtained maximum cellulose yield using molasses 5% (v/v) as a nutrient source (5.95 g/L). A study by Huang et al. (2016) showed that *Gluconacetobacter xylinus* was able to ferment wastewater generated after the lipid fermentation which had a high COD of 25,591 mg/L and produce 0.659 g/L BC after five days fermentation period. Revin et al. (2018) used acidic by-products of alcohol, and dairy industries (thin stillage and whey) was used, and *Gluconacetobacter sucrofermentans* B-11267 was effectively able to synthesise BC (6.19 g/L) from thin stillage and (5.45 g/L) from whey. Salari et al. (2019) utilised sugar beet molasses and cheese whey to produce BC nanocrystals of about (0.25 g/l d) from the strain *Gluconacetobacter xylinus* PTCC 1734. These studies show that several researchers have effectively utilised alternative nutrient source in place of standardised medium and synthesised BC, thus lowering the overall cost of production.

Table 2.5 BC synthesised from agricultural and industrial waste materials

Agro-waste used	Bacterial strain	Reference(s)
Molasses from sugarcane	*Acetobacter xylinum*	Keshk and Sameshima (2006) El-Saied et al. (2008)
Molasses from beet	*Gluconacetobacter xylinus* ATCC 10245, *K. lovaniensis*	Keshk et al. (2006) Bae and Shoda (2005) Coban and Biyik (2011)
Pineapple fruit wastes	*Acetobacter xylinum*	Junaidi and Azlan (2012)
Pineapple peel and Sugar cane juice	*Gluconacetobacter swingsii*	Castro et al. (2011)
Molasses	*Gluconacetobacter xylinus* FC01	Çakar et al. (2014)
Coconut water	*Rhodococcus sp*. MI 2	Tanskul et al. (2013)
Juices from citrus, apple and pear	*Acetobacter xylinum* NBRC 13693	Kurosumi et al. (2009)
Palm Date syrup	*Gluconacetobacter. xylinus* PTCC 1734	Moosavi-nasab et al. (2010)
Aqueous extract of grape skin	*Gluconacetobacter sacchari*	Carreira et al. (2011)
Kombucha tea	*Gluconoacetobacter xylinus*	Nguyen *et al*. (2008)
Corn steep liquor	*Gluconoacetobacter xylinus*	El-Saied *et al*. (2008)
Dry olive mill residue	*K. sacchari*	Gomes *et al*. (2013)
Straw from wheat fibres	*Gluconacetobacter xylinus*	Chen *et al*. (2013)
Thin stillage (rice wine)	*Gluconacetobacter xylinus*	Wu and Liu (2012)
Methanol, ethanol, glycerol, ethylene glycol	*Acetobacter xylinum 186*	Lu et al. (2011)
Wastes from citrus fruits	*Gluconacetobacter intermedius CIs26*	Yang et al. (2013)
Watermelon peels	*Komagataeibacter kombuchae MCM B- 967*	Kumbhar et al. (2015)
Sulphite liquor, whey of cheese and crude glycerol	*Gluconacetobacter xylinus*	Vazquez *et al.* (2013)

2.11.2 Industrial wastes generated from Breweries

These industries generate volumes of wastewaters and byproducts on a day today basis, which remains a problem for the management so as to effeciently treat the wastewaters (Fillaudeau et al. 2006). The wastes generated are rich source of carbon and nitrogen, hence these can be utilised by microorganisms as nutrient media. Several byproducts produced from brewery industries have been used for cellulose production. Hyun et al.(2014) reported that makgeolli sludge, which is generated as a byproduct from rice wine distilleries was a suitable carbon nutrient source for the growth of the bacterium *G. xylinus* to synthesise BC. It was revealed the medium was composed of nitrogen amount of 0.81 g/L, the glucose of 10.24 g/L, alcohol (0.93% v/v) which supported the bacterial growth. The chemical and structural features of BC obtained from makgeolli sludge medium displayed dense microfibrillar networks along with the peaks which were significant features of cellulose I type.

Waste beer yeasts (WBY), another brewery waste which is generated in huge amounts in are either discarded into the environment or used as a feed for animals. After chemical hydrolysis, WBY hydrolysate was used as a production medium for *G. hansenii* CGMCC 3917. Results showed that BC productivity gradually increased up to 6 times with dense BC membrane production. The morphological characterisation revealed that cellulose microfibrils produced were randomly arranged with large pores (Lin et al. 2014). Grape bagasse, another by-product of wine production, was also assessed as a medium for BC production. The characterisation studies revealed that the BC microfibrils displayed fibre diameter of 35–70 nm and thickness of 13–24 nm, the other features of Cellulose I was reported as well (Vazquez et al. 2013). Another waste, namely citrus peel and pomace were treated enzymatically and this enzymatic hydrolysate was used for cellulose synthesis. The results showed 46.15% higher BC production over the standard HS medium.

Fan et al. (2016) also reported that there were no much variations in BC surface morphology, FTIR peaks, and CI and size in comparison to the BC obtained from HS medium. Hence, the huge quantities of waste produced from this industry can be used

either as a complete or as a supplement along with a portion of standard nutrient medium for BC production.

2.11.3 Sugar, paper and pulp industries

Sugarcane is one of the primary contributors of sugar and jaggery. A rich source of cellulose, fodder for cattle's, and a source of energy in the form of fuels. In general, major byproducts of sugar and related industry consist of molasses, bagasse and effluent waters (Yadav and Solomon 2006). From the economic value and nutritional point of view, this biomass and waste can be utilised to produce several value-added products by chemical and biotechnology methods (Poddar and Sahu 2017; Tyagi and Suresh 2016).

Various reports are available on molasses as a nutrient medium for the production of Bacterial Cellulose. Keshk and Sameshima (2006) used the sugar cane molasses residue as a carbon nutrient source instead of glucose in standard medium. The results demonstrated enhanced BC production than that of the standard medium. Also, many of the physicochemical features of BC obtained were similar to that of BC derived from standard medium indicating a typical cellulose type I form. The heat-treated and acid (sulphuric acid) pretreated molasses were used as a medium for cellulose production. The results showed that the cellulose synthesised from the HS medium and acid pretreated molasses-HS media depicted almost same FTIR and XRD spectra. Moreover, the Bacterial Cellulose synthesized from only molasses showed a higher mechanical propert (102 ± 16.8 MPa) over that of BC derived from standard medium (mechanical strength 74 ± 4.7 MPa).

Another study reported by Qi et al. (2017) demonstrated that the acid and enzymatic hydrolysates of bagasse were suitable to be used as feedstock for BC synthesis. The physicochemical and structural analysis revealed that these fibres displayed high water holding capacity and lower mechanical strength. A study by Pinto et al. (2016) proved that BC obtained from sugarcane molasses did not display any level of cytotoxicity indicating its capability to be used a biomaterial for biomedical applications.

Residual materials obtained from pulp mills contains huge amount of waste sludge, pulp derived wastes and hot wood water extract. The byproducts predominantly have cellulose and hemicellulose polymers. Hence, these wastes can be converted into commercially useful products (Alonso et al. 2017). Due to highly complex structural framework, usually enzyme and chemical mediated processes are used to break down to simple sugars and also to detoxify any harmful components. The sweet lime pulp waste was studied for BC production under batch and fed-batch fermentation conditions. The FTIR revealed prominent peaks of cellulose with certain new peaks which were formed due to variation in the structure of BC synthesised from the waste broth medium. The microstructural analysis of Cellulose showed randomly distributed nanofibrils with almost same fibre size. XRD revealed type I crystallinity (Dubey et al. 2018).

The biocompatibility feature of Bacterial Cellulose serves a major parameter for various biomedical applications. The biocompatibility studies performed using BC synthesised from jaggery scum medium using skin fibroblast cells revealed the adherence of the cells onto surface of cellulose film, further inferring its non-toxic nature (Khattak et al. 2015 a, b).

2.11.4 Textile industrial wastes

The demand for sustainable textile/fibre waste production and utilisation, recycling and management of waste effluent has gained importance over the past few decades. As, we know fibre/textile generated from these industries are a huge source of cellulosic fibres waste and hence, the produced wastes may be subjected to chemical treatments and converted into simpler products which can then be utilised for further applications (Pensupa et al. 2017).

A study by Hong et al. (2012) showed that wastes obtained cotton fibre that were initially pretreated using an ionic liquid and later subjected to enzyme treatment. The lysate produced as a result of this process displayed a high carbon concentration of 17 g/L. BC synthesized using this waste derived lysate gave a high yield of 10.8 g/L and displayed a good tensile strength (0.070 MPa) over the cellulose derived from the standard HS medium which used glucose as carbon source.

Previous literatures have also reported that the textile wastes which contain huge amount of cellulose were pretreated using chemical and enzymes and end product generated was suitable to be used as growth medium for the synthesis of BC. Kuo et al. (2010) used this as a medium for cellulose production and obtained 1.88 g/L of BC.

2.12 Purification of Bacterial Cellulose

The cellulose obtained from the culture medium has to be further treated using chemicals to remove any bacterial cells adhered to the film, to remove media components and any other impurities to finally obtain pure cellulose devoid of any contaminants (Moosavi-Nasab and Yousefi 2010). The most common process of treatment involves alkali treatment (usually NaOH) as the first step for purification. Post alkali treatment the Bacterial Cellulose is further subjected to treatment with a weak acid to neutralise the effect of the base. This step is followed by intermittent washing using deionised water. This step is repeated until the filtrate reaches neutral pH (Chawla et al. 2009).

The bacterial cells of adhered to the cellulose mat were treated with NaOH/KOH/Na_2CO_3 for about 15 – 20 mins at the temperature of 90°C to destroy the cells. This cellulose mat was then given acid wash for about 5-10 minutes, and then the pellicle was thoroughly rinsed with deionised water until neutral pH was attained. This purified pellicle was later dried in an oven for about 30 mins, and its dry weight was recorded (Jung et al. 2005; Kongruang 2008). Some researchers have treated the Bacterial Cellulose pellicle using sodium dodecyl sulfate solution, followed with alkaline and acidic washing and later rinsed repeatedly with distilled water. Later, dried at 60-80°C temperature until a constant weight was recorded (Jung et al. 2005; Sakairi et al. 1998; Chawla et al. 2009).

The attention is focused on the purification step is because the Bacterial Cellulose synthesised have been majorly used in several medical applications. Therefore, care to need to be taken to get the pellicle rid of impurities or bacterial cells, which could otherwise elicit immune reactions (Thomas et al. 2013). The medical applications needed purification steps which involved the processing of the pellicle absorbent filter

papers such that 80% of water is removed. Then the mat was immersed in NaOH (3%) for 12 hours. This step was repeated thrice; post which the pellicle is immersed in HCl (3%) late was thoroughly washed in deionised water and pressed in between filter papers remove the excess water. The purified BC mat was sterilised under ^{60}Co radiation or steamed under 121°C for 30 mins. It was concluded that this radiation treated pellicle only had around 1-50 nanograms of endotoxins in contrast to conventionally treated, which had 30mg endotoxins (Chawla et al. 2009; Thomas et al. 2013). Several studies have been carried out by drying the BC in hot air oven, vacuum oven. Some of the rent studies have been reported to use conventional air drying and lyophilisation/freeze-drying as a suitable method of drying the Bacterial Cellulose (Bae and Shoda 2005).

2.13 Structural properties of Bacterial Cellulose

The morphological structure of Bacterial Cellulose entirely depends on the culture conditions during fermentation. In static fermentation culture, the bacteria synthesised the thick cellulose mat at the air interface of the fermentation medium (Czaja et al. 2004; Chawla et al. 2009, Dubey et al. 2002; Czaja et al. 2007 ; Czaja et al. 2004). The thick gelatinous pellicle is made up of by a 3-D network structure of ultrafine nanofibrils, which are uniaxially aligned (Meftahi et al. 2009). However, BC produced from agitated cultures are irregular or spherical shaped, having perpendicular or parallel orientations, which make them suspended in the broth medium (Gu and Catchmark 2012; Oshima et al. 2008).

The cellulosic microfibrils are which are synthesised in the cytoplasm and translocated to the extracellular space in the form ribbon-shaped fibrils (Huang et al. 2014; Ruka et al. 2013). These fibrils were 110 times thinner when compared to the plant cellulose fibrils (Czaja et al. 2007; Ross et al. 1991). Yamanaka and Sugiyama (2000) reported the dimensions of the BC ribbons were approximately 4.1 x 117 nm. The length of the ultrafine densely packed (due to H bonding) microfibrils of BC range was from one to nine µm (Czaja et al. 2006). Scanning electron micrographs revealed well defined 3D picture of the fibril structure with the fibrils in arranged in mesh like network pattern whereas the cellulose produced in agitated condition were

twisted with strands with larger width of around 0.2 μm as compared to the BC synthesised via static fermentation (0.05 - 0.10 μm) as studied by Johnson and Neogi (1989). Morphological properties of BC synthesised in agitated condition can be modified by the addition of certain substances such as pectin, xylan, xyloglucan etc. to the production medium and continually varying the agitation speed (Hu and Catchmark 2010; Gu and Catchmark 2011).

Several methods have been used to study morphology as well as structural properties of cellulose namely scanning electron microscopy (SEM), atomic force microscopy (AFM), Field-emission gun scanning electron microscope (FEG-SEM) and transmission electron microscopy (TEM). These instrumental methods give various insights about structure, morphological and internal fibrillar arrangement of BC. Several studies have been done using these techniques to study the arrangement of fibrils and to study their features as produced by static or agitated mode of fermentation. However, several other properties of BC such as degree of polymerization, degree of crystallinity, crystal size, thermal stability value, viscosity, Young's modulus and mechanical properties etc. were analyzed by methods such as X-ray diffraction, CP/MAS ^{13}C-NMR analysis, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), Viscometry etc (Surma-Slusarska et al. 2008; Hsieh et al. 2008; Cheng et al. 2009).

To analyse the morphological and physical properties of BC and to differentiate the parallel and antiparallel chains of cellulose I and II respectively, the nuclear magnetic resonance (NMR), X-ray diffraction, FT-IR techniques were employed (Czaja et al. 2004; Keshk and Sameshima 2006; Mondal 2013). CI and CrS values were obtained from XRD spectra (Aytekin et al. 2016). Properties such as thermal stability, WHC, mechanical strength and Young's modulus were determined by conventional methods (Iguchi et al. 2000; Schrecker and Gostomski 2005). Thompson et al. (1988) investigated the morphological features under scanning electron microscopy (SEM) and analysed both structural and internal morphology of cellulose and observed that at higher magnification the fibrils appeared irregularly arranged clusters whereas internally they were well organised, and well-defined cross-section was seen. Luo et al. (2014) studied the morphological features of BC such as fibre structure, fibre

diameter, porosity, light transmittance capacity and thickness of the fibres. However, these parameters varied slightly over the days of the incubation period, but the structural morphology remained unaffected all through the fermentation period.
Goh et al. (2012) characterised the BC produced from Kombucha extract by employing SEM, FTIR, X-ray diffractometry and reported that that BC film was made of an ultrafine network of fibrils forming mesh like structure. The FTIR spectra and X-ray diffraction confirmed the purity of the cellulose and degree of crystallinity. The degree of crystallinity was found to be 93% as compared to agitated mode derived BC is 51%. The morphological structure was viewed under SEM and reported that BC produced under static condition is densely and thick with cellulose microfibrils tightly held whereas, in shaking culture more loosely woven with a larger porosity (Sarkono et al. 2014; Vazquez et al. 2013; Kazim 2015).

Yim et al. (2017) investigated the properties BC cellulose fabrics using scanning electron microscopy (SEM) and X-ray diffraction (XRD) and observed the good crystallinity ratio of 74.3 % and well-distributed cellulose fibril network. The Fourier transform infrared (FT-IR) spectra revealed characteristics peaks of cellulose. The tensile strength of BC fabric was two times greater than the thickness of leather. Machado et al. (2016) used various analytical methods to study the physicochemical properties of BC namely thermal degradation, crystallinity index, crystallite size and water holding capacity. Also, morphological analysis was performed by using scanning electron microscopy (SEM) and atomic force microscopy (AFM). Huang et al. (2016) characterised the BC synthesised by *Gluconacetobacter xylinus* from lipid fermentation wastewater by using FE-SEM, FT-IR, and XRD to confirm the morphological and chemical properties and compared to the BC synthesised from standard medium.

Dayal et al. (2016) studied the effect of additives like pectin, carboxymethyl cellulose(CMC), corn starch, corn steep liquor(CSL) and gelatin at various concentrations(1%, 3% and 5%) on BC. X-ray diffraction (XRD) and field emission scanning electron microscopy (FESEM) was used to characterise the dry pellicle. The crystallinity and crystal size of Bacterial Cellulose was found to be lower when carboxymethyl cellulose (CMC) and gelatin were added while the addition of pectin

only reduced the crystal size. The morphology of Bacterial Cellulose was examined under field emission scanning electron microscopy (FESEM) and observed the increased microfibril aggregation in Bacterial Cellulose pellicles.

The antibacterial composite was made from Bacterial Cellulose invitro. This was further characterised by scanning electron microscopy (SEM), Fourier transformation infrared spectrum (FTIR), X-ray photoelectron spectroscopy (XPS) and X-ray diffraction (XRD), and reported that the SiO_2 coated Cu particles were uniformly distributed on the cellulose surface (Ma et al. 2016). De Oliveira et al. (2017) synthesised the hyaluronic acid-based polymeric composites with BC. The characterisation of the composites revealed that the biocompatibility of BC with hyaluronic acid as an extracellular matrix component met the requirements as an essential biomaterial for tissue engineering applications.

2.13.1 Degree of Polymerization

The degree of polymerisation (DP) is the main parameter which depends on the length of the microfibrils and determines the crystallinity of the cellulose (Gardener et al., 2008). The Bacterial Cellulose has been studied and reported to have a higher degree of polymerisation (DP) due to their long nanofibrils in comparison to the plant cellulose (Habibi et al. 2006; Siró and Plackett 2010). The degree of polymerization (DP) of BC usually lies in the ranges between 2000 to 8000 (Jonas and Farah 1998; Klemm et al. 2005 ; Watanabe et al. 1998) Wanichapichart et al. (2002) found that DP and molecular mass of a single BC fibre is approximately around 793 and 142.73 kDa, respectively. The two major factors that influence the DP of BC are the fermentation factors and media components.

Watanabe et al. (1998) stated in a study that DP is higher for cellulose produced from static fermentation than that of shaking culture condition. The study also reported that mutated strain of *Acetobacter xylinum subsp. Sucrofermentans* BPR3001 showed higher DP than the native strain. Keshk (2006) studied the effect of lignosulphonate on BC synthesis, and it was reported that viscosity of Bacterial Cellulose increased to 76.98 cP from 36.48 cP, viscosity of BC in standard medium, indicating higher DP value. BC synthesised using glucose or mannitol as carbon nutrient source displayed a

higher DP (~1700) in contrast to BC produced from other sugar sources, which had a lower DP of 1050.

Tsouko et al. (2015) reported the degree of polymerisation of BC ranges from 1889 – 2673. The Bacterial Cellulose synthesised by using the crude glycerol as nutrient source with modified HS medium after replacing glucose, showed the highest DP (2673), whereas the BC produced from by-products of the biodiesel and confectionery industry waste showed the DP of 2391 and 2176, respectively.

2.13.2 Degree of Crystallinity

The crystallinity of BC is a very distinctive and unique feature, which is one of the major aspects of the characterisation of cellulose. The degree of crystallinity in BC is much higher at approx. Around 60–80% (Vitta and Thiruvengadam 2012; Meftahi et al. 2009; Klemm et al. 2005; El-Saied et al. 2004). As we already know, cellulose exists in two allomorphs: cellulose I and cellulose II (Saxena and Brown 2005; Brown 2004). The crystallite size and I_α/I_β content of the cellulose play an important role in determining the crystallinity index of cellulose. These two forms were characterised and distinguished by techniques like XRD, NMR, and IR spectroscopy (El-Saied et al. 2004). It was observed that β-1, 4-glucan chains are arranged uniaxially in Cellulose I but the same chains were arranged in an antiparallel manner in cellulose II. This antiparallel arrangement gave higher stability along with better hydrogen bonding. The Bacterial Cellulose has approximately 70-80 % cellulose of type I_α as compared to plants cellulose that was which is composed of only 30% I_α content (Huang et al. 2014, Mohite and Patil 2014, Park et al. 2014).

However, in higher plants and tunicates cellulose I_β type prominently found (Moon et al. 2011; Yoshinaga et al. 1997). Watanabe et al. 1998, for the first time, revealed the presence of two distinct forms of cellulose I present in the BC using CP/MAS ^{13}C-NMR analysis. A study by Czaja et al. (2004) revealed that the agitated culture conditions affected the smaller crystalline size resulting in a decrease of cellulose type I content in Bacterial Cellulose. The study concluded that media components and type of fermentation conditions largely determined the ratio of cellulose I_α and I_β as more I_α cellulose content was higher in the static mode of fermentation than the under

agitated mode. The ratio of two allomorphs (I_α: I_β) of Cellulose type I in Bacterial Cellulose showed lower ratio using agitated fermentation than using static mode of fermentation (Sugiyama et al. 1991).

Although, both the modes of fermentation produced Bacterial Cellulose with the almost similar or equal crystalline size of cellulose $I_{\alpha,}$ but it was observed that crystalline index of Bacterial Cellulose I_α calculated varied widely (Watanabe et al. 1998). Some studies have shown the addition of the supplements had an impact on the crystallinity index of BC. A study by Keshk and Sameshima (2005) reported that BC had a higher crystallinity index and higher I_α ratio due to the addition of lignosulphonate 1.0 % (v/v). Similarly, Cheng et al. (2009) stated that the production medium containing carboxymethyl cellulose/CMC (1%) affected the crystallinity index and crystal size Bacterial Cellulose synthesised. Kim et al. (2011) reported inclusion of chitosan to the fermentation broth decreased the crystallinity ratio from 82 % to 61 %. Gu and Catchmark (2012) reported that addition of xylan and xyloglucan components resulted in decreased crystallinity index and crystalline size of BC along with an increase in I_β type of cellulose which is usually lower in Bacterial Cellulose.

2.14 Mechanical properties of Bacterial Cellulose

The Bacterial Cellulose produced in static fermentation has much higher mechanical properties (Young's modulus and tensile strength) than that synthesised via agitated cultures (Cheng et al. 2009; Krystynowicz et al. 2002). As mentioned earlier, even the mechanical properties depend on culture conditions.

A study by McKenna et al. (2009) reported increased fermentation period gradually enhanced the tensile strength of the BC. However, too long fermentation period lead to a decrease in the tensile strength. Young's modulus was highest after three days of fermentation, which dropped after 96 hours. Hsieh et al. (2008) recorded Young's Modulus of 114 Gpa for single BC fibril by using the method of Raman Spectroscopy. Several studies have reported that Young's modulus and tensile strength was slightly higher in BC in comparison to the cellulose strand derived from plant cellulose (Wang et al. 2011; Schrecker and Gostomski 2005; Iguchi et al. 2000).

Yamaka et al. (2000) studied mechanical strength of BC, and high Young's modulus value could be attributed to the uniform nanofibrillar network structure which upon compression tends to get aligned bi-dimensionally leading to increase in Young's modulus (Torres et al. 2012).

Limited reports are available on the methods of studying mechanical strength of BC fibrils. Guhados et al. (2005) used Atomic Force Microscopy (AFM) method to estimate a value of Young's modulus (78 ± 17 GPa) of BC nanofibers. A study by Kim et al. (2011) reported decreased tensile strength of Bacterial Cellulose was attributed to the addition of chitosan as an additive to the medium. Grande et al. (2009) reported the method of drying BC also plays an important role in determining the mechanical strength. It was observed that hot air-dried/ pressed BC possessed high tensile strength 241.42 ± 21.86 MPa, Young's modulus of 6.86 ± 0.32 GPa and a maximum elongation strength of 8.21 ± 21.86 MPa, respectively. The addition of BC fibres to commercial starch reportedly heightened the mechanical properties as observed by Nainggolan et al. (2013). Retegi et al. (2009) studied the effect of compression pressures on BC sheet and concluded that the pressure enhanced the elongated break and tensile strength of the cellulose sheet. The reasons for the improved strength could be attributed to the reduction in pores between the fibrils, which in turn lead to the increased intramolecular fibrillar bonding of BC.

In a study reported by Gayathry and Gopalaswamy (2014), the tensile strength of BC synthesised using the standard HS medium was 120 MPa. Soykeabkaew et al. (2012) prepared nanocomposite using a starch matrix with the help casting over with Bacterial Cellulose. The composite displayed at a high tensile strength of 58 MPa and Young's modulus of 2.6 GPa more than original starch matrix by 20 fold and 106 fold respectively. This casting with BC also notably increased thermal degradation temperature of the nanocomposite to 348.46°C. Tsouko and coworkers (2015) investigated the mechanical properties of lyophilised BC pellicle and found that the tensile stress and Young's modulus were in the range of 72.3 ± 6 to 139.5 ± 12.6 MPa and 0.97 ± 0.05 to 1.64 ± 0.20 GPa respectively varied on the strains which produced BC.

2.15 Thermal Properties

Thermal stability of BC is yet another important parameter that determines its stability strength. The thermogravimetric analysis method was used to study the stability index. The stability region was observed up to a temperature of 300°C. Beyond this temperature, gradually decomposition phase began and reached maximum reported at 350 – 370°C. Studies have shown that BC has thermal stability, which declines after a temperature of 300°C. Alkali treatment notably lengthened the thermostability time of the BC membrane (between 343 and 370 °C) (Surma-Ślusarska et al. 2008). Halib et al. (2012) performed the thermal degradation study of BC synthesised in coconut water as a nutrient medium, and observed that BC was more thermostable; stable weight till the temperature of 343°C whereas, Whatman paper (plant-derived cellulose) showed weight loss around 330-335°C. Literature studies have shown maximum weight loss temperature of 333°C for barley straw cellulose and 350°C for Kraft paper (Sun et al. 2005, Soares et al. 1995).

Mohammadkazemi et al. (2015) investigated the thermal degradation of BC films produced from different nutrient mediums. The study showed that the around 75-80% weight loss was observed around at 360-390°C temperatures. Media composition also played an essential role in determining the thermal stability index of Bacterial Cellulose. Addition of honey as an additive to the production media increased the thermal stability of the BC to the extent of 450 °C. Likewise in another study, Cheng et al. (2009) showed that thermal stability gradually increased in by around 20°C with the addition of carboxymethyl cellulose (CMC) to the fermentation broth than in the medium devoid of CMC. Similarly, a study by Cai et al. (2009) revealed that BC synthesised in the medium containing chitosan had higher thermal stability as over the standard medium derived Bacterial Cellulose.

The temperatures gradually increased as the concentration of chitosan increased, i.e., from 1.2 % to 4.5 % (w/v), the thermal degradation temperature raised from 263 to 366ºC. George et al. (2011) designed a BC nanocomposite film with the addition PVA to BC matrix. This step raised the thermal stability index of the nanocomposite, in addition to this melting temperature (Tm) was also found to be higher than the pure

BC or pure PVA. Vazquez et al. (2013) studied the thermal stability of BC produced from low-cost agro wastes medium. They reported that the initial degradation phase began from 150°C, and the final phase of thermal degradation occurred at 200 to 400°C.

2.16 Water Holding Capacity

Water holding capacity (WHC) of BC makes it a promising raw material for several medical applications. Thompson and Hamilton (2001) considered BC as hydrated membrane due to its high hydration capacity of 99.2%. In another study, Gelin et al. (2007) estimated that 89% of water was tightly bound to the BC fibrillar network, and the remaining 10% is considered to be free water. The good water holding capacity can be attributed to the hydrophilic nature, surface area and porous fibril arrangement of BC (Dahman 2009; Tsouko et al. 2015).

BC exhibited higher WHC than plant derived cellulose with nearly hundred times the weight (Chawla et al. 2009). Like all other features/properties previously discussed, this property as well depends on the media cultured and strains used. Tsouko et al. (2015) reported that the BC synthesise in crude glycerol medium showed the highest water holding capacity (138 ± 9 g/g) followed by BC produced from other carbon sources. However, in contrast to crystallinity index, water holding capacity is higher in case BC produced from the agitated culture in comparison to the static cultured (Krystynowicz et al. 2002; Watanabe et al. 1998).

Seifert et al. (2004) studied the effect of water-soluble additives namely carboxymethyl cellulose (CMC) and methylcellulose (MC) on BC and concluded that the inclusion of these components to the fermentation medium increased the water holding capacity of BC up to 96 % with the addition of carboxymethylcellulose (2.0 %). In a similar study, Ul-Islam et al. (2012) reported that BC, with increased water holding capacity, was produced with the addition of chitosan to the production medium. Krystynowicz et al. (2002) observed that BC produced in rotatory disc bioreactor was able to hold five times more water than that of Bacterial Cellulose synthesised via static mode. Torres et al. (2012) observed that the phenomenon of

hydration was irreversible meaning BC films when completely dehydrated, cannot be rehydrated again.

2.17 Biocompatibility

The biodegradable and biocompatible nature of BC makes it ideal raw material for wide range of biomedical applications like wound dressing, tissue engineering and antimicrobial activity (George et al. 2014; Wu et al. 2014; Torres et al. 2012; Wang et al. 2011; Grande et al. 2009; Czaja et al. 2006). Biocompatibility is the ability of any material to act as suitable and most promising host without eliciting any immune reaction during any application. Petersen and Gatenholm (2011) evaluated the biocompatibility nature of BC for tissue engineering studies and found that it was structurally similar to the extracellular matrix of tissue. Most of the tissues like collagen had the same diameter identical to that of BC and hence helpful in preparations of artificial tissues/scaffolds. In this regard, many studies have been done to improve and enhance the biocompatibility of BC with the incorporation of polymers such as chitosan, hydroxyapatite, polyvinyl alcohol, polymethyl methacrylate etc. (Kim et al. 2011; Torres et al. 2009; Wan et al. 2009; Yin et al. 2011; Grande et al. 2009; Wan et al. 2006; Olsson et al. 2010; Hagiwara et al. 2010). Chitosan has been proved to be one of the best polymeric materials for incorporation in Bacterial Cellulose. Silva and coworkers (2014) evaluated the addition of diclofenac sodium salt and glycerol in BC films as nanostructured transdermal delivery systems. The BC film incorporated with diclofenac salt showed six times higher swelling capacity when compared to normal Bacterial Cellulose. To improve the antimicrobial property of Bacterial Cellulose, some modifications such as the addition of polymeric materials or nanoparticles. The commonly used additives for such modification, silver nanoparticles, titanium dioxide, diclofenac salt, ibuprofen, chitosan, benzalkonium chloride and poly 3-hydroxybutyrate (PHB) (Berndt et al. 2013; Feng et al. 2014; George et al. 2014; Wu et al. 2014; Li et al. 2015; Ruka et al. 2014). Cai et al. (2011) designed a nanocomposite for tissue engineering application by incorporation of Bacterial Cellulose into PHB polymer. Similarly, another study by Cai and Yang (2011) showed that thermal stability index of PHB notably increased

from 195 to 250°C, tensile strength to the extent of 150 % and Young's modulus by 120 %.

2.18 Applications of Bacterial Cellulose

The BC is a synthetic polymer displaying enormous potential in different types of applications in different domains of interest (food, textile, paper, cosmetics and medical). The unique standout features such as greater purity, greater CI and DP, good mechanical property, excellent WHC, flexibility, and larger cross-sectional surface area (Brown 2004; El-Saied et al. 2004; Shah and Brown 2005; Czaja et al. 2006; Torres et al. 2012; Thomas et al. 2013).

2.18.1 Bacterial Cellulose in Healthcare (medical and pharmaceutical applications)

An in-depth review of Bacterial Cellulose states that BC has the extensive application in healthcare domain for skin therapy, preparation of vascular scaffolds, help in tissue regrowth, bone and connective tissue repairs, scaffold preparation and drug release tool. BC membranes have been used for wound healing procedures as early as the 1980s. Due to its unique nanofibrilled 3D network structure, BC has been used as wound dressing materials, artificial skin, vascular grafts, scaffolds for tissue replacements artificial blood vessels, medical healing pads, and dental implants etc. (Alvez et al. 2010; Legeza et al. 2004; Czaja et al. 2006; Solway et al. 2010; Peterson and Gatenholm 2011; Fu et al. 2013; Shah et al. 2013)

2.18.1.1 Bacterial Cellulose as Vascular Grafts

Klemm et al. (2001) and Helenius et al. (2006) projected BC to be a new biosynthetic vascular graft/ membrane as it elicited a shallow inflammatory response and antigen-antibody reactions. Also, BC befitted newly design vascular graft over the traditional ones may cause intimal hyperplasia, reduced blood flow and surface thrombogenicity. Millon and Wan (2006) evaluated BC-PVA biocomposite, which mimicked the physiological behaviour of heart valves leaflets. They concluded from their study that the stress-strain properties of BC-PVA biocomposite were similar to that of heart's aorta.

Similar to this study, Mohammadi (2011) has also developed BC-PVA composites for the replacement of heart valve leaflets. Schumann et al. (2009) performed a microsurgical study on rats by implanting biosynthetic BC membranes to study artificial defect on arteries of the heart. The study was carried over a year concluded that wound healing actively took place along with the growth of new fibroblasts cells in the artery. Brown et al. (2012) prepared artificial blood vessels by designing tiny tubes of BC-fibrin biocomposites, which were treated with glutaraldehyde to bring about crosslink between the two polymers and served as a suitable replacement for native blood vessels of small diameter.

2.18.1.2 Role of BC in bone/tissue engineering

Bacterial Cellulose is considered one of the best materials in recent times for tissue engineering and related applications (Fu et al. 2013). Torgbo and Sukyai (2018) reported that BC were manipulated with hydroxyapatite to mimic the three dimensional framework of bone tissues and also facilitated the incorporation of nanoparticles for cell proliferation and differentiation.

Azuma et al. (2007) proposed that BC-poly dimethyl acrylamide networked structure mimicked and fulfilled the needs of the connective tissue and hence was a suitable replacement aid. A study by Millon et al. (2009) also showed that BC polyvinyl alcohol biocomposites displayed tensile strength, elasticity and other physiological parameters similar to the natural cartilage. Few researchers have also evaluated the BC as a vascular graft for the dental tissue regeneration process. De Macedo et al. (2004) demonstrated the usage of BC in the treatment of bone injury by means guided tissue regeneration protocol. Since Bacterial Cellulose membrane has WHC (~99%) and hence it provides space for cell growth and proliferation. Bäckdahl et al. (2006) studied the interaction between BC membrane and smooth muscle cells. The results concluded that Smooth muscle cells were able to adhere to the BC film and proliferated in numbers on the BC matrix. Extremina et al. (2010) designed and developed BC and cellulose triacetate composite membranes with the antibiotic imipenem absorbed within the BC matrix. This helped them to evaluate in vivo study with new features anti-adhesive and anti-proliferative contributed by the polymer-antibiotic blend.

2.18.1.3 Wound Healing

Regeneration of any tissue is a vital requirement for wound healing. The wound healing process involves the complex mechanisms which takes places between different body cells and other connective tissues. It is essential that wound healing material need to have excellent biocompatibility, good mechanical strength and elasticity, transparency, ability to maintain a moist environment for longer duration near the site of injury/wound, and ability to absorb exudates during the inflammatory phase. Chawla et al. (2009) reviewed BC sustaining all these features of wound healing and hence found to be effective. The biocompatibility of BC is attributed to its distinctive nanofibrilled structure, which eliminated pain to a more significant extent and facilitated better absorption of drug (Czaja et al. 2006). Winter (1962) proposed the very first application of BC as material for wound healing. The study concluded that wound healing occurred faster when the injury on tissue remained moist for long durations. Xcell®, Bioprocess® and Biofill® are some of the examples of commercially available BC-based bioadhesives for medical applications.

Jonas and Farah (1998) demonstrated in their research that BC could function as temporary skin substitute to heal skin burns and skin replacement and hence coined the term "Biofill®". Also, the Biofill® showed positive effects on post-surgery discomfort, faster healing along with lessened pain and overall reducing the treatment time and cost (Fontana et al. 1990; Jones et al. 2002). Kucharzewski et al. (2003) evaluated product Bioproces® (Bacterial Cellulose wound dressing) and found it effective for wound healing. Solway et al. (2010) reported BC in bioadhesive Dermafill® used for dressing material for injured skin was efficient in the healing tear/injuries over the conventional dressings. Trovatti et al. (2011) designed an antibacterial membrane using BC by incorporating the membranes with lidocaine, an anaesthetic and evaluated the ability of BC in topical drug delivery and concluded BC to be suitable drug delivery membrane. Meftahi et al. (2009) reported BC film with cotton fibre composite, which displayed higher water retaining capacity and wound healing ability over regular cellulose films. Table 2.6 shows some of the medical application, where BC has been used in the form of composites for various applications.

2.18.1.4 Role of BC in drug release

Several studies have been performed using BC membranes as a matrix for drug release by transdermal and oral methods. BC composites have been prepared using different polymeric materials, which further has been used for controlled drug delivery applications. A study by Trovatti et al. (2011) has evaluated the therapeutic potential of BC membranes by *in vitro* diffusion study on human abdominal skin cells using two types of drugs, namely lidocaine hydrochloride and ibuprofen. Lidocaine hydrochloride incorporated BC membranes showed the lowest penetration rate over the other conventional formulations. Another study by Halib et al. (2012) showed BC membranes irradiated with electron beam radiations at various intensities showed slower diffusion of the drug than that of the non-irradiated BC membranes. This result was attributed to the fact that pore sizes significantly reduced in irradiated membranes.

A study by Silva et al. (2014) showed that transdermal patches prepared using BC membrane loaded with diclofenac drug showed similar permeation rate on the human epidermis as that of commercial patches available in the markets. Bhavana et al. (2014) studied the in *vitro* drug loading and release activity of the BC membrane incorporated with various dosages of benzalkonium chloride drug. These studies conclude biocompatibility of BC membrane along with more flexibility, good absorbing, and releasing capacity makes the membrane a suitable carrier for drug delivery application.

Table 2.6 Medical applications of Bacterial Cellulose

Biocomposite/s	Applications	Reference/s
Bacterial Cellulose-hyaluronic acid silver composites	Wound dressing material	Wang et al. (2011)
BC membrane	Artificial skin graft	Farah (1990)
Bacterial Cellulose film and collagen composites	Skin tissue repair	Saska et al. (2012)
BC hollow tube (CMC-BC composite)	Artificial blood vessel	Yamanaka et al. (1989)
Reabsorbable composites using BC, collagen and hydroxyapatite	Bone and connective tissue repair	Saska et al. (2011)
PVA and BC composites	Antibacterial mask	Barud et al. (2011)
Carboxymethyl- Bacterial Cellulose composite	Blood purification	Oksman et al. (2016)
Bacterial Cellulose hydrogel loaded with cosmetic	Therapeutics and cosmetic preparation	Pacheco et al. (2018)
Bacterial Cellulose hydrogel	Cold packets	Cacicedo et al. (2018), Mohite et al. (2014)
Bacterial Cellulose microfibrils	Oil in water emulsion	Sai et al. (2015)

2.18.2 Food Applications of Bacterial Cellulose

Bacterial Cellulose has been used in food processing industries due to the distinct soft texture and high fibrous content. The gel-like property and complete indigestibility in the human intestinal tract made this an attractive diet rich in fibres (Budhiono et al. 1999). Table 2.7 summarizes few food applications of BC.

Table 2.7 Applications of Bacterial Cellulose in the food industry

Applications	Salient features	Reference/s
Edible antimicrobial packaging	Retains freshness for a long time	Padrão et al. (2017), Wan et al. (2016)
Stabiliser and Thickener	High water absorption capacity	Ullah et al. (2016)
Food additive	Thickening, gelling, stabilising, emulsifying binding agent. High stability during freeze-thawing	Feng et al. (2015), Budhiono et al. (1999), Shi et al. (2014), Azeredo et al.(2017)
Yoghurt and ice-cream	It has a strong tendency to resist syneresis	Sukara (2014)
Packaging material	Good resistant to oxygen and air	Esa et al. (2014)
Enzyme immobilisation	Increases enzyme stability	Wu and Lia (2008)
Fruit cocktail and jelly	Strong tendency to blend and form a gel with sugar syrup	Halib et al. (2012)
Immobilisation of probiotics strains	Efficient to hold strains in encapsulated form	Baldikova et al. (2017)
High fibre supplement for nata coco	Lowers body lipid levels	Mesomya et al. (2006)
Ingredient for salads, low-calorie desserts	Blends with desserts	Shirai et al. (1994)
BC in a health drink	Rich in fibres and low-calorie drink	Keshk (2014)

In 1992, the microbial cellulose-based drink was introduced in Japan. This health drink is even consumed today by the trade name Kombucha, or Manchurian tea. Shirai et al. (1994) in their study reported the thick cellulose mat due to its fibrous texture can cold be used as a new culinary ingredient for salads, low-calorie desserts, and fabricated foods. Also, Bacterial Cellulose has been certified as safe and edible (GRAS) as per IPTS report (1997) and also approved by the FDA, USA (Garcia and Bontoux 1997).

2.18.3 Role of BC in Animal Cell Culture

Various researchers have studied the evaluation of BC film as a substratum for animal cell culture. Svensson et al. (2005) demonstrated the growth of bovine chondrocytes cells on BC matrix. Bovine chondrocyte proliferation was observed at the rate of 50% more than the normal collagen matrix. Sanchavanakit et al. (2006) developed a protocol to culture human keratinocytes and fibroblasts on the artificial substrate like Bacterial Cellulose. Their results showed that the number of the live keratinocytes cells and fibroblast cells seeded on Bacterial Cellulose substratum was similar to that of plated on a polystyrene culture plate but with enhanced properties.

2.18.4 Some other applications of BC

2.18.4.1 Acoustic Transducer Diaphragm

The Bacterial Cellulose with good dimensional stability paved its way for sound transducing membrane capable of sustaining high sonic velocity over a wide range of frequencies. Audio speaker diaphragms were first developed by collaborations of two companies Sony Corporation and Ajinomoto (Japan) using BC, although they are commercially priced costly (Iguchi et al. 2000). Ciechańska et al. (2002) and El-Saied et al. (2004) also demonstrated exceptional shape retention ability and good tensile strength of BC which made it feasible raw material for designing speaker diaphragms.

2.18.4.2 Paper Manufacturing

The properties of BC such as excellent tensile strength, higher thermal stability and uniform texture made it useful for making papers (El-Saied et al. 2004; Chawla et al. 2009). Brown (2004) used BC as a binding agent to increase the strength and durability of pulp for making papers (Table 2.8). Iguchi et al. (2000) observed a four-fold increase in the endurance of the new polymer blend paper with the addition of 15.0 % BC when compared to pulp derived paper. It was also stated that value of Young's modulus increased by 1.5 GPa.

A study by Cheng et al. (2011) also proved increased tensile strength and Young's modulus over the regular paper. The high reflectivity, moldability/flexibility, lightweight, ease of carry over and transporting brought out new perspectives in applying BC for advanced technology such as digital printing or electronic paper (Shah and Brown 2005).

Table 2.8 Some other applications of Bacterial Cellulose.

Applications	Features	Reference/s
Immobilisation matrices	Immobilisation of denitrifying bacteria, yeast and enzyme	Rezaee et al. (2008), Akduman et al. (2013), Yao et al.(2011)
Paper making	High-quality durable papers	Surma-ślusarska et al. (2008), Basta and El-Saied (2009)
Packaging material	Bacteriostatic sausage casing film	Zhu et al. (2010), Dobre et al. (2011)
Reinforcing material	Reinforcement for thin fibres and polymeric matrices	Lee et al. (2009), Eichhorn et al. (2010)
Electronics	Flexible displays, electronic paper, acoustic membranes, photo catalyst and super capacitors.	Barud and Ribeiro (2013), Shah and Brown (2005), Markiewicz et al.(2004), Wang et al. (2013)
Biosensors	H_2O_2 biosensor, Humidity sensors	Zhang et al. (2010), Hu et al. (2011a,b)
Aerogels	Controlled release gel matrices and light weight flexible	Haimer et al. (2010), Wu et al. (2013)

2.19 Research gaps

The in-depth review of literature analysis throws light on various aspects of BC. The following were the literature gaps identified from the literature review:

- The production of Bacterial Cellulose in commercialised media, which is made up of various chemical components; hence, the use of large quantities commercial media for BC synthesis on industrial scale makes it an expensive process.
- High capital investment and high operating costs, which is one of the main reasons why industrial production of BC is limited.
- The period for a proper and a complete BC production (static fermentation) approximately takes longer time duration. Thus, the rapid production of BC is a short period is difficult.
- Existing methods still face problem to enhance BC yield. However, to some extent, fermentation in a bioreactor has addressed the yield issue and the lowering production time of BC.
- The protocol for evaluation of purity standard for medical applications of BC remains unexplained.

SCOPE OF THE RESEARCH WORK

The major limitations faced by large scale production of BC are the high cost of the fermentation media and low production rate on an industrial scale. The viable alternative option to this problem is by experimenting using several renewable and cheap carbon nutrient sources as raw materials for BC synthesis. The conversion of waste materials and byproducts obtained from industries to BC can act as both economical and eco-friendly approach. Recent researches are on the lookout for the implementing new methods to recycle or reuse the waste generated along with production of value-added products. Clean-biotechnology employs new methods to recycle and reuse large quantities of cheap and cost effective substrates and wastes materials obtained from various industrial sectors for the synthesis of Bacterial Cellulose. This can help in large scale production with low-cost raw materials and better commercialisation of Bacterial Cellulose.

The microbial production of cellulose can be made cost-effective and eco-friendly if synthesised by utilisation of agro-industrial by-products like molasses, thin stillage, whey, corn steep liquor, fermentation wastewaters etc. Although there are limitations in the Bacterial Cellulose production process, concerning the energy, cost and purifications procedures, it is a better alternative over plant-derived cellulose. Hence, Bacterial Cellulose is the one step towards sustainable development and preservation of plant resources for future generations. Therefore, the current study aims at utilising a waste resource to produce wealth out of it, i.e., to use wastewater to synthesise BC. As a result, we reuse the waste resources and make it easier for further treatment/ disposal and also produce a biopolymer which could be utilised in various applications.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

In this chapter, various materials and methods that were used for the production, purification, characterization and application of Bacterial Cellulose along with the different media composition and reagents that were used in the during the course of research work are described. In addition to this, some protocols have been discussed in relevant sections in the next chapter.

3.1 General

All the experiments were carried out in sample processing laboratory, Department of chemical engineering, National Institute of Technology, Surathkal, Karnataka.

3.1.1 Chemicals and Reagents

Chemicals such as glucose, fructose, sucrose, maltose, citric acid, acetic acid, disodium hydrogen phosphate (Na_2HPO_4), purified glycerol and calcium carbonate were obtained from Merck, India. Starch, agar powder bacteriological, peptone bacteriological, yeast extract powder were purchased from Himedia, India. Calcofluor white stain was purchased from Sigma Aldrich, USA. Cycloheximide was procured from Sigma Aldrich, USA. Sodium salt of ibuprofen was procured from Sigma Aldrich, USA. All the experiments were performed using chemicals and reagents of analytical grade. Deionized water was used for carrying out the experiments.

3.1.2 Stock solutions and buffer

- Normal saline

Normal saline solution (0.9%) was prepared by dissolving 0.9 g of NaCl in 100 mL of deionized water and sterilized by autoclaving.

- Hydrochloric acid (HCl) (1.0 N)

1.0 N of Hydrochloric acid solution was prepared by adding 1.0 ml concentrated HCl to 10 mL of sterilized deionized water.

- Sodium hydroxide (NaOH) (1.0 N)

1.0 N of Sodium hydroxide solution was prepared by dissolving 4.0 g of sodium hydroxide pellets in 100 mL of sterilized deionized water.

- Phosphate Buffer (pH 7.4) (0.1 M)

Phosphate Buffer of 0.01M was prepared by dissolving 10.98 of sodium phosphate dibasic, and 2.71 g of sodium phosphate monobasic monohydrate to 1000 mL sterilized deionized water

Note: HCl and NaOH solutions were used for adjusting the pH of the culture media.

3.1.3 Collection of samples for experiments

Rotten fruits (apple, banana and green grapes) were purchased from local Surathkal market. Distillery effluent was procured from UB breweries limited, Baikampady, Mangalore, India.

3.1.4 Sterilization procedures

All glassware were washed and sterilized in a hot air oven at 80°C for 8-10 hours. All growth and culture media, broth and deionized water, were sterilized in an autoclave at 121°C at 15 psi pressure for 15 mins. Buffer, drug solutions were filter sterilized using 0.25 microns cellulose nitrate filters. Isolation, inoculation and other microbiology assays were performed in a laminar airflow chamber.

3.2 Methodology

3.2.1 Isolation of the bacterial isolates from rotten fruits

Rotten fruits of banana, apple, and green grapes were chosen for isolation of bacteria. Rotten/decayed fruits were chosen for the present study as they are found to accommodate the larger population of acetic acid bacteria (Mateo et al. 2014). The rotten fruits were gently cleansed with distilled water to remove the dirt and dust on

the fruits. They were cut into small pieces, five grams was weighed and transferred into 100 mL conical flasks containing sterilized Hestrin–Schramm (HS) broth medium comprising of 20 g/L D-glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L sodium hydrogen phosphate, and 1.15 g/L citric acid (Schramm and Hestrin 1954). The pH of the growth media was adjusted to 6 with 1N HCl before autoclaving. Autoclaving was carried for about 20 minutes at 121°C. Once the medium was sterilized, 0.02% (w/v) Cycloheximide was added to the medium to avoid fungal/mold growth. The flasks were kept at a temperature of 30°C for 7-8 days in a static incubator. After the incubation period, the Erlenmeyer flasks were observed for thin film production at the air-liquid interface. Later, 0.1 ml volume of the broth sample was withdrawn from these flasks and transferred to the Petri dishes containing HS agar (1.5 % agar) by the spread plate technique. The Petri dishes were incubated at 30°C for 72 hours. The bacterial growth development was evaluated in spread Petri dishes white- to cream-shaded mucous apparent colonies were observed. The stock bacterial plates were stored at 4°C. They were routinely subcultured for further study.

3.2.2 Morphological characterization of isolates

3.2.2.1 Colony characters

The colony characterization was carried out by simple visualization of colour, the shape of bacterial colonies which were grown on HS medium.

3.2.2.2 Gram staining

The differential staining of the potential isolates was performed as per the protocol described by Jones (2007).

3.2.3 Biochemical characterization of potential isolates

Biochemical characteristics were evaluated by oxidase, catalase, indole and cellulose solubility tests which were performed as follows:

3.2.3.1 Oxidase test

A Single bacterial colony was spread onto the oxidase disc and observed for colour change reaction within 5-10 seconds. Development of a deep violet colour after 10 sec indicated positive and development of a no colour indicated negative result (Collins and Lyne 2004).

3.2.3.2 Catalase test

Potential cellulose producing isolates were spread onto a clean glass slide and flooded with 3 per cent hydrogen peroxide. Production of rapid effervescence indicated a positive result for catalase test (Collins and Lyne 2004).

3.2.3.3 Indole production

The indole production test was performed by inoculating the bacterial colonies into tryptone broth. 3-4 drops of Kovac's reagent (p-Dimethylaminobenzaldehyde) was added to this 48 hours old culture broth and observed for cherry red colour formation at the surface, which indicated indole positive (Collins and Lyne 2004)

3.2.3.4 Cellulose dissolution test

BC pellicle was mixed with 4% cupriethylenediamine hydroxide solution, and complete dissolution of cellulose indicated positive for cellulose producing isolate (Halib et al. 2012).

3.2.4 Screening tests

- HS-Calcofluor white stain method was performed by addition of Calcofluor white stain (0.02 mg/100 mL) to HS agar plates and later observed for UV fluorescence.

- Visualization of the zone of clearance on $CaCO_3$ supplemented medium confirmed that the isolate was a cellulose producer. The $CaCO_3$ medium is made up of 0.5g/L glucose, 3g/L peptone, 5 g/L yeast extract, 15g/L $CaCO_3$, 20 g/L agar, and 15g/L ethanol (Aydin 2009).
- Water-soluble polymer analysis test was performed to affirm that the produced biopolymer was cellulose. This step was performed by mixing supernatant (5000 g for 5 min) of the bacterial culture broth obtained by with 90% ethanol in the ratio 1:3. This mixture was shaken well and observed for precipitate formation (Ishida et al. 2002).

3.3 Identification of the bacterial isolate

The phenotypic characterization was done by 16S rRNA gene sequencing method, which was carried out by Eurofins genomics, Bangalore. Amongst all the isolate obtained, only one bacterium was sent for identification which was chosen based on aforesaid mentioned criteria. Later the sequenced data obtained were aligned and identified with the help of NCBI search homology tool. The identified BLAST sequence data were further run by CLUSTAL X omega tool. The final retrieved sequence data was used to build the phylogenetic tree using MEGA7 software.

3.4 Production of Bacterial Cellulose

A loopful of bacterial culture was inoculated into 250mL Erlenmeyer flasks containing 100mL of HS medium. Later the Erlenmeyer flasks were kept at static incubation at normal room temperature (30 ± 2°C). After two days of incubation, a thin gelatinous film was observed floating at the air-liquid interface that inferred the production of Bacterial Cellulose; the pellicle/mat gradually became denser as the incubation time increased. The pellicle was later removed after a week of completion of the incubation period.

3.4.1 Harvest and purification of BC pellicle

The pellicle was removed from the flask and gently washed using distilled water. The film was then boiled in 1% NaOH at a temperature 90°C for 90 mins to remove the bacterial cell mass adhered to the pellicle. Further, the film or mat was again rinsed

with deionized water several times till the water reached neutral pH. The rinsed and purified pellicles were then dried until the weight of the film/pellicle was constant. Later, the dry weight of Bacterial Cellulose was recorded (Chawla et al. 2009).

3.4.2 Drying methods

The features wet BC pellicle obtained was evaluated by drying using the following methods:

- Drying at room temperature was performed by placing the BC pellicle on glass Petri plate and dried at room temperature until weight remained constant.
- Drying by Hot air oven was carried out by placing the pellicle in the oven at 40°C for 5 hours until the weight remained constant.
- Freeze drying by Lyophilizer was done by freezing the BC pellicle for 48 hours before freeze drying. Later, the wet BC was freeze-dried at condenser temperature of -53°C and vacuum pressure of 15 mTorr/hour.

3.4.3 Media optimization for production of BC

All the shake flask trials were carried out in 250ml Erlenmeyer flasks, incubated at 30°±2 C in a static condition, unless otherwise stated. The unoptimized medium composition, as mentioned in section 3.1.1. One factor trial was used to optimize the medium. To evaluate the highest concentration of BC, different carbon sources such as a monosaccharide, disaccharides, polysaccharides, and sugar alcohols were taken at a concentration of 2% (w/v); nitrogen sources, including both organic and inorganic sources at a concentration of 0.5% (w/v). pH and temperature were also optimized during the production of BC. The pH study was carried out from the range of pH 2.0 to 8.0 adjusted using 1N NaOH and 1N HCl, along with the prior optimized factors (carbon and nitrogen sources). In addition to these, the temperatures and inoculum size were also optimized from 20°C to 45°C and from 2% to 8% respectively. A kinetic study was performed based on BC production along the incubation period to calculate the overall BC productivity (g/100 mL. h) and yield coefficient.

3.4.3.1 Pre inoculum preparation

The bacterial strain was stored and subcultured on Hestrin Schramm (HS) solid agar plate medium at the temperature of 4°C. Pre inoculum was prepared by inoculating single colony from the culture plate into 50 mL of optimized HS broth, the composition of which includes 20 g/L of mannitol, 2 g/L of yeast extract, 2 g/L of peptone bacteriological, 2.7 g/L sodium hydrogen phosphate, and 1.15 g/L citric acid and 1 L of deionized water, pH was adjusted to 5. The broth culture was placed in a static incubator set at 30°C for about 72 hours. The inoculum suspension used for the study was 5% (v/v).

3.4.4 BC yield and productivity

The BC yields, rate at which BC was produced and carbon consumption rate evaluated during kinetic study of BC were calculated by the following equations.

$$\text{Bacterial Cellulose yield}(\%) = \frac{\text{Dry weight of BC(g)/Volume of medium (L)}}{\text{initial sugar conc. (g/L)} - \text{final sugar conc.(g/L)}} \quad (3.1)$$

$$\text{Bacterial Cellulose productivity} = \frac{\text{Dry weight of BC(g)}}{\text{Volume of medium(L)*time period(days)}} \quad (3.2)$$

$$\text{Rate of BC production} = \frac{\text{weight of cellulose produced } (\frac{g}{L})}{\text{cultivation period(hour)}} \quad (3.3)$$

$$\text{Carbon consumption rate} = \frac{\text{carbon } \left(\frac{g}{L}\right) \text{ at time } t_o \text{-carbon } \left(\frac{g}{L}\right) \text{at time } t_n}{\text{time } t_n \text{(days)}} \quad (3.4)$$

3.5 Characterization of the BC

Various analytical techniques were used to study the chemical properties and structural morphology of synthesized BC.

3.5.1 Attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectral analyses were done to bring out the similarities between the functional groups of the test sample and the reference. Bruker ALPHA FTIR with diamond ATR probe spectrophotometer was used for performing 24 scans from the range of 4000 – 400 cm^{-1} with a resolution of 4 cm^{-1}.

3.5.2 Scanning electron microscopy (SEM)

The surface morphological study of BC was investigated using SEM. The dried pellicle was cut into small pieces, processed by auto coating with gold. The prepared samples were observed under a Jeol JSM 6380 microscope which was operated at 20 kV to obtain micrographs at a magnification of 25 kX.

3.5.3 X-ray diffraction (XRD)

The crystallinity index of the produced BC was evaluated using X-ray diffraction method. The diffractograms were noted from 10 to 80° 2θ range at the scan rate of 2°min^{-1} using the Cu X-ray radiation of the wavelength of 1.54Å, the voltage of 40 kV and current of 15mA with a step of 0.02 (Bruker Corporation, Germany). The crystallinity index of BC was calculated by the Segal method (Nam et al. 2016), as shown below:

$$\text{Crystallinity Index(CI)} = \frac{I_{200} - I_{AM}}{I_{200}} \qquad (3.5)$$

I_{200} represents the highest intensity of the 2θ peak (200) which is around at 22.5°, and I_{AM} represents to the intensity of peak situated between (110) and (200) peaks that is at 18°.

The crystal interplanar distance was calculated by applying Bragg's equation (Osorio et al. 2019), as shown below:

$$d=[\lambda/(2 \sin \theta)] \qquad (3.6)$$

where θ corresponds to the diffraction peak angle and λ is equivalent to 1.54 Å

The type of cellulose produced by the bacteria was calculated by the Z discriminant function, wherein Z value corresponds to the type of cellulose based on its source. For instance, Z>0 indicated the type cellulose that could be algal or bacterial origin (I_α) type whereas Z<0 represented the cellulose obtained from the cotton or wood (I_β) type (Wada et al. 1993) as given below:

$$Z = [(1693 \times d_1) - (902 \times d_2) - 549] \qquad (3.7)$$

The d-spacing of $(1\bar{1}0)$ and (110) peaks are designated as d_1 and d_2, respectively.

3.5.4 Thermo Gravimetric Analysis (TGA)

The thermogravimetric analysis (TGA) of Bacterial Cellulose using powdered cellulose sample approximately 5 mg was placed in an aluminium pan. The instrument was set with a heating rate of 20°C min^{-1} under nitrogen atmosphere at the rate of 50 mL min^{-1}. The temperature range was selected from 0 to 600°C. The weight loss curve and its derivative weight loss percent were obtained.

3.5.5 ^{13}C Solid-state Nuclear Magnetic Resonance (^{13}C NMR)

^{13}C CP/MAS NMR analysis was performed on ECX- JEOL 400(S), AVIII400 (L) NMR Spectrometers at 25°C. The lyophilized BC samples were subjected to the magnetic field of 9.389 T, proton frequency of 399.78 MHz and carbon frequency of 100.52 MHz. The sample was packed in 4 mm rotors, spun at 8 kHz, with the pulse of 0.1μs and recycle delays of 5s. Each spectrum was recorded after 334 scans.

3.5.6 Enzymatic digestion of BC by Cellulase

The purity of BC was examined by performing enzymatic digestion using Cellulase enzyme (Sigma Aldrich, USA). The Bacterial Cellulose (100mg) was treated with the enzyme prepared in (100mM) sodium citrate buffer at pH 5 and kept at 50 ºC for about 1 hour. The reaction mixture was then subjected to centrifugation at 5000 rpm for 10 mins, and the supernatant was used for further analysis. The supernatant was used for DNS assay for estimation of reducing sugars (glucose) concentration (Miller, 1959).

3.5.7 Thin layer chromatography of BC

The purity of synthesized biopolymer was evaluated by the method of thin layer chromatography (TLC). BC was dissolved in concentrated H_2SO_4 for about 45 minutes. After acid hydrolysis of the polymer, the hydrolyzed BC and standard glucose sample were placed on the TLC plate. The mobile phase was made up of solvents ethyl acetate: propanol (65:35). Standard glucose was used as a reference. The bands on the TLC plate were detected using iodine vapours (Mohite and Patil 2014).

3.6 Preparation of BC-ibuprofen film

The purified and lyophilized BC membrane was cut into small discs of the diameter of 2 cm and thickness of 50µm. Ibuprofen sodium salt solutions prepared at various concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1%, 5% and 10%) by dissolving 0.2-10 mg of IbuNa in freshly prepared phosphate buffer. The membranes were immersed into 10 ml of ibuprofen solution prepared using appropriate dissolving concentrations in phosphate buffer and left for overnight soaking for 24 hours, the BC film discs were removed from the solution, and the excess of the solution was wiped out with filter paper.

3.6.1 Swelling rate of BC

BC samples were taken and cut into equal size pieces and dried to constant weight. The initial weight was measured, and the sample was then immersed in deionized water at room temperature. The swelling rates were determined by gravimetrically at intervals of 30 minutes. The swelling rate at time t was calculated as follows:

$$\text{Swelling rate} = \frac{W_t - W_d}{W_d} \text{X } 100 \qquad (3.8)$$

where, W_t signifies the weight of BC after 30 minutes of immersion in water and W_d is the dry weight of BC.

3.6.2 Drug encapsulation efficiency and loading capacity

The drug encapsulation efficiency (EE) and loading capacity (LC) of the BC membrane was determined by knowing the initial drug concentration before suspending the dry BC. The final drug concentration was measured after 24 hours of incubation. The EE and LC values were calculated from the following equations (Zhang et al. 2006):

Encapsulation efficiency (EE)

$$EE(\%) = \frac{\text{Total drug added-amount of free drug in the solution}}{\text{Total drug added}} X\ 100 \qquad (3.9)$$

Loading capacity (LC)

$$LC(\%) = \frac{\text{Total drug added-amount of free drug in the solution}}{\text{The initial mass of dry BC}} X\ 100 \qquad (3.10)$$

3.6.3 Drug release assay

In vitro drug release tests were carried out by immersing BC-ibuprofen sodium salt loaded films in 10 mL phosphate buffer solution (pH 7.4) at room temperature (32°C) glass beakers which were maintained under 50 rpm for maximum release of the drug. At fixed time intervals, the amount of IbuNa released was determined by measuring the absorbance at a wavelength of 221 nm using a UV-Vis spectrophotometer (Labindia 3000), based on the standard ibuprofen sodium salt calibration curve. Release studies were performed in duplicates, and the average values were recorded (Silva et al. 2014). The cumulative release of IbuNa was calculated using the following equation:

$$\text{Cumulative drug release } (\%) = \frac{M_t}{M_\infty} X\ 100 \qquad (3.11)$$

where, M_t is the amount of ibuprofen sodium salt released at time t and M_∞ is the amount of ibuprofen sodium salt loaded in the BC film. UV–Visible quantitative analysis of Ibuprofen sodium salt was performed on a UV spectrophotometer

(Labindia analytical UV3000) at 221 nm. A calibration curve (y = 0.0968x + 0.179; R^2 = 0.9967) for Ibuprofen sodium salt was plotted in the range of 5–25 μg/mL at 221 nm.

3.6.4 Antimicrobial assay

The antimicrobial activity of ibuprofen drug loaded BC disc was tested against two strains of bacteria, namely *Escherichia coli* and *Staphylococcus aureus* by the disc diffusion method. The Mueller-Hinton agar plates were spread with a 0.1 ml test inoculum of dilution of 10^{-3}(CFU/mL), and the ibuprofen loaded BC discs were placed on the plates. The discs were gently pressed on to the agar plates and kept undisturbed for incubation at 37 °C for 24 hours. The plates were examined for a zone of bacterial growth inhibition around the disc.

3.6.5 Cytotoxicity studies of BC films

The cytotoxicity of BC films was studied by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Mosmann 1983). The drug-loaded BC samples were sterilized by exposure to UV light. The A549 (Human small cell lung carcinoma) cells were seeded in a 96-well microplate at a cell density of 1 x 10^4 with 100 μl complete medium per well. The plate was incubated at 5% CO_2, 37 °C, 95% humidity for 24 h till the culture in the plate was 70%-80% confluent. After 48 hours, the test samples were added to the medium at different concentrations i.e, 500 μg/mL, 250 μg/mL, 125 μg/mL, and 62.5 μg/mL. 100 μl of each concentration was added to the designated wells. The plate was incubated. MTT assay was performed after 72 hours of drug treatment. 2mg/mL MTT reagent was prepared in PBS and filter sterilized. Post 72 hours of incubation, 50 μl MTT reagent was added to the wells including the controls. The plate was incubated for 2-4 hours (incubation time varies with the type of cell lines used for the study). After the formation of formazan crystals during incubation, 50 μl of solubilization agent such as DMSO (dimethyl sulphoxide) was added to the wells. The plate was gently tapped and incubated for 10 minutes to ensure proper solubilization of the formazan crystals in DMSO till uniform deep purple colour was obtained.

The plate was taken for absorbance reading at 540 nm using spectrophotometer or ELISA plate reader. Percentage cytotoxicity was a representation of cells killed by the sample. This was compared with the cell control values.

$$\text{Cytotoxicity percentage (\%)} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \text{ X } 100 \qquad (3.12)$$

3.7 Statistical analysis

The results were expressed in terms of mean ± standard deviation (SD) by performing the experiments in triplicates. Statistical analysis was done using the software Origin Pro 8, Graph Pad Prism7 and Minitab 18 (Minitab Inc., State College, PA, USA). The data obtained were further compared by the method of one-way analysis of variance (ANOVA) along with Tukey's post hoc test with a 95% confidence interval ($p<0.05$) as significant.

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

This chapter gives a detailed account of the experimental results on various aspects of BC production such as the isolation of cellulose producing bacteria, optimisation of different process parameters for BC production, the use of the alternative medium as a source for BC production and application of produced bacterial cellulose. The results are divided into three parts; the first section discusses the isolation, screening, and optimisation of potential BC producer. The second and third parts discusses the use of crude distillery effluent as an alternative medium, characterisation of BC along with the use of BC pellicle in the evaluation of *in vitro* drug release and absorption potential respectively. All the experiments were carried out in triplicates, and the data presented are the mean value of the triplicates.

The results of various experiments have been explained in the following sections:

PART ONE: This part describes the isolation of potential isolates from different rotten fruits. These were further subjected to screening tests to confirm the production of BC. The most effective bacterial strain was identified by molecular sequencing method. This identified strain was used for further BC studies. The strain was cultivated in standard HS medium, and later, various process parameters were optimised by one variable at a time approach and were experimentally evaluated. This part also discusses the analytical techniques used for assessing BC pellicle based on chemical and structural aspects.

PART TWO: As discussed in the earlier chapters, the standard media makes the overall production process expensive. Therefore, this part focuses on the replacement of the HS medium with a cheap and cost-effective nutrient medium such as crude distillery effluent and evaluation of various aspects of BC production. This part also explains on the COD and BOD aspects of the wastewater and their reduced values after the fermentation process, along with the production of Bacterial Cellulose. The BC produced was characterised based on various analytical techniques such as SEM,

FTIR, XRD, NMR, TLC and was compared to that of the one obtained from the standard medium.

PART THREE: The produced BC was further used to perform the *in vitro* evaluation of drug absorption and release potential using anti-inflammatory drug Ibuprofen and to study the antimicrobial activity of BC.

PART ONE

4.1 Isolation and screening of bacterial cellulose producers

A total of 4 isolates were obtained from banana (*Musa paradisiaca*), apple (*Malus domestica)* and green grapes (*Vitis vinifera*) and evaluated further for BC production (Figure 4.1). The morphologies of the bacterial colonies were characterised by visual observation of the colonies on HS agar plates. The growth of colonies confirmed that the isolates were able to grow on HS medium and displayed distinct features as summarised in Table 4.1. Further, assays were performed to evaluate the biochemical aspects of the potential isolates and presented in Table 4.2. Although all the isolates were potential BC producers, isolate BC1, which was obtained from rotten green grapes produced thick and dense BC pellicle when compared to others (Table 4.3). Therefore, isolate BC1 was chosen for further screening, characterisation and identification process. Further, differential staining of the isolate was performed and was found to be a gram-negative strain (Figure 4.2).

Table 4.1 Morphological characteristics of isolates

Isolates	Shape and elevation	Colour	Texture	Margins
Isolate BC1	Circular convex	Creamish white	Smooth	Round margins
Isolate BC2	Flat	Pale yellow	Smooth	Round margins
Isolate BC3	Circular convex	Milky white	Smooth	Irregular margins
Isolate BC4	Small circular	White	Smooth	Irregular margins

Table 4.2 Biochemical characterisation of isolates

Isolates	Gram staining	Shape	Oxidase test	Catalase test	Indole test	Water soluble polymer test
Isolate BC1	Negative	Small rods	Negative	Positive	Negative	No precipitate
Isolate BC2	Negative	Small rods	Negative	Negative	Negative	No precipitate
Isolate BC3	Negative	Small rods	Negative	Positive	Negative	No precipitate
Isolate BC4	Positive	Small rods	Positive	positive	Negative	No precipitate

Table 4.3 Preliminary screening of BC isolates based on wet yield

Isolates	Wet yield of BC(g/100ml)
Isolate BC1	50.41g
Isolate BC2	12.45g
Isolate BC3	14.75g
Isolate BC4	21.04g

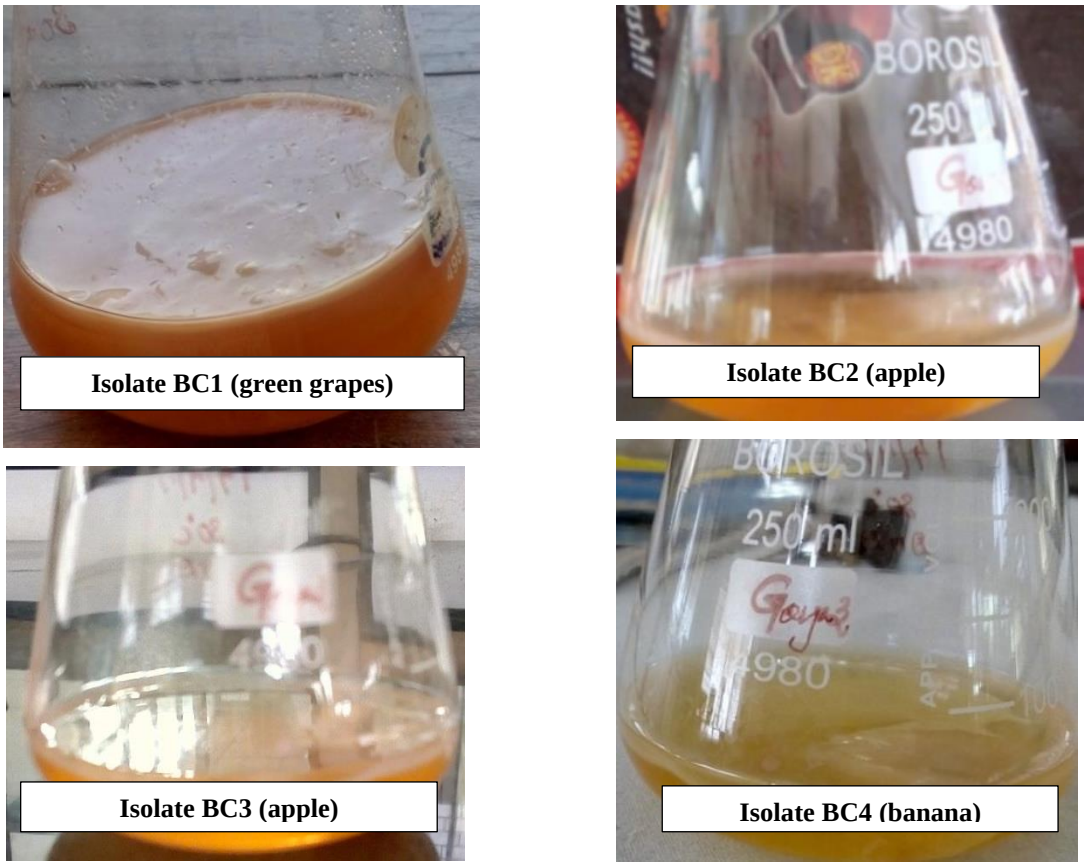


Figure 4.1 Bacterial isolates showing pellicle formation in HS broth medium

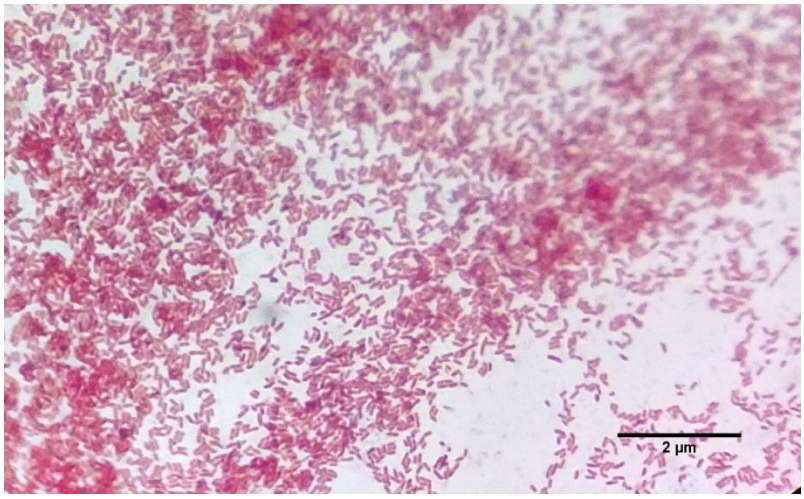


Figure 4.2 Differential staining of isolate BC1

The screening of BC producer was performed using the following tests viz., Calcofluor white stain test, water-soluble polymer analysis and $CaCO_3$ agar test.

(a) The colonies were streaked on HS agar plates supplemented with Calcofluor white stain and incubated for 24-48 hrs. Later, fluorescence was observed under UV transilluminator. The colonies were found to fluorescence under UV light (Figure 4.3a). Calcofluor white stain reversibly binds to β-D glucan chains of cellulose as a result of which cellulose producing strains were able to fluorescens under the UV light. Bielecki et al. (2002) reported similar results, i.e., blue coloured fluorescent fibrils were observed when the BC pellicle stained with Calcofluor white stain was observed under the UV light (355nm) using an epifluorescent microscope. The study also reported that binding of the stain to cellulose microfibrils was responsible for the phenomenon of fluorescence. Studies have shown that Calcofluor white is commonly used dye to stain polysaccharides and cellular components which are made of β (1,3) or β (1,4) linkages, and have a strong association with polymers cellulose (Hughes and McCully 1975; Hawkins et al. 2017).

(b) Water-soluble polymer analysis was performed by mixing the bacterial culture broth and ethanol. This mixture was shaken vigorously and observed for precipitation. The mixture remained clear and transparent, indicating no precipitate formation. The results of the water-soluble polymer analysis were confirmed either by white precipitate formation (stated the presence of acetan polymer along with cellulose) or

no precipitation (presence of only cellulose polymer). Therefore, this test confirmed the presence of the cellulose polymer alone. A study by Ishida et al. (2002) showed that acetan polymer was precipitated by addition of ethanol to the supernatant of BC culture broth obtained after centrifugation. They concluded that strain *A.xylinum* BPR 2001 produced acetan polymer along with cellulose with this test. Another study by Mugesh et al. (2016) confirmed that all bacterial broth samples showed no precipitation indicating the presence of only cellulose polymer.

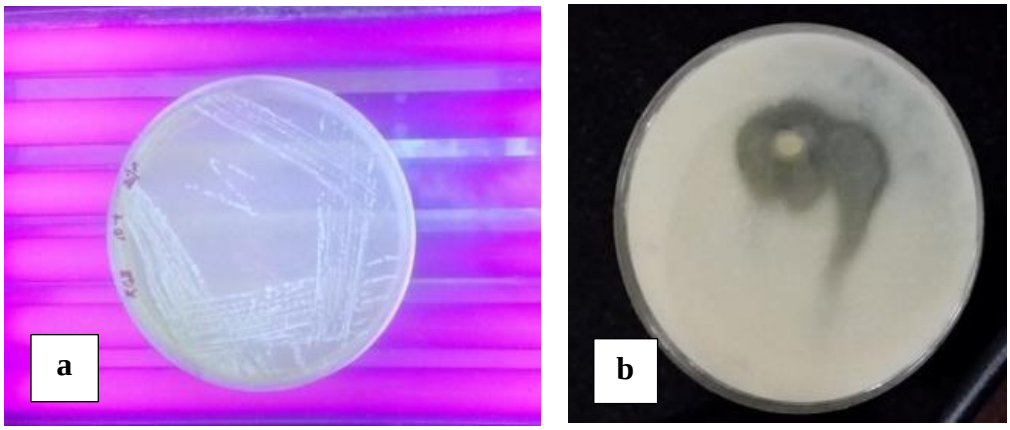


Figure 4.3 Visual identification of cellulose producing bacterial strain. (a) Bacterial colonies tend to fluorescence under UV light. (b) Zone of clearance around a colony on $CaCO_3$ agar.

(c) Visualisation of the zone of clearance around a bacterial colony on $CaCO_3$ medium is depicted in Figure 4.3b. The zone of clearance is caused due to fermentation of sugars into acids (acetic acid) by the acetic acid bacteria. This confirmed that the isolate belonged to the class of acetic acid bacteria and hence could be a potential cellulose producer. Berlanga (2010) reported that HS agar medium containing ethanol and $CaCO_3$ showed zone of hydrolysis around the bacterial growth, which could be attributed to the dissolution of $CaCO_3$ by acetic acid synthesised during the oxidation of ethanol by the bacteria. Aydin and Aksoy (2009) reported similar observations wherein *Gluconacetobacter* species isolated from vinegar produced clear zones in the $CaCO_3$ medium.A study by Tyagi and Suresh (2012) confirmed that isolate *Gluconoacetobacter intermedius NT* which was streaked onto $CaCO_3$-ethanol-agar medium showed the formation of clear zones around

bacterial colonies due to acid production, confirming that the isolate was a potential cellulose producer. Hence, by performing the screening tests, it was confirmed that the isolated bacterial strain was a potential Bacterial Cellulose producer. Most of the studies reported in the literature have confirmed that the Bacterial Cellulose producers were predominantly gram-negative strains and almost all the cellulose-producing bacteria come under the class of acetic acid bacteria (Stasiak and Błaejak 2009). Predominant BC producers, as reported from earlier studies, belonged to the genera *Acetobacter, Alcaligenes, Aerobacter, Rhizobium* and *Gluconobacter* (Jonas and Farah 1998; Chawla et al. 2009).

In the present study, bacterial strains were isolated from fruit residues. Amongst the fruit residues selected, the green grapes gave the maximum yield of BC. Several studies have been carried out using fruits and vegetable to isolate potential BC producers. Isolation of cellulose producers has been reported from rotten fruits and vegetables, and vinegar (Park et al. 2003; Aydin and Aksoy 2009; Young-Jung Wee 2011). Rani and Appaiah (2011) had isolated *Gluconacetobacter hansenii* UAC09 strain from contaminated grape wine. Similar to the current work, a cellulose-producing strain *Enterobacter amnigenus* GH-1 has been isolated from rotten apple (Hungund and Gupta 2010). Jahan et al. (2012) also have isolated BC producer *Gluconacetobacter sp.* F6 from a rotten apple. In addition to this, Neera et al. (2015) isolated *Gluconacetobacter xylinus* strains from five fruits such as apple, watermelon, mango, orange and banana. Rangaswamy et al. (2015) isolated cellulose producing bacteria from rotten fruits and vegetables. Kim et al. (2017) isolated *Gluconacetobacter sp.* gel_SEA623-2 from citrus fruit juice. Tyagi and Suresh (2012) have used the orange pulp as a source for isolation of BC producer *Gluconoacetobacter intermedius.*

Previous studies have also confirmed the BC production by the formation of a thin floating mat or pellicle at the air-liquid interface of the fermentation media (Hestrin et al. 1947; Brown 1985; Toyosaki et al. 1995; Bielecki et al. 2002; Brown 1886). Few studies have also reported on the evaluation of the BC pellicle by an iodine test. Iodine solution and conc. H_2SO_4 were used to stain the Bacterial Cellulose pellicle. The blue-violet colouration of the pellicle confirmed the presence of cellulose

polymer (Aterman 1976). The amount of bacterial cellulose was calculated in g/L as per the method stated by Ishihara et al. (2002); Bae and Shoda (2005).

4.1.1 Molecular characterisation of the isolate

16S rRNA gene is known to be the conserved and hypervariable regions present in the bacterial kingdom. Therefore, it acts as a genetic marker and helps us in studying bacterial taxonomy and phylogenetic relationships. Hence, by performing the sequencing method, the bacterial strain was identified as *Komagataeibacter saccharivorans* strain BC1.

5’TTCAGGACGAACGGCTGGCGGCGTTGCCTTAATAACATTCCAGTTCGAA
CGAACTTTAGTCTTTGATAGCGTAGTAGTTTGATCATGATCAGAGCGAACG
CTGGCGGCATGCTTAACACATGCAAGTCGCACGAACCTTTCGGGGTTAGT
GGCGGACGGGTGAGTAACGCGTAGGGATCTGTACATGGGTGGGGGATAA
CTTTGGGAAACTGAAGCTAATACCGCATGACACTGAGGGTCAAAGGCGCA
AGTCGCCTGTGGAGGAACCTGCGTTCGATCAGCTAGTTGGTGGGGTAAAG
GCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGATGATCAGCCACA
CTGGGAATGAGACACGGCCCAGTCTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGCAAGCGTGATCCAGCAATTCCGCGTGTGTGAAGA
AGGTTTTCGGATTGTAAAGCACTTTCAGCGGGGACGATGATGACGGTACC
CGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA
GGGGGCAAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGG
TTTTAACAGTCAGATGTGAAATTCCTGGGCTTAACCTGGGGGCTGCATTTG
ATACGTTGAGACTAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGA
GGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCAAC
CTGGCTCATTACTGACGGTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGATGTGTGCTGGATGTTGGGT
GACTTTGTCATTCAGTGTCGTAGTTAACGCGATAAGCACACCGGCC 3’

Table 4.4 Molecular Characterisation results

Sl.no	Closest Strain name	Accession number	Pairwise similarity (%)	Diff/Total nucleotide
1	*Komagataeibacter saccharivorans* strain JCM 25121	NR113398.1	99.65	5/1411

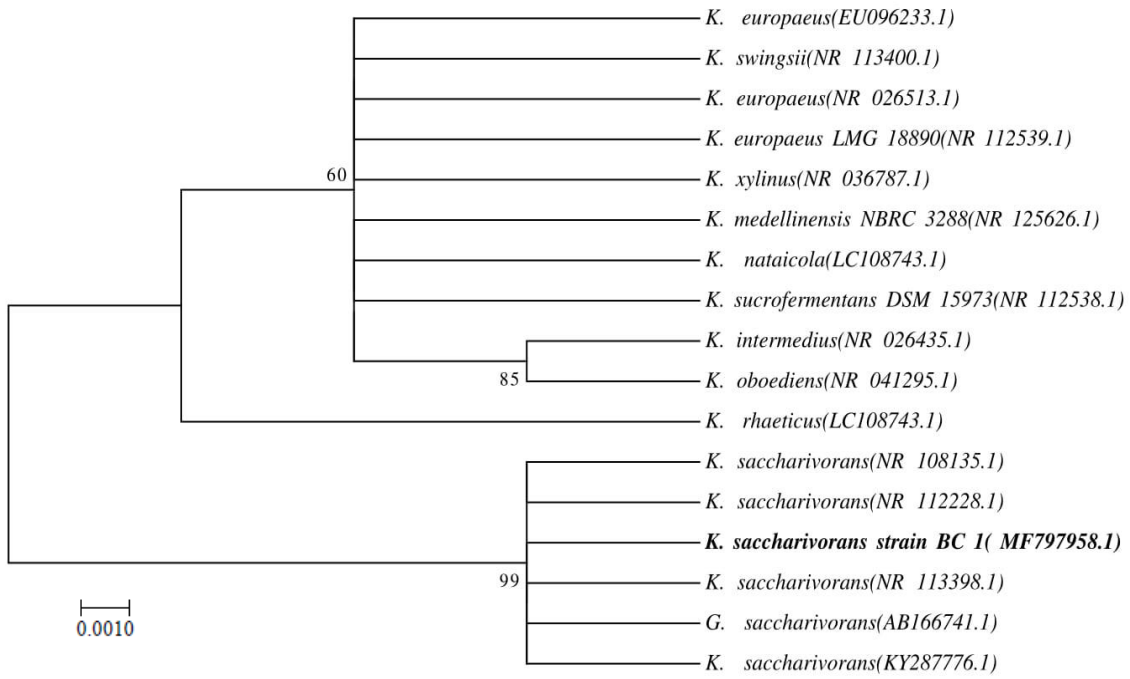


Figure 4.4 Phylogenetic tree of *K.saccharivorans* strain BC1

The isolated strain sequence and data were submitted to the GenBank database at NCBI as a direct submission and designated by accession number MF797958.1. The phylogenetic tree gives insights about evolutionary relationships of the novel isolated strain to that of previously existing/isolated similar strains or species. The phylogenetic tree illustrates that the closest taxa situated to the isolate were 99.65% identical, namely *Komagataeibacter saccharivorans* strain JCM 25121 (Table 4.4). The tree was constructed with the help of Maximum likelihood method as this method depends on multi-alignments of sequences, creating a phylogenetic tree of high accuracy (Figure 4.4).

Komagataeibacter is another genus that belongs to the class of AAB which are capable of producing crystalline cellulose as an extracellular product which can be recovered directly from a culture medium, and this was accounted for the first time by Yamada et al. (2000). Earlier acetic acid bacteria were classified into two main genera *Acetobacter* and *Gluconobacter*, but with more diversity in taxonomy, the classification has been revised and updated to fourteen genera and several of them recently was categorised under *Komagataeibacter* genus. The *Gluconacetobacter* genus was further divided into two categories, one of them is genus *Komagataeibacter*, and the species *Komagataeibacter xylinus* has been explored for its potential to synthesise cellulose. So far BC productions from *Acetobacter* and *Gluconacetobacter* strains have been extensively studied. However, minimal reports are available in the literature on the new strain *Komagataeibacter saccharivorans* and their ability to synthesise BC.

4.1.2 Production of Bacterial Cellulose using standard medium

Production of Bacterial Cellulose was carried out in the Erlenmeyer flasks under the static condition at normal room temperature (30 ± 2°C). A thin gelatinous film was observed floating at the air-liquid interface, confirming the production of BC. The pellicle/mat gradually became denser as the incubation period increased. The pellicle was later removed after a week of the incubation period and purified and used for further analysis.

4.1.3 Process optimisation for enhanced yield by one factor approach

Optimisation of physiological and nutritional parameters is an essential step as they play a pivotal role in the production of any bioprocess product (Junaidi and Azlan 2012). A significant amount of research has been carried out on studying the effects of several parameters that play a crucial role in BC production. However, there are only a few reports available on the study of BC production by *Komagataeibacter* strains.

To begin with, in the current study, HS medium has been used as a standard medium for cellulose production under unoptimized conditions for the screening of microorganisms. So to obtain the maximum yield of BC using the best carbon and nitrogen source, optimisation of the medium was performed. Hence, one variable at a time approach was used to optimise the media components. This method is a close-ended one for process optimisation and is applied for optimisation of medium components along with process conditions as this approach is one of the classical methods wherein of one independent variable is alone changed while fixing all others at a particular/constant level (Patidar et al. 2005).

4.1.3.1 Effect of different carbon sources

Various carbon sources were taken at a concentration of 2% (w/v) (standard concentration of carbon source for HS medium) to evaluate the highest BC production. The carbon sources selected for the study were glucose, fructose (monosaccharide), sucrose, maltose (disaccharides), starch (polysaccharide), mannitol, sorbitol (sugar alcohol) and glycerol. Figure 4.5 shows that mannitol gave the highest concentration of 1.725 ± 0.02 g/100mL of BC followed by glycerol of 1.692 ± 0.06 g/100 mL whereas the other carbon sources gave a lesser BC concentration i.e., glucose of 0.51 ± 0.44g/100 mL, sucrose of 0.19 ± 0.08g/100 mL, maltose of 0.28 ± 0.18g/100 mL, fructose 0.33 ± of 0.62g/100 mL, starch of 0.15 ± 0.02g/100 mL, and sorbitol of 1.51 ± 0.06g/100mL BC.

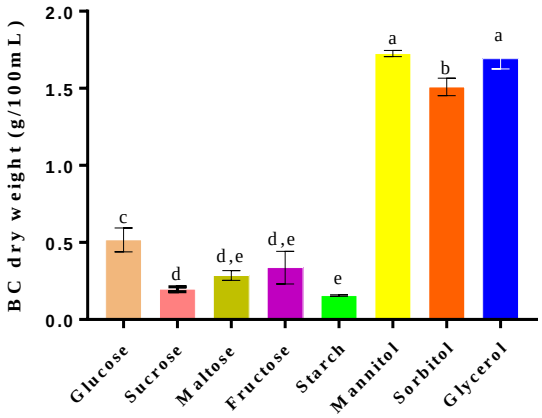


Figure 4.5 Concentration of BC using various carbon sources. Bars with different letters represent $p < 0.05$ significance

Metabolic pathways within bacterial cells involve oxidation of sugar alcohols into sugars which are later utilised by the acetic acid bacteria. Mannitol is broken down into fructose, which can then pass through the cell membrane and take part in the synthesis of cellulose polymer (Mamlouk and Gullo 2013). Another strain type that belongs to the same genus namely *K.saccharivorans* PE5 has been studied, which gave the highest concentration of cellulose, 11.74 g/L after 17 days of fermentation using glucose as carbon nutrient substrate on a modified Glucose–ethanol Acetic acid Medium (GAM) (Hassan et al. 2015). Other strains, namely *A.xylinum* that gave the highest concentration of 5 g/L BC using Mannitol as a carbon source (Ramana et al. 2000). *Acetobacter* sp.DR-1 strain produced 1.38 ± 0.22 g/L of BC (Raghunathan et al. 2013) Strain *Acetobacter xylinum* KU-1 also produced 4.6 mg/ mL of cellulose from D-mannitol (Oikawa and Ohtori 1995). Table 4.5 summarises various *Komagataeibacter* strains used for BC production along with mannitol as a suitable carbon source.

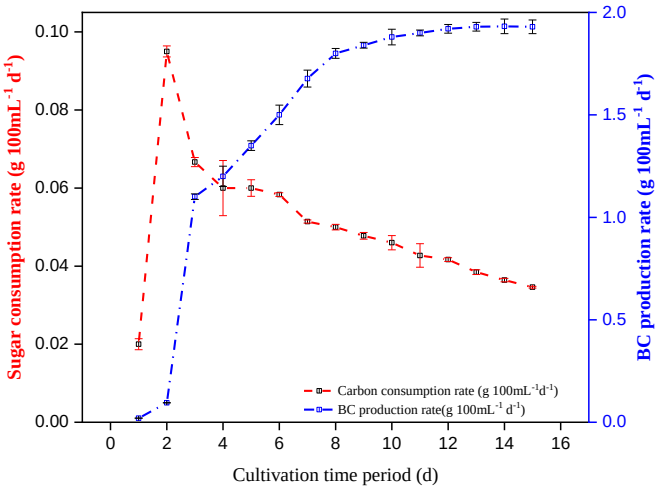


Figure 4.6 Kinetic studies on the production of BC by *K.saccharivorans*

A kinetic study was performed to know the relation between carbon source (mannitol) consumption rate and the BC production rates (Figure 4.6). On day 2 the carbon source consumption rate and BC production rates were 0.095 ± 0.001 g/100 mL/d and 0.085 ± 0.07 g/100 mL/d, respectively. On day 4, BC production rate spiked to 1.2 ± 0.045 g/100 mL/d and gradually increased up to 1.92 ± 0.033 g/100 mL/d. However, the sugar consumption rate gradually decreased to 0.05 ± 0.003 g/100 mL/d on day 5, further dropped down to 0.034 ± 0.002 g/100 mL/d on day 15 of the cultivation

period. This could be due to the conversion of mannitol to gluconic acid over a period, which causes the pH to drop, as a result of lesser sugar availability.

Table 4.5 Literature summary of different *Komagataeibacter* strains used for BC production.

Bacterial Strain	Source of Isolation	Carbon source (g/L)	Nitrogen source (g/L)	Supplement/s	Cultivation period (days)	Yield	Reference
A. xylinum DR-1	Temple wash waters	Mannitol (10)	Peptone(5)	–	6	1.38 g/L	Raghunathan (2013)
K.saccharivorans PE5	Rotten each	Mannitol (15)	Tryptone (6)	Ethanol, Nicotinic acid	17	11.74 g/L	Abdelhady et al. (2015)
A.xylinum	NCIM, India	Mannitol (70)	Yeast extract(5)	–	7	5g/L	Ramana et al. (2000)
K.sucrofermentans DSM15973	German culture collection, DSMZ	Crude glycerol (18)	Yeast extract(5), peptone (5)	Sunflower meal hydro-lysate	15	13.3 g/L	Tsouko et al. (2015)
K. europaeus SGP37	Rotten black grapes	Fructose (20)	Yeast extract(5), peptone (5)	Ethanol	16	9.98 g/L	Dubey et al. (2017)
K. rhaeticus P 1463	Kombucha	Glucose (20)	Yeast extract(5), peptone (5)	–	5	4.4 g/L	Semjonovs et al. (2017)
K. xylinus (KX)	Kasetsart University Thailand	Glucose (20)	Yeast extract(5), peptone(5)	–	7	1.84 g/L	Singhsa let al. (2018)
K.saccharivorans BC1 strain	Rotten green grapes	Mannitol (20)	Yeast extract(5), peptone(5)	–	15	2.01 g/100 mL	Current study

The study also showed faster production of BC over the rate of assimilation of carbon source. The average carbon consumption rate and the BC production rate were calculated and were found to be 0.045g/100 mL/d and 1.47 ± 0.001 g/100 mL/d, respectively. Therefore, from the study, it could be estimated that around 0.27 g of bacterial cellulose could be produced per gram of carbon source. The initial gluconic acid concentrations on day 2 and day 7 were found to be 0.152 ± 0.01 g/100mL and 0.167 ± 0.01 g/100mL, respectively. Therefore, the choice of evaluating the best carbon source is an utmost important step for the production of BC, as it serves as the main substrate during the fermentation process (Bielecki et al. 2002; El-Saied et al. 2004). Several studies have shown glucose as the best carbon source, followed by fructose. The remaining sugars, viz., lactose, xylose mannitol, galactose, sorbitol, arabitol and inositol, gave lower cellulose yields. However, in few studies similar to the current study carbon sources like sugar alcohols namely glycerol and mannitol have proven to be a better carbon source for cellulose production (Oikawa and Ohtori 1995; Ishihara et al. 2002; Keshk and Sameshima 2005; Kim et al. 2006).

4.1.3.2 Effect of different nitrogen sources

Peptone, yeast extract, casein, beef extract, tryptone, urea, sodium nitrate, ammonium chloride were taken at a concentration of 0.5% (w/v) (standard concentration of nitrogen source for HS medium). Figure 4.7 shows that yeast extract gave the highest concentration of 0.58 ± 0.07 g/100 mL, followed by peptone, which gave the BC concentration of 0.52 ± 0.32 g/100 mL. Ammonium chloride gave the least concentration of 0.03 ± 0.29 g/100 mL in the category of inorganic nitrogen. However, other inorganic sources namely urea, sodium nitrate showed no BC production indicating these bacterial cells were incapable of metabolising the inorganic sources of nitrogen. Several studies have reported that yeast extract has been an ideal nitrogen source along with other supplements such as corn steep liquor, asparagine (Dubey et al. 2017).

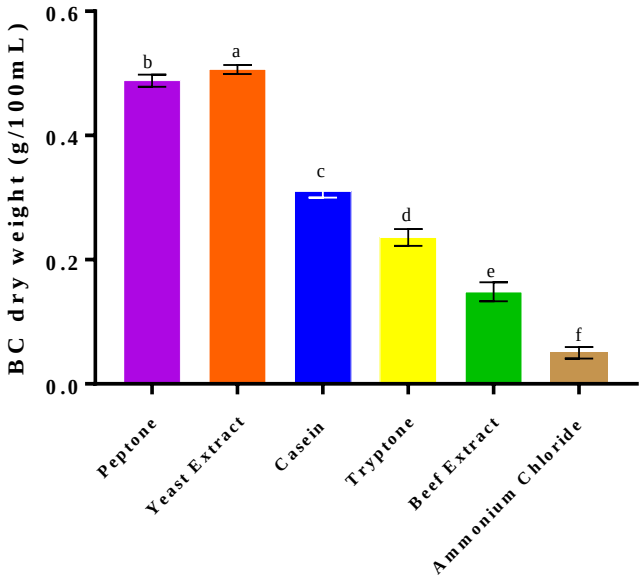


Figure 4.7 Effect of different nitrogen sources on BC production. Bars with different letters represent $p < 0.05$ significance

Although the nitrogen sources do not directly take part in the synthesis of cellulose biopolymer, they play a significant role in designing bacterial protein and nucleic acid, which are essential for bacterial growth and survival. Yeast extract is a robust source of vitamin B12 complex and other amino acids such as glutamic acid which act as growth stimulators and contributes to the better productivity of BC by the bacterial strain. Literature studies have shown, BC fermentation medium containing yeast extract and peptone as nitrogen source supports optimal BC production of *Acetobacter* strains (Pourramezan et al. 2009). A recent study by Singh et al. (2017) has shown that *Gluconacetobacter* strain C18 showed maximum yield using peptone and yeast extract as a nitrogen source. Mohammadkazemi et al. (2015) found that yeast extract and peptone were indispensable nitrogen sources for cell growth and BC production using *G. xylinus* strain PTCC 1734 in different media.

4.1.3.3 Effect of Inoculum size

Inoculum size is an important measure of the bacterial population. Different inoculum sizes ranging from 2% - 10% were chosen for inoculation into the fermentation medium. Figure 4.8 shows that the BC production gradually increased from 2 to 5%, at 5% the yield was around 1.83/100 mL. The yield slowly started dropping to 1.62g/100ml for 7% and finally to 1.01g/100mL for 10%.

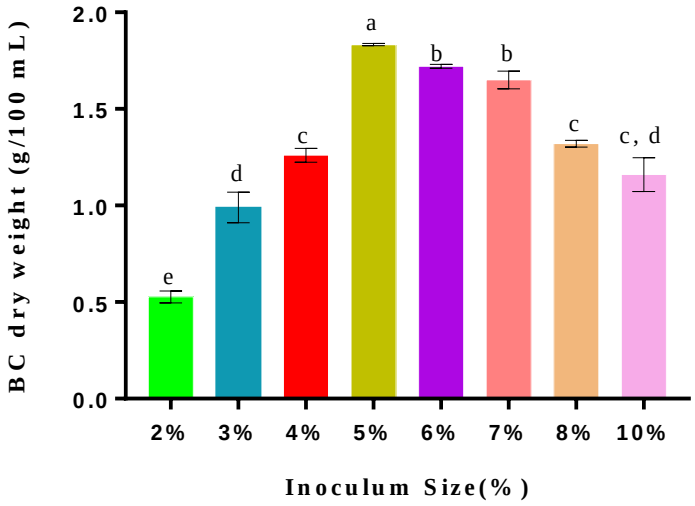


Figure 4.8 Effect of inoculum size on BC production. Bars with different letters represent $p < 0.05$ significance

The study showed that the inoculum ratio had a significant influence on cellulose production. As the inoculum size increased more than 5%, there was a decrease in BC production. This could be due to the reason that excess of inoculum density increased the bacterial population, which then competes for nutrients resulting in disruption of both growth and BC production (Yanti et al. 2018). A study by Rangaswamy et al. (2015) shows 5% as the optimised inoculum concentration for the production of BC by *Gluconacetobacter sp*. RV28. Another study by Tanskul et al. (2013) showed that a strain of *Rhodococcus* was able to produce maximum BC at 5% inoculum concentration.

4.1.3.4 Effect of pH

The pH of the medium plays an important role in determining the growth and production of BC. The strain was evaluated for its cellulose production over a pH range of 2–8. Figure 4.9 shows that maximum BC production of 1.82 ± 0.65 g/100mL was observed in the medium, which had pH 5. Studies have shown the pH ranging from slightly acidic to neutral was feasible for BC production. Due to fermentation of sugars to acids, there is a reduction in overall pH in the medium as a result of which cell viability decreases, hence the production of BC is affected (Castro et al. 2012). The initial pH of the production medium was altered from 2 to 8 using HCl and NaOH, respectively, and output was monitored over a week.

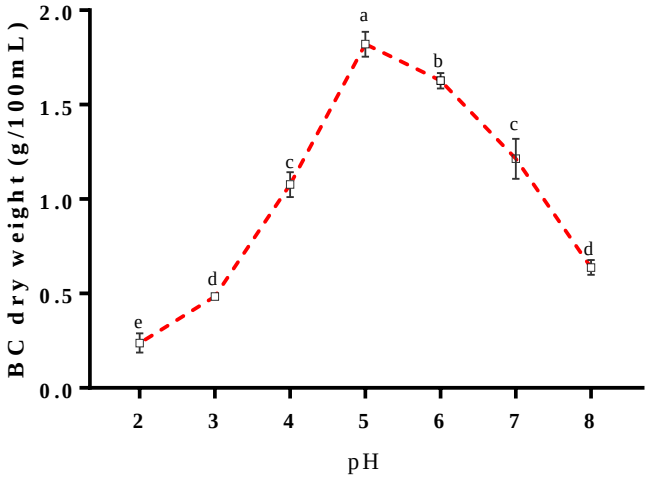


Figure 4.9 Effect of pH on BC production. Means with different letters represent p < 0.05 significance

Studies have shown that maximum BC production of 4 g/L at was observed at pH 5 by *Leifsonia sp.* CBNU-EW3 (Velmurugan et al. 2015); *K.europaeus* SGP37 produced the highest concentration of BC (6.07 ± 0.40 g/L) at pH 5 (Dubey et al.2017). The findings of this study are consistent with the previous studies of Ishikawa et al. (1995); Zahan et al. (2015); Costa et al. (2017); Dirisu and Braide (2018); Lima et al. (2017); Jung et al. (2005), which reported pH 5 as the optimal for BC production. However, some others have also reported the maximum cellulose production from the pH range of 5.5-7 (Hungund and Gupta 2010; Masaoka et al.

1993; Son et al. 2001, Panesar et al. 2009; Jung et al. 2010; Junaidi and Azlan 2012). Studies by Oikawa et al. (1995) and Embuscado et al. (1994) have reported that from 3-3.5, there was no BC production.

4.1.3.5 Effect of temperature on BC production

The synthesis of BC requires a specific temperature as the process involves activities of several enzymes, which are temperature dependent. *K.saccharivorans* strain was cultivated under various temperatures ranging from 20°C to 45°C. Figure 4.10 shows that at 30°C, maximum cellulose production of 1.82 ± 0.01 g/100 mL was observed. This could be due to the reason that at 30°C incubation period, the growth of *K.saccharivorans* would have been faster (prolonged log phase), which in turn facilitated better BC production.

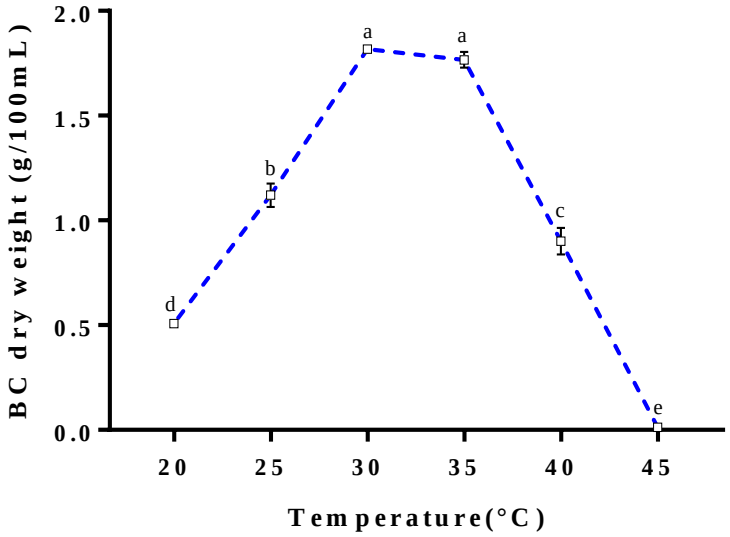


Figure 4.10 Temperature versus BC production. Means with different letters represent $p < 0.05$ significance.

At the temperatures, 35°C and 40°C slow growth of BC was observed indicating the bacterial population could be still in lag phase. As a result of a longer lag phase, only cell density increased, and there was no increase in biomass production. At 45°C, no BC production was observed. The results of the temperature study were in agreement with previous studies of Son et al. (2003); Kongruang (2008); Costa et al. (2017);

Hutchens (2007); Kaewnopparat (2008) which reported 30°C as the preferred temperature for BC production. Studies by Gomes et al. (2018); Raspor and Goranovič (2008) showed optimum growth between 25-30°C. Although some thermotolerant strains which can survive up to 40°C have also been isolated, one of them being *Acetobacter tropicalis* SKU1100 which have been reported to produce BC at high temperature (Perumpuli et al. 2014; Deeraksa et al. 2005). The genus *Komagataeibacter* is known to produce BC at the temperature of 25-30°C (Lavasani et al. 2017; Molina-Ramírez et al. 2017).

4.1.3.6 Time course of BC production

The production of cellulose by *K.saccharivorans* was studied over two weeks using the optimised production medium. The medium used for time course study had been optimised earlier for carbon and nitrogen sources (modified HS medium), i.e., mannitol as a carbon source and yeast extract and peptone as a nitrogen source, pH was adjusted to 5 and grown at a temperature of 30°C under static incubation. Figure 4.11 shows that BC production followed a sigmoidal growth curve pattern. The initial concentration was 0.003g/100mL of BC on day 1, and the cellulose synthesis gradually accrued, with maximum BC concentration of 2.01 ± 0.05 g/100mL on the 15th day (after 360 hrs.), after which the bacterial cellulose production slowly started declining, indicating that the microorganisms might have crossed the exponential phase and stepped into the stationary phase of their growth (after 360 hrs.). The overall BC productivity (PBC) which was found to be 0.01 ± 0.03 g/100 mL/h, and the 1.71 as yield coefficient (Yp/s) that is the yield of the product on the substrate.

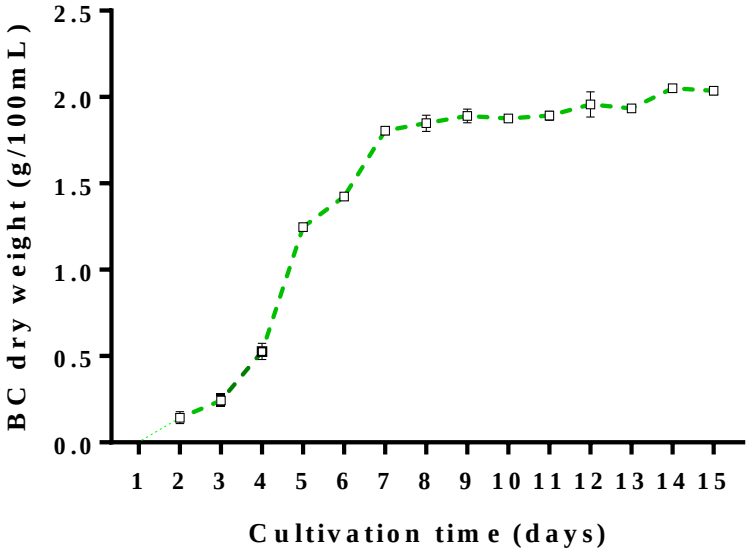


Figure 4.11 Time course of BC production

4.1.4 Characterization of BC pellicle

The pellicle obtained was dried and cut into small pieces (~1mm) size, were analysed to study structural and chemical properties**.** Standard cellulose obtained from Sigma Aldrich was used as the reference sample. The samples showed similarity to commercial sample in chemical aspects, although morphologically they differed.

4.1.4.1 ATR-FTIR (attenuated total reflection Fourier transformed infrared spectroscopy) analysis

ATR method was used for performing FTIR spectroscopy as it offers a rapid analysis, non-destructive method, and suitable for real-time online monitoring system, namely for studying the growth of bacterial cell in a culture media, the formation and development stages of a biofilm (Schmitt and Flemming 1998). Figure 4.12 illustrates FTIR spectral peaks of the BC pellicle having various functional groups. The hydroxyl groups were observed at 3406.90 cm^{-1} to 3300 cm^{-1}, which indicated (–OH) stretching vibrations (γ). The broader peak bands indicated the presence of intermolecular hydrogen bonding in the cellulose molecule. Also, these hydroxyl groups are responsible for facilitating substitution in the molecule; similar kind of results was reported in earlier studies (Guzel and Akpınar 2018; Kondo et al. 2016).

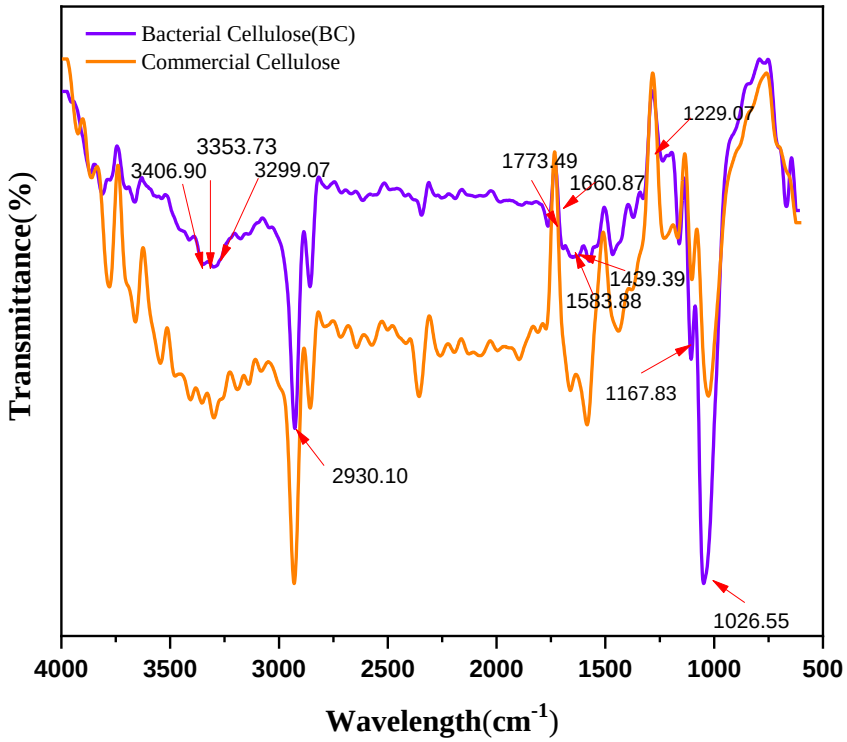


Figure 4.12 FTIR spectra of Bacterial Cellulose

The peak at 2930 cm^{-1} represents symmetric (–CH) stretching vibrations (γ) indicating the presence of methyl/methylene functional group. 1660.23 cm^{-1} revealed a peak water molecule associated with cellulose (absorbed H_2O) bending vibrations (δ). Peak 1439.39 cm^{-1} inferred the presence of symmetric ($–CH_2$) bending vibrations(δ), also regarded as crystallinity band of cellulose indicating the higher intensity of this band indicated an increase in crystallinity, also this peak is characteristic of cellulose type I (Popescu et al. 2007). 1379.33 cm^{-1} indicated the presence of (–CH) plane bending vibrations (δ). 1167.83 cm^{-1} peak inferred the presence of (C–O–C) asymmetric bridge stretching vibrations (γ) in the cellulose, which is responsible for holding together the monomeric units of glucose through the β-1, 4 glycosidic linkages. 1102.49 cm^{-1} shows the presence of C–C ring asymmetric valence vibration (present in polysaccharide cellulose) and 1026.55 cm^{-1} showed the presence of (C–O) stretching vibration (γ) in the polymer (Park et al. 2010; Coates 2006). These significant peaks confirmed that the produced pellicle is a cellulose polymer. The hydrogen bonds present in cellulose is distributed in both crystal and amorphous regions. The hydrogen bonds for cellulose I include two intramolecular bonding, namely, O2H---O6 bonding and O3H---O5 bonding and one intermolecular bonding,

O6H---O3. In the present study, BC exhibited Cellulose I type which showed a peak at 3406.9 cm^{-1}, indicating the presence of intra hydrogen bonding mainly between O2H---O6 (Gardener and Blackwell 1974). The bacterial cellulose sample peaks were compared with commercial microcrystalline cellulose (Himedia) that has been used as the reference sample. The study concluded that the functional groups present in BC were similar to those present in commercial cellulose, confirming the polymer produced was cellulose.

Table 4.6 Comparison of peaks and their assignments

Standard Cellulose Peaks	Assignments	Bacterial Cellulose peaks	Assignments	Reference/s
3350cm^{-1} 3400-3500cm^{-1}	O-H stretching	3406.9 cm^{-1}	O-H stretching	Guzel and Akpınar, (2018); Kondo et al. (2016)
2800-2900cm^{-1}	C-H stretching	2930.1 cm^{-1}	C-H stretching of CH_2 and CH_3 groups	Popescu et al. (2007)
1600cm^{-1}	OH bending of absorbed water	1660.87 cm^{-1}	OH absorbed	Coates (2006)
1300cm^{-1}	C-H bending	1439.39 cm^{-1}	CH_2 bending	Coates (2006)
1400cm^{-1}	CH_2 bending	1379.33 cm^{-1}	C-H bending	Park et al. (2010)
1160 cm^{-1}	C-O-C stretching	1229.07cm^{-1}	C-O-C stretching	Park et al. (2010)
1060 cm^{-1} ~1070 cm^{-1}	C-O-C pyranose ring skeletal vibration	1167.83 cm^{-1}	C-O-C stretching	Coates (2006)
1035cm^{-1}-1060 cm^{-1}	C-O stretching	1026.55cm^{-1}	C-C, C-OH, C-H ring	Coates (2006)

4.1.4.2 Scanning electron microscopy (SEM) analysis

SEM analysis was performed to study the morphological appearance and arrangement of cellulose microfibrils. Around a hundred randomly distributed fibrils were measured for their average width. Figure 4.13a shows the arrangement of cellulose fibrils was intertwined tightly packed with less interfibrillar spaces Due to the thin fiber diameters; the surface area is increased hence facilitating smoother surface for better absorption, lesser air permeability. BC diameters were measured and were ranging from 40.65 to 58.62 nm, with the average fiber diameter of about 30 nm (Figure 4.13b).

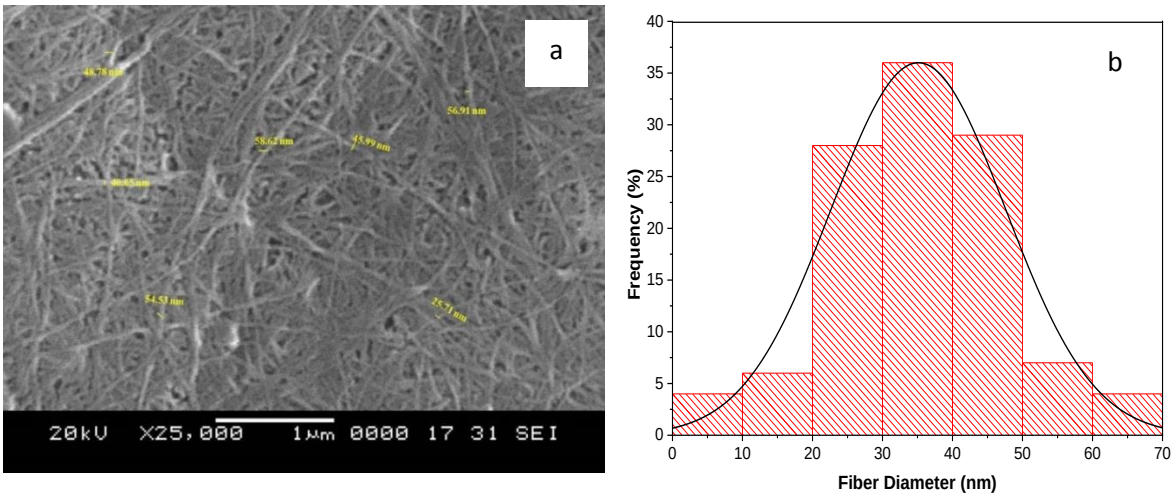


Figure 4.13 (a) SEM image of dry BC polymer magnified at 25000X (b) fiber diameter distribution

Similar to this, a study by Wang et al. (2017) showed that BC fibrils produced by the strain *Komagataeibacter sp* W1 displayed fibril diameter in the range of 40-60 nm with the maximum number of fibers of 35 nm in width. Another study by Yang et al. (2013) showed that BC nanofibers synthesised by *Acetobacter xylinum* NUST5.2 had a diameter of about 30nm, which showed good absorption of silver nanoparticles. The results were consistent with previous studies.

4.1.4.3 X-Ray Diffraction (XRD) analysis

X-ray diffractogram was performed to study the crystallinity ratio and the type of cellulose produced by the bacterial strain. Figure 4.14 shows that the produced BC depicted three prominent peaks at 2θ angles =14.98, 16.48, and 22.78. These peaks can be represented as a crystallographic plane of the polymer lattice which are14.98 (100), 16.48 (010), and 22.78 (110), which indicated cellulose I_α type. The β allomorph of cellulose is produced in higher plants and is absent or rare in bacterial derived cellulose (Park et al.2010).

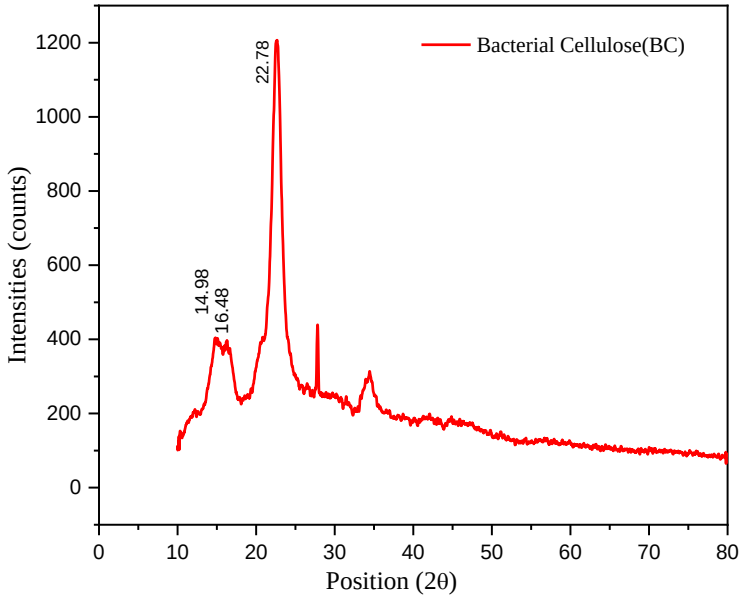


Figure 4.14 XRD profile of BC showing the peaks of cellulose

Cellulose I exists in two other allomorphic forms, namely Cellulose I_α (triclinic structure) and Cellulose I_β (monoclinic structure). The amounts of celluloses I_α and I_β differ with the sources from where the cellulose is obtained, with the I_β form being dominant in higher plants and Iα in bacterial and algal sources of cellulose (Popescu et al. 2007). Cellulose I_α is made up of parallel chains (1→4) linkage in with intermolecular hydrogen bonding between the planes that have progressive shear to chain axis stabilised by Van der Waals interaction and has a triclinic structural arrangement of the lattice (Goelzer et al. 2009).The BC produced by the strain had the Crystallinity index of 82.2%. The crystallinity index of Bacterial Cellulose refers to the relative amount of crystallinity in the cellulose polymer (as the polymer consists of both crystalline and amorphous regions). The crystallinity index of the synthesised

cellulose depends on several external factors such as the mode of fermentation, the physiological and the nutritional parameters, method of drying (Park et al. 2010).

Table 4.7 shows the d-spacings which refers to distance "d" which separates the atomic planes apart in a molecule. As this d spacing is unique to each entity, expressing the diffraction peaks in terms of d-spacings, aids in the better identification of the component. The interplanar distances (d-spacings) were calculated with Bragg's law, and the value of Z discriminant function was greater than 0, indicating that Bacterial cellulose had I_α form of cellulose. This type of cellulose is produced only by algae and bacteria, as mentioned before (Mohite and Patil 2014). Hence, from this study, it was evident that cellulose produced is from the bacterial origin and exhibits cellulose (I_α) crystalline form.

Table 4.7 d-spacings and crystallinity percent of BC.

d-Spacing (Å)			**Z-value**	**CrI (%)**
d_1 ($1\bar{1}0$)	d_2 (110)	d_4 (200)	4.5	82.2
5.90	5.374	3.9		

4.1.4.4 Thermogravimetric analysis (TGA) analysis

TGA reveals the thermal stability and degradation behaviour of BC. Other than material characterisation, this method is also used to study degradation mechanisms of solids, the organic or inorganic content of solid samples or chemical samples. Figure 4.15 shows the thermal degradation study in BC. The initial degradation began at a temperature of 25°C. There was a quick drop in weight observed at 210°C (84%). This could be attributed to the cleavage of glycosidic bonds present in the polymer (Manfredi et al. 2006). The weight loss of 50% was observed at around 300°C. Also, the weight loss at 100°C could be attributed to the volatilisation of water molecules in the polymer.

The pronounced degradation of BC ranges from 210°C to 310°C. The differential thermogravimetric (DTG) curve showed that the temperature of the initial weight loss for BC began at 50 °C. Further sharp degradation was observed from 250 °C to 320 °C, showed a sharp peak at 255 °C with a degradation rate of 350 µg min^{-1}, which could be due to depolymerization, decomposition of the residue, second phase wherein degradation occurred at 450°C, owing to the charred carbon residue formation due to oxidation (Dubey et al. 2017; El-Saied et al. 2008).

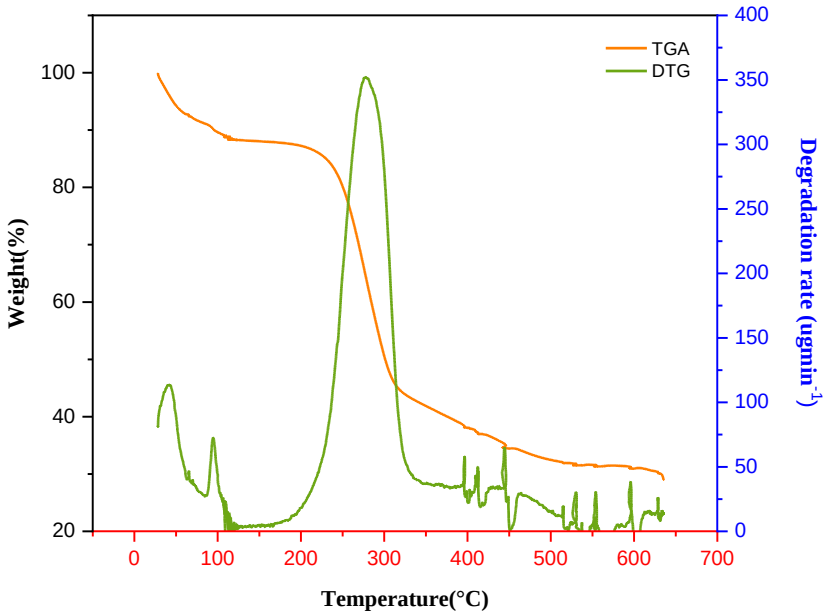


Figure 4.15 TG-DTG curve of BC synthesized from HS medium

The measurement of TGA relies on two major factors, namely mass change and temperature change. A typical TGA plot of bacterial cellulose shows weight loss in two phases, the first phase begins from room temperature to 200°C due to volatilization of water and solvent molecules and the second phase is characterized by the weight loss from 200° C to 500°C, this loss in weight could be attributed to organic compound decomposition and charring of the material (Calahorra et al. 1989). The amount of weight loss and the temperature of degradation of BC/BC composites vary on the type of BC fermentation, the culture medium used, and with other polymeric materials or additives. A study by Albu et al. (2014) showed that modifying the BC fibres with a blend of collagen displayed high thermostability over the native BC fibre alone. Another study by Seifert et al. (2004) illustrated that the

initial weight loss observed in the range of 200°C to 360°C is attributed to the removal of functional groups such as hydroxyl and methyl hydroxyl groups. The second phase of weight loss can be observed from 360°C to 600°C, which indicated the degradation of polymeric chains and the pyranose ring structure. This study showed that BC displayed good thermostability nature up to 300°C.

4.1.4.5 Purity of BC produced from the standard medium

The purity of BC was evaluated by performing enzymatic hydrolysis of BC by cellulase enzyme. The supernatant of the digested mixture was analysed by DNS method using UV spectrophotometry for the glucose concentration. One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per mL per minute during the reaction. The cellulase enzyme showed an activity of 0.96 IU/mg. The results of enzyme activity confirmed that the substrate was cellulose, which was hydrolysed to glucose molecules by the action of enzyme cellulase.

Summary

The study describes the isolation of potential BC producer *K.saccharivorans* from rotten green grapes. The production and optimisation for BC were carried out using standard HS medium. Mannitol and yeast extract were found to be the best carbon and nitrogen sources, respectively. The BC produced was later characterised using SEM, FTIR, TGA and XRD to study the morphological and structural aspects of BC.

PART TWO

The scope for BC production on a large scale is still limited; one of the main reasons being the production cost. The usage of commercial media makes the production process costlier. Therefore, the synthesis of BC was carried out from a cheap and cost-effective carbon source using the same microbial strain. The medium used here was distillery effluent.

Distilleries generate wastewaters from distilling units, spent lees and other effluent waters discharged during fermenter wash, cleaning processes (Jahan et al. 2018). India has around 298 distilleries producing 3.2 billion litres of alcohol and 45 billion litres of spent wash annually. Amongst these, spent wash is the main wastewater stream. The Spent wash has the following characteristics. It has a BOD of around 30,000 mg/L to 60,000 mg/L and COD of 80,000 mg/L to 100,000 mg/L. The pH of the spent wash ranges from 3.5-5 (acidic) (Bharagava et al. 2017). The dark brown colour of the effluent is due to the organic load. The high TDS content of spent wash makes it unsafe to discharge due to its adverse effect on the environment. Also, this effluent water cannot be used for drinking, and irrigation purpose as the alkaline pH affects the plant growth. The disposal of effluent containing suspended solids into water bodies causes frothing and decreases the light penetration affecting the aquatic life (Chowdhary et al. 2017). Due to the heavy organic load in the wastewater, anaerobic decomposition leads to the production of harmful gases such as methane, hydrogen sulphide, and ammonia (Patil Anagha et al. 2018). Therefore, the effluent water needs to be effectively treated using a proper treatment such that targeted pollutants load remain within safe limits for further disposal. Generally, biological, chemical and thermal methods are used to treat the spent wash. Activated sludge and microbial mediated anaerobic digestion methods are the most common ones used for the treatment of effluent. Another way of treating them would be to utilise them as nutritional supplements for the production of high-value biomaterials.

Thus, several fermentation studies have been performed using the wastewater as a nutrient broth to obtain commercially useful products such as bio-ethanol, enzymes, and other biofuels with the help of microorganisms (Ahn et al. 2011; Hsieh et al. 2005; Wu and Liu 2012). In our study, an effort was taken to reuse the raw distillery effluent to produce BC from an isolated bacteria *K.saccharivorans* strain BC1. Over the past few decades, many studies have been performed on different aspects of BC production, such as yield and productivity, structural and rheological properties, fermentation conditions, and applications. However, due to low production yield and high cost, commercialisation of BC on an industrial scale is limited.

Therefore, the large scale production of the biopolymer would depend on designing effective yet cost-efficient strategy. One such approach is the utilisation of byproducts and wastewaters of fermentation industries as raw materials for the synthesis of biopolymer (Salari et al. 2019). Also, there is no data available on *K.saccharivorans* fermenting wastewater. The literature reports of *K.saccharivorans* employed in fermentation studies and bioprocesses are also limited (Hassan et al. 2015). Therefore, the present study demonstrates the synthesis of cellulose biopolymer along with the reduction in the COD value of wastewater which acts as the primary nutrient for microbial growth, in turn making the effluent less toxic during the disposal process. Hence, this study could prove not only as a helpful step in the reduction of pollutants load but also in designing a fermentation process with inexpensive raw materials.

4.2 Distillery wastewater

The distillery wastewater was procured from M/s UB breweries limited, Baikampady, Mangalore, Karnataka State, India. The effluent was centrifuged at 5000 rpm for 15 mins and filtered through 0.45-micron cellulose acetate filters. This filtrate was used for studying various effluent parameters, as shown below, as depicted in Table 4.8. Protocols were referred from APHA (2012).

Table 4.8 Raw Distillery effluent characteristics

Parameters	**Values**
pH	4.3
TDS (Total dissolved solids)	50.45 (g/L)
TSS (Total suspended solids)	31.42 (g/L)
BOD (Biochemical oxygen demand)	20,700 (mg/L)
COD (Chemical oxygen demand)	87,433 (mg/L)
TOC (Total organic carbon)	15,420 (mg/L)
Nitrogen	2333 (mg/L)
Phosphorus	90 (mg/L)
Sodium	370 (mg/L)
Magnesium	182 (mg/L)
Potassium	190 (mg/L)
Zinc	6.4 (mg/L)
Colour	Creamish brown

The raw distillery effluent procured showed a high BOD and COD with a low pH. This pH was unfit for bacterial growth; therefore, the pH was adjusted to 5 for the growth of the isolated strain in the culture medium. The effluent was monitored for BC production, and simultaneously, COD study was performed. The BOD and COD values before and after the cultivation period were recorded.

4.2.1 BC production and COD degradation study

The objective of this work was to assess the conversion of low-cost raw material (distillery effluent) to the valuable product (BC) by *K.saccharivorans*. The crude distillery effluent was used as such for the production of BC with no media alterations. The pH was adjusted to 5 (optimised from the previous study) with 1N NaOH. Figure 4.16 shows that *K.saccharivorans* could produce 1.24 g/L of BC over eight days using effluent medium along with the reduction of COD up to 66831 mg/L.

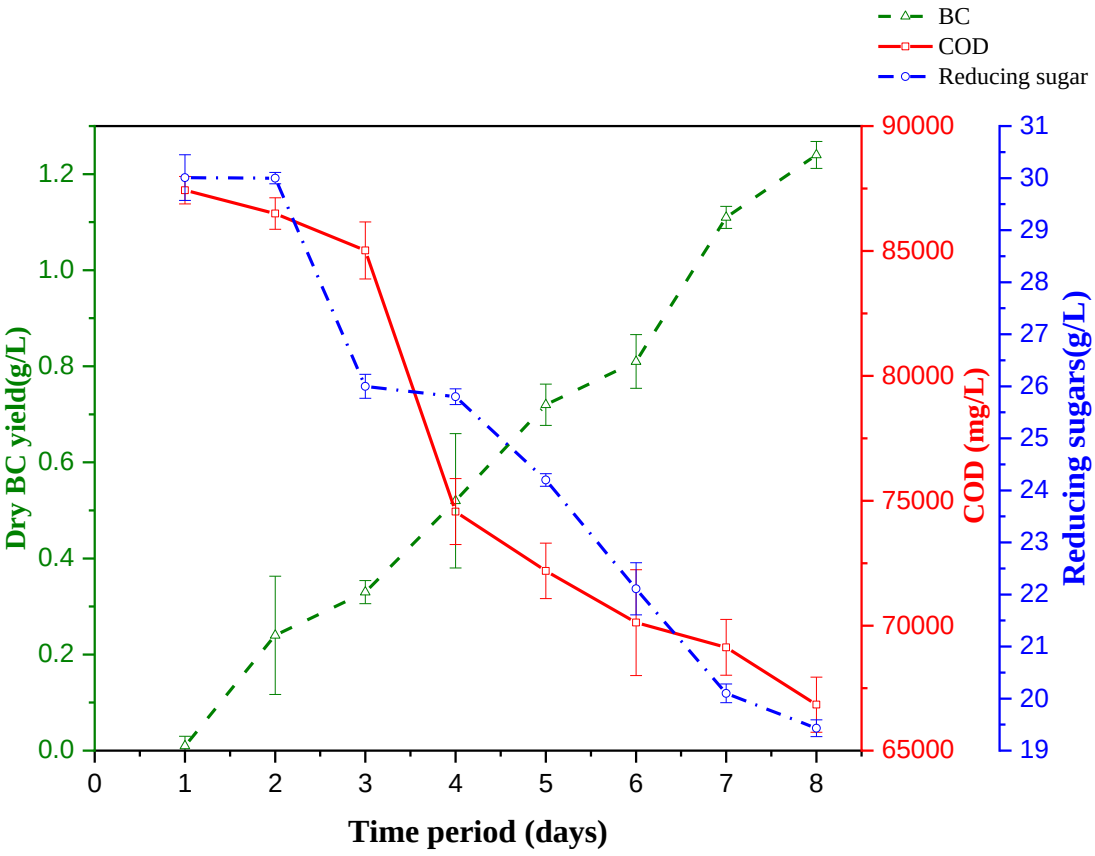


Figure 4.16 BC production and COD degradation from distillery effluent by *K.saccharivorans*: Dry BC yield (g/L), COD (mg/L) and reducing sugar concentration (g/L)

There was no cellulose production of BC on Day 1 after inoculation of culture. After 48 hours, thin pellicle was observed at the air-liquid interface of the culture medium (0.21g/L). The BC production steadily increased each day, reaching 1.24 ± 0.03 g/L on the 8th day, as illustrated in Figure 4.16. The utilisation of sugars was also

observed with the initial sugar concentration of 30 ± 0.44 g/L to 19.43 ± 0.16 g/L on day 8. Until day 2, there was no significant drop in sugar concentration. On day 3, a significant decline was observed up to 26 ± 0.23 g/L. Therefore, *K.saccharivorans* strain BC 1 was capable of utilising the residual sugars present in the effluent. Thus, this study confirmed that the novel isolated strain bacterium was capable of utilising the effluent as a nutrient medium to produce the biopolymer. The maximum BC production yield and productivity was calculated over 8 days. The BC yield of 11.7% and productivity of 0.155 g/L d was recorded, in contrast, the BC production rates from HS medium was found to be 1.47 ± 0.001 g/100 mL d, and yield of 0.27g of cellulose was produced every gram of carbon source (Gopu and Govindan 2018).

During the fermentation period, the COD reduction study was also monitored. The (initial) COD of the medium before the beginning of fermentation was found to be 87433 mg/L and at the end of 8 days was recorded to be 66831 mg/L with reduction of 23.6% in COD as depicted in Figure 4.16. Hence, this study confirmed that crude distillery effluent could be reused as a substrate for microbial fermentation. Although the decrease ratio is low, the bacterial strain was able to utilise the organic load and produce the biopolymer making the overall process cheap and feasible. 36% of reducing sugar was consumed after the fermentation period was complete.

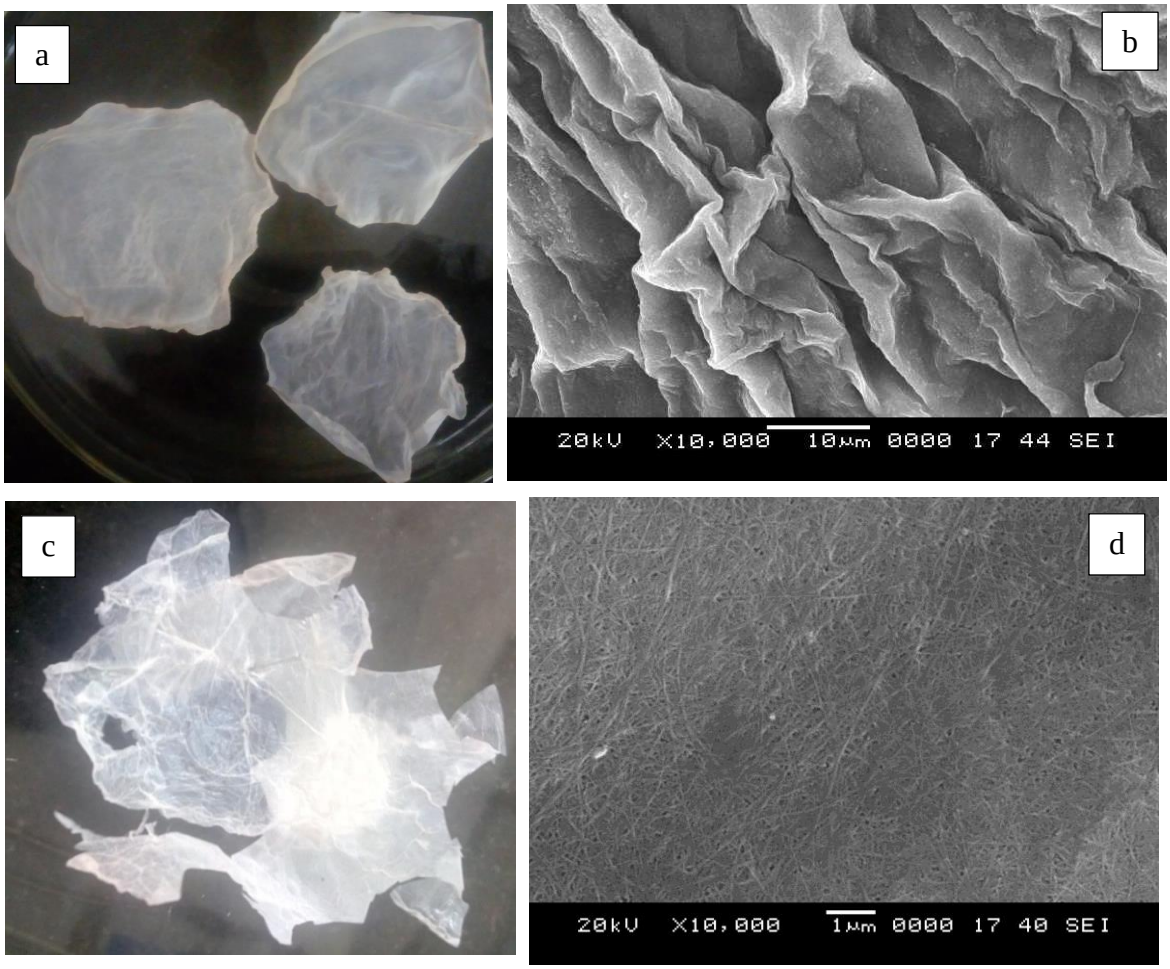
A study by Zhao et al. (2018) also showed that fermented wastewater of polysaccharide could be used as nutrient source BC production of 1.17 g/L along with COD reduction of 18.9%. Huang et al., (2016) showed that from lipid fermented wastewater as effective as a carbon source, with cellulose production of 0.659 g/L and 30% COD reduction. Another study by Huang et al., (2015) revealed that 1.34 g/L of BC synthesised with 14.7% reduction in COD using acetone- butanol – ethanol (ABE) fermented wastewater broth. Also, the BOD of the wastewater after fermentation was recorded as 18220 mg/L with a reduction of 11.9%. So far, very few studies have been carried out using distillery effluent as a nutrient source for the production of BC. A study by Jahan et al. (2018) showed that 0.85 g of BC was synthesised from 100mL of crude distillery effluent. Another study by Wu et al. (2013) showed that replacement of HS medium by 50% of thin stillage wastewater produced the highest yield of 6.26 g/L of BC.

4.2.2 Characterisation of BC

The BC synthesised from the effluent was removed after the cultivation period. This pellicle was rinsed with distilled water and then boiled in 1N NaOH for about 90 mins at 90°C. The BC film was then removed and rinsed several times until the pH of the filtrate reached 7. The film was then dried and used for further characterisation.

4.2.2.1 Drying of BC

Three different methods were employed for drying of Bacterial Cellulose fibrils, namely air drying or drying at room temperature, freeze drying using lyophilizer and oven drying in a hot air oven at 40°C for 5 hours.



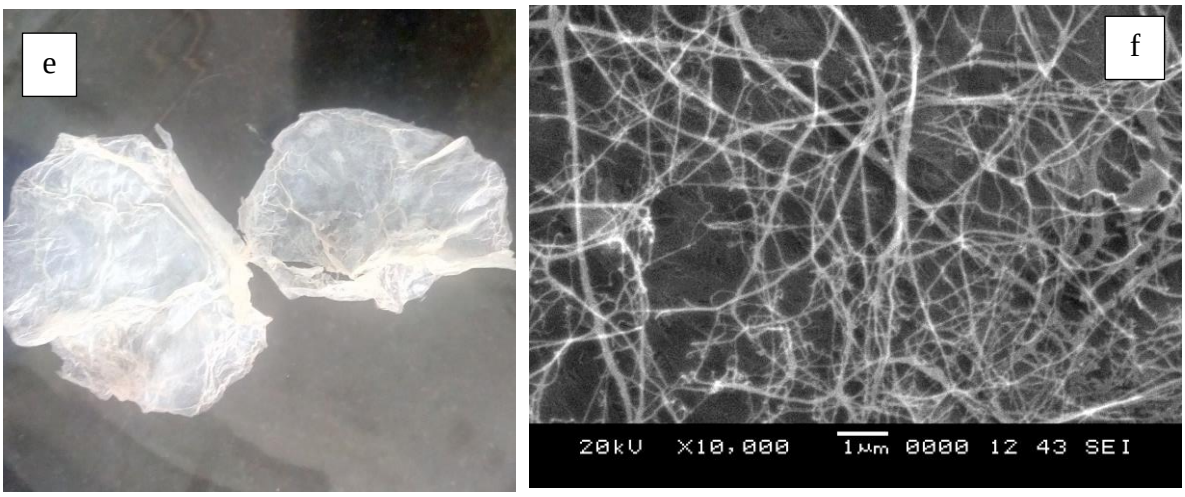


Figure 4.17 BC films with morphological features (a) and (b) air-dried BC films, (c) and (d) BC films oven dried and (e) and (f) freeze dried BC films respectively.

Figure 4.17 shows the BC pellicle dried under various conditions showed different fibrillar morphology. The air-dried films displayed densely packed fibres, whereas in case of oven dried films depicted thick fibrils with less interfibrillar spaces. The freeze-dried BC fibrils showed thin, slender fibres with large porosity, providing more insights on the fibre width and arrangement.

The thickness of films can be arranged in the increasing order of thickness with oven dried films<freeze dried films<air dried films with 30 micrometres, 60 micrometres and 640 micrometres respectively. The appearances of the BC dried films were from translucent to opaque. When BC films were dried by the method of oven drying, moisture losing was fastened; however, the homogeneous fibrillar structure retained intact. Under vacuum freezing conditions, moisture in the gelatinous membrane was frozen to ice initially, which later was transformed into vapour under vacuum conditions, this made the structure devoid of any water molecule and gave a better visualisation of BC fibrillar networks (Zhang et al. 2011).

4.2.2.2 FTIR spectra analysis

FTIR spectra of synthesised BC from distillery effluent (BC-D) and BC produced from HS medium (BC-HS) depicted similar functional groups, as shown in Figure 4.17. Peaks were interpreted and analysed based on the previously available literature reports related to the present study. The peak at 3341.61 cm^{-1} displayed a broad and sharp band which inferred the hydroxyl (O3H---O5) functional groups (-OH) which exhibited stretching vibrations (γ). This also reflected the existence of inter-molecular hydrogen bonds in the BC polymer (O6H---O3) (Wu et al. 2012; Thorat et al. 2018; Coates 2006).

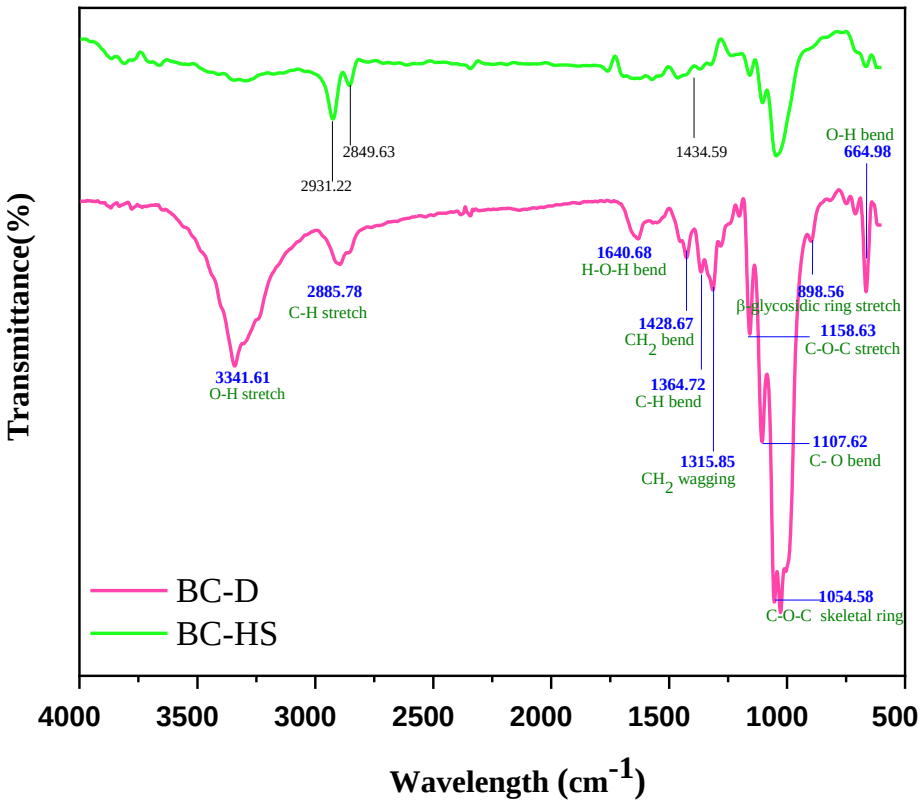


Figure 4.18 FTIR spectra of BC produced from distillery effluent and HS medium

The peak at 2985.78 cm^{-1} was attributed to ($-CH_3$) and ($\succ CH_2$) functional groups displaying symmetric or asymmetric stretching vibrations (γ) (Wang et al. 2018). The peak at 1640.68 cm^{-1} was assigned to an adsorbed water molecule (H_2O) that displayed bending vibration (δ) (Wang et al. 2018; Dubey et al. 2017). The peak at 1428.67 cm^{-1} is one of the main principal peaks of cellulose Iα type, which is associated with symmetric ($-CH_2$) bending vibrations (δ) (Thorat et al. 2018). The peak at 1364.72 cm^{-1} represented C-H bending vibration (δ) (Oh et al. 2005). The peak at 1315.85 cm^{-1} was assigned to CH_2 functional groups wagging (out-of-plane) (Colom et al. 2002). Weak peaks of C-H bending vibrations (δ) were detected at 1281.68 cm^{-1} and 1203.06 cm^{-1} (Singhsa et al. 2017).

The peak at 1158.63 cm^{-1} inferred stretching vibration (γ) C-O-C bond of 1, 4 - β glycoside pyranose ring (asymmetric) (Shao 2015). The peak at 1107.62 cm^{-1} attributed to C-O bending vibration (δ) or C-C bonds present in the monomer of a polysaccharide. The peak at 1054.58 cm^{-1} was assigned to C–O–C pyranose ring skeletal vibration. The peak at 898.56 cm^{-1} represented asymmetric out-of-phase ring stretching vibration (γ) of glycosidic linkages of the polymer or angular bond deformation (Shao 2015). The peak at wavenumber below 664 cm^{-1} was associated with O-H bending (out-of-phase) (Wang et al. 2018). There were two other peaks observed in cellulose synthesised from HS medium at a narrow, sharp peak at 2931.22 cm^{-1} and 2849.63 cm^{-1} inferring symmetric stretching vibrations(γ) of (–CH) functional groups and broad peak at 1434.59 cm^{-1} indicating($-CH_2$) bending vibrations(δ) (Gopu and Govindan 2018). Rest peaks were similar in both the spectra. This study confirmed that cellulose produced from distillery effluent displayed similar structure as that of cellulose obtained from HS medium.

Table 4.9 Comparison of BC peaks from Standard medium (HS) and distillery medium (D)

BC-D peaks	BC-HS peaks	Assignment	Reference/s
3341.61 cm^{-1}	3406.9 cm^{-1}	O-H stretching (O3H---O5) (O6H---O3)	Wu and Liu (2012); Thorat and Dastager (2018); Coates (2006)
2985.78 cm^{-1}	2930.1 cm^{-1}	C-H stretching vibrations	Wang et al.(2018)
1640.68 cm^{-1}	1660.87 cm^{-1}	H_2O absorbed	Wang et al. (2018); Dubey et al. (2017)
1428.67 cm^{-1}	1439.39 cm^{-1}	CH_2 bending vibrations	Thorat and Dastager (2018)
1364.72 cm^{-1}	1379.33 cm^{-1}	C-H bending vibrations	Oh et al. (2005)
1315.85 cm^{-1}	1328.47 cm^{-1}	CH_2 functional groups wagging (out-of-plane)	Colom and Carrillo (2002)
1158.63 cm^{-1}	1167.83 cm^{-1}	C-O-C stretching glycoside pyranose ring (asymmetric)	Shao et al. (2015)
1107.62 cm^{-1}		C-O bending vibration (δ) or C-C bonds present in the monomer	Wang et al. (2018)
1054.58 cm^{-1}	1026.55cm^{-1}	C-O-C pyranose skeletal ring	Wang et al. (2018)
898.56 cm^{-1}	852.12 cm^{-1}	out-of-phase ring stretching vibration (γ) of glycosidic linkages	Wang et al. (2018)
664 cm^{-1}	652 cm^{-1}	O-H bending(out-of-phase)	Wang et al. (2018)

4.2.2.3 Scanning electron microscopy (SEM)

Scanning electron micrographs provided details on the microfibrillar arrangement and fibrillar properties of BC produced from the effluent. Figure 4.19 shows the SEM image of BC from HS medium and the BC from distillery effluent. 100 fibrils were randomly measured using ImageJ software, and fibre diameter distribution was plotted. Fig.4.19a and 4.19b represent the BC fibrils produced from standard HS medium and dried by hot air oven drying method, exhibited dense fibre distribution and less porosity, with the fibres ranging from 14 to 70 nm (Gopu and Govindan 2018) and average fibre diameter 30nm. Fig.4.19c and 4.19d show the SEM images of same BC fibres obtained from HS medium but dried using vacuum freeze-drying method, fibres were loosely entangled, and diameters of the fibres ranged from 20-180 nm. The average fibre diameter was found to be 33 nm. Fig.4.19e and 4.19f depict the SEM image of BC produced using distillery effluent as the medium at a magnification of 20 kX, thin fibrillar network with more porosity. The diameter of fibres ranged from 19-195 nm, with the average fiber width of 60 nm. It is important to note that the methods of drying the BC influence the morphology of the fibrillar network (Costa et al. 2017). A study by Santos et al. (2015) showed that subjecting the fibres to high temperatures resulted in shortening of inter-fibre distances and affected the porosity (Santos 2015). This could be taken as one of the reasons for more porous fibres (Figure 4.19c and 4.19e), as the latter fibres were freeze-dried in contrast to the former, which was oven dried (Figure 4.19a).

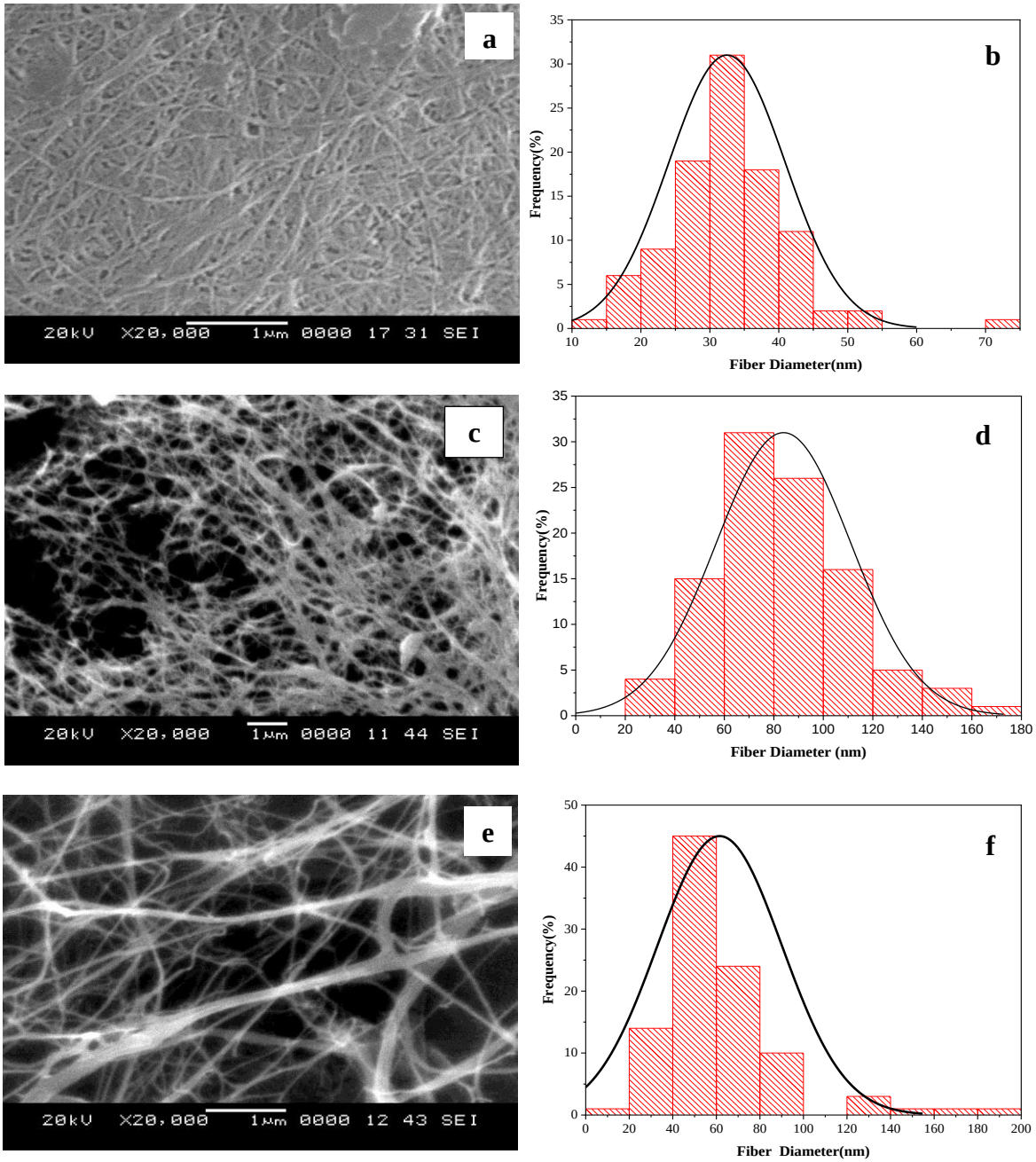


Figure 4.19 SEM micrographs of BC fibrils (a) BC produced from HS medium dried by oven drying method (b) Fiber diameter distribution of oven dried BC produced from HS medium (c) BC produced from HS medium dried by vacuum freeze-drying method (d) Fiber diameter of freeze-dried BC produced from HS medium (e) BC produced from distillery effluent (f) Fiber diameter distribution of BC from distillery effluent.

4.2.2.4 XRD analysis

XRD analysis provided insights on the crystalline and amorphous nature of the biopolymer. The data were used to interpret the value of the crystallinity index and crystallite size of BC. Figure 4.20 illustrates the peaks attributed to crystallographic planes which are represented as Miller indices 14.52(100), 16.48 (010), and 22.78(200) which depicted I_α type; this is the signature of bacterial cellulose. Also, I_β type, as mentioned in the previous section is absent or rare in bacterial derived cellulose (Park et al. 2010). The triclinic structural arrangement of cellulose I_α lies in between parallel chains which interact via intermolecular hydrogen bonding, stabilised over by Van der Waals interaction (Goelzer et al. 2009). The degree of crystallinity plays a significant role in determining the crystalline and amorphous area of a molecule (Auta et al. 2017). Cellulose type I is mostly synthesised by plants, bacteria and algae. This Cellulose is present in form two other allomorphs based degree of crystalline phases, namely Cellulose I_α and Cellulose I_β.

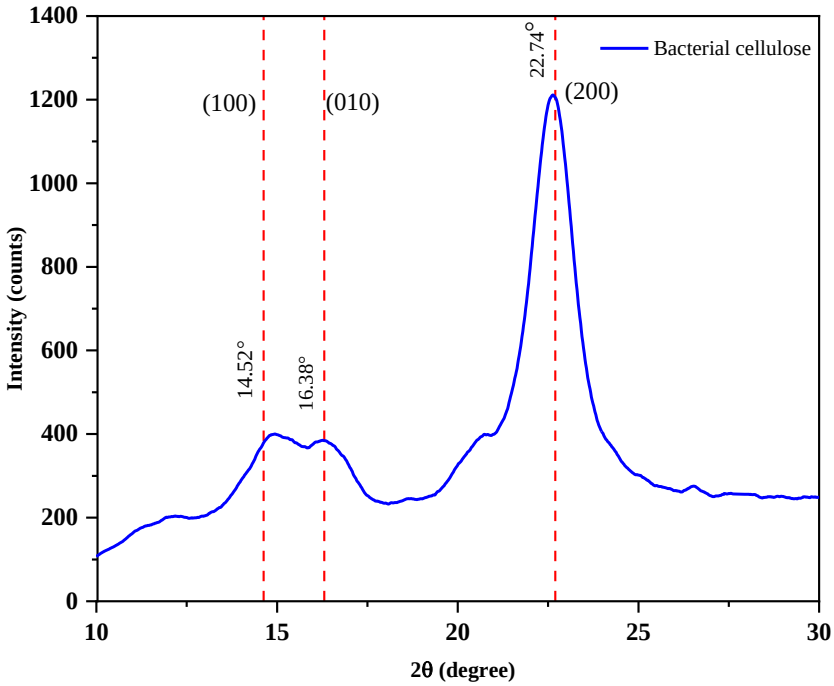


Figure 4.20 XRD profile BC synthesised from effluent wastewaters

These allomorphs exhibit triclinic and monoclinic structural arrangement, respectively (Tsouko et al. 2015). The ratio of celluloses I_α / I_β differs with the origin of cellulose,

with the I_β allomorph is found in angiosperms and gymnosperms of the plant kingdom and I_α allomorph in bacterial and algal-derived cellulose (Poletto et al. 2014). The crystallinity index (CI) in the current context is defined as to the crystallinity ratio in the cellulose polymer (as the macromolecule is made up of both crystalline and amorphous regions). Types of fermentation, the nutritional parameters and the types of drying play a significant role in determining the crystallinity ratio of BC (Poletto et al. 2014). Also, Crystallinity plays a significant role in determining the mechanical property of any macromolecule. Hence it is essential to determine the CI of any substance. In this study, synthesised BC showed a CI of 80.2%. Another parameter studied in XRD is the crystallite size. Cellulose fibres strength increases and their elasticity decreases when the crystalline ratio exceeds more than amorphous regions ratio. CI tends to increase with the increase in the crystallite size, as the crystallite surface area of the amorphous region decrease (Park et al. 2010).

Table 4.10 Crystallinity index, crystallite size and d-spacing of BC produced from distillery effluent

Sample	CI	CrS	2θ	d spacing
Bacterial cellulose	80.2%	8.36 nm	14.52	6.093Å
			16.38	5.41 Å
			22.74	3.94 Å

Hence, the present study confirmed that the polymer synthesised was cellulose and the BC synthesised using distillery effluent as medium produced three significant peaks, at 2θ angles of 14.52, 16.38 and 22.74 and exhibited 80.2% CI with 8.36 nm crystallite size, as presented in Table 4.10. Also, the study infers that the polymer properties were not affected by the media composition. Similar results were observed in the previous studies (Salari et al. 2019; Jozala et al. 2015; Revin et al. 2018).

4.2.2.5 Purity evaluation by TLC

The purity of BC was confirmed by performing thin layer chromatography. The BC lyophilised films were digested in H_2SO_4. Post-acid hydrolysis, the monomer unit glucose along with standard glucose as reference was plotted onto dry TLC plates.

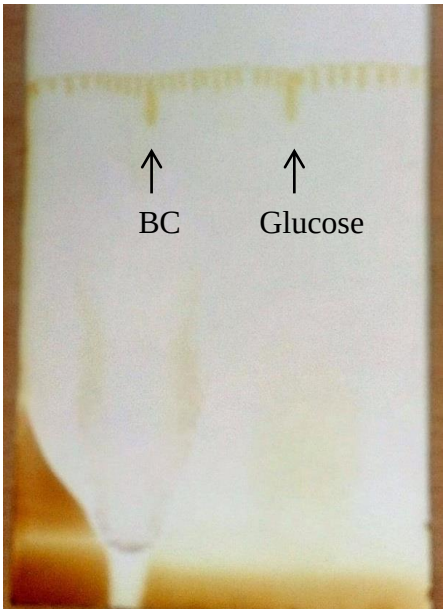


Figure 4.21 TLC plate of hydrolysed BC and standard glucose

Figure 4.21 shows the Rf value for standard glucose was found to be 1.83 and 1.81 for hydrolysed BC. Standard D-Glucose anhydrous purified was procured from Merck chemicals; ≥99.8 % purity. Hence, TLC analysis confirmed that the bacterial cellulose synthesised by the isolate *K.saccharivorans* strain BC1 was a homopolymer of glucose. Mohite and Patil (2014) have performed thin layer chromatography to confirm the purity of BC; the study reported Rf values of 1.9 and 1.81 for hydrolysed BC and standard glucose, respectively. Another study by Çoban and Biyik (2002) performed TLC of hydrolysed cellulose and found the peak corresponding to Glucose monomer. A study by Mugesh et al. (2016) reported TLC analysis of hydrolysed BC products corresponded to Rf values of that of standard cellulose, confirming BC is pure biopolymer made up of monomeric glucose units.

4.2.2.6 ^{13}C Solid-state Nuclear Magnetic Resonance analysis

NMR technique was performed to study the structural framework of BC. Figure 4.22 shows the peaks under C1- C6 carbon atoms. The C1 peak (strong resonance line) was evident at 105 ppm, and the C4 peak at 89.4 ppm as shown in, this indicated the allomorph cellulose Iα was dominant (Keshk and Sameshima 2006). Also, this single resonance peak infers crystalline phase in contrast to the doublets, which are prominent in cellulose Iβ rich allomorphs (Castro et al. 2011; VanderHart and Atalla 1984).

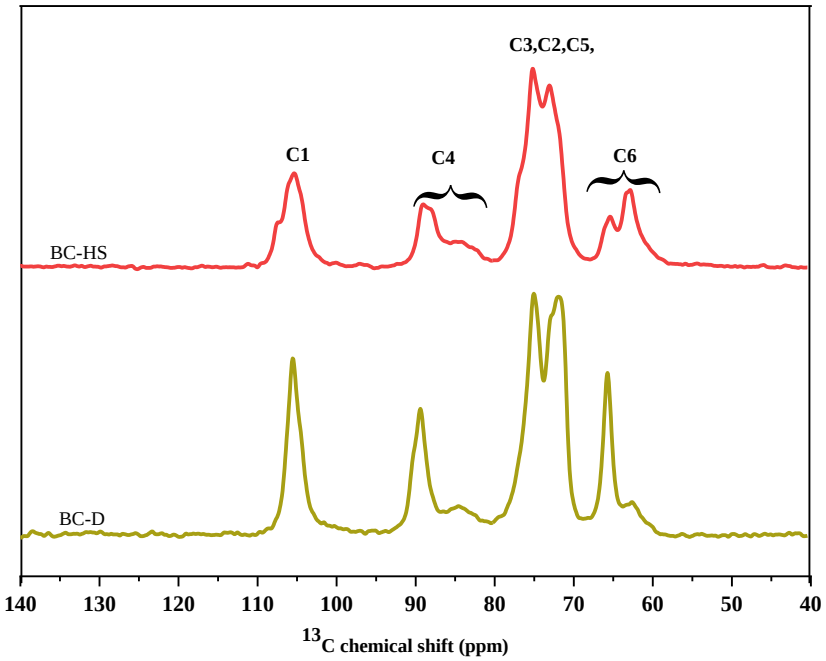


Figure 4.22 CP/MAS ^{13}C NMR spectra of BC

C2, C3 and C5 were grouped under one resonance cluster. From the previous literature studies, it could be inferred that amorphous cellulose regions were characterised by broad resonance peaks found at 84.6 ppm for C4 carbon and 62.57 ppm for C6 carbon (Atalla and VanderHart 2009; Kono et al. 2002). The peak at 71.8 ppm represented C2, and 72.9 represented C5. The peak at 75.04 ppm represented C3 peak. These results were similar to a study conducted by (Hesse-Ertelt et al. 2008; Numata et al. 2019; Meza-Contreras et al. 2018).

The ^{13}C solid NMR spectrum of BC is preferred over liquid state NMR analysis of cellulose is due to the reason that cellulose is insoluble in most of the commonly used NMR solvents. Hence, combinations of solvents or ionic liquids are used is required for liquid NMR spectroscopy of BC. The solvents used are DMAc/LiCl and DMSO/TBAF. ^{13}C CP/MAS solid-state NMR experiment involves the sequence of three steps (i) cross polarisation (ii) magic angle spinning and (iii) high power decoupling (Brown et al. 2014; Schmidt-Rohr and Spiess 2012). Solid-state NMR spectroscopy analysis can provides the chemical information on the atoms of the molecules but also ultrastructural details which are not available easily other non-destructive high-resolution spectral techniques (Fostan 2014). This makes solid-state NMR particularly useful in studying complex structural aspects of biological systems that involve biosynthesis mechanisms of several molecules (Auger 2000).

Some of the earliest studies which employed ^{13}C CP/MAS solid-state NMR are cellulose isolated by VanderHart and Atalla (1984) from various sources. In studies on cellulose isolated from various sources. It was observed that carbon chemical shifts for the C4 and C6 ring positions varied according to the origin of cellulose. A typical ^{13}C CP/MAS NMR spectrum of isolated cellulose showed broad resonances peaks over a range of 102–108, 80–92 and 57–67 ppm attributed to the C1, C4, and C6 signals (modelled as Gaussian or Lorentzian functions), respectively (Yamamoto and Horii 1993).

As we know, Cellulose I_α and I_β are the two types of crystalline cellulose polymorphs. The cellulose I_α (triclinic lattice arrangement) polymorph is typically associated with algae, bacteria, and lower plants, while plants/woods contain mostly cellulose I_α (monoclinic structure). The thermodynamic stability of cellulose I_β over cellulose I_α is evident in the ^{13}C CP/MAS NMR spectra of cellulose. Usually, the C4 peak in the carbon spectrum of cellulose is significant as it is used to extract primary structural information such as crystallinity percentage.

Broader bands under C4 around 80–85 ppm represented amorphous regions, while sharp resonance peaks from 85-92 represented crystalline regions (Foston 2014). Solid state ^{13}C CP/MAS NMR technique has been proven as an excellent analytical

technique for structural elucidation of other forms of cellulose such as *para* - crystalline cellulose and other non-crystalline forms such as amorphous cellulose at accessible and inaccessible lattice surfaces (Wickholm et al. 1998).

Summary

The results of the study have brought out the possibility of using crude distillery effluent for BC production. The characterisation techniques revealed that BC synthesised from the crude distillery was similar in both morphological and chemical aspects to that of BC produced from HS medium. Thus, BC, a useful polymer which was obtained from wastewater as medium with good properties can be suitable for various applications.

PART THREE

4.3 Ibuprofen as model drug for BC application

Non-steroidal anti-inflammatory drugs (NSAIDs) are a category of drugs which are largely prescribed all over the world. Ibuprofen is a drug under this category, which is widely used for inflammation, to relieve pain and fever. However, due to its vulnerability to short half-life and side effects of gastric irritations along with the need to provide and maintain drug levels for extended duration of time without any side effects, topical administration of ibuprofen is considered as a suitable option (Bushra and Aslam 2010).

Ibuprofen [2-(4-isobutylphenyl) propionic acid], a potent drug used generally for the treatment of acute and chronic arthritic diseases. This drug has pH dependent solubility and permeability property. Although ibuprofen has permeability through the stomach tissues and cells, its poor water solubility restricts its entry into blood and plasma circulation before elimination of the drug from the intestine. Ibuprofen enters the small intestine, but remains impermeable to intestinal membranes and excreted without incomplete absorption. Since the solubility of the drug is the rate-limiting step during the process of drug absorption and the poor water solubility property causes lesser bioavailability of the drug. In addition to absorption difficulties, as mentioned earlier oral formulations of ibuprofen triggers gastric mucosal damage, leading ulceration and bleeding of gastric lining damage (Aukunuru et al. 2011).

Hence topical drug delivery method is adapted as this method provides ease of drug delivery, less immunogenicity, avoidance of liver metabolism pathway, avoids gastro irritations, limits the metabolic method of degradation of drug, the larger surface area of exposure, and delivery of a large amount of drug over longer durations. These advantages makes the topical method better over oral administration (Lakshmi et al. 2011; Hu et al. 2014). Also, several studies have proven that topical administration of the drug ibuprofen has been equally effective as that of the orally administered ibuprofen and superior by providing an instant result. Studies have also shown gel formulation of the drug as a better vehicle for topical delivery over cream and ointment based formulations (Rainsford 2012; Herkenne et al. 2007).

Therefore, this part of the work describes *in vitro* study of BC loaded Ibuprofen sodium salt (IbuNa) as a model drug highlighting various aspects such as drug loading and release potential, antimicrobial and cytotoxicity study.

4.3.1 Swelling rate of BC

The swelling behaviour of lyophilised BC film was studied in distilled water at room temperature and was measured gravimetrically. Figure 4.23 shows that BC showed 100% swelling rate, within about 60 min and the swelling ability increased up to 600 % with increase in time. The pellicle exhibited the highest swelling rate of 616.55±15.01 % after 8 hours of water absorption. The water uptake by the polymeric matrix varies depending on the morphology, pore sizes, fibrillar cross-section. At the initial stage, they rapidly absorb water molecules in the hydrophilic regions. As the hydrophilic regions attain saturation, exchange of free water molecules occurs with external water molecules (Hoffman 2002). Water molecules remain entrapped on the BC surface and in between the BC fibrillar network. The highly porous and hydrophilicity nature exhibited by BC permits a faster absorption of the water molecules.

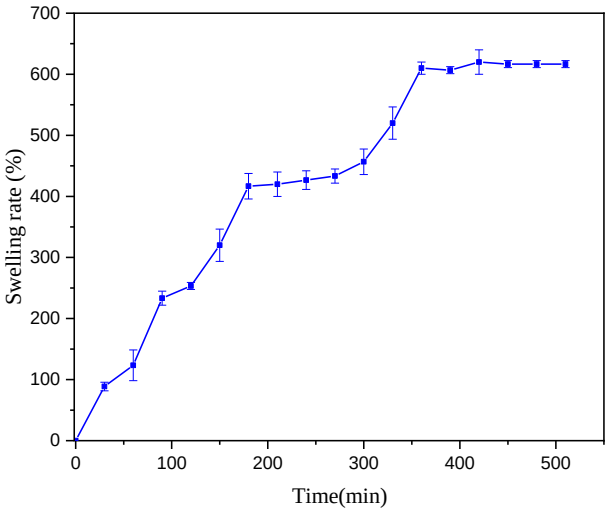


Figure 4.23 Swelling rate of BC in deionised water

The results of the present study are consistent with the reported values in the literature. A study by Mohite et al. (2014) also reported on the swelling behaviour of BC up to 600% over 6 hours. A study by Treesuppharat et al. (2017) showed that BC-gelatin hydrogel composites showed a 400-600% swelling rate after 48 hours of the period.

4.3.2 Preparation of ibuprofen-loaded BC membrane

The BC membrane (0.1g) was loaded with ibuprofen sodium salt (IbuNa) of various concentrations (0.2%, 0.4%, 0.8%, 1%, 5% and 10%) in phosphate buffer solutions. The mass of the pellicle was weighed before and after incorporation of ibuprofen-loaded membranes and further was measured by UV–Visible spectroscopy at 221 nm.

4.3.2.1 Drug loading efficiency and drug loading capacity

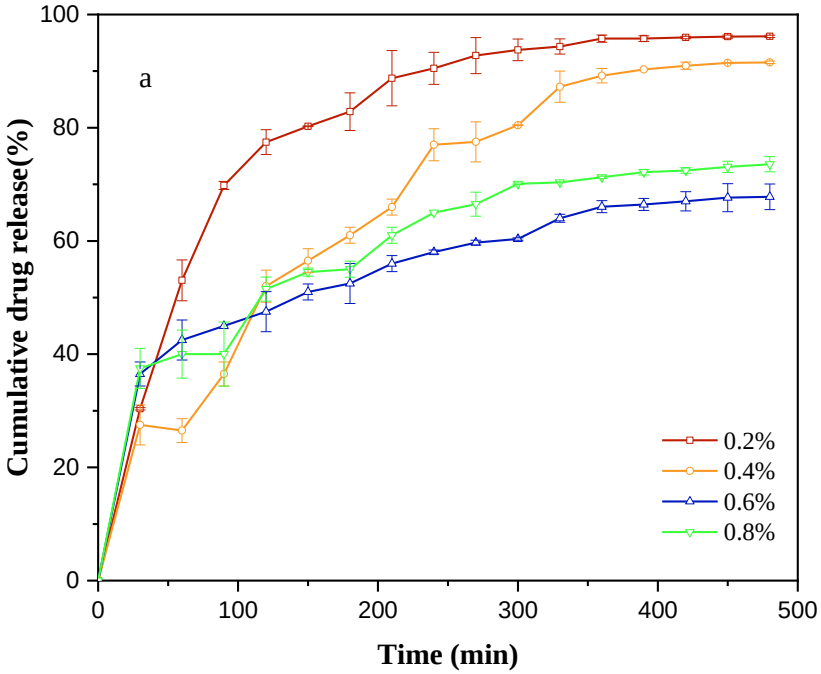
Table 4.11 shows the drug loading capacities and encapsulation efficiencies of BC increased with the increase in drug concentration. The drug loading capacity depends on the porous structure of the loading material, and the subsequent release depends upon the diffusion coefficient of the molecule. The maximum encapsulation efficiency of 99.64% was observed when the concentration of drug was 10mg/mL. The difference in the initial and final concentration of the drug showed the entrapment behaviour of BC. Hence, it was confirmed that Bacterial Cellulose was capable of absorbing drug molecules within the membrane.

Table 4.11 Percentage of IbuNa encapsulation and loading into BC matrix

Sample(mg/mL)	Encapsulation efficiency (%)	Loading Capacity (%)
0.2	80.45	12.94
0.4	87.27	14.32
0.6	90.43	19.82
0.8	91.65	19.81
1	93.93	25.31
5	98.42	34.31
10	99.64	38.22

4.3.2.2 Release of IbuNa from BC membrane

Drug release from BC matrices is dependent on various factors such as the affinity of the drug molecules to glucan chains, the solubility of the drug in a suitable solvent and swelling behaviour of BC (Brazel and Peppas 1999; Hoffman 2002). Figure 4.24 presents the experimental results of the elution of model drug ibuprofen at different concentrations from BC. For a drug molecule to show prolonged drug release, stable and good support is required. IbuNa was released gradually from BC up to 90% within 8 hours for drug concentrations 0.2-0.8%, but the release lowered down to 80% for higher drug concentrations, indicating that at higher drug concentrations has the release time considerably reduced.



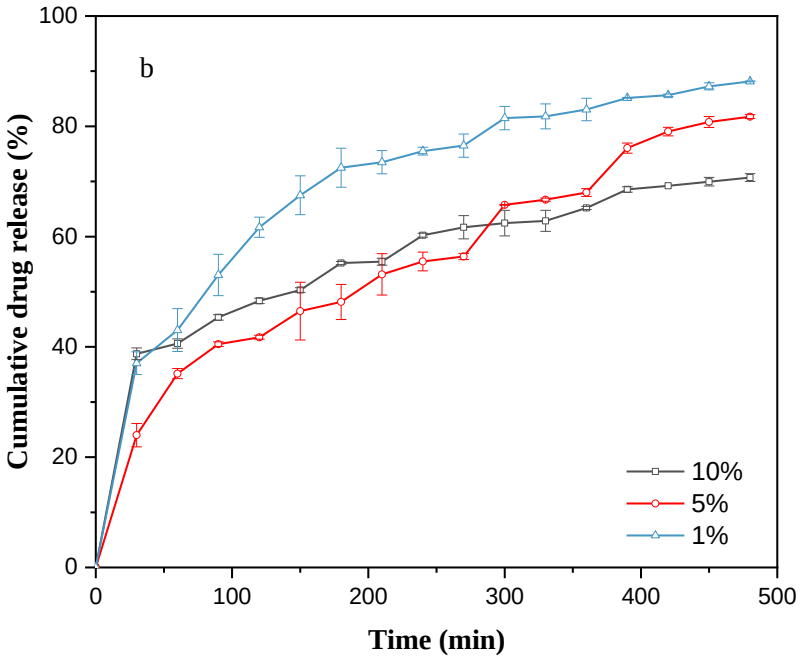


Figure 4.24 (a) Cumulative drug release of concentrations from 0.2 - 0.8% (b) concentrations (1, 5 and 10%)

The BC-IbuNa samples of 0.1g were immersed in a beaker containing 10mL of freshly prepared phosphate buffer solution and were maintained in shaking condition for maximum release of the drug (pH 7.4). Furthermore, all drug release profiles of the BC-IbuNa exhibited a small burst in drug release in the initially 1 hour and then slowed release at the constant rate was observed. This burst effect could be likely due to the presence of ibuprofen molecules on the surface of BC films (Harting et al. 2019). The rate of release of IbuNa from the BC films was higher during the initial 200 minutes, followed by the immersion of the drug-loaded membranes/films in the release medium. The difference between the concentrations gradient of the release medium and BC-IbuNa membranes during the initial stage could be considered as the driving force for the drug release (Bruschi 2015; Treesuppharat et al. 2017; Mohd Amin et al. 2012). At a later stage, the release rates decreased because of the gradual diffusion of the drug from the BC matrices. The slowed release could also be attributed to the porous microfibrillar network and large surface area of BC, which allowed gradual release of the drug (Wei et al. 2011). It was observed that the BC-IbuNa films took around 8 hours to release the absorbed drug. These results suggest that it is possible to control the release rate of IbuNa by varying the ibuprofen sodium

salt concentrations and hence could be used as a potential carrier for drug delivery applications.

4.3.3 Antibacterial activity using drug loaded BC discs

Although Ibuprofen is predominantly used as an antinflammatory drug, but for the current study drug entrapment and release property of BC discs were visually evaluated by performing antimicrobial test. This was performed by examining the drug-loaded BC to show resistance against microbial growth. Figure 4.25 shows that 10% of Ibuprofen loaded BC films showed bacterial resistance against two strains, namely *Escherichia coli* and *Staphylococcus aureus*. The antimicrobial activity was measured based on the diameter of zone of inhibition. Amongst the two strains, *S.aureus* displayed higher activity of 10.2 mm when compared to *E.coli* of only 4mm. There was no antimicrobial activity observed on control plates having only BC discs. This further indicated that the inhibition zone was attributed to the presence of drug ibuprofen, which was incorporated into the discs. Hence, the results of the study concludes that BC displays good drug entrapment and release potential that could be visually observed (by formation of zone of inhibition).

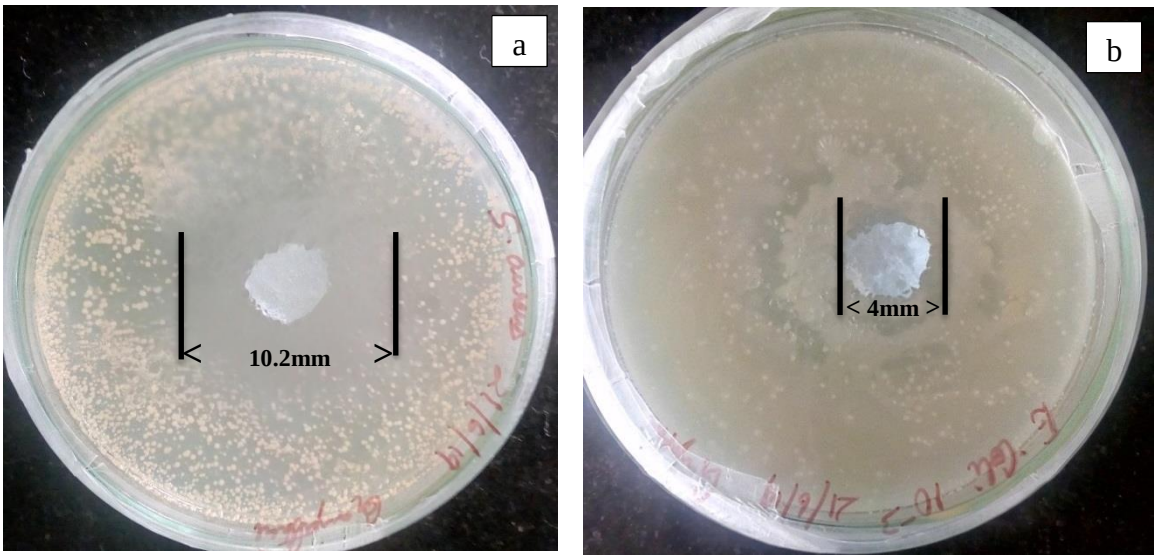


Figure 4.25 Antibacterial activity of BC disc loaded with ibuprofen (10%) concentration against two bacterial strains a) *Staphylococcus aureus* and b) *Escherichia coli*.

Although BC by itself does not show any activity against microbial growth but with the incorporation of ibuprofen into BC film showed potential antimicrobial activity against both gram-negative and positive strain, further inferring that the drug was well entrapped into the BC discs. Several researchers have evaluated the drug release potential of BC by performing antimicrobial assay (Mohite et al. 2014, Portela et al. 2019).

4.3.4 *In vitro* cytotoxicity studies of Bacterial Cellulose films

The cytotoxicity assay was performed to determine the therapeutic potential of BC. The capability of cells to survive in toxic environment forms the basis of cytotoxic assays. From figure 4.26, it could be concluded that BC film was non-cytotoxic. The cytotoxicity of BC films incorporated with Ibuprofen sodium salt (0.2%, 5% and 10%) was evaluated by MTT assay using Human small cell lung carcinoma A-549 cell lines. The cell viability was compared with BC film as control. 10% of BC-IbuNa showed the maximum IC50 value of 210 µg/mL and 0.2% showed the least value of 85µg/mL. In this assay, the measured absorbance was proportional to the viable cell number and inversely to the degree of cytotoxicity. Even at the highest concentration of 10%, almost 75% cell viability was observed.

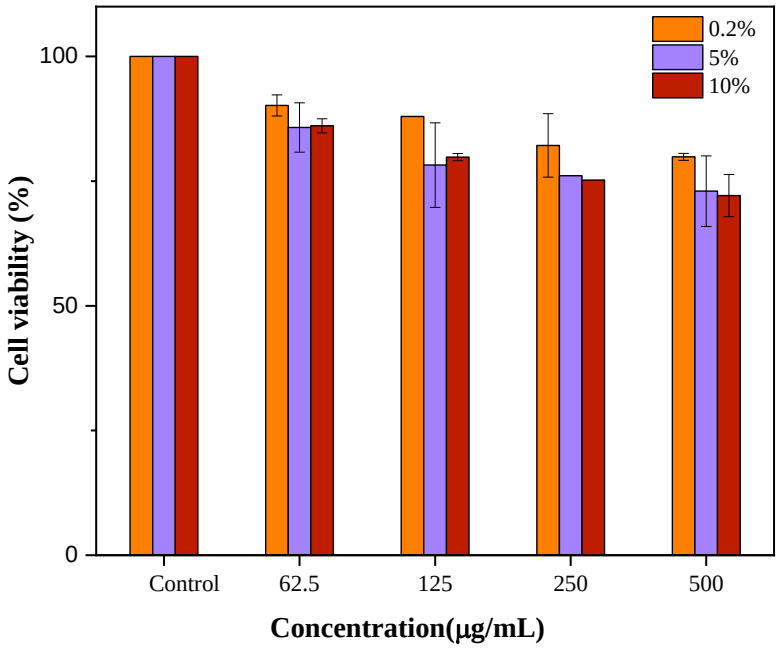


Figure 4.26 Effect of BC-IbuNa on cell viability percentage of A549 cells

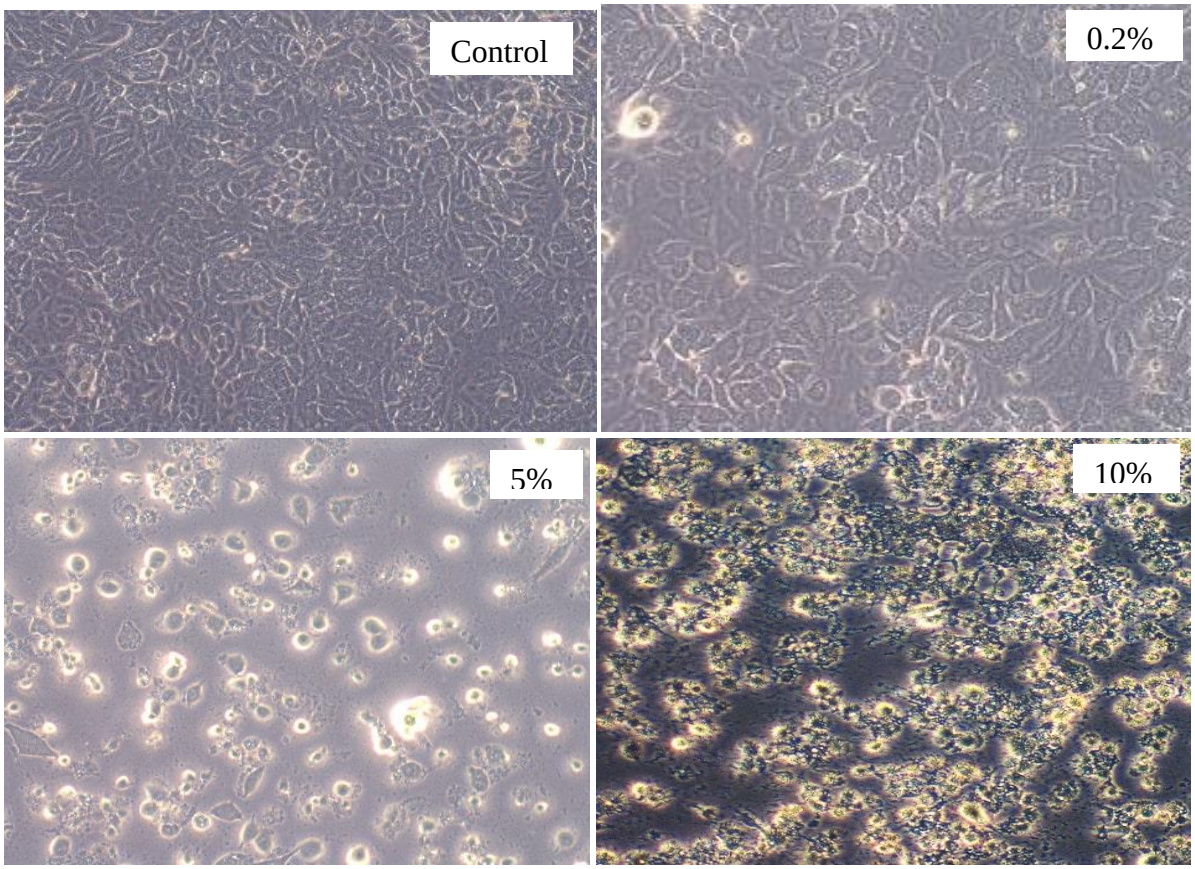


Figure 4.27 Cytotoxicity effect of BC-IbuNa at different concentrations (Control, 0.2%, 5% and 10%) on A549 cells

Figure 4.27 shows cytotoxicity effect at various concentrations; the purple coloured cells indicate viable cells, hence it could be concluded that at higher drug concentrations, lesser number of cells were viable than control. This is indicated by decrease in the number of purple coloured cells (5&10%) when the cells die, they lose the ability to convert MTT to formazan, thus purple colour is not produced (Stockert et al. 2012). The IC50 value is the half-maximal inhibitory concentration which is widely used to measure the efficacy of a drug. It gives the minimum quantity of the drug which is required to how much drug is needed to inhibit a biological process by half, thus providing a measure of the potency of a drug in pharmacological research (Aykul and Martinez-Hackert 2016). A study by Sabine Schnell et al. (2008) showed ibuprofen concentration of 150ug/mL inhibited cell proliferation. A study by Nguyen et al. (2018) showed cell viability of 75% with the concentration of 500mg/mL of ibuprofen-poly vinyl alcohol nanocomposites. Trovatti et al. (2012) evaluated the potential BC membrane for *in vitro* diffusion of lidocaine and ibuprofen drugs. The

study reported that permeation of ibuprofen (5% w/w) in the BC membrane was three times higher than other formulations. Hence the present study confirmed the non-toxic, biocompatible nature of Bacterial Cellulose along with good dissolution capacity of ibuprofen drug from the polymer.

4.3.5 Characterisation of BC-ibuprofen films

4.3.5.1 FTIR analysis

FTIR analysis depicted the structural characterisation of BC pellicle, Ibuprofen sodium salt (IbuNa) and BC-ibuprofen sodium salt. Figure 4.28 shows that the BC showed peaks at 3353.47cm^{-1} indicating O-H stretching, and IbuNa showed a sharp peak at 3363.66 $cm^{-1,}$ and BC-ibuprofen showed a peak at 3337.15 cm^{-1} indicating the presence of O-H stretching vibrations (Coates 2006). The peak at 2943.46 cm^{-1} and 2904.70 cm^{-1} indicated the presence of strong CH_3 asymmetric stretching vibrations. Ibuprofen sodium salt showed a sharp peak at 1552.10 cm^{-1} and similarly BC-ibuprofen film depicted a peak at 1556.38cm^{-1}, these peaks indicated the presence of aromatic C=C structure (Acharya et al. 2017). Ibuprofen sodium salt showed another distinct peak at 1421.35 $cm^{-1,}$ which indicated CH-CO deformation, but this peak was absent in both BC and BC-ibuprofen composite film (Horcajada et al. 2006). BC ibuprofen showed a peak at 1332 $cm^{-1,}$ which indicated C-H bending vibrations; this peak was absent in both ibuprofen sodium salt and BC. Another prominent peak was observed at 1032.15 cm^{-1} for BC-ibuprofen, 1036.23 cm^{-1} for BC and 1050.51cm^{-1} for ibuprofen. These peaks indicated cyclohexane ring vibrations. Ibuprofen showed another distinct peak at 725.14 cm^{-1} indicating C-H out of plane bending vibrations (Acharya et al. 2017).

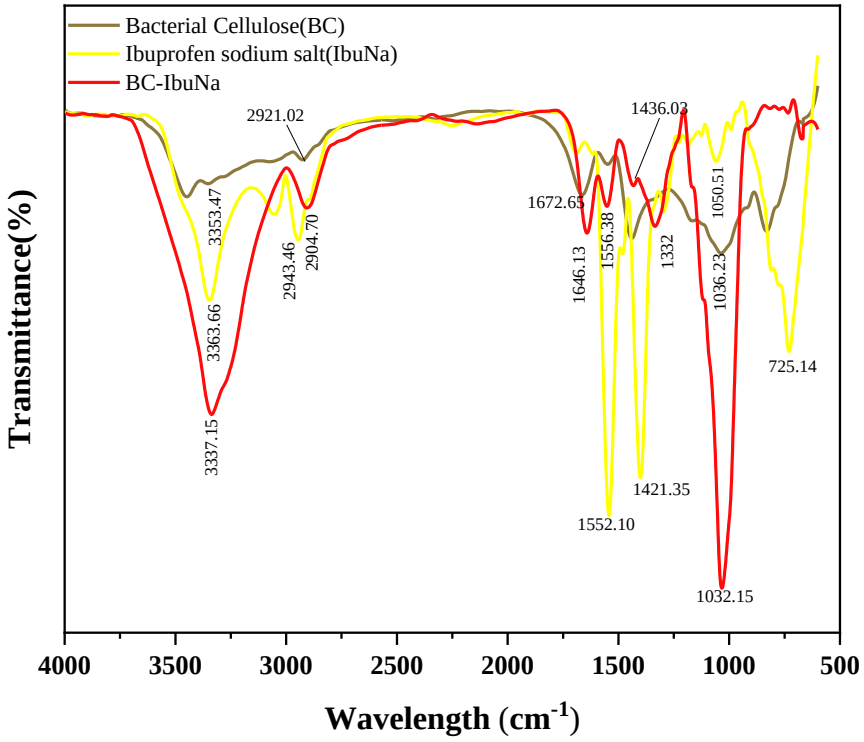


Figure 4.28 FTIR spectra of BC, IbuNa and BC-IbuNa

Table 4.12 Assignments of various peaks of BC, ibuprofen and BC-ibuprofen

BC peaks	IbuNa peaks	BC-IbuNa peaks	Assignments	Reference/s
3353.47 cm^{-1}	3363.66cm^{-1}	3337.15cm^{-1}	O-H stretching	Coates (2006)
2921.02 cm^{-1}	2943.46cm^{-1}	2904.70 cm^{-1}	CH_3 stretching	Coates (2006)
1672.65cm^{-1}		1646.13cm^{-1}	Carboxylate stretching	Coates (2006)
	1552.10cm^{-1}	1556.38cm^{-1}	Aromatic C=C structure	Acharya et al. (2017)
	1421.35 cm^{-1}		CH-CO deformations	Acharya et al. (2017)
	1332.0 cm^{-1}		C-H bending	Coates (2006)
1036.23cm^{-1}	1050.51cm^{-1}	1032.15cm^{-1}	Cyclohexane vibrations, C-O stretching	Coates (2006)
	725.14 cm^{-1}		C-H out of plane bending	Acharya et al. (2017)

4.3.5.2 SEM analysis

The BC-ibuprofen loaded membranes were further studied for their morphological features by SEM (Figure 4.29). The SEM images displayed a good distribution of ibuprofen molecules on the BC film surface. In pure BC membrane, the fibrils appeared loosely bound with large interfibrillar spaces (Figure 4.29c), and ibuprofen molecules appear to be randomly dispersed all over the dried membrane. The surface modified BC after drug loading (Figure 4.29d) showed tightly bound microfibrils with pore sizes between the fibres reduced. The micrographs of the BC-ibuprofen also showed dense coagulated appearance on the matrix with drug molecules bound to the surface. These surfaces of the micrographs (Figure 4.29 b and d) indicated successful loading of model drug ibuprofen onto the BC matrices.

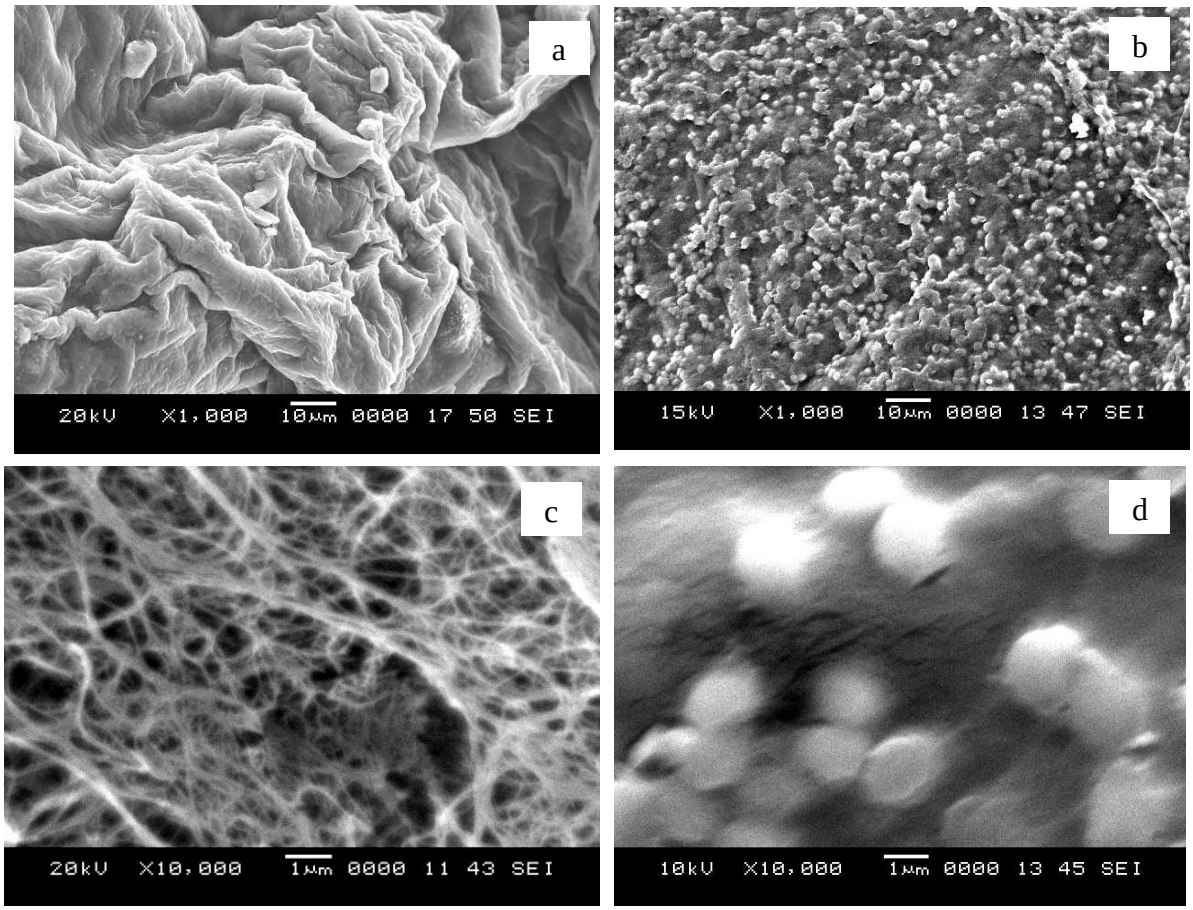


Figure 4.29 SEM images of BC fibres (a) 1000X magnification (b) Ibuprofen molecules dispersed on the BC membranes (c) and (d) BC fibres at higher magnification 10kX

4.3.5.3 XRD analysis

X-ray diffractogram of the BC and drug-loaded BC membranes have been shown in Fig. 4. The scanning angle was carried out from 10° to 50°. BC showed prominent and sharp peaks at 14.46°, 16.38° and 22.87° depicted the crystalline structure of Bacterial Cellulose (Park et al. 2010). The XRD pattern for BC-ibuprofen matrix showed a weak peak at 11.50°, and the peak at 16.35° was absent. Several short peaks were observed at 28.90°, 30.86° and 34.49°, which indicated the entrapment of drug in the BC membrane. Hence the study concluded that the BC surface modified with ibuprofen sodium salt showed similar XRD pattern as that of the produced BC but with lower intensity, indicating reduction in the crystallinity due to the entrapment of drug molecules.

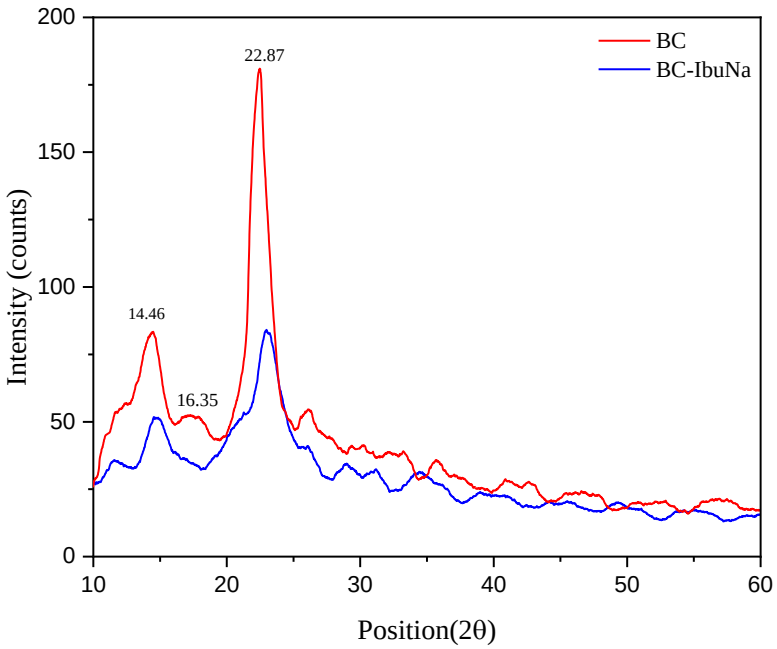


Figure 4.30 XRD profiles of BC and BC-IbuNa

Summary

This chapter describes the application of Bacterial Cellulose using Ibuprofen sodium salt (IbuNa) as a model drug. The study explored the potential properties such as swelling rates, drug absorption and release abilities of BC. The results suggested that IbuNa could be successfully entrapped into the BC matrices, retained and slowly released over some time. This finding suggests that BC films could be possibly used

for topical administration of drugs that requires continuous drug release at frequent intervals over a long periods. The results were further confirmed by visually examing the drug release activity BC films by performing antibacterial assay against both gram negative and positive bacterial strains. The *in vitro* cytotoxicity assay suggested that the films were non-toxic to cells. Therefore, the study concludes that a synthesised polymer such as BC has potential to act as a drug carrier and aid in studies related to drug delivery.

CHAPTER 5

SUMMARY AND CONCLUSIONS

CHAPTER 5

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Water is one of the most crucial elements for the existence of life on earth. Though renewable resource, only less than 1% of earth's freshwater is available to us. Water pollution has emerged as one of the widespread concern around the globe. Not only the water supply is rapidly deteriorating, but the quantity of potable safe drinking water is also diminishing. The toxic chemicals or pollutants, which either remain dissolved or suspended in the water, cause detrimental effects to aquatic life and human health. Therefore, several wastewater treatments have been adopted to remove the contaminants from the wastewater and make the water safe for reuse or recycle for purposes like irrigation, fire protection, industrial washings etc. Some of these industrial effluents are discharged into the environments.

The Central Pollution Control Board (CPCB) reported that only around 60% of the industrial wastewater generated from large scale industries is treated. In the majority of cases, the untreated wastewater which is let out either percolates into the ground and forms the potential pollutant of groundwater or is discharged into water bodies leading to the pollution along with the deterioration of water quality. Discharge of wastewater has led to 75% contamination of water bodies across India. Hence, CPCB has taken initiatives and implemented technologies which to remove the solid contaminants and organic loads up to a certain extent. Since the wastewater generated from industries contain huge amount of organic load, several wastewater treatment methods have been adopted such as primary, secondary and tertiary treatments along with processes like sedimentation, floatation, adsorption, filtration, coagulation, flocculation, ion exchange, disinfection, aerobic and anaerobic treatments. However, these wastewater treatments face several problems such as low sludge removal efficiency, energy utilisation, improper functioning of treatment plants due to poor maintenance, frequent electricity breakdowns, lack of technical manpower and no economic returns. Thus, new methods such as zero effluent discharge recycle and reuse the wastewater as resource offer new perspectives to utilise the waste as raw

materials and produce value-added products. The wastewater generated from the textile, paper and pulp, sugar, brewery and beverage industries contains a high organic load. These high amounts of organic load can be utilised as a nutritional source for the microorganisms. Literature has shown several studies carried out on the production of Bacterial Cellulose, a biopolymer which is extracellularly synthesised by certain species of bacteria. But there are few limitations in the production process such as low yield of BC, characterisation methods and identification of suitable fermentation medium. Usage of commercial media on a large scale leads to increased production costs. Therefore, there is a need to identify a suitable effluent source with high carbon source and possible microorganisms which can thrive on the medium and produce BC. The study was designed to use the distillery wastewater as a nutrient medium for microorganisms and to produce a valuable product from them.

5.1 Summary of the present work

The research work carried out explores the potential of a novel bacterial isolate *Komagataeibacter saccharivorans* BC1 in production of Bacterial cellulose both in standard and wastewater as a medium and suitable application of the biopolymer.

- *Komagataeibacter saccharivorans* BC1 (MF797958.1) strain was isolated and evaluated for BC production in standard HS medium and optimised for different parameters such as carbon source, nitrogen source, pH, temperature and inoculum size.
- Characterisation of BC using various analytical techniques such as FTIR, SEM, and TGA revealed morphological, chemical and thermal stability features of cellulose
- The strain was further evaluated for its ability to grow on cost-effective and cheap industrial wastewater medium (distillery effluent) and produce BC.
- The yield of BC grown in the distillery medium was compared with that of BC synthesised in HS medium along with BOD and COD values. Although the yield was a low, significant reduction in both BOD and COD values were observed over a week.

- Characterisation of BC fibres obtained from distillery effluent showed higher porosity with thin fibrillar structure. The ATR-FTIR spectra of both the former and latter BC pellicle depicted similar functional groups. ^{13}C NMR spectra provided more insights on the structural framework and positioning of the 6 carbons of cellulose. TLC analysis proved that hydrolysed BC was made up of glucose monomers.
- The BC produced was further investigated for drug absorption and release potential using ibuprofen sodium salt (IbuNa) as a model drug, and cumulative drug release was calculated. *In vitro* biocompatibility studies BC membranes performed by MTT assay using Human small cell lung carcinoma cell lines (A549).

5.2 Significant findings

- A gram-negative strain isolated from rotten green grapes, *Komagataeibacter saccharivorans* BC1, was characterised both by biochemical and molecular methods. The strain was identified as a novel cellulose producing strain as no research work has been carried on this particular isolate so far.
- Optimisations of BC production parameters in HS medium were comparable to the previous literature studies with respect to the pH and temperature. The maximum yield of 2.01g/100mL was obtained at pH 5 and the temperature of 30°C using mannitol as the best carbon source and yeast extract as the best nitrogen source.
- The yield of BC obtained from distillery medium was 1.24g/L, with 23.6% reduction in value COD and 11.9% in BOD value was recorded over 8 days of the fermentation period.
- SEM analysis revealed that BC fibers had higher porosity with an average fiber diameter than BC obtained from HS medium. XRD method was used to determine the crystallinity index and crystallite size of the sample. The sample had crystallinity index of 80.2% and crystallite size of 8.36 nm. This indicated highly crystalline nature cellulose which is comparable to that of the commercially available microcrystalline cellulose.

- IbuNa loaded BC films showed 99.64% encapsulation efficiency for 10mg/mL IbuNa concentration and maximum loading capacity of 38.22%. IbuNa was released gradually from BC up to 90% within 8 hours for drug concentrations 0.2-0.8%, but the release lowered down to 80% for higher drug concentrations, indicating at higher drug concentrations slowed release time. The slower release time indicated better and longer durations of drug retained in the BC films, which can be useful for several drug-related applications.
- *In vitro* cytotoxicity study at 10%, drug concentration showed 75% cell viability and the IC50 value of 210 µg/mL. The cytotoxicity property of drug-loaded BC has been comparable to the previous literature studies.

5.3 Conclusions

The demand for industries is rapidly increasing with the growth of population. Industries like sugar, distilleries, pulp and paper use an enormous amount of water for various process, hence generates wastewaters with high organic carbon. Although prevention is better than cure, it feels there is still a need for industries to develop better technologies to treat wastewater containing high amounts of BOD. The conventional methods of wastewater treatment involve oxidation of organic carbon, wherein a media transfer occurs mainly from a liquid solution to solid solution, which is not acceptable today. Technologies, where wealth can be generated from the waste materials, have been developed and transferred from lab to industrial scale. The present study is one such initiative to utilise organic carbon to obtain Bacterial Cellulose. A novel microbial strain was isolated and grown both in synthetic as well as wastewater medium. The growth of the microorganism was significant, and the parameters were optimised. The characteristics of the BC obtained were comparable with commercial cellulose, and this technology can be a suitable substitute for the existing one as this has economic importance, and the process is also an eco-friendly one.

The Bacterial Cellulose obtained in the present study has also been tested for its drug entrapment, release rate, *in vitro* cytotoxicity property. The results are encouraging to get the BC synthesised in an economical way which can be applied for beneficial

purposes. So the current research work presents a rational way of treatment of wastewater to reduce organic load and at the same time, obtain a value-added product ensuring the global requirement of sustainable development. Thus, a cheap and cost effective approach to reuse the waste to produce a value added product.

5.4 Scope for future work

- Optimisation and scaling up of Bacterial Cellulose production in fermenters using distillery effluent as the production medium
- Developing BC polymer composites/hydrogel and study its properties and application of the same.
- To carry out gene modification and study the overexpression of cellulose synthase gene to enhance BC production by the strain.

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APPENDICES

APPENDIX I

STANDARD CURVE OF IBUPROFEN

Standard curve of Ibuprofen was prepared using commercially available Ibuprofen sodium salt dissolved in Phosphate Buffer (pH 7.4) (0.1M). 100mg of Ibuprofen sodium salt was weighed and transferred to 100 ml volumetric flask containing phosphate buffer. The final volume was made up to 100 ml with the phosphate buffer to obtain a stock solution. This was further diluted to obtain concentrations from 5-25 µg/mL. The absorbance was recorded at the wavelength of 221 nm using UV-Visible spectrophotometer (LabIndia UV 3000) against phosphate buffer as blank solution. The standard curve was plotted using absorbance verses concentration and a slope was obtained. The amount of ibuprofen released at required time intervals was determined by extrapolating the standard curve.

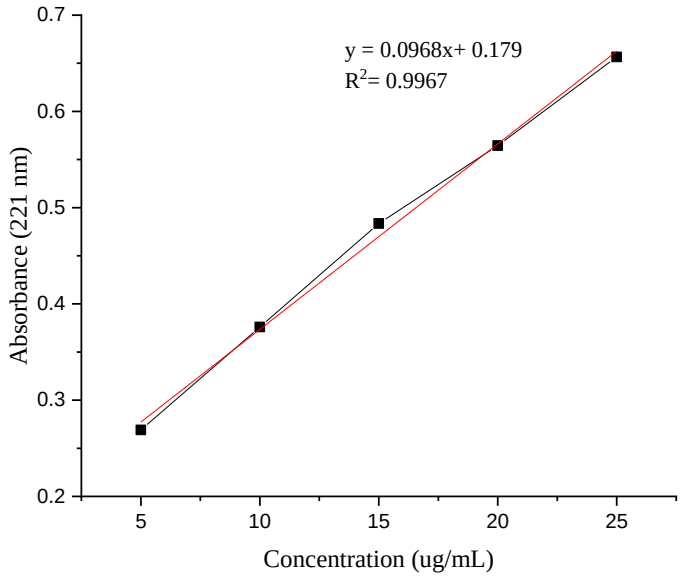

Figure AI Calibration curve of Ibuprofen

APPENDIX II

CHEMICAL OXYGEN DEMAND (COD)

COD determines the amount of oxygen required to oxidize the organic matter in in the waste water sample, using a specific oxidizing agent under controlled conditions. It is used to estimate the organic matter load in a given water sample.

Reagents: Standard potassium dichromate digestion solution (0.25N), Sulfuric acid reagent, Ferroin indicator solution, Standard ferrous ammonium sulfate (FAS) (0.1N)

Procedure (APHA 2012):

1. Take 2 ml of wastewater sample in COD tubes and to this add 1ml potassium dichromate reagent.
2. Carefully add 3 ml of Sulphuric acid reagent to the mixture.
3. Close the tubes tightly and place it in COD digester at 150°C for 2 hours.
4. After cooling the mixture to room temperature, transfer the contents to clean 100mL conical flask. Fill the burette with freshly prepared FAS solution. Add 2-3 drops of ferroin indicator to conical flask containing the mixture,
5. Titrate again the FAS until the colour changes to reddish brown.
6. Do the blank sample using the same procedure.
7. Calculate the COD concentration.

$$\text{COD (mg/L)} = \frac{\text{(Blank-titre value) X Normality of FAS(0.1N) X 8000}}{\text{Volume of sample taken (mL)}}$$

8000: equivalent weight of oxygen ×1000 mL/L

LIST OF PUBLICATIONS BASED ON THE CURRENT RESEARCH WORK

JOURNALS

1. Gayathri, G., and Srinikethan, G. (2016). "Review on production of Bacterial Cellulose from wastewater and its applications." *Research Journal of Chemical and Environmental Science,* 4 [4S], 25-30.
2. Gopu, G., and Govindan, S. (2018). "Production of bacterial cellulose from *Komagataeibacter saccharivorans* strain BC1 isolated from rotten green grapes." *Preparative Biochemistry and Biotechnology*, 48(9), 842–852.
3. Gayathri, G., and Srinikethan, G. (2018). "Crude glycerol as a cost-effective carbon source for the production of cellulose by *K. saccharivorans*." *Biocatalysis and Agricultural Biotechnology*, 16, 326–330.
4. Gayathri, G., and Srinikethan, G. (2019). "Bacterial cellulose production by *K.saccharivorans* BC1 strain using crude distillery effluent as medium". *International Journal of Biological Macromolecules,* 138, 950-957.

MANUSCRIPT UNDER REVIEW

1. Gayathri, G., and Srinikethan, G. (2019). "Characteristics of Ibuprofen loaded Bacterial Cellulose films in In vitro drug release application". *International Journal of Biological Macromolecules* (Elsevier publishers).

CONFERENCE PROCEEDINGS

1. Gayathri, G., and Srinikethan, G. (2016). "Review on production of Bacterial Cellulose from wastewater and its applications." *National conference on recent advances chemical, biochemical and environmental engineering (RACBEE-2016),* 1st October, OP-6, SDM college of Engineering and Technology, Dharwad.

2. Gayathri, G., and Srinikethan, G. (2018). “Crude glycerol as a cost-effective carbon source for the production of cellulose by *K. saccharivorans*.” *International conference on Bioengineering on Health and Environment (ICBHE-2018),* 8-10th January, Paper no OP-6B, Sathyabhama University, Chennai.

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PUBLICATIONS

- **G.Gayathri**, G. Srinikethan. (2016). "*Review on production of Bacterial Cellulose from wastewater and its applications*." Res. J. Chem. Environ. Sci., 4(4S), 25–30.
- Mittal, R., and **Gayathri, G.** (2016). "*Role of Yarrowia lipolytica in production and bio-modification of lipids*." Bio-Chemiae Acta, 1(1), 10–15.
- Parween, N., **Gayathri, G**., Saroha, T., and Chaudhry, N. (2016). "*Mechanistic insight of disease regulation in Breast cancer by miRNA*." Bio-Chemiae Acta, 1(1), 23–29.
- **Gayathri, G**., and Srinikethan, G. (2018). "*Crude glycerol as a cost-effective carbon source for the production of cellulose by K. saccharivorans*." Biocatal. Agric. Biotechnol., 16, 326-330.

- **Gopu, G.**, and Govindan, S. (2018). "*Production of bacterial cellulose from Komagataeibacter saccharivorans strain BC1 isolated from rotten green grapes*." Prep. Biochem. Biotechnol., 48(9), 842-852.
- **Gayathri, G.,** and Srinikethan, G. (2019). "*Bacterial cellulose production by K.saccharivorans BC1 strain using crude distillery effluent as medium.*" Int. J. Bio. Macromol., 138, 950-957.

CONFERENCES /WORKSHOPS

- Presented a research paper titled "Review on production of Bacterial Cellulose from wastewater and its applications" at the National Conference on "Recent Advances in Chemical, Biochemical and Environmental Engineering (RACBEE-2016)" held at SDM college of Engineering and Technology, Dharwad, Karnataka, India (October 1, 2016).
- Presented a research paper titled "Crude glycerol as a cost-effective carbon source for the production of cellulose by *K. saccharivorans*" at the "International Conference on Bioengineering on Health and Environment (ICBHE-2018)" held at Sathyabama Institute of Science and Technology (Deemed to be University), Chennai, Tamilnadu, India (January 8-10, 2018).
- Attended Certificate courses on Forensic Science and Women health hygiene conducted at Mount Carmel College, Bengaluru, Karnataka, India (2014-15). Attended Workshop on Personality and Skill development held at National Institute of Technology, Mangalore, Karnataka, India (August 27-28, 2016).

DECLARATION:

I hereby declare that the above furnished details are true to the best of my knowledge.

PLACE: Surathkal

DATE: **G.Gayathri**