Development of chitosan biopolymer films by fungal fermentation of waste substrates

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REFERENCE DECLARATION

I, Mr. K.K.R. Naidoo – 21313741 and Prof Kugen Permaul, Dr. Algasan Govender, and Dr. Adarsh Puri do hereby declare that in respect of the following dissertation:

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AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was conducted in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof. Kugen Permaul, Dr. Algasan Govender** and **Dr. Adarsh Puri**.

Student's signature

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ABSTRACT

Zygomycetes are known for their relatively high chitosan content (approximately 10% m/m) in comparison with other fungal genera. In this study, *Mucor circinelloides* was grown on the following industrial waste substrates: corn steep liquor (CSL); soft drink overflow spillage waste (DBW); and sugarcane molasses (MOL). Biomass production on waste substrates was statistically optimized by Plackett-Burman design in conjunction with Response Surface Methodology, followed by validation of the model. DBW hindered fungal biomass growth and was found to be a statistically insignificant variable and therefore omitted from further optimizations. The validated model produced a biomass of 77.87 g/L, a 2.65-fold increase over the highest-yielding unoptimized medium. Fungal biomass obtained after batch fermentation was subjected to acid-alkaline treatment for chitin extraction from the cell wall and deacetylation of the chitin to chitosan. A yield of 8-9% chitosan was obtained from the fungal biomass. FTIR spectroscopic analysis was conducted on the extracted fungal chitosan to compare extracted chitosan against commercial chitosan and chitosan monomer. The waste-grown, fungal-derived chitosan profiles were similar to those of commercial crustacean chitosan. The extracted chitosan was used in conjunction with additives and solvent systems to create biopolymer variants with differing properties. A library of data from the chitosan biopolymer variants was generated with considerable differences in characteristics based on their composition. Improvements in sample #11 (the most modified formulation) in contrast to the most common chitosan biopolymer film composition used in literature (sample #9), included a 3.37-fold improvement in the static force required to break the film. There was a 3.39-fold increase in tensile strength and an 11-fold reduction in elongation (%) and elongation rates. The creation of these variants will allow the use of these chitosan biopolymers for specific industrial applications.

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CHAPTER 1 : INTRODUCTION

Of the world's total oil production, about 4% is synthesized into plastics for use as consumer, commercial and industrial products. Processing industries use another few per cent due to the substantial amounts of energy required to manufacture oil-based plastics. As oil reserves are being depleted and the use of fossil fuel-based energy systems become more costly, the demand for alternative sources of raw material for the production of essential plastics becomes a higher priority (Chen, 2013). Of all polymeric materials about 80% are manufactured by the petrochemical industry and are therefore produced from fossil fuel (non-renewable) resources. Co-incidentally with the escalating use of plastics, the environmental strain has proportionately amplified. Furthermore, due to the environmental impacts caused by the production of petropolymers, there is an increasing encumbrance of waste generated by products discarded after use (Wei and Zimmermann, 2017). As a consequence, two main threats have been well-identified: greenhouse gas effects and environmental pollution, which have deleterious impacts on climate, biodiversity, and human health (Carro et al., 2013). With humankind responsible for both of these threats, interventions are needed. There is an ever-increasing demand for plastic in most sectors and the finite amount of fossil fuels is continuously being depleted. The synthesis of fossil fuel-based plastics has many adverse effects upon the environment and requires high energy input and labour-intensive fractional distillation of fossil fuels. The use of fossil fuels alone has resulted in many man-made catastrophes involving crude oil and fracking. The inherent non-biodegradability of traditional plastics also contributes to the pollution of the environment and poses a threat to all living organisms. There is a need for petropolymer substitutes, which are sustainable, environmentally friendly, and inexpensive to produce.

Biomass is a naturally abundant source of sustainable biopolymers (Portes et al., 2009). Great efforts have been made to produce biodegradable plastics with the aim of alleviating the environmental problems created by the discarding of synthetic plastics (Sreekumar et al., 2012). Chitosan polymers are semi-synthetically derived amino-polysaccharides that have unique structures, multidimensional properties, and highly sophisticated functionality (Dash et al., 2011). Chitosan-based biopolymers are therefore a potential substitute and solution to this ongoing problem.

Chitin is a natural biopolymer derived from the exoskeletons of crustaceans and from the cell walls of fungi and insects. Approximately 45% of seafood is composed of chitin, primarily present in the exoskeleton of crustaceans, and this is used as the primary source of chitosan. Chitin is structurally identical to cellulose except for the hydroxyl group replaced by an acetamide group at the C-2 positions. This is also true for chitosan and cellulose, with the hydroxyl group being replaced by an amino group (Figure 1.1). Resembling cellulose and highly insoluble, chitin is known for its biocompatibility, biodegradability and non-toxicity as well as its antimicrobial activity and low immunogenicity (Boonsongrit et al., 2006). Chitosan is a derivative of chitin, obtained by the deacetylation of chitin. Seventy percent deacetylated chitin is regarded as the minimum percentage for the biopolymer to be deemed as chitosan. This heteropolysaccharide is composed of *N*-acetyl-glucosamine (NAG) and D-glucosamine. Chitosan is soluble in acidic solutions; the solubilisation occurs by protonation of the NH_2 functional group on the C-2 position. It is used in many applications (food, cosmetics, biomedical and pharmaceutical) and provides major functionality towards biotechnology needs and this explains its greater potential compared to chitin for use in different applications (Bhattarai et al., 2010; Ji et al., 2010; Mao et al., 2010).

Chitosan films can possess either a clear or coloured appearance depending on the application. Chitosan biopolymers have been used in various fields such as edible biofilms to prolong the shelf life of food, to act as a physical barrier for packaging and use of its inherent anti-microbial activity as well as the addition of nutritional value to food content (Aider, 2010). Currently, the bulk of the commercial chitosan industry is dominated by processes based on extraction from shrimp shells. This is not surprising because historically shrimp and crab shells have always produced the highest yields of chitosan and are the most readily available. However, it is increasingly becoming difficult to obtain substantial amounts of shellfish waste due to competition from other sectors, especially manufacturers of animal feed. Another issue is the ever-increasing decline in natural fisheries which, in future, will make shellfish harder to procure. Whereas shellfish waste was previously available in substantial amounts at practically no cost at all, manufacturers of chitosan are now being forced to look for alternative sources of raw material. In this regard, the production of chitosan from fungal cell walls is a feasible alternative, since fungal chitosan is bereft of all the problems associated with crustacean chitosan, like the presence of inorganic materials and the need for demineralization

treatments. Fungi can be cultured year-round in controlled environments, unlike shellfish which are seasonal. Utilizing waste sources for the growth of fungi will enable a sustainable crustacean-independent chitosan supply. Using multiple waste substrates (WS) for fermentation will dramatically increase the feasibility of chitosan production and once scaled for industrial production, will result in an environmentally friendly source of chitosan (Liu et al., 2016). The number of applications of chitosan is steadily increasing, but the number of sources of chitosan to supply the demand has not increased (Sahoo et al., 2012).

The properties of biopolymer films are determined by the orientation of the microstructures of its polymer matrix subcomponents. Additives may fundamentally change both the composition and arrangement of the polymer molecules. The use of additives in composite films alters the chitosan biopolymer film properties such as elasticity, rigidity, insulation, conductivity, insolubility and optical properties (Vlacha et al., 2016). Different additives enhance polymers for specific applications. With relatively simple extraction procedures from a natural renewable source, biologically synthesized chitosan is an ideal substitute in the petropolymer industry. Modified chitosan-based biopolymers offer infinite possibilities in use for applications which are traditionally dominated by fossil fuel-derived petropolymers. Tailored application-based formulations will be able to match and even outperform conventional petropolymers specifications and have none of the associated stigma.

The current study involved the growth of a fungus having a high chitosan content on several waste carbon sources and subsequent extraction and characterization of the chitosan. Biopolymer films were developed with and without additives to create variants with different properties.

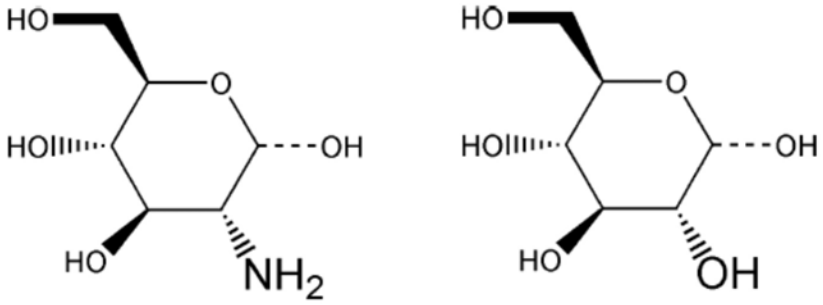


Figure 1.1. Structure of glucosamine (monomer of chitosan) and glucose (monomer of cellulose) (Pillai et al., 2009).

CHAPTER 2 : LITERATURE REVIEW

2.1 Chitosan

The use of chitosan in distinct areas has increased quickly in recent years due to its notable properties namely: (a) a defined chemical structure; (b) it is polycationic, innocuous, biodegradable and biocompatible; (c) it is physically and biologically active; (d) it is amenable to chemical or enzymatic modification and; (e) it can be processed into numerous forms, e.g., flakes, beads, powders, membranes, gels, sponges, kinds of cotton, films, and fibres (Dhillon et al., 2013; Muxika et al., 2017; Madni et al., 2019).

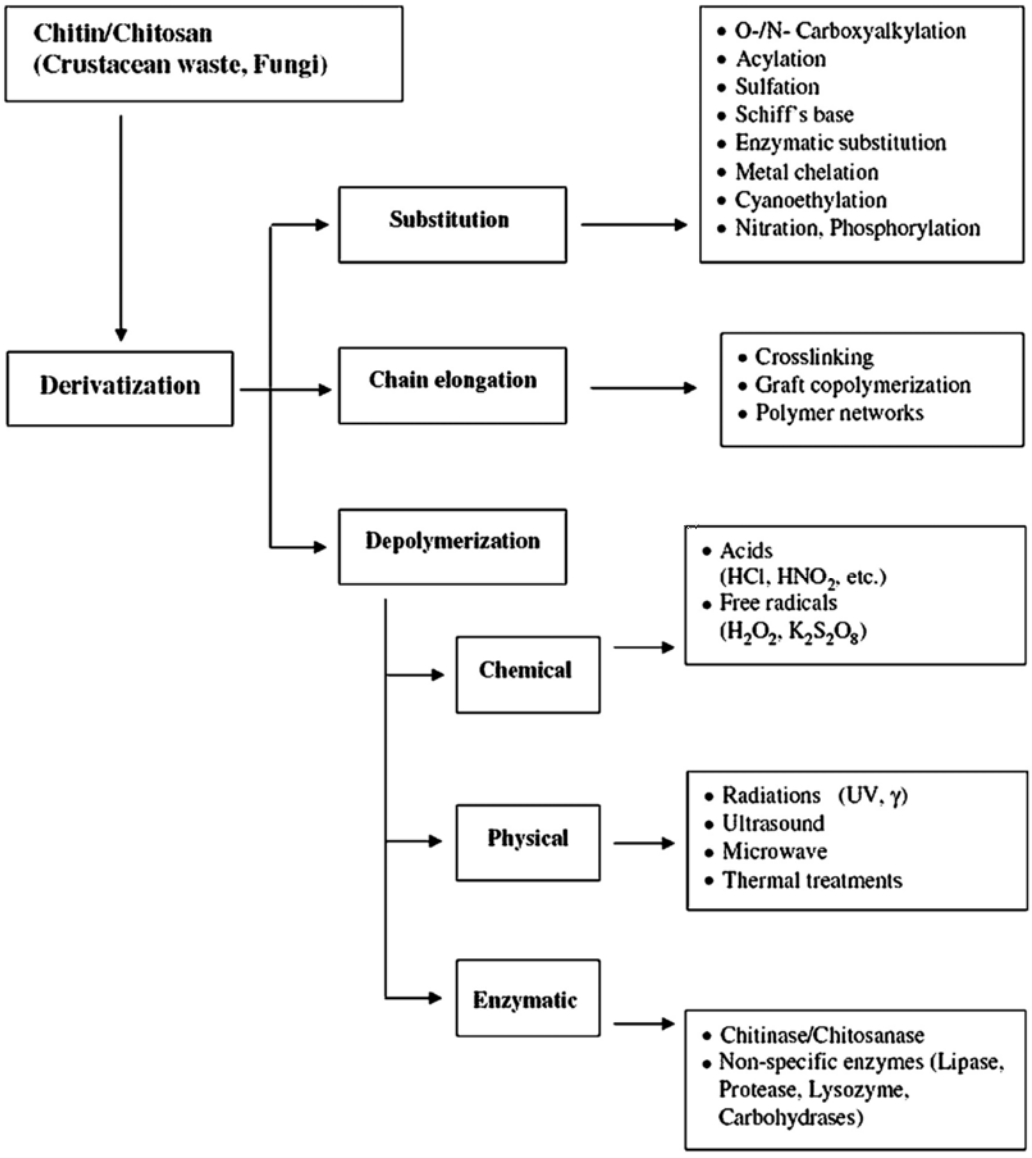


Figure 2.1 Category-based applications of chitin and chitosan (Sahoo et al., 2012).

Derivates of chitosan have comparable properties. Chitooligosaccharides can be used in several applications based on their monomer structure and degree of deacetylation (DDA) (Hsiao et al., 2008). Chitosan has been utilized in enzyme immobilization, wastewater treatment, as a food additive, in anti-cholesterolemic applications, for wound healing, and in pharmaceuticals as drug delivery systems (Donnelly et al., 2013). The broad range of the applications of chitin and chitosan is illustrated in Figure 2.1. Chitosan is commercially manufactured from the shells of shrimp, lobster and crab by chitin deacetylation, which is a finite and seasonally dependent source (Tajdini et al., 2010).

2.1.1 Structure and properties

Chitosan is a linear copolymer of beta-(1–4) linked 2-acetamido-2-deoxy-beta-D-glucopyranose and 2-amino-2-deoxy-beta-D-glycopyranose (Dash et al., 2011). The chemical structure of chitin is similar to cellulose, except that the hydroxyl group in the C-2 of cellulose is replaced by an acetamide group in chitin (Dhillon et al., 2013). After refinement, chitosan has a rigid crystalline structure through inter- and intra-molecular hydrogen bonding. The characteristic features of chitosan are: being cationic; hemostatic; and insoluble at neutral to high pH. The linear unbranched structure and high molecular weight of chitosan makes it an excellent viscosity-enhancing agent in acidic mediums, and it acts as a pseudoplastic material, demonstrating a reduction in viscosity with increasing rates of shear. It is soluble only in dilute inorganic and organic acids with a pH lower than chitosan pKa, (~6.3). Upon dissolution in acidic media, the amino groups of the polymer become protonated, rendering the molecule positively charged. Chitosan behaves as a stabilizer of hydrocolloids and lipid mixtures, enhancing emulsion formation and interfacial stabilization because its molecules are composed of both hydrophilic and hydrophobic sections (Pereda et al., 2012). Applications based specifically on the unique properties of chitosan have been explored mainly under biomedical applications (Table 2.2) as well as applications per sector (Table 2.1).

2.1.1.1 Antimicrobial properties of chitosan

Chitosan is known to have antimicrobial properties. The positive charge on the amino group is attracted to other negatively charged polymers, e.g., the cell membrane of microorganisms, cholesterol, and proteins. Upon exposure of chitosan to microorganisms, the proteinaceous

and other intracellular constituents are induced to leach out from the cell which causes the death of the microorganism (Portes et al., 2009). Secondly, chitosan behaves as a chelating agent by selectively binding to essential metals and nutrients, thus inhibiting microbial growth.

Table 2.1 Principal applications for chitosan (Rinaudo, 2006)

Agriculture	Defensive mechanism in plants Stimulation of plant growth Seed coating, frost protection Time release of fertilizers and nutrients into the soil
Water and waste treatment	Flocculant to clarify water (drinking water, pools) Removal of metal ions Ecological polymer (eliminate synthetic polymers) Reduction of odours
Food and beverages	Not digestible by humans (dietary fibre) Binds lipids (reduce cholesterol) Preservative Thickener and stabilizer for sauces Protective, fungistatic, antibacterial coating for fruit
Cosmetics and toiletries	Maintains skin moisture. Treats acne. Improves suppleness of hair. Reduces static electricity in hair. Tones skin Oral care (toothpaste, chewing gum)
Biopharmaceutics	Immunologic, antitumoral Hemostatic and anticoagulant Healing, bacteriostatic

The antimicrobial activity of chitosan depends on certain factors, i.e., the type of chitosan (deacetylation degree and molecular weight), the pH of the medium and temperature (Rodríguez-Núñez et al., 2012). Chitosan has been used in conjunction with carvacrol to inhibit the mycelial growth and spore germination of *Aspergillus flavus*, as a coating on cherry tomato fruits and in laboratory media (de Souza et al., 2015).

Table 2.2 Principal properties of chitosan in relation to its use in biomedical applications (Rinaudo, 2006)

Potential Biomedical applications	Principal characteristics
Surgical sutures	Biocompatible
Artificial skin	Renewable
Corneal contact lenses	Hydrating agent
Time-release drugs for animals and humans	Nontoxic, biological tolerance
Encapsulating material	Hydrolyzed by lysozyme, wound healing properties

2.1.2 Characterization of chitosan

Infrared (IR) spectroscopy is one of the main and broadly utilized analytical technique accessible to scientists working on chitin and chitosan (Kumirska et al., 2010). The infrared spectrum is commonly obtained by passing infrared electromagnetic radiation through a sample that possesses a permanent or induced dipole moment and determining what fraction of the incident radiation is absorbed at an energy level. The energy of each peak in an absorption spectrum corresponds to the frequency of the vibration of a molecule constituent, thus allowing qualitative identification of certain bond types in the sample. The total spectrum is analysed by an interference process and converted into the frequency or wavenumber range employing Fourier-transform infrared (FTIR) spectroscopy. This has improved the quality of data produced by infrared spectra and minimised the time needed to acquire data. Common conditions for the FTIR spectroscopic analysis of chitosan show peaks at 3000-3500 cm^{-1} (NH bond) and at 1400-1650 cm^{-1} (C=O bond) (Jafari et al., 2016). The FTIR spectra are generally recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} in the

absorbance mode for 8 to 128 scans at ambient room temperature. The samples for FTIR analysis are prepared by grinding the dry blended powders with powdered KBr, often in the ratio of 1:5 (sample: KBr) and then compressed to form discs. Spectra can be measured using a deuterated triglycerine sulphate detector (DTGS) (Thanpitcha et al., 2008) or on films using an attenuated total refraction (ATR) method in an IR spectrometer (He et al., 2016a). Diffuse reflectance infrared Fourier-transform (DRIFT) spectroscopic analysis is also applicable.

2.1.3 Deacetylation

Chitin is a biopolymer composed of beta-(1–4) linked 2-acetamido-2-deoxy-d-glucose residues (Di Mario et al., 2008). The polymorphic forms of chitin differ in the packing and polarities of adjacent chains in successive sheets (Pillai et al., 2009). Chitin can be enzymatically or chemically deacetylated to chitosan, a more flexible and soluble polymer (Dhillon et al., 2013), either by homogeneous or heterogeneous alkaline *N*-deacetylation or by enzymatic deacetylation. The parameters used in deacetylation determines the polymer molecular weight and the degree of deacetylation (DDA) (Dash et al., 2011). There are varying grades of chitosan according to the level of deacetylation, normally ranging from 70% to 100%. The change in functional groups facilitated by deacetylation is illustrated in Figure 2.2. A change in the functional groups would impart changes to the physico-chemical properties of the molecule which affect solubility, film-forming ability as well antimicrobial properties (Chiu et al., 2007). Commercially available chitosan when compared with laboratory extracted chitosan may possess some negligible impurities pending the extraction method, specific solvent and concentration of the solvent.

2.2 Sources of Chitosan

Chitin biosynthesis in crustaceans differs considerably from that of fungi. However, they do follow similar biosynthetic steps and the enzymatic machinery exhibits comparable catalytic regulation (Tharanathan and Kittur, 2003). Chitin has three different crystalline allomorphs: the α-, β- and γ-forms with the difference being the orientation of the micro-fibrils. The most abundant form of chitin is α-chitin which is present in fungal and yeast cell walls, in krill, lobster and crab tendons and in shrimp shells, as well as in insect cuticles (Younes and Rinaudo, 2015). Typically, on an industrial scale, chitosan is predominantly derived from the waste product of

crustacean exoskeletons obtained after the industrial processing of seafood, such as shrimp, crabs, squids, and lobster shells by chemical deacetylation. In terms of being environmentally friendly, fungal chitosan is more attractive as chemical extraction from fungi uses milder procedures than from crustaceans (Dhillon et al., 2013). The advantages and disadvantages per source are listed In Table 2.3.

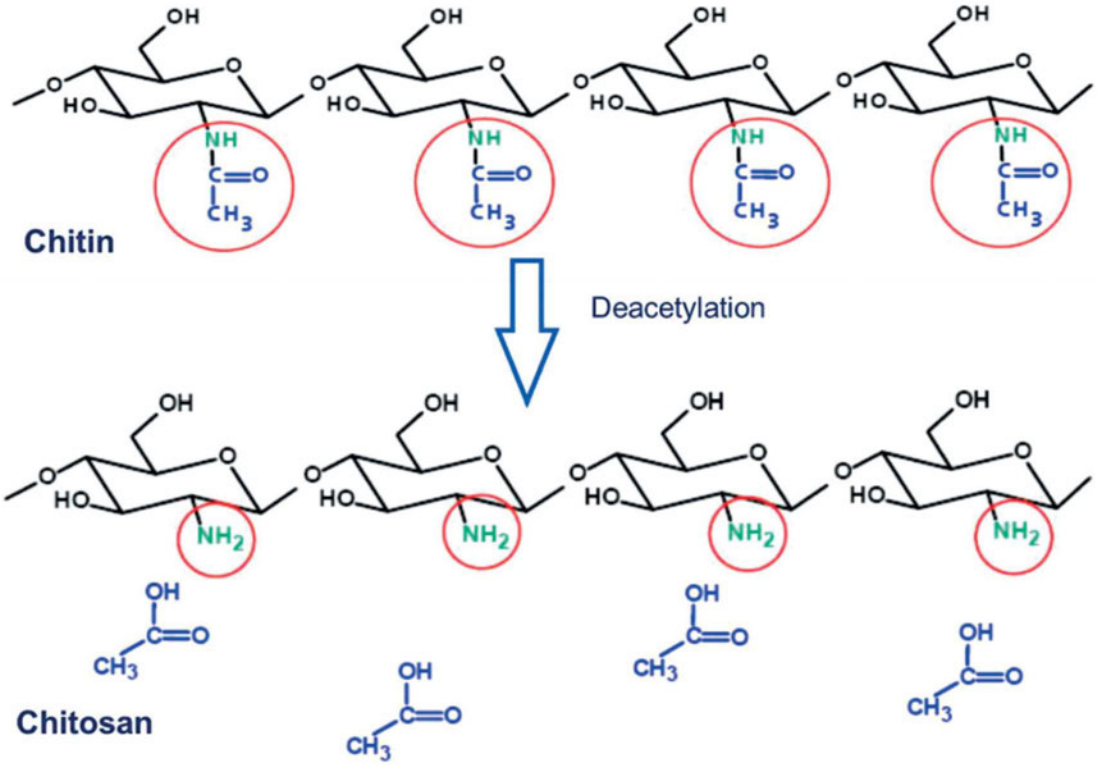


Figure 2.2 The structure of chitin and its deacetylated product chitosan (Kaur and Dhillon, 2014). Glucosamine is the monomer of chitosan while the monomer of chitin is *N*-acetyl glucosamine. The carboxylic acid, acetic acid (blue molecules) is the product formed from the deacetylation reaction. In the C-2 position (highlighted by the red rings) on the polymer chain, chitosan has an amine group while chitin has an amide group.

2.2.1 Crustacean sources

In crustaceans, where α-chitin is produced, the chitin is found to occur as fibrous material embedded in a six-stranded protein helix. Due to the nature of crustacean chitin, the earlier methods of extraction employed the use of harsh chemicals to facilitate extraction (Figure 2.3). New methods are being developed to obtain chitosan from chitin, due to the disadvantages and drawbacks of the traditional extraction process. Phyto-mediated chitosan synthesis using a Graviola extract has been investigated and recovered chitosan nanofibres from commercial

chitin flakes and chitosan particles directly from solid marine wastes of crab, shrimp and squids (Gopal et al., 2019). Base-free preparation of chitin from crab shell, the raw crab shell was treated with hydrochloric acid under different conditions and concentrations, as a means of demineralization and deproteinization. The results of the study indicate that the demineralization of a crab shell is completed in minutes, while the deproteinization needs at least 180 min to acquire a high-purity product (Zhang et al., 2018).

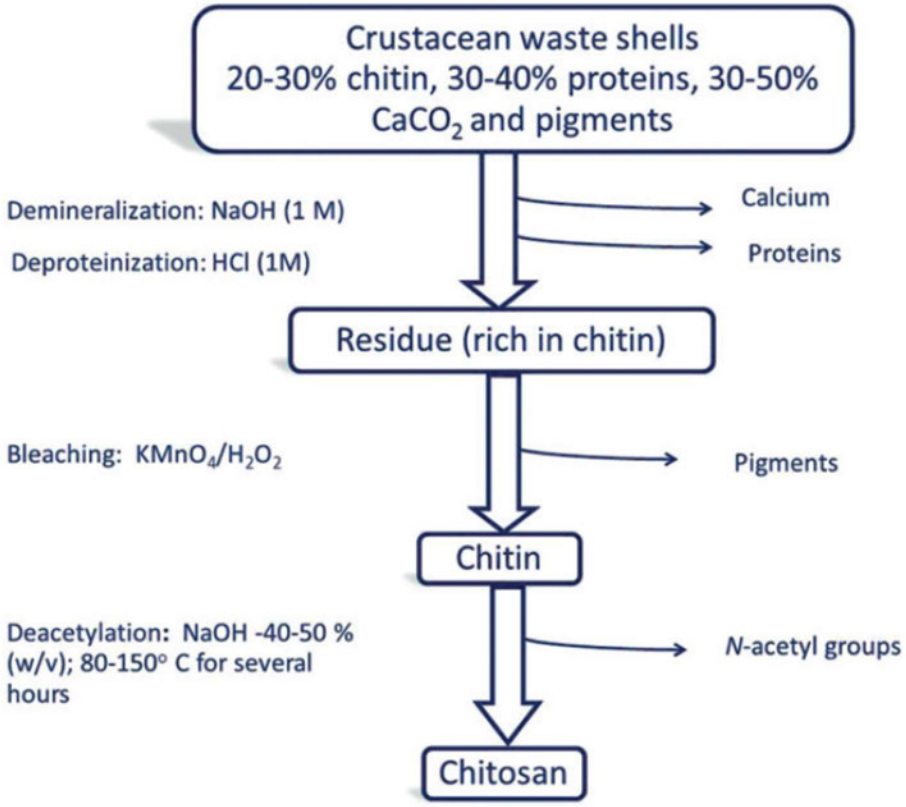


Figure 2.3. Extraction of chitin and deacetylation to chitosan from crustacean shells by conventional methods (Kaur and Dhillon, 2014).

2.2.2 Fungal sources

Chitin is present in and is a defining component for the following taxonomical fungi groups: Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes (Muzzarelli et al., 2012). In fungi and yeast, chitin occurs in nature as ordered crystalline microfibrils forming structural components in the cell walls. Chitin is synthesized as chains of β-1,4-linked *N*-acetylglucosamine residues in fungal cell walls (Dhillon et al., 2013). Fungal mycelia can be

produced by simple fermentation regardless of geographical location or season. Processing and purification of chitosan from the cell walls of fungi grown under controlled conditions offers the advantage of being environmentally friendly and imparts a greater possibility for a more consistent result. Fungal biomass can be cultivated on commercial media or a suitable waste material. Laboratory media will typically cost more and add expense to the production process while waste material obtained at little to no cost will reduce the production expenses. So long as the waste material has fermentable sugars and carbohydrates, it can be used as a fermentation substrate. Waste material which has been reported in the literature ranges from corn steep liquor (de Souza et al., 2015), molasses (Chatterjee et al., 2005), and yam beans (Fai et al., 2011) to animal and human wastewater (Liu et al., 2016). The methods of biomass growth as well as the nutrient value of the waste substrate used will determine the biomass yield.

In nature, *Mucoraleae* fungi (Zygomycetes) possess chitosan in the cell walls and specifically amongst the Zygomycetes class, the Mucorales order is the only one that has both chitin and chitosan in the cell walls. Noting the above the extraction process is higher yielding to obtain chitin/chitosan from the cell wall when compared with other fungal classes using a similar extraction process. There are two main fungal chitin extraction methods one using sulphuric acid (H_2SO_4) and the other using acetic acid (AA). The H_2SO_4 method is shorter in terms of time required to perform (±4 hours) but yields poorer quality chitosan while the AA method requires a longer time period (±8 hours) but produces better quality chitosan (Tajdini et al., 2010; Zamani et al., 2010; Naghdi et al., 2014). The extraction and purification processes must be conducted properly, especially in the case of medical applications where impurities may cause allergies or adverse reactions. The higher the level of purity the smaller the immune responses and the converse is true as low-purity fungal chitosan from *C .albicans* presents as an antigen therefore prompting an immune response (Alvarez, 2014). The choice of extraction method will depend on the desired specifications required for the application of the chitosan. *Mucor rouxii* has been thoroughly investigated in literature, as a fungal source for chitosan production, showing chitosan yields ranging from 6–9% of the dry cell mass (Chatterjee et al., 2005). The mycelium of *Mucor circinelloides* is a reliable source of chitin and chitosan. The highest levels of chitosan (64 mg/g) from this fungus have been reported by Fai et al. (2011). The extraction of chitosan from fungi is accomplished by mild alkaline and acidic treatments as when

contrasted with the standard chemical extraction method from crustaceans and it can be deemed as a greener method.

Table 2.3 Advantages and disadvantages of chitosan from different sources (Dhillon et al., 2013)

Source	Advantages and disadvantages
Crustacean shells	Advantages
	Well-established method for industrial production of chitosan
	Disadvantages
	Seasonal and limited supply, high cost and laborious process and not environmentally friendly
	Large quantities of chemicals, such as alkali and acids, higher temperatures and long processing time are needed for extraction. Usually, an alkali concentration of 30–50% w/v and a temperature of 100°C is needed
	Demineralization treatment is needed to remove calcium carbonate which constitutes 30–50% of crustacean shells
	Possesses high molecular weight and protein contamination which restricts its implementations in biomedicines
Fungi	Advantages
	Medium to low molecular weight that is appropriate for numerous biomedical applications
	Higher DDA can be obtained
	Free of allergenic shrimp protein
	The molecular weight and DDA of fungal chitosan can be controlled by varying the fermentation parameters
	Supply of fungal biomass is limitless, largely from the biotechnological and pharmaceutical industries
	Low-cost biowastes can be used as economic substrates for culturing fungi
	Disadvantages
	Processes not scaled up to the industrial level

2.3 Waste Materials

Millions of volumetric tons of waste are produced annually most of which end up in landfill sites, rivers which flow into the oceans, and storage sites. All these destinations incur costs in the form of labour, transport, and energy consumption. Also, there are potential issues with harming the environment and the consequences related to polluting the environment, affecting both biotic and abiotic factors. Toxic waste sometimes affects almost all forms of life, as many microbiota cannot use the waste as substrates or their toxic analogues are not utilizable and can kill the organisms (Huang et al., 2010). Upon first exposure to xenobiotic compounds, organisms are affected and sometimes killed. Those that survive then adapt but exist in an ecologically imbalanced state. Some of the killed organisms are the missing links in the ecological food chain. An examples is eutrophication, whereby certain pollutants from effluent streams create ecological imbalances. Such instances are found in fertilizers, antibiotics, waste effluent with high fermentable sugars and fermentable carbohydrates. Other conditions such as pH, COD (chemical oxygen demand), and BOD (biological oxygen demand) of the effluent are also important as these alter the conditions of the natural environment. Every industry contributes to the total waste as effluent, be it solid or liquid form. There is a need to reduce the amount of wastes released into the environment and to find alternative uses for these wastes.

2.3.1 Types of waste

Waste classification is extremely broad. Waste can be generated from any process as is defined as it no longer has any contribution to the rest of the process, thereby a waste product. Ranging from toxic to non-toxic, “wastes” can be incredibly diverse in nature, therefore using something which is a waste and has little to almost no value and converting it into something with value and function is worth exploring.

Organic and inorganic waste are the two broadest categories of wastes. Heavy metals, radioactive isotopes, aluminium shavings, and spent batteries are examples of inorganic waste. Molasses, agricultural produce waste, lignocellulosic materials, and biomass fermentation supernatant are examples of organic waste (Iandolo et al., 2011b). Looking at both the environmental benefits and the potential sustainable products, waste valorization is a well-

motivated theme of research. Waste can also be classified as primary, secondary, tertiary, and quaternary. The categories indicate how many processes the waste has been through in the original production process. Many primary and secondary waste materials still have components which could be utilizable e.g., sugar production from sugarcane results in molasses. Molasses still have 45 to 50% fermentable sugars. With tertiary and quaternary waste, there are even less usable components. In ethanol production using molasses as the fermentation substrate, the final beer produced still has 2-5% sugar content. After distillation, the remaining solution would be referred to as tertiary waste, but many organisms would still be able to grow in that concentration of fermentable sugars. Quaternary waste is a type of waste where almost all potential uses of a certain compound have been exhausted. Usually, combustible quaternary waste can be used to heat water and produce steam as an energy production scheme.

2.3.2 Waste material conversion

Repurposing waste material and finding waste applications is a central theme of biotechnological processes. Worldwide waste recycling has been utilized to effectively reduce the carbon footprints of production processes. In almost all cases, a viable option has been found. The only waste material not being repurposed is spent radioactive fuel. Many organic wastes still possess components which fungi and other microorganisms can metabolize, thereby allowing growth. *M. circinelloides* has also been used to produce oil due to the high lipid content in the cell as well as animal feed (Mitra et al., 2012). Once established, the organisms can be coaxed to produce desired products. Enzyme production is an example of one of the largest biotechnological industries, in which many of the processes use waste material as the substrate for culture growth (Dave et al., 2012). Fungi are again useful in enzyme production given their characteristic saprophytic attributes with a plethora of enzymes being produced to digest organic matter, especially plant-based/derived material. They are also able to grow in both yeast and filamentous mycelial form depending on the environmental conditions. Enzymes can be produced individually from various fungal cultures, conversely, a variety of multiple enzymes can also be produced from a single fungal strain (Romo Sanchez et al., 2015; Wang et al., 2015; Zhang et al., 2015). All of these processes can use waste material as the starting substrate, e.g., corn-ethanol stillage, Agri-industrial waste, and lignocellulosic waste material. A main focus and application is to use waste material to grow the cultures

which produce enzymes that aid in the detoxification of process effluent and bioremediation efforts (Pant and Adholeya, 2007; Huang et al., 2010; Iandolo et al., 2011a; Evirgen and Acikel, 2013; Michailides et al., 2015).

2.3.3 Biological conversion

Using a bioconversion process vs. a chemical process has many advantages such as using far less energy and being less labour intensive, using processes which are environmentally friendly as they are much less toxic and milder therefore posing less of a hazard to the environment (Prigione et al., 2012). Fungi are eukaryotes which are largely known as saprophytes, a key role in recycling of organic matter. They can grow on numerous substrates making them a versatile tool for converting low-value compounds into high-value compounds. As with many fungi, both yeast and mycelia can be used but depending on process-specific limitations or growth requirements one of the forms will be preferable. As in the case of antibiotic or ethanol production, specific strains are used which are optimized to ensure optimal production of the target compound. When using fungi as the medium of bioconversion, the fungi will exhibit a preference for certain substrates as components within the WS and this will enhance or inhibit waste substrate utilization (Pant and Adholeya, 2007). Bioremediation is an environmentally directed example of bioconversion. These processes target the waste which have become pollutants in the environment, and toxic components which adversely impact the local fauna and flora (Ahluwalia and Goyal, 2013).

2.3.4 Waste substrate fermentation

With fermentation, the waste can be defined or complex substrates depending on their origin and composition. The majority of chemical inorganic wastes can be used as growth media, with dilution to the appropriate concentrations for fermentation. Organic waste is mostly complex media resulting from the biological deterioration of the original substrates, creating derivatives of the waste. Many studies have used fungi in fermentation, with various sources as the substrate of the fermentation. Production of useful products is dependent on the type of fermentation and the desired outcome, as well as using the best-suited fungi to facilitate the process (Mendes et al., 2013). The fermentation can be solid state or submerged liquid fermentations. In some studies, the consortia of fungi are used to further improve the

degradation or conversion of the waste depending on the desired outcome. This is due to the specialized enzymes produced by the distinct species which work synergistically to achieve a better process yield and reduce the time required for the fermentation, in comparison to sequential multi-species fermentations. Large-scale fermentations would generally be best facilitated using continuous fermentation, in certain instances fed-batch is required with multistage fermentation which first produces a high biomass yield and then changes substrate to produce bioproduct. When there are compositional differences in the substrates used across media batches using a gradient blend is best for implementing new blends of media, especially when changing the substrate entirely to allow a transition period for the culture to adapt. Issues may arise concerning optimal conditions maintained in closed systems, but open-air fermentations have shown the best results (Mohammad et al., 2013).

2.4 Biopolymer Films

A bioplastic is a plastic that is made partly or wholly from polymers derived from biological sources (Chen, 2013). This means that the production of biopolymers has a lesser carbon footprint in contrast to petrochemical-based plastics and has lower energy costs during manufacturing since high temperatures and high pressures are no longer needed. The key differing characteristics of these two processes have been outlined in Table 2.4. Creating a composite which is stronger than its individual components is key when producing a film. The individual components contribute to the properties of the structure; usually, specific components are used to impart a specific property to enhance a related application (Dang and Yoksan, 2016). Blends exhibit economic advantages and better properties than completely natural polymers, due to the properties that are imparted by the commercial polymer when used as a blending component (Morro et al., 2016). There are a wide variety of biopolymers currently being produced in various research fields, including lipid, protein, and polysaccharide-based films. Variants or combinations of the three major types can have improved properties due to different subcomponents. Xanthan gum was the first to be produced at the industrial scale and still is one of the most significant biopolymers currently on the market (Kreyenschulte et al., 2014). The sources, substrates, and applications of microbially produced biopolymers are shown in Table 2.5, demonstrating multiple potential options for sustainable alternatives.

Table 2.4 Comparison of biopolymers with petroplastics (Chen, 2013)

	Bioplastics	**Petroplastics**
Renewable	Yes, or partially	No
Sustainable	Yes	No
Breakdown in the environment	Biodegradable and/or compostable	Some degradable by polymer oxidation
Polymer range	Limited but growing	Extensive
GHG emissions	Generally low	Relatively high
Fossil fuel usage	Generally low	Relatively high
Arable land use	Currently low	None

2.4.1. Film formation

There are two main methods of film formation, namely wet processes and dry processes. Wet processes dissolve the components in the solvent, and then remove the excess solvent by drying. Casting is the most common method used for producing biopolymer films due to the majority of biopolymer solutions containing high moisture levels in their matrix. It is consequently more complex to create films from biopolymer solutions using traditional petropolymer manufacturing methods. Dry processes make use of plasticizers with bioplastic constituents and are blended together using heat, e.g. extrusion by an extruder (Davidovich-Pinhas et al., 2014). Compression moulding is similar in process to extrusion but after blending a force is applied to the polymer in a mould of desired dimensions. Injection moulding uses the biopolymer before curing and with relatively high temperature and high force, the polymer is injected into the desired mould. The potential use of protein-based injection moulding to make multiple kinds of shaped products will encourage new arguments in favour of opting for these biopolymer materials as an alternative to synthetic plastics for different applications (Félix et al., 2014).

Table 2.5 List of microbial biopolymers on a commercial scale (Kreyenschulte et al., 2014)

Polymer Class	Source	Substrates	Applications
Polyamides Cyanophycin	*Cyanobacteria, Acinetobacter* sp., *Bordetella* sp., *Desulfitobacterium hafniense, Nitrosomonas europaea*	arginine, $(NH_4)_2SO_4$ protein hydrolysate	Water softener Metal ion-exchange system Hydrogels Synthesis of chemicals Nutrition
y-Polyglutamic acid	*Bacillus* sp., *Staphylococcus epidermis, Natrialba aegyptiaca, Natronococcus occultus, Fusobacterium nucleatum*	glycerol, L-glutamic-acid, citric acid	Biodegradable plastics Fertilizer Food thickener Hydrogels Medical adhesives Nanoparticle drug/gene delivery Skincare Tissue scaffolds Wastewater treatment
Poly-L-lysine	*Streptomyces albulus lysinopolymerus*	Glucose, $(NH_4)_2SO_4$	Coating material Dietary agent Drug/Gene delivery Emulsifying agent Endotoxin removal Food preservative Hydrogels Interferon inducer
Polyanhydrides Polyphosphate	Eukaryotic and prokaryotic cells	sodium acetate, KH_2PO_4 and NH_4Cl e.g., from wastewater	Antibacterial agent ATP substitute Food additive Insulating fiber

Polyesters Polyhydroxy-alkanoates	Prokaryotes	carbohydrates, starch, alcohols, industrial waste products	Biodegradable plastics Drug delivery Tissue engineering
Polysaccharides Alginate	*Pseudomonas* and *Azotobacter* sp. (mostly *A. vinelandii*)	sucrose	Cell immobilization Drug delivery Food additive Textile/paper industry Wound dressing Water treatment
Bacterial cellulose	*Gluconacetobacter, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Escherichia, Rhizobium, Sarcina*, and *Salmonella* sp.	glucose, sucrose, and other carbohydrates	Food additive Membrane material Oil recovery Paper industry Wound dressing
Curdlan	*Agrobacterium, Rhizobium* and *Cellulomonas* sp.	glucose, sucrose, other carbohydrates	Food additive Concrete additive Drug delivery Immune stimulator Heavy metal removal
Dextran	*Leuconostoc, Streptococcus* and *Lactobacillus* sp. (mostly *L. mesenteroides*), *Gluconobacter* sp., *Pediococcus pentosaceus*	sucrose maltodextrins	Blood-plasma substitute Molecular sieves (Sephadex) Heavy metal removal Cosmetics

			Emulsifying and thickening agents
Gellan	*Pseudomonas elodea, Sphingomonas* sp. (mostly *S. paucimobilis* ATCC 31461)	carbohydrates, industrial waste products	Agar substitute Cell immobilization Food additive Gel electrophoresis Tissue engineering
Hyaluronic acid	*Streptococcus zooepidemicus, S. equi, Pasteurella multocida*	glucose, amino acids, nucleotides, salts, trace elements and vitamins	Cosmetics Drug/gene delivery Viscosupplementation Wound dressing
Levan	*Zymomonas mobilis, Bacillus sp., Streptococcus sp., Alcaligenes viscosus* and other prokaryotes	sucrose, lactose	Blood-plasma substitute Cosmetics Emulsifying agent Food additive
Pullulan	*Aureobasidium pullulans, Tremella mesenterica, Cytaria* sp.*, Cryphonectria parasitica, Rhodototula bacarum*	carbohydrates, industrial waste products	Blood-plasma substitute Cosmetics Enzyme immobilization Flocculating agent Food additive Pharmaceutical coating
Scleroglucan	*Sclerotium rolfsii* and *S. glucanicum, Schizophyllum commune, Botrytis cinerea, Epicoccum nigrum*	glucose, sucrose	Cosmetics Drug delivery Immune stimulator Oil recovery Pharmaceutical coating

Succinoglycan	*Sinorhizobium meliloti, Agrobacterium* sp*., Alcaligenes faecalis* var*. myxogenes, Pseudomonas* sp*.*	sucrose and other carbohydrates	Food additive Oil recovery
Xanthan gum	*Xanthomonas campestris*	glucose, sucrose, and other carbohydrates	Agricultural products Coatings Cosmetics Food additive Oil recovery Paper industry Thickener

2.4.2. Polysaccharide-based films

Many polysaccharides possess numerous hydroxyl groups which encourage hydrogen bonding. These hydroxyl groups also act as sites for active and functional modifications to the molecule. For the food industry, it is advantageous to manufacture polysaccharide-based films due the to; numerous sources available; no toxic solvents are required; low-cost; and having a relatively simple production process. Ample effort has been invested in polysaccharide-based films for food packaging applications (Aider, 2010; Higazy et al., 2010; Lago et al., 2011; Kasirga et al., 2012; Al-Naamani et al., 2016).

2.4.2.1 Starch films

Starch is one of multiple carbohydrate polymer options for producing films. It can be derived from a variety of crops, It is natural, renewable and inexpensive. The functionality and characteristics will vary based on the origin, molecular weight and the relative proportions of amylopectin and amylose. Starch granules can be synthesized into a thermoplastic form by mechanical and thermal treatments with the use of plasticizers (Sreekumar et al., 2012). A prominent trend is the blending of polymers, be it biopolymer or petro-plastic-producing hybrid or multi-compositional polymers (Morro et al., 2016). This allows the characteristics from both polymers to be imparted into the film matrix in the optimal blend ratio to produce the desired

properties. The main difficulty with employing this approach is the inter-compatibility of the polymers, ergo the use of either alteration of the primary or secondary polymer or plasticization (Dang and Yoksan, 2015). Apart from the manipulation of the polymer matrix to yield altered physico-chemical properties, the impact of antioxidant addition has also been investigated (Bonilla et al., 2013).

2.4.2.2 Cellulose films

Cellulose films have also been studied extensively. Cellulose has been used in polymer films as a primary polymer, co-polymer component or as an additive. Modified cellulose has also been used in film research, namely methylcellulose (Peressini et al., 2003), whereby the main modulator of properties of the film was determined by the concentration of the primary polymer. With the use of a more modified base polymer in conjunction with additives, the range of properties of the film exhibits vastly distinctive characteristics in contrast to the original polymer. Beeswax (BW) was used with hydroxypropyl methylcellulose (HPMC) as a topical fruit coating to evaluate the impact of BW on HPMC utilizing the fruit quality and shelf life over a 4-week period (Navarro-Tarazaga et al., 2011). The preparation of SiO_2/cellulose nanocomposites has been reported as exhibiting encouraging characteristics for distinct potential applications (Carro et al., 2013). While cellulose biodegradation in itself is particularly good, once polymers are synthesized from cellulose-based molecules, their biodegradability profile may change, either improving or being hindered.

2.4.2.3 Chitosan films

Chitosan as an amply available and low-cost biomaterial can be simply formed into various semi-solid and solid structures under mild conditions (Szymanska and Winnicka, 2015). Chitosan is a very encouraging biopolymer due to its environmental friendliness because it is biodegradable and displays exceptional film-forming properties. Chitosan is soluble within acids with a maximum pH of six, allowing many organic and dilute inorganic acids to be used in the production of films. Pairing chitosan with organic acids produces films in line with the general theme of being eco-friendly as well as biocompatible and meeting the grade required in terms of the use of the films. Mostly these wholly biologically derived films have applications in the biomedical, food, pharmaceutical and biotechnological industries (Table 2.1). While the

use of harsher solvents may produce more robust films, many of the niche biologically inclined properties of chitosan are no longer usable due to the presence of allergenic, irritant components which have a degree of toxicity.

Casting is the most popular method to produce chitosan films (Portes et al., 2009). For films in which chitosan is an additive or lower total content secondary copolymer, other means of film production become viable due to the properties of the primary polymer. Chitosan has been used with a plethora of compatible matrix modulators, which in turn, has produced a wide variety of chitosan-based polymers (Lewandowska, 2015). Many of the studies in the literature focus on the use of chitosan's innate antimicrobial activity, which has been incorporated into the produced films with varying degrees of effect (Dutta et al., 2009; Higazy et al., 2010; Martínez-Camacho et al., 2010; Rodríguez-Núñez et al., 2012; Leceta et al., 2013; Escarcega-Galaz et al., 2018).

Chitosan biodegradability is similar to that of cellulose, which is not surprising given the overall similarity in their structure. Biodegradation studies usually use bacteria, enzymes, simulated composting as well as a combination of all three to assess the biodegradability of the biopolymer films (Harish Prashanth et al., 2005; Sztuka and Kołodziejska, 2007). In contrast to the above, the further modified the polymer that is used, the more likely the biodegradation profile will change as additions and modifications are mostly aimed at improving the physico-chemical properties, which in turn will likely make the biopolymer more resistant to degradation. This will mostly depend on the methods employed in the polymer alteration. The functional groups which can be used as possible modification reaction sites are shown in Figure 2.4. Functionalization based on the reactive primary and secondary hydroxyl (–OH) and amino (–NH2) functional groups permits the following options of chemical modifications; acylation; alkylation; carboxymethylation; phthaloylation; quaternization; sulfation; thiolation and graft co-polymerization (Pokhrel and Yadav, 2019). While functionalization is highly dependent on the additives used in the given polymer matrix, the availability of the required functional group site specifically the modification reaction may not be viable as the functional group site may be already occupied by an additive or crosslinking. Differently, functionalized chitosan polymers with altered composition will have varied properties, therefore the desired application may change dramatically purely based on the preparation and components.

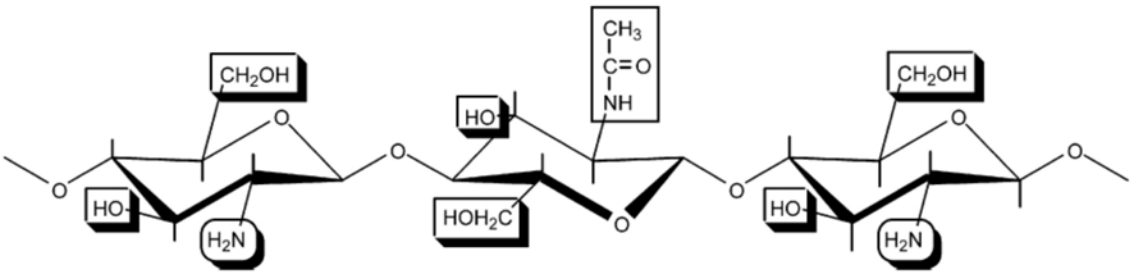


Figure 2.4 Illustration of the possible reaction sites in chitin and chitosan (Pillai et al., 2009), indicated by the boxes.

2.4.3 Characterization of polymers

A polymer's function is dependent on its characteristics. Characterization of said properties elucidates the relationship between the processing methods and the film components when manipulating the formulation.

2.4.3.1 Mechanical strength

Texture profile analysis (TPA) is a double mechanical compression test, usually used for food-based samples. Hardness, Cohesiveness, Viscosity, Elasticity, and Adhesiveness main textural properties determined by TPA. The compression of a sample by a set distance provides the measurements by analysing the force it takes for the sample to deform. TPA has configurable many parameters which is one of the main advantages (Paredes et al., 2022). The two most common test methods used for films and sheets are tensile strength and elongation at break. The mechanical properties of the sample are investigated by applying forces in opposite directions which will aid in understanding the behaviour of the material (Siracusa et al., 2008). To calculate the tensile strength of the sample, divide the maximum load at the fracture of the film by the original minimum cross-sectional area. Calculation of the elongation can be determined by dividing the elongated film by the initial length. (Peressini et al., 2003; Ku et al., 2011; Prommakool et al., 2011). When rheological and texture profile analyses data are used conjunctively, they enhance the definition of the properties of polymers which help in determining procedure parameters for managing them during processing. Mechanical testing data in the form of stress/strain curves, supply information about the elongation, flexibility, and toughness that can be utilized to anticipate the films' performance (Hernandez-Izquierdo and Krochta, 2008). TPA has limitations for sample preparation and the environmental

conditions for the duration of testing. Environmental characteristics like relative humidity and temperature are detrimental to certain susceptible films. Films may absorb moisture from the environment or release water into the environment during analytical testing. In the instance of such occurrences, properties may change and end-use behaviour and may impact by altering the mechanical properties of the film. While a reduction in moisture concentration may increase the brittleness, given that a small amount of water can function as a plasticizer within a polymeric structure (Boesel, 2015).

2.4.3.2 Physical properties

Water solubility is a fundamental characteristic, given the requirement for polymers to be insoluble. The water solubility must be sufficiently low enough (5% and below) to maintain rigidity for cases when they are used as a packaging material or storage vessel, which may also react with the contents if water vapour condenses on the surface of the film. The thickness of the film depends on the specific application as it is also needed to determine many more characteristics, in conjunction with density which indicates the mass per volume ratio of the polymers in the final film, as grading of polymers in some instances is based on the density. Mass and density can be used to infer the level of deacetylation in specific relation to chitosan and chitin. Chitosan of varying degrees of deacetylation will possess a different molecular weight, as per the percentage of the polymer with or without the acetyl group on each molecule of chitosan. 100% deacetylated chitosan has had all acetyl groups removed whereas 75% deacetylated chitosan still has 25% of acetyl groups present. Solids content indicates the total number of solids of the polymer after exposure at 105°C, as it gives an indication of the polymer composition after formation. Solvent content indicates the total number of solids of the polymer after exposure at 105°C, as it gives an indication of the polymer composition after formation just as the solids content with looking at polymer phase as it is not solid nor liquid but a combination.

2.4.3.3 Thermal properties

Transition zones are phases when a polymer is heated from a low to a high temperature, it passes through these phases sequentially, described as rigid, thermoelastic, and thermoplastic (Trimukhe and Varma, 2009). Specific properties that are representative of that material will

be demonstrated in each zone. Transition temperatures are the temperatures at which these properties change. When a polymer is heated at a controlled rate from low to high temperatures, it will experience the glass transition temperature (Tg), followed by the melt temperature (Tm). Before reaching Tg, the material is described as in a glass state (brittle) at this point there is no movement in its molecular structure. Beyond the Tg, vibrational movements of the functional groups, the polymeric chain occurs and/or segmental mobility. At this phase, the polymer is known as being in an elastic state. With a further rise in the temperature, the material begins to flow as the polymeric chains have become adequately disarranged, this point is known as the melt temperature (Tm). Crystalline polymers have another phase, the crystal-melt range, which is usually between Tg and Tm. In the case of amorphous polymers, Tm is generally called the softening point. The characteristics of the polymer constituents are generally considerably affected by the temperature change. Heat sensitivity is common for most polysaccharide-based materials and exceeding appropriate drying temperature may cause the browning reaction to occur. The temperature will affect the dehydration of protein polymeric chains and protein degradation. Additionally, high temperatures may result in lipid oxidation to lipid-based films (Saurabh et al., 2015).

2.4.3.3.1 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is mostly utilized to distinguish distinctive polymeric materials. Polymers and their composites or blends have unique thermal properties which supply valuable information concerning stiffness, toughness, stability, and miscibility with other compounds. Weight changes in a sample are measured under controlled conditions during the TGA temperature program (Ou et al., 2010). Inside the TGA instrument, a sensitive scale is used to precisely determine weight differences in the sample during the test. TGA is used to understand the correlations between sample weight differences and temperature. The smooth decreasing lines of the weight-loss curves indicate the percent weight changes and sharp peaks identify the temperature where weight loss is most apparent. Weight differences may be a result of material decomposition, release of volatiles and water loss (Moussout et al., 2016). TGA has been utilized to investigate the thermal degradation of chitosan-based polymers (Ou et al., 2010; Moussout et al., 2016). TGA temperature range may start from ambient room temperature and up to as high as 1000°C. The testing conditions must be precisely controlled, and the accuracy of the system continuously monitored using standards

for the entire duration of the test. Sample preloading is not allowed, especially in the case of sensitive samples, the water content in the sample may change during the waiting period. The differences in the sample weight over the test temperature range are shown in the TGA thermogram. It is best advised to study the peak transition areas and compare differences in their areas, intensities, and shapes to fully understand the thermal degradation patterns. DSC (Differential scanning calorimetry) and TGA are utilized conjunctively to obtain a clearer understanding of the thermal properties of the material (Monteiro et al., 2012).

2.4.3.3.2 Dynamic mechanical analysis (DMA)

When DMA is illustrated, the response of the sample to an applied deformation force in terms of stress, temperature, and frequency can be viewed. DMA is based on the fundamentally distinctive feedback of viscous and elastic components in the sample at a controlled temperature. It applies a vertical force (controlled strain or controlled stress) to the material and measures the viscoelastic characteristics of the sample. It is like texture profile analysis as it determines recovery and shape change (Lodi and Vodovotz, 2008). Additionally, DMA can report Damping, which is the amount of energy that is absorbed by the material to display elastic behaviour and Creep-recovery, which is a test that determines how much strain had recovered in the sample after omitting the applied force (Costa et al., 2016). DMA capabilities extend to determining the glass transition temperature by indicating the changes in heat capacity and the breadth of the transition. In more complex polymeric matrices, DMA indicates more visible transitions and better sensitivity which is required for detecting Tg in comparison to DSC. Furthermore, DMA is a better tool for assessing the thermal characteristics of a material in comparison to DSC and texture profile analyser methods. It has a controlled system for both stress/strain and temperature of the material (Pereda et al., 2014; Dang and Yoksan, 2015).

2.5 Additives to Biopolymers

As with traditional petro-plastics, there are ways to improve their innate properties namely using certain components and/or processes to either change the original polymer matrix such as cross-linking the intra-polymer chains (Harish Prashanth and Tharanathan, 2006). The additions are physically, chemically, or enzymatically introduced into the polymer matrix. Either by interacting between the existing polymer chains or by incorporating them into the original polymer matrix chain (Sahoo et al., 2012). Multiple additives may be used simultaneously to employ all the above methods in a single polymer.

2.5.1 Lipids

A main aim of lipid inclusion into composite films has been to lessen the water vapour permeability of hydrophilic materials. A multitude of lipids have been used, essential oils have been used to improve the water-based applications of biopolymers as well as the antioxidant and antibacterial characteristics (Bonilla et al., 2011). Buriti oil has been incorporated into chitosan films to serve as an active packaging material (de F. Silva et al., 2016)

2.5.2 Polysaccharides

Polysaccharides, already being a polymer of monosaccharides, would technically be co-polymers when used as additives to the primary polymer matrix. Using nanocrystals and nanoparticles to fortify nano-biocomposite films has been conducted (Fortunati et al., 2014), where silver nanoparticles and cellulose nanocrystals were used. Cellulose has also been used as whiskers to reinforce composites. The impact of cellulose on the composite yielded significant improvement in the tensile strength but has varying physico-chemical properties depending on the cellulose whisker concentration (Bras et al., 2010). Cellulose nanocrystals were also used to enhance mechanical properties (Pereda et al., 2014).

2.5.3 Proteins

Similarly, proteins are polymers of amino acids as opposed to monosaccharides. They again would act as co-polymers to the primary polymer matrix. There are a multitude of candidate proteins that can play this role. Proteins which can be used as additives in biopolymers include

collagen, corn zein, gelatin, milk proteins, myofibrillar proteins, soy protein and wheat gluten (Hernandez-Izquierdo and Krochta, 2008). Due to their ability to form films, they can function as an additive or stand-alone polymer but in the case of chitosan-compatible protein-based additives are limited due to the nature of chitosan and the production process of chitosan-based biopolymers. To enhance the barrier, functional and physical properties of both chitosan and whey protein films, laminating and blending these two materials has been suggested (Kurek et al., 2014). The results showed the potential production of transparent WP/CS bilayer films with improved mechanical resistance and higher water vapour barrier efficiency.

2.5.4 Inorganics

The addition of inorganic molecules such as nanoclay to composite films has also been investigated. Montmorillonite (MMT) is the most commonly investigated type of clay which is a hydrated alumina-silicate layered clay (Kasirga et al., 2012). This nanocomposite film is aimed at improving the mechanical and physical properties of biopolymer films (Abdollahi et al., 2012). Polymer/clay composites often display multiple attributes, including biodegradable, thermal and mechanical characteristics that are better than conventional composites (Lewandowska et al., 2014). Magnesium stearate is an example of an additive used in starch biopolymers. It is needed for dry processes such as extrusion (Davidovich-Pinhas et al., 2014). This procedure includes the grafting of polymeric side chains to chitosan, a known antibacterial biopolymer, to improve its affinity with bulk plastic material.

2.5.5 Solvent

In wet processes for biopolymer formation, solvents contribute greatly to creating a proper distribution of the composite constituents in the film matrix solution. When casting the biopolymers, the rate of evaporation of the solvent will impact the time required and the curing process. It has been reported that the drying rate can be improved by up to 30% by increasing ethanol concentration in the polymer film matrix. The viscosity of the chitosan film solution is directly affected by the ethanol concentration and organic acid species. The inclusion of ethanol did not alter thermal characteristics, but the organic acid type utilized will have a greater effect on TGA degradation patterns e.g., chitosan films made with acetic acid versus lactic acid have differing weight loss in the 115-120°C range. (Lin, 2012).

2.5.6 Plasticizer

A plasticizer is an additive which normally is incorporated to make the polymer malleable and amenable to process parameters. Plasticizers can also be used to alter the properties of the film. Some react with the primary polymer by influencing its' innate properties while others co-polymerize with the original polymer forming a hybrid polymer chain (Hernandez-Izquierdo and Krochta, 2008). Amidst the numerous plasticizers, water is generally a good plasticizer, but it is very problematic to control its quantity during heat transformation (Sreekumar et al., 2012). The role of plasticizers is to assist with the mobility of the polymeric chains and to reduce the glass transition temperature (Félix et al., 2014). Glycerol in conjunction with water reduces the mechanical properties of films in the casting of films but improves, in certain ratios, dry processes of extrusion bioplastic formation, with no effect on thermal stability (Chen et al., 2018).

2.6 Applications of Biopolymers

Biopolymers can substitute for all current plastic applications in various industries such as bioactive films, using additives or functionally active groups or as inert films. The recent trend of producing biopolymer-petrochemical plastic blends yields significant improvement. The inclusion of 0.37–1.45% of chitosan improved thermal stability, strength (tensile strength increased ~8–97%), and stiffness (Young's modulus increased ~40–154%) (Dang and Yoksan, 2015; Lewandowska, 2015).

2.6.1 Food processing applications

Biopolymer films, coatings and packaging supply additive-releasing functions, protection and convenience without altering the original components or the processing procedure for food items. They can be affixed to the product or become incorporated into food products. Biopolymer packaging can be utilized to protect a food item from ageing, microbial contamination, and moisture loss, to improve appearance, and mechanical characteristics and to fortify nutritional value (Dutta et al., 2009). Nutritional components such as vitamins can be added into the film matrix thereby improving the nutritional value of the food.

2.6.2 Biomedical applications

Tissue engineering using biopolymers as bone tissue matrix scaffolds, and chitosan biopolymer-based stents are only but a few of the biomedical applications. Implementations in patients proved adequate in terms of good healing indicating satisfactory biocompatibility and tissue reaction. Acute mutagenicity, toxicity, and pyrogenicity were negative in all respects (Pillai et al., 2009). The chitosan monomer, NAG (N-acetyl glucosamine) can be used in medical supplements for joint and cartilage damage repair, for example, in homoeopathic medicines like “OsteoEze” and allopathic supplements such as “OsteoFlex.” Both contain a significant content of NAG. For corneal tissue engineering, Ultrathin chitosan–poly(ethylene glycol) hydrogel films have been tested in research with promising results (Ozcelik et al., 2013).

2.6.3 Industrial applications

Coagulants and altered solubility for membranes are facilitated by modifications of the hydroxyl and amino functional groups on the chitosan polymer (Pillai et al., 2009). Transparent and hydrophobic silica-chitosan hybrids can find use-case in the optics industry (Carro et al., 2013).

2.6.4 Pharmacological applications

Film-based drug delivery systems whereby adhesive biopolymers diffuse desired drugs through the skin of the patient into the bloodstream with controlled release over time, are facilitated by the degradation of chitosan by the skin microflora. The creation of controllable release carriers using sodium cellulose sulphate (NaCS) and polyelectrolyte complex (PEC) of chitosan was investigated and yielded positive results (Zhu et al., 2010). The release time was varied and was dependent on the composition of the film as such drugs with specific targeted gastrointestinal tract locations as well as drugs which require slow release can be used in drug delivery systems.

2.6.5 Biotechnological applications

Chitosan can serve as carriers for active molecules like antioxidants (Jung and Zhao, 2012). Electrolyte-complexed chitosan can also be used in biosensors (Upadhyaya et al., 2013).

Construction biotechnology involves using biopolymers as additives in structural components for building infrastructure. These inclusions increased the strength of concrete by 10–20% after 3 days of curing, irrespective of different mixing methods or curing parameters (Stabnikov et al., 2015). Using chitosan as an admixture biopolymer, has reduced drip erosion, improved mechanical properties and contributed to both hydrophobicity along with resistance to water erosion (Aguilar et al., 2016).

2.6.6 Cosmetic applications

The use of producing cosmetic packaging, which is more tactile and apo-allergenic for individuals who have sensitivity issues with traditional petrochemical-derived plastics. Chitosan in polymer form could be used as a thickening agent in hair care products (Rinaudo, 2006).

2.6.7 Bioremediation, wastewater and water purification

Chitosan as a membrane, a component of membrane, or as a membrane-coating are the major forms in which chitosan is used in this field (Shanthana Lakshmi et al., 2017). Mercury removal from groundwater has been investigated by use of the chelating property of chitosan (Miretzky and Cirelli, 2009). Source and DD were the main factors in the adsorption capacity of the chitosan and were compared to that of other low-cost adsorbents.

2.7 Scope of the Study

Given the limitations mentioned above of the supply of crustacean chitosan and proven in previous unpublished work, fungal chitosan is indeed comparable to crustacean chitosan. It was hypothesized that the fungus *M. circinelloides* would be able to grow on WS containing fermentable carbohydrates and sugars. The chitosan was extracted from the fungal biomass after being grown in different waste substrates. The quantity of the chitosan was determined and characterized by various physical methods. Biopolymers were made with the obtained chitosan and analyses were conducted to determine any differences in their composition. Additives were used to further improve the characteristics of the produced fungal chitosan biopolymers, namely thermomechanical, physico-chemical, morphological and barrier properties.

CHAPTER 3 : FUNGAL FERMENTATION OF WASTE SUBSTRATES AND CHITIN EXTRACTION, DEACETYLATION

3.1. Introduction

Microorganisms can utilize a range of substrates as carbon sources depending on their availability through preferred metabolic pathways for the production of vital metabolites. This allows for the utilization of waste substrates (WS) as a cost-effective alternatives to costly laboratory medium components for the generation of biomass and bioproducts (Cazetta et al., 2007; Jiang et al., 2009; Teclu et al., 2009). Fungal saprophytes with specialized enzymatic machinery to degrade decaying organic matter, are good candidates for utilizing agricultural waste residues (Mohammad et al., 2013; Xin et al., 2013; Saratale et al., 2014). Determining the optimum nutritional and physical parameters are essential to achieving maximum microbial growth and bioproduct yield using complex substrates. Screening viable substrates depends on the compatibility of the culture with the growth medium (Pradeep and Reddy, 2010). Fungi prefer an acidic pH with good aeration and moderate agitation. Waste substrates would need to be treated or diluted sufficiently to be used as the primary fermentation medium component. Many components may be inhibitory or unavailable to the culture when used in their original form due to the individual compositional form of the substrates (da Silva et al., 2012).

Fermentable sugar and carbohydrate content in the media are key for fungal biomass yield, and hence in chitin production. Chitin and chitosan in certain fungal genera are produced as structural cell wall components. The chitosan polymer chain and monomer unit are remarkably similar to cellulose, a glucose polymer. The difference is that chitosan is an aminated glucose polymer. Without an appropriate quantity and quality of carbon source in the medium, both biomass and chitin yield will be minimal. Higher concentrations of sugar in the medium are inhibitory to most microbes. Therefore, the formulation of a fermentation medium with the precise quantity of sugar required for the fermentation period is necessary (Singh et al., 2016). Fermentation scale ranges from small to large (industrial/commercial) with generally three main methods of operation: batch; fed-batch; and continuous fermentation. Batch fermentation was used in this study.

Using laboratory media for the culturing of strains has certain caveats, as it may not be the best medium for growth and expression of a strain's characteristics. In many instances, a natural medium is more appropriate, except for fermentations aimed at yielding high biomass. Strains cultured only on defined media may also lose certain properties and characteristics over generations of sub-culturing such as the ability to metabolize certain substrates, thermal regulation systems, and plasmids obtained from the natural environment (Rosano and Ceccarelli, 2014). Laboratory media is also significantly more expensive during scale-up operations, hence the most cost-effective medium is sought after for mass production. Agri-waste substrates possess the necessary components for both high biomass fermentation and maintaining the innate wild-type strain characteristics. Processed plant material contains fermentable carbohydrates/sugars, amino acids, and nitrogenous compounds, all of which can be utilized for culture growth. Blends of various agri-wastes can be used to obtain an optimal fermentation feedstock.

Zygomycetes are known for relatively high chitosan content in comparison with other fungal genera (Zamani et al., 2010; Fai et al., 2011). *Mucor* species contain chitin deacetylases which causes the cell wall composition to vary between a chitosan/chitin polymer, as the chitin is deacetylated to produce chitosan (Dhillon et al., 2013). This allows easier, rapid and low-energy intensive extraction and deacetylation of chitin to chitosan, in contrast to traditional crustacean chitosan extraction. Crustacean chitosan extraction requires the use of multiple harsh reagents at higher concentrations with more processing stages when compared to that of fungal chitosan extraction (Kaur and Dhillon, 2014).

This chapter focuses on the physico-chemical analysis of MOL, CSL, DBW, statistical optimization of fermentation parameters of the WS using *M. circinelloides* and the extraction and deacetylation of the fungal chitin to chitosan.

3.2 Materials and Methods

3.2.1 Strain and media

Mucor circinelloides ZSKP (Zininga et al., 2019) was obtained from the Enzyme Technology Research Group, Department of Biotechnology and Food Science, Durban University of Technology. Potato dextrose agar (PDA, Sigma) plates were used for the growing the culture. Agar blocks (1 cm^3) of *M. circinelloides* ZSKP were used for the inoculation of agar plates and incubated for 3 days in an incubator (Infors HT) at 30°C (Fai et al., 2011). Potato dextrose broth (PDB, Acumedia) was used for liquid cultivation. Furthermore, the strain was stored as a glycerol spore suspension. The glycerol spore suspension was preserved at 4°C for short-term storage and frozen at -80°C for long-term storage. A freshly sub-cultured agar plate and spore suspensions adjusted to 1×10^9 and 1×10^5 spores/ml for upper and lower limit, for each fermentation.

3.2.2 Physico-chemical characteristics of waste substrates

Three substrates were used as fermentation media: beverage waste from a local beverage company in Durban (DBW); corn steep liquor from Tongaat Hulett Starch in Germiston, Johannesburg (CSL); and sugarcane molasses from the Sugar Milling Research Institute (SMRI) in Berea, Durban (MOL). The solids content was calculated using the mass of dried (24 h at 105°C) substrate divided by the initial weight. The density (g/ml) was calculated using 100 ml of the WS, after determining the mass using an analytical balance. Solvent content was calculated using the difference in mass of dried substrate divided by the initial weight (Beigzadeh Ghelejlu et al., 2016). The pH of WS was determined with a pH meter.

3.2.3 Nutritional, minerals and metals analyses

The nutritional, minerals, and metals analyses (NMM) of the WS was outsourced to Food and Cosmetic Technologies, Durban (Fact Labs). The following components were quantified: moisture; ash; fat; dietary fibre; protein; nitrogen; sucrose; fructose; glucose; lactose; maltose; total sugars; total carbohydrates; energy; sodium; calcium; magnesium; potassium; phosphorus; iron; copper; and zinc. The methods that were used are listed in Table 3.1.

Table 3.1 Nutritional, minerals and metals analyses conducted on the waste substrates.

Analysis	Method Description
Moisture	Determination of moisture using the air oven method – AOAC 925.10
Ash	AOAC 942.05
Protein	Kjeldahl method
Fat	Acid hydrolysis - AOAC 922.06
Nitrogen	Kjeldahl method
Dietary fibre	AOAC 991.43
Total sugars	Determination of sugars using HPLC
Energy	Determination of energy using the Atwater System by calculation
Total carbohydrates	Determination of carbohydrates using the Atwater System by calculation
Sodium	Determination of sodium content using flame photometry
Minerals and Metals (Ca, Mg, K, P, Fe, Cu, Zn)	ICP – MS

3.2.4 Fermentation and biomass production

One millilitre of *M. circinelloides* spore suspension (1×10^9 spores/ml) was inoculated into 500 ml shake flasks containing 150 ml medium for 5 days at 30°C, under submerged fermentation at 150 rpm. The fermentation feedstocks consisted of a single waste substrate at multiple concentrations and a 1:1 ratio blend of MOL and CSL (Figure 3.3). DBW was omitted after initial fermentations as the yield obtained was minimal, as both a blend and as a standalone fermentation substrate. The fermentation medium was filtered using a 180 µm filter to recover the biomass and the supernatant was discarded. The biomass was dried to constant weight in an oven at 45°C. The dry biomass was measured on a weighing balance.

3.2.5 Plackett-Burman Design, statistically significant parameters identification

To study the effect of waste substrates (MOL and CSL), alternative carbon and nitrogen sources and varying physico-chemical conditions on biomass production were tested. Two levels of each variable were used. Design Expert software (Version 6.0, Stat-Ease Inc.) was used for Plackett-Burman Design (PB). The design comprised of twelve experimental runs in triplicate with nine variables and two unassigned variables, as indicated in Table 3.6. Biomass was quantified in triplicate after each experimental run and the effects of variables were determined using the following equation:

$$E\ (Xi) = \frac{2\ \sum C_i^+ - \sum C_i^-}{2}$$

where C_i^+ and C_i^- are the biomass values produced levels from the experiments where the variable (X_i) was present at upper (+1) and lower (-1) limits, respectively. N is the total number of experiments. Student's t-test was used to determine the significance (p-value) of each variable (Singh et al., 2016).

3.2.6 Optimization of biomass production by Response Surface Methodology

The most significant variables affecting biomass production, identified by PB, were chosen as independent variables. Thereafter, Response Surface Methodology (RSM), using central composite design (CCD), was used to investigate the interaction between these significant variables (Kara Ali et al., 2017). A total of twenty experiments were carried out in triplicate using several combinations of the variables, as listed in the CCD shown in Table 3.8. The conditions predicted by the statistical model for biomass production were validated by conducting fermentations in 150 ml shake flasks.

3.2.7 Chitosan extraction

A modified approach combining the methods of Naghdi et al. (2014) and Zamani et al. (2010) was adopted for the extraction of chitosan. Acids, (H_2SO_4 and HCL) and alkali (NaOH) at (varying) concentrations were used. Changes were also implemented to the time periods and repetition of steps to ensure complete extraction. Further modifications to the extraction method were employed from the method described by Abdou et al. (2008). For the preparation

of alkali-insoluble material (AIM) of biomass, dried mycelium of *M. circinelloides* was treated with 0.5 M NaOH (30 ml/g) at 121°C for 20 min. Alkaline insoluble material was separated from the mixture by centrifugation (15 min, 4000 × g), washed with distilled water to neutral pH, and stored at 4°C until use. Extraction of chitosan from phosphate-free AIM, was accomplished by extensive depolymerization of chitosan during dissolution in hot sulphuric acid and exploiting the insolubility of chitosan in sulphuric acid at lower temperatures. The AIM was first treated with 0.1 N H_2SO_4, (100 ml acid per gram of dry AIM), at room temperature for 30 min to remove phosphates. Without this step, phosphates forms random bonds with the polymer units when the chitosan is soluble (Zininga et al., 2019). In the next step, the phosphate-free cell wall derivative was subjected to extraction with hot sulphuric acid (120°C) for 45 min to extract the chitosan. Finally, the dissolved chitosan was precipitated at an alkaline pH (8) and recovered at a lowered temperature (8°C). The solid phase was separated by centrifugation (10 min, 4000 × g) and washed three times with distilled water. The chitosan was dried at -80°C (Alpha 2-4 LDplus freeze dryer, CHRIST) and the weight of the dry chitosan was determined using an analytical balance.

3.2.8 FTIR analysis

The chitosan produced was characterized by Fourier-transform infrared (FTIR) spectroscopy in KBr pellets using an infrared spectrophotometer in the range of 400 to 4000 cm^{-1} (Abdou et al., 2008). The transmission spectra were compared to the spectrum produced by commercial chitosan extracted from crustacean shells (Sigma). The FTIR analysis was outsourced to the School of Chemistry and Physics at the University of KwaZulu-Natal, Westville.

3.2.8.1 Degree of deacetylation (DDA) of chitosan

Data obtained from FTIR spectra was used to calculate the DDA of chitosan using the following equation (Mohanasrinivasan et al., 2014):

$$\mathrm{D}\ (\%) = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \times \frac{100}{1.33}\right]$$

where A_{1655} is the absorbance at 1655 cm^{-1}, corresponding to the amide-I bond, indicating the amount of N-acetyl groups. The absorbance at 3450 cm^{-1} (A_{3450}) represents the hydroxyl groups. The theoretical value for the A_{1655}/A_{3450} ratio for fully deacetylated chitosan was assumed to

be zero. A linear correlation was reported between the absorbance ratio (A_{1655}/A_{3450}) and the DDA of chitosan (Baskar and Sampath Kumar, 2009; Kaya et al., 2014).

3.3 Results

3.3.1. Physico-chemical properties of waste substrates

The results for the physico-chemical properties of WS influenced the selection of future methods based on the intrinsic properties of WS. Substrates with characteristics not compatible with fermentation parameters were diluted, detoxified, pH-adjusted and manipulated for use as a feedstock. All three WS samples showed acidic pH values, with DBW having the lowest pH of 3.25. CSL was the next most acidic substrate with a pH of 3.97, while MOL was the least acidic substrate with a pH of 6.0. The solids content of the substrates ranged considerably from 6.78% for DBW, to 49.38% for CSL and to 74.63% for MOL. The aqueous content values also showed a varied distribution with 93.22% for DBW, 50.62% for CSL and 25.31% for MOL. The density of all substrates was within 0.4 g/ml of each other. DBW had the lowest density of 0.98 g/ml. CSL had a density of 1.12 g/ml while MOL had the highest density of 1.38 g/ml. The results of the physico-chemical analysis are shown in Table 3.2.

Table 3.2. Physico-chemical characteristics of waste substrates

Substrate	pH	Solid's content (%)	Aqueous content (%)	Density (g/ml)
CSL	3.97	49.38	50.62	1.12
DBW	3.25	6.78	93.22	0.98
MOL	6	74.63	25.31	1.38

3.3.2 Nutritional, minerals and metals analyses

The NMM profiles of MOL and CSL consisted of twenty-two characteristics (Table 3.3). The percentage sugar content of MOL is tabulated in Table 3.4. Analysis of DBW was omitted due to poor biomass yield and its insignificant effect in preliminary PB experiments.

Table 3.3 Nutritional, minerals and metals profile of the waste substrates

Analysis	MOL	CSL
Moisture (g/100 g)	20.8	54.7
Ash (g/100 g)	9.8	5.9
Fat by acid hydrolysis (g/100 g)	0.7	0.3
Dietary fibre (g/100 g)	0.9	2.8
Protein (g/100 g)	4.2	21.5
Nitrogen (g/100 g)	0.7	3.4
Sucrose (g/100 g)	25.6	< 0.3
Fructose (g/100 g)	3.9	< 0.2
Glucose (g/100 g)	1.4	< 0.1
Lactose (g/100 g)	< 0.3	< 0.3
Maltose (g/100 g)	< 0.3	< 0.3
Total sugars (g/100 g)	31.0	< 0.3
Total carbohydrates (g/100 g)	63.6	14.8
Energy (kJ/100 g)	1114.9	613.4
Sodium (mg/100 g)	41.8	20.4
Calcium (mg/100 g)	8.7	0.2
Magnesium (mg/100 g)	436.1	492.1
Potassium (mg/100 g)	63.4	32.6
Phosphorus (mg/100 g)	72.1	1199
Iron (mg/100 g)	8.5	7.5
Copper (mg/100 g)	0.2	0.3
Zinc (mg/100 g)	0.4	8.0

Moisture content in MOL was 20.8%, which was less than half of CSL (54.7%). The fat content of MOL was 0.7% while CSL was 0.3% which was less than half of MOL. Dietary fibre in CSL (2.8%) was 3.11 times higher than that in MOL (0.9%). There was 0.4 times more ash content

in MOL (9.8%) than in CSL (5.9%). Protein content was significantly lower in the MOL (4.2%) than in CSL (21.5%) which is also true for nitrogen at MOL (0.7%) and CSL (3.4%). MOL has 0.2 times the amount of CSL's nitrogen and protein. MOL contained 41.8 mg/100 g of sodium component in the substrate, and 20.4 mg/100 g for CSL. MOL contained double (2.05 times) the amount of sodium present in CSL.

The proportion of sugars present in MOL was much higher (>10-fold) than in CSL. Total sugars (TS) present in MOL and CSL were 31% and <0.3%, respectively. Sucrose concentration was highest among total sugars in MOL (25.6%), while it was less than 0.3% in CSL. Lactose and maltose were present at the lowest concentrations (<0.3% each) in CSL and MOL. MOL (3.9%) contained 19.5 times the amount of fructose compared to CSL (<0.2%). The glucose content present in MOL was 1.4% and it was <0.1% in CSL. Sucrose constituted the largest proportion (82.58%) of TS in MOL (Table 3.4), followed by fructose (12.58%) and glucose (4.52%). The total carbohydrate content (TC) of MOL was 63.6% (Zheng et al., 2015) which was 4.3 times the amount in CSL with 14.8%. MOL showed a significantly higher energy value of 1114.9 kJ, which was approximately 55% more than the energy value of CSL with 613.4 kJ. The ratio of mass to the energy of MOL was 1 g: 11.15 kJ and for CSL was 1 g: 6.13 kJ.

Table 3.4 Percentage of sugar content in MOL

Sugars	Percentage
Sucrose	82.58%
Fructose	12.58%
Glucose	4.52%
Lactose	< 1%
Maltose	< 1%

Calcium was one of the lowest mineral components quantified in both substrates from the NMM analysis (Table 3.3). MOL has a magnesium content of 436.1 mg/100 g, while CSL has 492.1 mg/100 g. There was only a ±0.05% difference in magnesium present in the two substrates. The analysis showed 63.4 mg/100 g of potassium in MOL, and 32.6 mg/100 g in CSL. CSL has half (0.51 times) of MOLs' potassium component. CSL phosphorus content was the

biggest outlier from the minerals and metals quantified with a value of 1199 mg/100 g. MOL had a phosphorus result of only 72.1 mg/100 g. Iron content in mg/100 g was 8.5 and 7.5 for MOL and CSL, respectively. Copper in MOL was equally as low as calcium in CSL. CSL had copper content slightly higher than MOL. Copper content in CSL and MOL was the closest analyses conducted with a difference of only ±0,0001%. MOL had twenty times less zinc than CSL.

3.3.3 Plackett-Burman Design (PB)

Optimization of biomass yield is an important prerequisite for achieving efficient chitosan production. Various parameters that could influence biomass production were investigated (Table 3.6). A PB with nine parameters was used for this study. Each parameter was studied at an upper and lower limit. A high variability in biomass data (12.1 to 63.2 g/L) was observed, which indicated the importance and suitability of PB for optimization experiments. Exp. 5 yielded the highest biomass of 63.2 g/L, while exp. 2 produced the second-highest biomass yield of 52.9 g/L. *M. circinelloides* was examined after every experiment to observe morphology, either as yeast or a combination of filamentous and yeast forms in flasks. Temperature, CSL, and MOL were identified as the three most significant ($p<0.05$) factors affecting biomass production (Table 3.5). The model F value of 14.21 was obtained for the best-fit linear regression model. The p value (Prob>F) of the PB model was 0.0258, and the coefficient of determination (R^2) was 0.9743.

Table 3.5 The significant factors and their p-values

Variables	p-value
CSL	0.0056
Temperature	0.0090
MOL	0.0458

Experiments 2 and 5 produced the highest biomass yields but differed in certain physico-chemical parameters. Exp's. 2 and 5 however had have identical parameters for MOL, CSL, soluble starch, peptone concentration and temperature. The differing parameters are pH, agitation, inoculum size and the incubation period.

Table 3.6 Plackett-Burman Design showing biomass production*

Exp. No.	MOL (%)	CSL (%)	Soluble starch (%)	Peptone (%)	pH	Temperature (°C)	Agitation (rpm)	Log inoculum size (spores/ml) (1×10^n)	Incubation period (h)	Biomass production (g/L)
1	8	1	1	0.25	5	27	250	9	168	51,1
2	8	10	0.25	1	5	27	50	9	168	52,9
3	1	10	1	0.25	8	27	50	5	168	46,6
4	8	1	1	1	5	37	50	5	72	14,3
5	8	10	0.25	1	8	27	250	5	72	63,2
6	8	10	1	0.25	8	37	50	9	72	47,2
7	1	10	1	1	5	37	250	5	168	33,5
8	1	1	1	1	8	27	250	9	72	36,2
9	1	1	0.25	1	8	37	50	9	168	12,1
10	8	1	0.25	0.25	8	37	250	5	168	15,4
11	1	10	0.25	0.25	5	37	250	9	72	36,17
12	1	1	0.25	0.25	5	27	50	5	72	19,33

*Exp. 2 (blue) and Exp. 5 (green) had the highest biomass production.

3.3.4 Effect of different factors on biomass yield

The effect of each variable was calculated as mentioned in section 3.2.5. Temperature, CSL, and MOL showed the highest effects on biomass production. CSL and MOL showed marked positive effects at their respective upper limits, while temperature negatively affected the biomass of *M. circinelloides* towards the lower limit. Overall, CSL showed the greatest effect on biomass production (Figure 3.1).

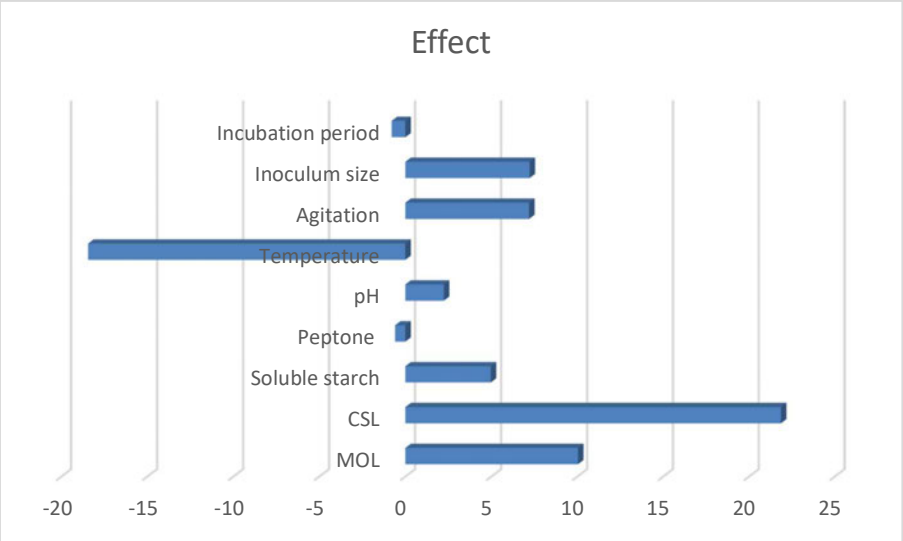


Figure 3.1 Pareto-graph showing the effect of each variable on the production of *M. circinelloides* biomass.

3.3.5 Response surface methodology using central composite design.

A central composite design was used to implement RSM with the intention to study the interaction between selected independent variables for enhanced biomass production. Twenty experimental runs for biomass production were conducted using selected significant factors. The observed responses of the CCD experiments are presented in Table 3.7, with the highest biomass production of 83.57 g/L obtained in run 10 and the lowest production was 18.25 g/L in run 9. The following polynomial equation explained the biomass production as a function of CSL (A), temperature (B) and MOL (C) as independent variables:

$$\textbf{Biomass production (g/L)} = 34.49 + 19.60A + 0.70B + 8.57C + 5.89A^2 - 0.76B^2 + 4.00C^2 - 3.97AB + 5.12AC + 4.25BC$$

Table 3.7 Central Composite Design

Run	A	B	C	Biomass (g/L)
1	4.03	27.03	2.82	20.47
2	9.97	27.03	2.82	57.99
3	4.03	32.97	2.82	21.1
4	9.97	32.97	2.82	42.05
5	4.03	27.03	8.18	18.88
6	9.97	27.03	8.18	76.2
7	4.03	32.97	8.18	35.83
8	9.97	32.97	8.18	77.92
9	2.00	30.00	5.50	18.25
10	12.00	30.00	5.50	83.57
11	7.00	25.00	5.50	30.24
12	7.00	35.00	5.50	33.94
13	7.00	30.00	1.00	30.77
14	7.00	30.00	10.00	60.36
15	7.00	30.00	5.50	34.91
16	7.00	30.00	5.50	34.87
17	7.00	30.00	5.50	33.87
18	7.00	30.00	5.50	34.18
19	7.00	30.00	5.50	36.00
20	7.00	30.00	5.50	33.20

*A, CSL (%); B, Temperature (°C); C, MOL (%)

Table 3.8 ANOVA analysis for optimization of biomass production using response surface methodology.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value (Prob>F)	Significance
Model	7448.68	9	827.63	1037.84	< 0.0001	significant
Residual	7.97	10	0.80			
Lack of Fit	3.23	5	0.65	0.68	0.6582	not significant
Total	7456.65	19				

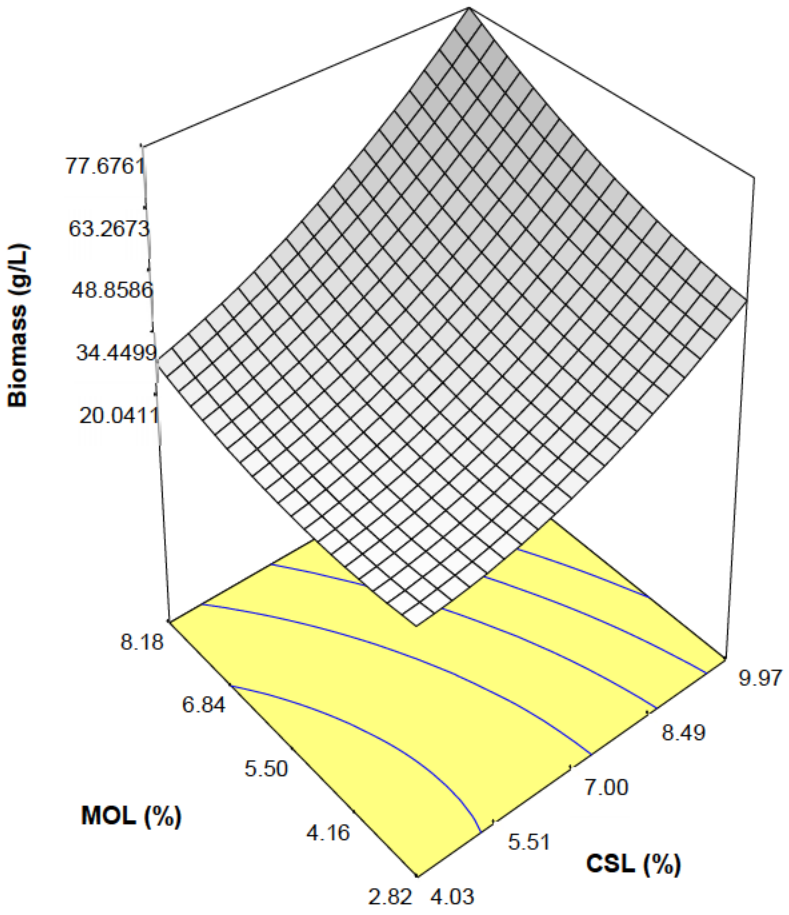
R^2 : 0.9989

Adjusted R^2 : 0.9980

Pred R^2 : 0.9958

The model F values of 1037.84 and 'Prob>F value' of 0.0001 implied the model was highly significant (Table 3.8). There was only a 0.01 % chance that the model F value could occur due to noise.

3D contour plots were generated from the RSM software analysis as a representation of the interaction of the two variables with the other variable being kept fixed at the central level. The RSM contour plot shown in Figure 3.2a shows the interactions between MOL and CSL. Optimal biomass production of 77.68 g/L was achieved with an MOL concentration of 8.18% and a CSL concentration of 9.97%. The second RSM contour plot (Figure 3.2b) shows the interactions between temperature and CSL. Optimal biomass production of 62.50 g/L was possible at a temperature of 27.03˚C and CSL concentration of 9.97%. Optimal biomass production is reflected by the apex point illustration on the contour. Any further increase in these pairings of variables hindered biomass production for both contours.


77.6761
63.2673
48.8586
34.4499
20.0411
Biomass (g/L)
8.18
6.84
5.50
4.16
2.82
MOL (%)
4.03
5.51
7.00
8.49
9.97
CSL (%)

(a)

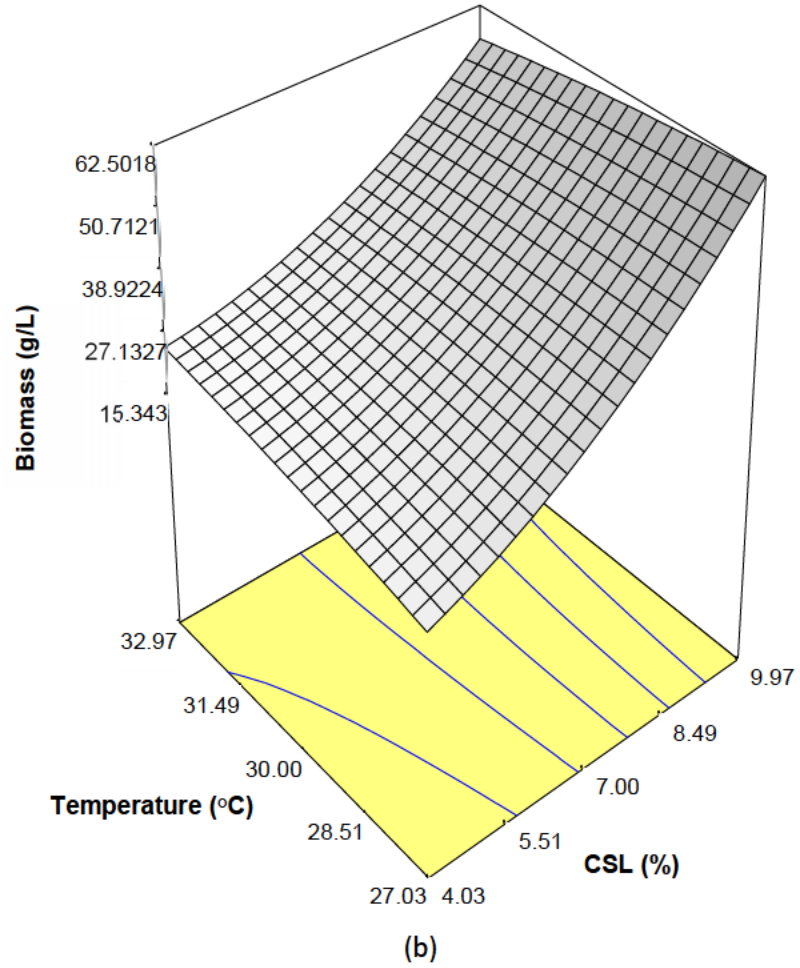


Figure 3.2 Response surface contour of the interaction between (a) MOL and CSL and (b) temperature and CSL

Validation of the RSM model was on the premise of comparing experimental yield against model predictions. Triplicate sets of experiments for the three significant variables were generated by the Design Expert software. Exp. Number 1 was exceptionally concurrent with the predicted value of 78.06 g/L and the observed biomass of 77.87 g/L, which represented an accuracy value of 99.76% (Table 3.9).

Table 3.9 Validation of RSM

Exp. Number	CSL (%)	Temperature (°C)	MOL (%)	Predicted Biomass (g/L)	Observed Biomass (g/L)	Accuracy (%)
1	9.97	30.02	8.18	78.06	77.87	99.76*
2	9.97	28.97	8.18	77.82	72.27	92.87
3	9.97	28.19	8.18	77.71	65.87	84.76

*Exp. 1 (green) had the highest biomass and best accuracy

3.3.6 Biomass and chitosan yield

The biomass obtained from the unoptimized waste substrate fermentations (UWSF) vs. PB, RSM and model validation fermentations varied interestingly (Figure 3.3). Exp. number 1 (Validation) biomass production of 77.87 g/L was a 2.65-fold increase over the highest yielding UWSF (2.5% MOL + 2.5% CSL). Single-component UWSF yielded lower biomass, ranging from 14.45 to 22.97 g/L. The combination of CSL with MOL yielded more biomass of 29.47g/L. DBW produced negligible biomass and was therefore omitted.

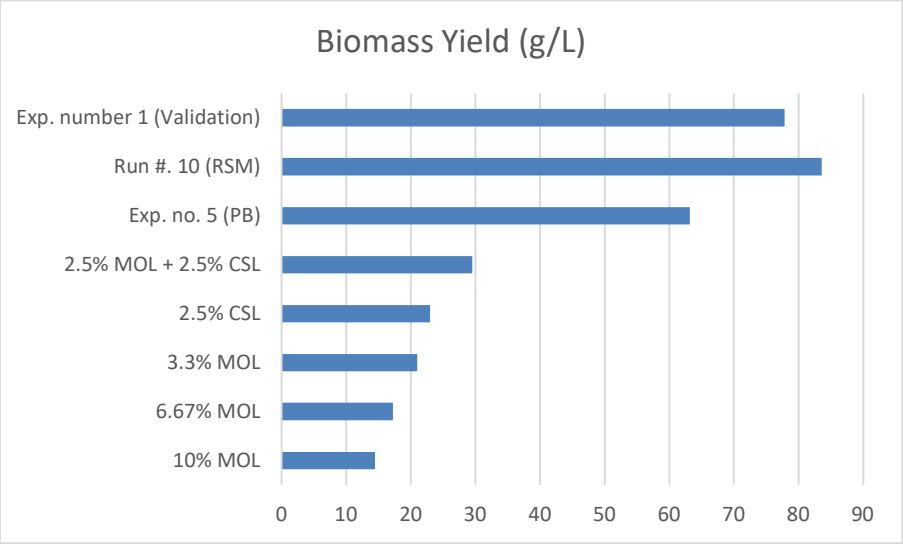


Figure 3.3 Effect of optimization on biomass yield

Table 3.10 Chitosan yield

Biomass (g)	Chitosan (g)	Chitosan/Biomass (%)	Chitosan/Substrate (%)
27.74	2.45	9.64	12.25
29.47	2.61	8.83	13.04
24.52	2.37	9.67	15.80

The fungal chitosan yield averaged consistently to 8-9% of the biomass produced. Highest observed chitosan yield was 9.67% (Table 3.10).

3.3.7 FTIR Spectra

FTIR spectroscopic analysis was conducted on (C) fungal chitosan, (A) NAG – monomer, and (B) crustacean chitosan to characterize the chitosan (Figure 3.4), as well as to calculate the DDA. The functional groups of chitosan were indicated by FTIR analysis. The NH bond was observed between 3000-3500 cm^{-1} and the C=O bond was observed between 1400-1650 cm^{-1}. The spectra shown in Figure 3.4b for commercial crab shell chitosan, confirm that the wavenumber at around 1000 cm^{-1}, unique to NAG which is usually used to confirm the structure of chitosan, is clearly visible. Other bonds which can be used for confirmation, are the NH bond and the C=O bond mentioned above. The other characteristic spectral features were the OH stretching at around 3400 cm^{-1}, the NH stretching at around 3100 cm^{-1}, symmetric CH_3 stretching and asymmetric CH_2 stretching, at around 2900 cm^{-1}, the amide I at around 1650 cm^{-1} and amide II between 1560 to 1540 cm^{-1}. The Saccharide structure-related peaks are near 1080 and 1370 cm^{-1}. The inter- and intra-molecular hydrogen bonding of the $-NH_2$ and -OH stretching vibrations correspond to the peaks at 3440 cm^{-1}. The absorption peak close to 1635 cm^{-1} is associated with the carbonyl groups and the peak around 1655 cm^{-1} corresponds to the N-acetyl groups of chitin molecules, which indicates partial deacetylation (Thanpitcha et al., 2006).

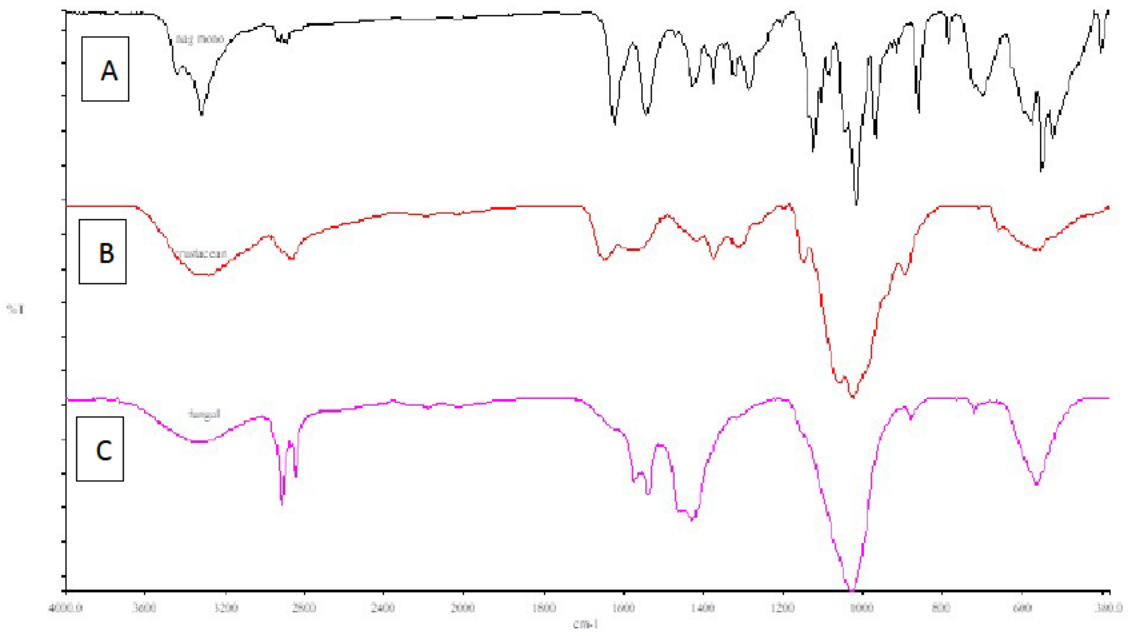


Figure 3.4. FTIR spectra of (A) NAG monomer, (B) commercial crustacean chitosan, (C) fungal chitosan from *Mucor circinelloides*.

3.4 Discussion

M. circinelloides is reported to grow in and on various substrates ranging from plant matter to human hosts as an opportunistic pathogen (Vellanki et al., 2018). The substrates were diluted with water, supplemented with soluble starch and peptone, pH adjusted, and autoclaved before fermentation. The pH of all WS used before blending was acidic, which is an advantage as *M. circinelloides* grows better in low-pH environments.

Molasses as a waste substrate has unique properties. The high sugar content and high density are advantageous as it presents a difficult viscous medium for spoilage microbes to grow in. Undiluted molasses has been tested by DNA extraction and analysis to be "virtually sterile" (Scoma et al., 2017).

Components quantified in MOL and CSL are generally conversely varied, either high in CSL or low in MOL and vice versa. However, magnesium, iron and copper appeared in comparable amounts in both CSL and MOL. The 55% higher energy per 100 g of MOL may be attributed to significantly higher TC and TS content in MOL than in CSL. The cumulative total of TC and TS was approximately 94.6 g/100 g, which contributes to almost 95% of the energy-containing molecules in MOL. As expected, sucrose was the highest saccharide quantified as sugarcane is primarily harvested for the production of sucrose with molasses as the by-product. Fructose and glucose in MOL were less than 5%, which is consistent with the typical composition of fresh samples. Lactose and maltose were negligible (<0.3%). These saccharides are generally not known to be present in plant-derived substrates. Although CSL contained a minimal proportion of saccharides, it had a considerably greater protein and nitrogen content than MOL. This was also expected, because CSL is a by-product of corn flour milling, which is known for its high protein content. The innate salts, metals and minerals content was similar to concentrations supplemented in typical fermentations, with certain components exceeding the typical dosage in the validated medium (McIntyre et al., 2002).

Other components present in the waste substrate may be inhibitory and hinder and/or completely prevent the growth of the culture. With DBW in particular, there may have been preservatives present which needed elimination from the substrate before use. DBW was

omitted due to poor biomass yield in preliminary PB experiments. The initial compositions suggests that MOL could be the preferred carbon source and CSL the nitrogen source with minimal need for external supplementation of sodium, metals and minerals for biomass production. The characteristics determined of MOL and CSL are similar to that in the literature, with some minor variances in the composition. This was likely due to the species, age, location and type and degree of processing these substrates have undergone before use (Ren et al., 2006; Scoma et al., 2017).

The individual waste substrate fermentations produced more biomass at relatively low concentrations of the WS, as higher concentrations showed a detrimental effect on biomass production. With MOL as a sole fermentation medium, any increase in substrate concentration above 3.3% resulted in a decrease in biomass, which justified the need for optimization. CSL was the best standalone WS, and in terms of blends, MOL + CSL was the best medium. UWSF biomass yields varied across the waste substrate blends used; the highest yielding biomass was the 1:1 for MOL and CSL. This blend likely had the best yield due to CSL having a wider spread of fermentable components/nutrients required for biomass growth, in conjunction with a high content of MOL. The CSL had more macronutrients at lower concentrations required for biomass growth. This may be the reason for the higher biomass yield than MOL fermentations at similar substrate concentrations. This was despite TS content being considerably higher, as seen in Table 3.3.

Higher biomass is directly proportional to higher chitosan yield (Zininga et al., 2019). This is due to the nature of the chitosan progenitor, chitin, being part of the structural component of the fungal cell wall. Therefore, the simplest method to maximize chitosan production is to maximize biomass production of a fungal strain having high chitosan content.

MOL, CSL concentrations, and temperature were the key variables identified by the PB methodology. The Pareto-graph (Figure 3.1) based on PB results indicated a highest positive effect by CSL, inferring an increase in the upper limit would be beneficial to biomass production. The next most significant factor was temperature. Conversely, it showed a negative effect which implied that a reduction in temperature may enhance biomass production. Many fungi have been reported to grow optimally at 30˚C (Herath et al., 2014; Janveja et al., 2014; Gaind,

2016), while they struggle to grow at higher temperatures. Pure molasses and molasses at high concentrations are known to be impervious to spoilage and microbial colonization, therefore an appropriate amount was required to maximize biomass production.

RSM experiment run ten, yielded a higher biomass production when compared to both the highest yielding experiment runs for PB and validation. This result is likely due to the specific set of factor values which enabled a higher biomass yield. The model predicted based on the output of maximal biomass, yet the biomass predictions did not reach the RSM biomass yield due to the upper limit of CSL being 9.97% and the run 10 CSL concentration was 12%, with a MOL concentration of 5.5%. With an upper limit of 9.97% for CSL, the predicted maximal biomass output was 78.06 g/L. The validation experiment yielded 77.87 g/L of biomass, which represents a difference of only 0.24%. Future studies could increase the upper limit of CSL in the fermentation feed to find the true upper limit of CSL.

The data produced is consistent across the optimization stages. Exp. no. 5 (PB) differed by 2.67 g/L when compared to Exp. number 3 (Validation). A difference of only 1˚C for the significant factor (temperature) was found, while the non-significant factors had differing values. This further reaffirms the impact that an identified significant factor had.

The validated fermentation medium composition was appropriate for *M. circinelloides* cultivation. Stressful conditions usually result in a yeast form of growth in this dimorphic fungus. In certain flasks, *M. circinelloides* morphology was filamentous while in the other flasks it was entirely yeast or a combination of both forms. All of the flasks that had filamentous growth present in RSM Run #'s: 2, 4, 6, 7, 8, 10, 14 and Validation Exp. #'s: 1 & 3 produced higher biomass than the flasks having solely non-filamentous growth.

The successful extraction of the chitosan from the fungal biomass grown on WS and the resultant nearly identical FTIR spectra conclusively showed that the use of compatible waste substrates for fermentation is a viable process. Further optimizations would be needed for real-world application and scale-up to evaluate the impact of fermentation media on all product parameters and quality standards. The yield of chitosan was similar to that reported in the literature (Zamani et al., 2010; Naghdi et al., 2014), which ranged between 8 to 9%. A well-

established chitosan extraction procedure was followed with minor adjustments to allow each step to complete the desired reaction. Whatever the source of the chitin, provided the raw material is processed properly (removal of non-desired/process inhibitory components) for the extraction and deacetylation, the chitosan yield will be relatively consistent within a small degree of error (Abdou et al., 2008; Mohanasrinivasan et al., 2014). The FTIR spectra of the fungal chitosan against the purified commercial crustacean chitosan and NAG-monomer validates the use of WS as a viable medium for biomass production of *M. circinelloides* for chitosan production.

In summary, the processing of WS into chitosan can be cost-effective. Despite lower yield, fungal chitosan is a potential alternative to the traditional commercial crustacean chitosan. Fungal chitosan extraction is less labour and chemical-intensive, with an overall simpler procedure that uses less hazardous chemicals, with less waste production during the extraction process. As refined methods and optimizations of the processes used are developed and implemented, potentially higher yields will enable easier ingress into this waste valorization system. Finding the right waste substrate or blending WS to obtain an optimal fermentation medium is vital. Other relevant issues are the over-harvesting of naturally occurring crustaceans bordering on driving certain species to extinction, the dependence on a seasonal supply of crustacean seafood waste and limited crustacean farming to supply the demand. With many countries moving toward a more holistic minimal environmental impact-based system and an increasing reluctance to consume animal products, the use of animal-based food waste or solely crustacean shell harvesting for long-term chitosan production would be inadvisable. Fungal chitosan is therefore a long-term sustainable alternative to consider.

CHAPTER 4 : CHITOSAN BIOPOLYMER DEVELOPMENT AND PROTOTYPE ANALYSIS

4.1 Introduction

Polymers are molecules which are made of identical monomeric subunits in repeating arrangements. Polymers in use by humankind are mainly derived from petrochemicals or are chemically synthesized. Polymers have found multiple applications in our daily lives; from the chairs we sit on to various components of our smartphones. This is largely due to the ease of production and durability of the polymers. The major drawback of these polymers is their long life span after use and their minimal biodegradability, lasting up to approximately 1000 years before fully decomposing in natural environments (Siracusa et al., 2008).

Biopolymers are polymers which are produced by living organisms. The polymer essential to life itself is DNA (deoxyribonucleic acid). Biopolymers have the potential to be equally successful if not more successful than petrochemical polymers given their inherent properties. They are useful in many more biological-inclined applications, have little to no adverse immunogenic responses, and additives can confer improved properties. Biopolymers/biopolymer composites are largely regarded as the only viable petropolymer alternative for several reasons, such as: the environmental impact of the utilization of fossil fuels; lower energy and costs required for the production of an equivalent biopolymer; and the sustainability of the biopolymer (Hanninen et al., 2018; Danso et al., 2019; Yadav et al., 2020). To transition from wholly petrochemically derived polymers to entirely biopolymer-based alternatives, production processes will have to change for many applications. The creation of biopolymer/petropolymer blends is a sign of this transition. The characteristics of biopolymers can be developed, optimized, and blended with other polymers and additives to replicate and surpass the required operating parameters of the petropolymers (Vasile et al., 2013).

Most petropolymers have extremely poor biodegradability, unlike biopolymers which originate from biological sources and are completely degraded in natural environments. This is in stark contrast to the xenobiotic nature of the petrochemically-derived and synthesized polymers. Biopolymers have been the subject of many previous studies with varying sources, as listed in

Table 2.5. Microbially-generated biopolymers are the most effective means of commercial upscaling of production, whereby the polymer need only be harvested. Another approach is the synthesis of polymers from natural oils which is preferable for chemically produced polymers using biologically derived oils. Examples are castor, corn, fish, linseed, soybean and tung oil (Sharma and Kundu, 2006). These processes can be implemented using existing infrastructure, with minor alterations.

Chitosan is an aminated carbohydrate polymer, derived from chitin when it is deacetylated. Chitosan is a linear, semi-crystalline polysaccharide composed of (1 - 4)-2-acetamido-2-deoxy-β-D-glucan (N-acetyl D-glucosamine) and (1 - 4)-2-amino-2-deoxy-β-D glucan (D-glucosamine) units (Croisier and Jérôme, 2013). Chitosan can be produced from two main sources, namely, crustaceans and fungi, while there are other sources like insects and lower plant species. The former two are the highest-yielding sources, ranging from 30% to 10% for crustaceans and fungi, respectively. Much of the research conducted is directed at the antimicrobial activity and the biocompatibility of chitosan. The other prevalent theme of research is metal chelation for bioremediation and chitosan-based drug delivery systems. Polymer-based research is mostly directed at food packaging, with more varied chitosan biopolymer formulations being developed for non-food and non-biota-related applications.

Additives and solvent systems used in specific polymer variants have large impacts on the final properties of the cast polymer, whereby they alter the intermolecular reactions between the polymer components in the matrix. Solvents and additives change the polymer matrix structure and arrangement formed during casting (Vlasov et al., 2009; Aider, 2010). Excess solvent is used to make the polymer malleable in a viscous precast solution, in which the additives are incorporated. Upon drying the cast polymer, the excess noncomplexed solvent is evaporated.

Multiple types of analyses can be performed on polymers, including: thermal; physical; physico-chemical; mechanical; and visual. All of these measure the respective properties of the polymer which help determine potential applications (Trimukhe and Varma, 2009; Martínez-Camacho et al., 2010; Lago et al., 2011).

This chapter focuses on the biopolymer formulation, compositional manipulation and testing of the chitosan biopolymer film prototypes produced from chitosan isolated from *M. circinelloides*.

4.2 Materials and Methods

4.2.1 Chitosan extraction

Dried chitosan was prepared as described in section 3.2.7.

4.2.2 Chitosan biopolymer film prototypes preparation

Biopolymer film pre-cast slurries were prepared at 22 ± 1°C and at 1024 mBar by mixing chitosan powder ranging from 1.0 - 2.5% (w/v) in 0 - 30% ethanol-water suspensions. Acetic acid (Sigma), lactic acid (Merck) or formic acid (Merck) were incorporated proportionally (to chitosan weight) into the suspensions and mixed on a magnetic stirrer (1250 rpm). Additives such as food-grade sodium benzoate (SB) (Sigma), (Falguera et al., 2011), K10 Montmorillonite nano clay (MMT) (Sigma), and polymer-grade cellulose (CELL) (Sigma) at concentrations ranging from 0.1 - 0.5% were also added to the slurries. Films were prepared by casting them into glass petri plates. The chitosan solutions were cast at 35°C for 16-24 hours in an air-circulating oven set to maximum airflow, to obtain solid films (Abdollahi et al., 2012). Chitosan formulations change phase within the above-mentioned time-temperature configuration, as the excess solvents volatize (Pereda et al., 2014). The individual prototype composition determines the exact drying time, as well as the ambient environmental conditions. The chitosan biopolymer film prototype formulation process is summarized in Figure 4.1.

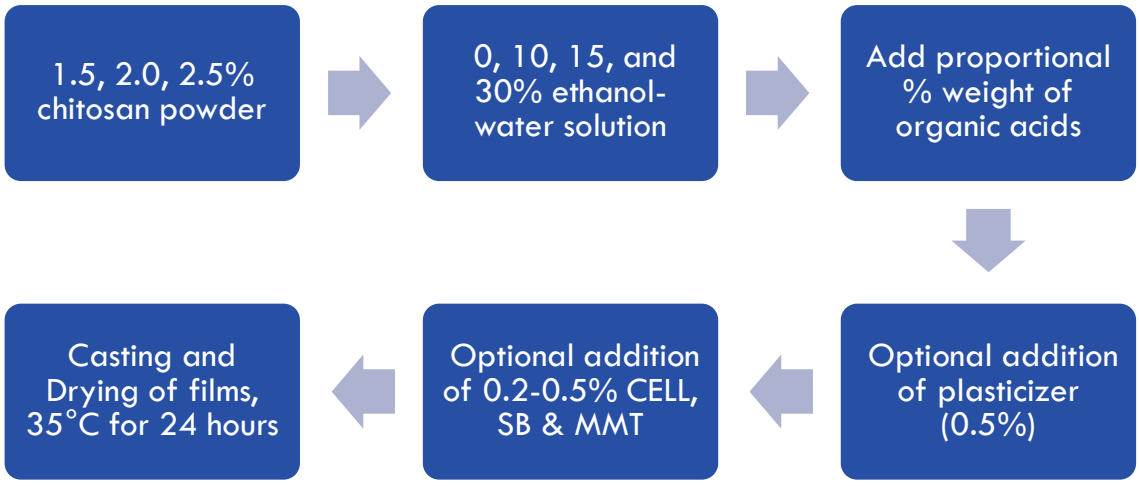


Figure 4.1 Biopolymer film prototype formulation. CELL, SB and MMT refer to polymer-grade cellulose, food-grade sodium benzoate and K10 Montmorillonite nano clay, respectively.

The properties of the chitosan biopolymer film prototypes (CBFPs) are determined by matrix composition. The selection parameter for successful filmogenic formulations was the formation of a monodispersive film, able to maintain integrity upon removal from the Petri plate. An inability to form a film post-drying, as well as poor integrity of the polymer post-drying was regarded as a failure. Of the multiple prototypes produced, the majority of the films were unsuccessful with only seven prototypes meeting the above requirements. Prototypes which produced negligible differing characteristics with marginal differences in composition were omitted. These successful prototypes were then tested using multiple analytical techniques. The composition of the seven biopolymer prototypes is listed in Table 4.1.

Table 4.1. Chitosan-based biopolymer film prototype matrix compositions

Prototype	Constituents
#9	2% chitosan, 0.2% SB, 2% acetic acid
#10	2% chitosan, 0.2% SB, 2% acetic acid, 10% ethanol
#11	2.5% chitosan, 0.25% MMT, 0.25% CELL, 2% acid (1:1:1 acetic acid, formic acid, lactic acid)
#12	2% chitosan, 2% formic acid, 1% glycerol, 30% ethanol
#13	2% chitosan, 0.3% MMT, 2% lactic acid, 0.3% CELL
#14	2.5% chitosan, 0.2% SB, 2% acetic acid
#15	2% chitosan, 2% acid (1:1:1 acetic acid, formic acid, lactic acid)

4.2.3 Chitosan biopolymer film prototype (CBFP) testing

4.2.3.1. Dynamic mechanical analysis (DMA)

The mechanical characteristics of the films including Static Force (N), Tensile Strength (MPa), Elongation (mm) and Percentage elongation (%) (Nouri et al., 2018) were determined at 30°C using a DMA Q800 (TA Instruments) equipped with an 18 N load cell. The experiments were conducted in the force rate mode. The CBFPs were cut into strips (10 mm × 10 mm) which were fixed to both arms of the tensile clamp in the instrument and the measurements were obtained by following the manufacturer's instructions.

4.2.3.2 Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was conducted to investigate the thermal properties of CBFPs. TGA decomposition profiles of dry samples were determined using an SDT Q600 thermal gravimetry analyser (TA Instruments) utilizing a heating rate of 10°C/min in the temperature range from 25–600°C. Thermogravimetric weight-loss curves elucidate the temperature of initial decomposition (Tdi), the temperature at maximum decomposition rate (Tmax) and characteristic temperatures of decomposition of the CBFPs (de Araujo et al., 2017). These tests were done at the Department of Mechanical Engineering at the Durban University of Technology, Steve Biko Campus.

4.2.3.3 Thickness and density of films

The thickness of the films was measured for each sample utilizing a manual digital micrometer (Mitutoyo, Japan) with an accuracy of 0.01 mm. Each CBFP was measured at four different sites along the strip (Prateepchanachai et al., 2017). The density was calculated using 4 cm^2 pieces after weighing the CBFPs on an analytical balance.

4.2.3.4 Solvent and solids content

Pre-weighed samples of the films (4 cm^2 pieces) were placed in an air-circulating oven at 105°C for 24 h. Solvent content was calculated using the difference in mass of dried polymer divided by the initial weight (Beigzadeh Ghelejlu et al., 2016). The solids content was calculated using the dried mass of biopolymer film divided by the initial weight.

4.2.3.5 Film solubility in deionized water

The dried films (4 cm^2 pieces) were initially weighed on an analytical scale. The pieces were placed in sterilized jars, and 30 ml of deionized water was poured into each jar. The jars were placed on a rotary shaker at 100 rpm for 24 h. Whatman no.1 filter paper was pre-weighed, and the contents of each jar were filtered through the filter paper. Residue on the filter paper represented the insolubilized fraction while that which passed through the filter paper was the solubilized fraction. The insolubilized fraction was dried in an air-circulating oven for 24 h at 105°C. Water solubility was calculated using the following equation:

$$\left[\frac{W_t - W_o}{W_o}\right] \times 100$$

where W_t and W_o are the weights of the specimen containing water and the dried specimen, respectively (Martín-Alfonso et al., 2014).

4.2.3.6 Scanning electron microscopy (SEM)

The microstructure of the films was obtained using a field-emission scanning electron microscope, located at the MMU Department, University of KwaZulu-Natal, Westville. Images were captured at 5 kV, using the Zeiss Ultra Plus FEG SEM. Before measuring, all films were submerged in liquid nitrogen and sputter-coated with gold for 30 s (Zhuang et al., 2019).

4.3 Results

4.3.1 Dynamic mechanical analysis

Pure chitosan biopolymers are usually ductile, but CBFPs #11, #14 and #15 experienced less than 10% strain and did not fracture. CBFPs #9, #10, #12 and #13 deformed considerably, experiencing more than 50% strain followed by fracture (Figure 4.2 & 4.3). Static Force (N), Tensile Strength (MPa), Elongation (mm), Percentage Elongation (%), and Elongation Rate ($mm.min^{-1}$) of CBFPs are illustrated in Figures 4.3 – 4.8.

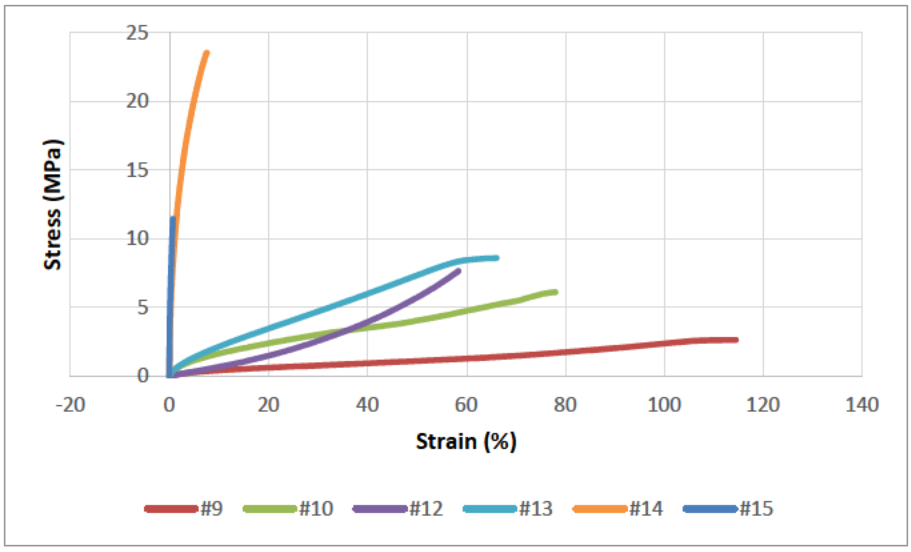


Figure 4.2 DMA Stress vs. Strain curve of CBFPs. #11 was omitted due to only 0.092% strain.

The Static Force at break (SFAB) measurements range from 3.81 N (#9) to 17.99 N (#15). #15, #13, and #11 have the closest SF values, all of which approach the limit of the instrument. The SFAB values for #15 represents a 3.37-fold increase over values for #9. CBFPs #10 and #12 were examples where the use of additives and formulation change did not improve the SFAB values. This is in contrast to #9, where the SFAB value reduced from 5.33 N to 4.7 N and 3.8 N, which is an approximately 12% and 29% reduction, respectively (Table 4.2). With the SFAB values of #9 being a reference point for the formulation changes, the force required to fracture the film was improved three-fold in the cases of CBFPs #11, 13, 14 and 15 from 5.33 N to 17.98 N, 17.96 N, 16.47 N and 17.99 N, respectively (Figure 4.4). In particular, the SFAB values of #14 show a stark contrast as to how a change in concentration of chitosan by 0.5% affects the force required to fracture the film.

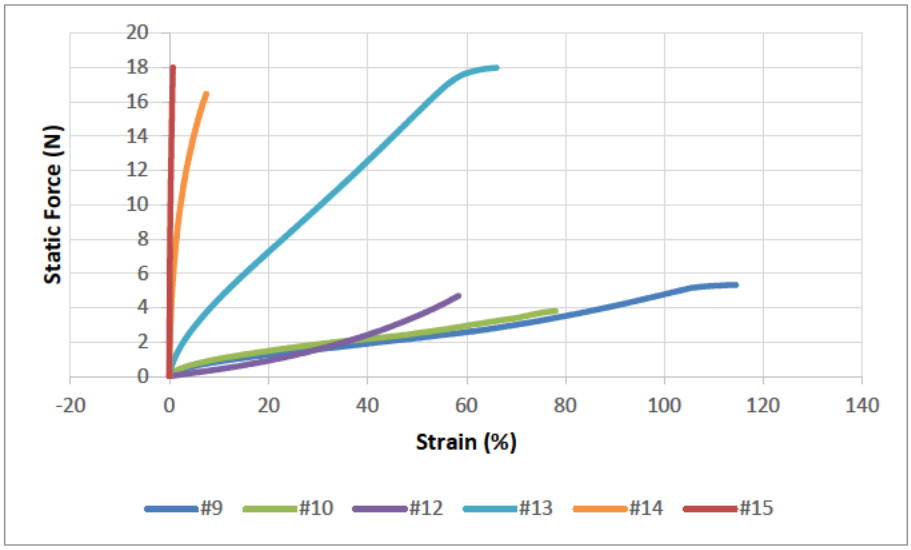


Figure 4.3 DMA Static Force vs Strain curve of CBFPs, #11 was omitted due to only 0.092% strain.

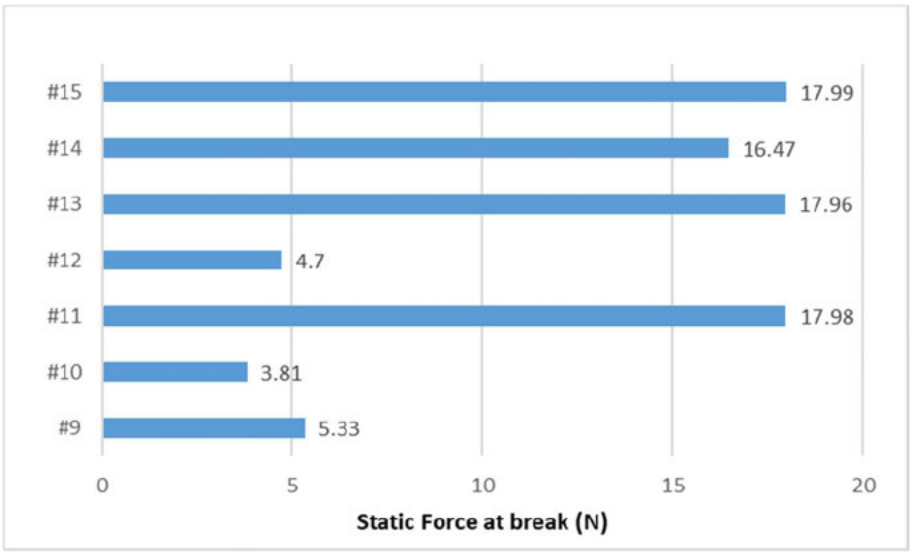


Figure 4.4 The Static Force (N) measurements of CBFPs #9 to #15.

The chitosan films produced did not return to their original form after the application of the physical force, i.e., they deformed and remained in that morphology, with little to no elasticity observed in the prototypes. The tensile strength (TS) for the tested prototypes ranged from 2.66 MPa (#9) to 23.53 MPa (#14), which is an 8.85-fold improvement with just a change in chitosan concentration (Figure 4.5). The use of additives has improved the TS of #9 by a minimum increase of 2.3-fold, with a change in formulation comprising the simple addition of 10% ethanol (#10). The TS values of #12, #11 and #13 differ by less than ±1 MPa, in ascending order.

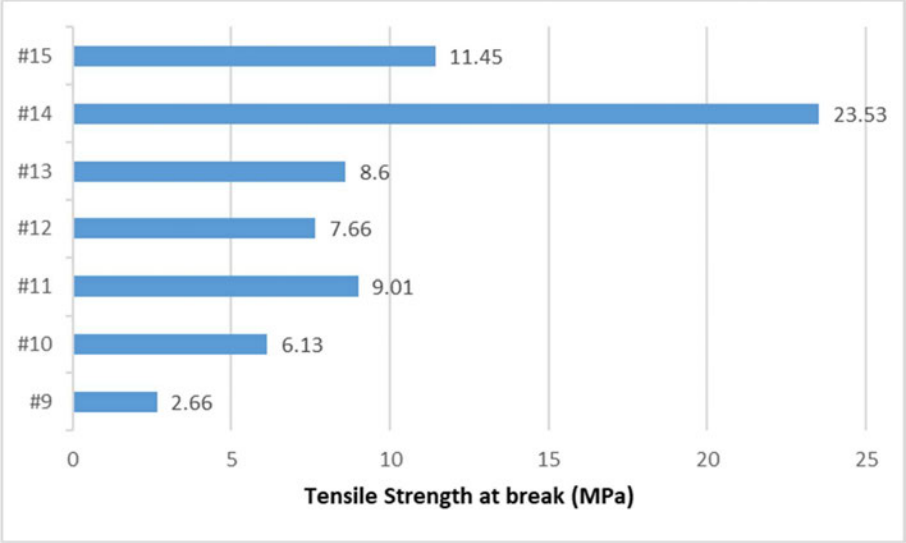


Figure 4.5 Tensile Strength (MPa) measurements of the CBFPs #9 to #15.

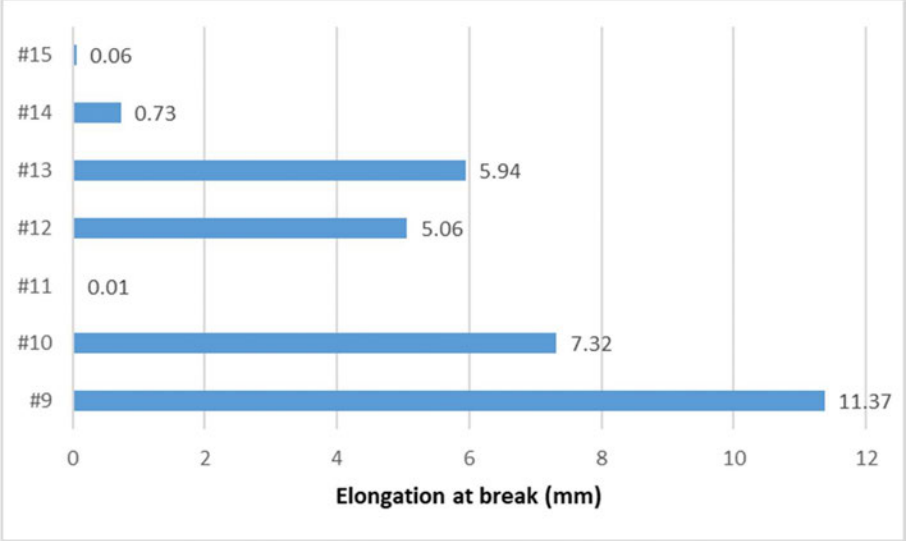


Figure 4.6 Elongation values (mm) of the CBFPs #9 to #15.

The elongation measurements of the prototype's ranges from 0.01 mm (#11) to 11.37 mm (#9), which is 0.09% and 114.42%, respectively (Figure 4.6). Elongation values of #9 when compared to #11 represents a 1.14-fold reduction in elongation. CBFP #9 deformed the most by 11.37mm (114.42%). The film is largely unchanged for CBFPs #11 and #15, with values of 0.01 mm (0.09%) and 0.06 mm (0.67%) of deformation, respectively (Figure 4.7). The use of additives and the improvement which can be obtained is best illustrated by the elongation data set. #13 and #12 both have a 7.73% difference in elongation but with vastly different compositions.

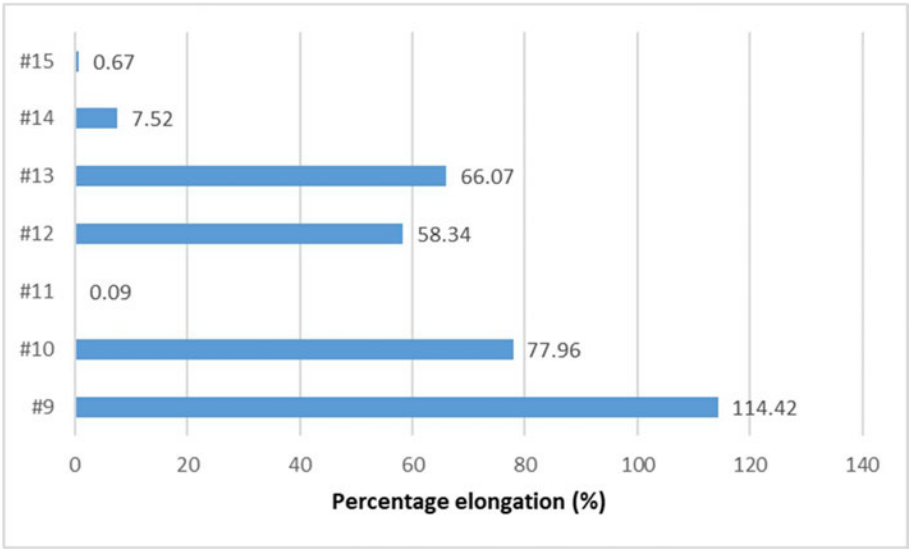


Figure 4.7 Percentage elongation (%) of the CBFPs #9 to #15.

The addition of 10% ethanol to the solvent for CBFP #10 improved certain properties, namely, the tensile strength which increased from 2.66 MPa to 6.13 MPa (2.3-fold improvement), while the force required to fracture the film decreased from 5.33 N to 3.81 N (0.7-fold reduction). The elongation decreased from 114.42% to 77.96% (36.46% reduction) and the elongation rate decreased from 1.02 $mm.min^{-1}$ to 0.9 $mm.min^{-1}$, (0.88-fold reduction) which the difference is almost directly proportional to the concentration of ethanol in the matrix.

The elongation rate of the CBFPs ranged from 2.74 x 10^{-4} $mm.min^{-1}$ (#11) to 1.02 $mm.min^{-1}$ (#9). CBFP #9 deformed the most, and also had the quickest deformation rate at approximately 1 mm per minute. CBFPs #11 and #15 had much smaller elongation rates of 2.74 x 10^{-4} $mm.min^{-1}$ and 1.67 x 10^{-3} $mm.min^{-1}$, respectively. CBFP #14 (0,02 $mm.min^{-1}$) had a 46.27-fold reduction in elongation rate when compared to CBFP #9. CBFP #13 had elongation similar to CBFPs #10 and #12, but the rate of elongation was substantially slower at 0.16 $mm.min^{-1}$ compared to 0.90 $mm.min^{-1}$ and 0.51 $mm.min^{-1}$, respectively (Figure 4.8).

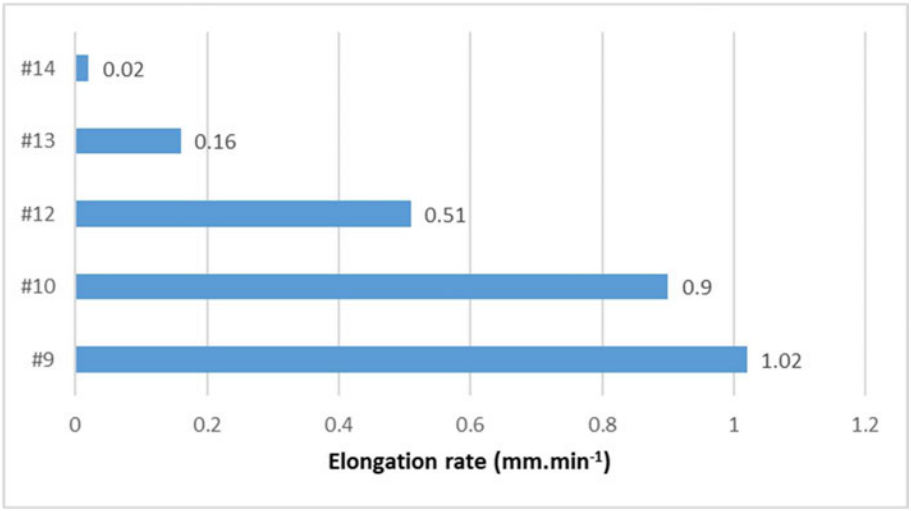


Figure 4.8 Elongation rate (mm.min^{-1}) of the CBFPs. CBFPs #11 and #15 were not shown due to negligible elongation.

Table 4.2 CBFPs DMA measurement values at break/end of the test

Sample	Static Force (N)	Tensile Strength (MPa)	Elongation (mm)	Percentage elongation / Strain (%)	Elongation rate (mm.min^{-1})
#9	5.33	2.66	11.37	114.42	1.02
#10	3.81	6.13	7.32	77.96	0.90
#11	17.98	9.01	0.01	0.09	2.74×10^{-4}
#12	4.70	7.66	5.06	58.34	0.51
#13	17.96	8.60	5.94	66.07	0.16
#14	16.47	23.53	0.73	7.52	0.02
#15	17.99	11.45	0.06	0.67	1.67×10^{-3}

*#11 was the most robust CBFP produced.

4.3.2 Thermogravimetric analysis

The CBFPs displayed the characteristic chitosan melting peak of ±220°C (Abdou et al., 2008). With initial weight loss starting at ±200°C for #9, #11, #13, #14, #15, with #10 & #12 being the exceptions at ±150°C. CBFPs #9, #10, #11, #13, #14, and #15 had single-stage degradation

patterns, while CBFP #12 had a two-stage degradation pattern, with two distinct peaks at 140°C and 265°C followed by reducing to a stable mass after 315°C (Figure 4.9.D).

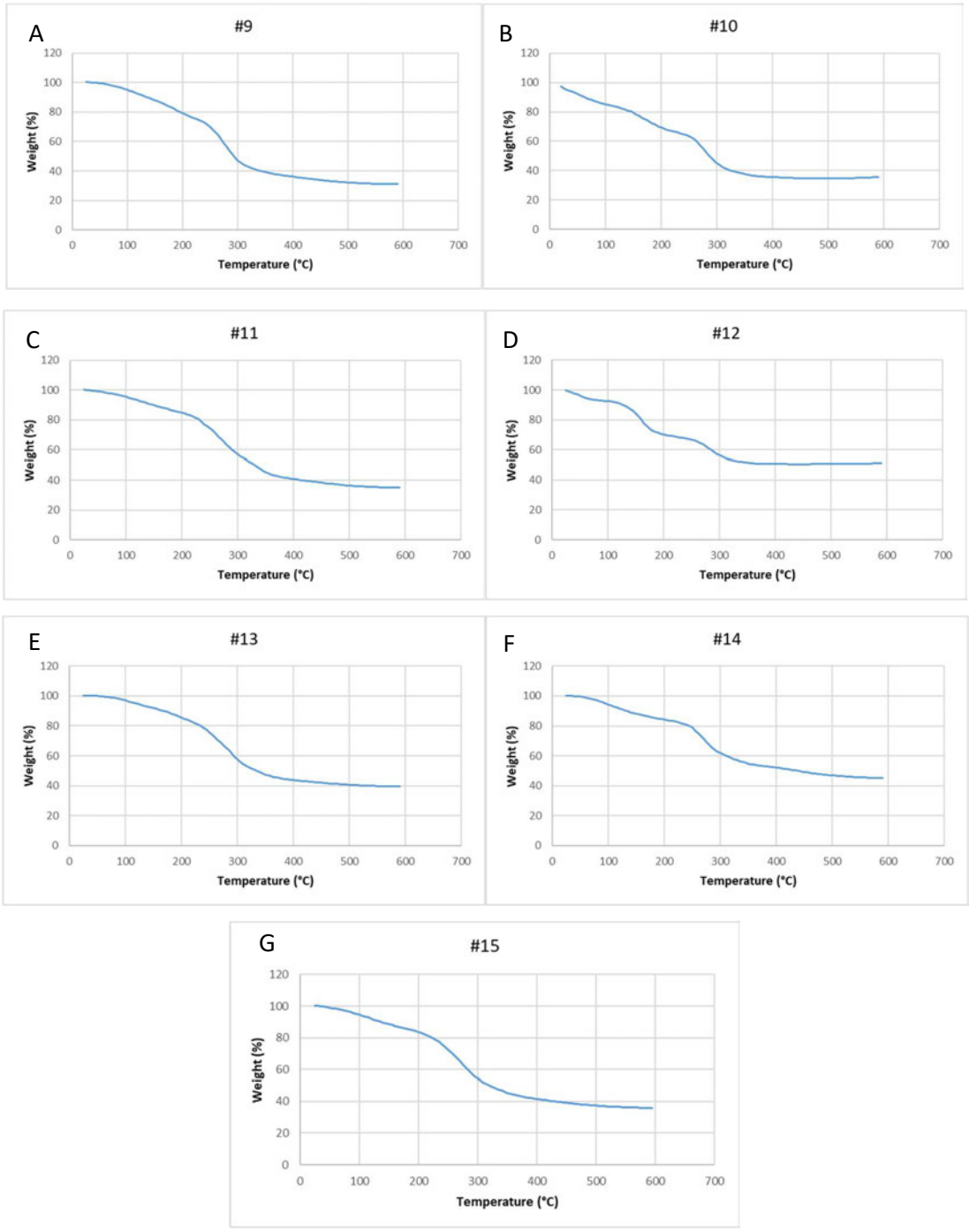


Figure 4.9 Thermograms of CBFPs (A) #9, (B) #10, (C) #11, (D) #12, (E) #13, (F) #14, (G) #15.

It has been reported previously that chitosan polymers with a sole solvent and mainly chitosan composition exhibit single-stage decomposition curves (Trimukhe and Varma, 2009). CBFP #10

had a fairly consistent gradient in the 25 to 100°C range, followed by a sharp decline at ±250°C. This is likely due to the ethanol present in the variant (Figure 4.9.B). #11 presents with a slower degradation curve prior to 230°C, after which increased weight-loss until 350°C (Figure 4.9.C). CBFP #14 had a Tdi point of 250°C, which is the highest temperature for retaining 80% weight of all tested CBFPs (Figure 4.9.F).

In the overlay showing all the curves (Figure 4.10), the effect of additives and solvent composition alteration can be compared more effectively. #9 had a quicker degradation trend in relation to most of the other variants. CBFP #10 did not conform to the above trend in the 25 to 400°C range, and after this interval, the degradation rate was slower than for CBFP #9. CBFP #14 in contrast to CBFP #15 showed differences in weight loss in the 500 to 600°C range. CBFPs #10 and #9 degradation patterns are most similar in the 275 to 475°C range, whereas CBFPs #11, #13, #14, and #15 degradation patterns are most similar in the 25 to 300°C. The weight-loss percentage of the prototype polymers deviated by no more than 20%.

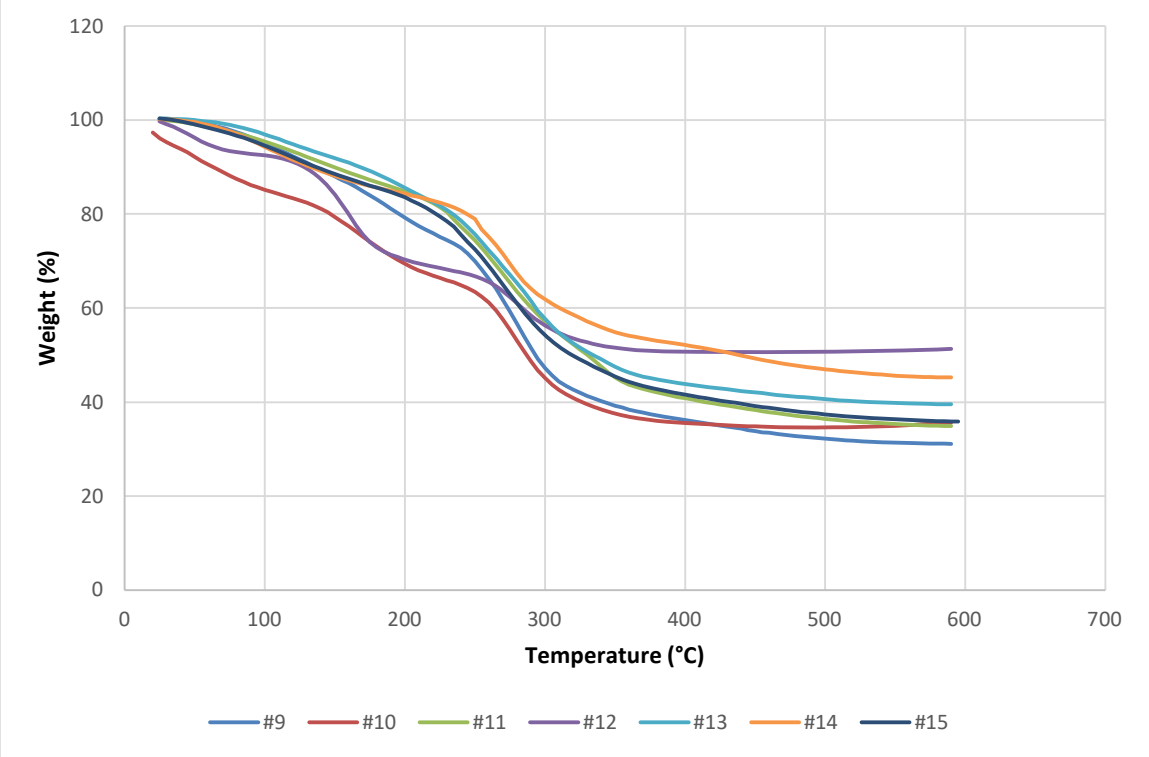


Figure 4.10 Thermogram overlay of CBFPs #9 to #15.

There is a 100°C difference between the 20% and 50% weight-loss intervals in CBFP #9 as opposed to a 140°C delta for #10 (Table 4.3). There is an equivalent difference in weight-loss intervals for CBFP #11 with a differing final weight to that of #9 (31.09%) and closer in final weight to #10 (35.64). The CBFP with the highest final weight was #12 (51.27%), along with the biggest difference in weight-loss intervals of 290°C. The same weight loss trend of CBFPs #9 and #11 is also true for #13 with a difference in weight-loss intervals of 100°C, with a higher final weight of 39.5%. The second-largest difference in weight-loss intervals of 190°C occurred with CBFP #14, as well as having the second-highest final weight (45.23%). This indicates a pattern of "the larger the difference in weight-loss intervals, the higher the residual mass." The later the onset of the initial degradation peak the greater the weight-loss of that peak despite an increase in temperature at a constant rate. The difference in weight-loss intervals of 95°C (CBFP #15) is similar to the most common difference in weight-loss intervals of 100°C across the prototypes and had a final weight of 35.87% which was also similar to CBFPs #10 and #11. The residual weight of the CBFPs ranges from 31.09% to 51.27%. The use of additives and varying solvents did alter the thermal degradation of the biopolymers, the highest being a ±20% deviation when compared with CBFP #9 (Table 4.3).

Table 4.3. Degradation percentage temperatures and stable weights of CBFPs

CBFP	#9	#10	#11	#12	#13	#14	#15
The temperature at 20% loss (°C)	195	145	230	160	235	245	225
Temperature at 50% loss (°C)	295	285	330	450	335	435	320
Final weight (%)	31.09	35.64	34.87	51.27	39.5	45.23	35.87

4.3.3 Density and thickness of CBFPs

Thickness was used to determine the volume of the CBFPs and therefore the density. The density ranged from 0.78 mg/mm^3 (#12) to 1.46 mg/mm^3 (#13) (Figure 4.11). #9 (1.3633 mg/mm^3) and #11 (1.3585 mg/mm^3) had the closest density values with a difference of 0.0048 mg/mm^3. #10, #14, #15 had density values of 1.0876 mg/mm^3, 1.0141 mg/mm^3 and 1.1538 mg/mm^3, respectively.

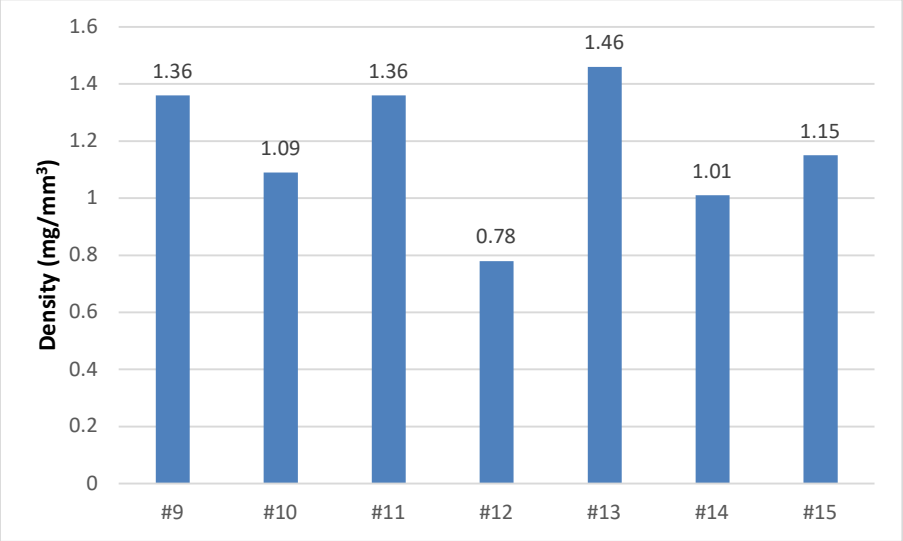


Figure 4.11. Density (mg/mm^3) of CBFPs #9 to #15.

The average thickness of the CBFPs ranged from 0.071 mm (#10) to 0.274 mm (#11). Film #11 was 1.45-fold thicker than #9 (0.189 mm) (Figure 4.12). #13 (0.228 mm) and #14 (0.221 mm) had a similar thickness, with only a 0.007 mm difference even though they had different compositions. #12 (0.097 mm) and #10 were the thinnest CBFPs. CBFP #10 was 2.66-fold thinner than CBFP #9. #15 had a thickness of 0.201 mm which was the closest to #9.

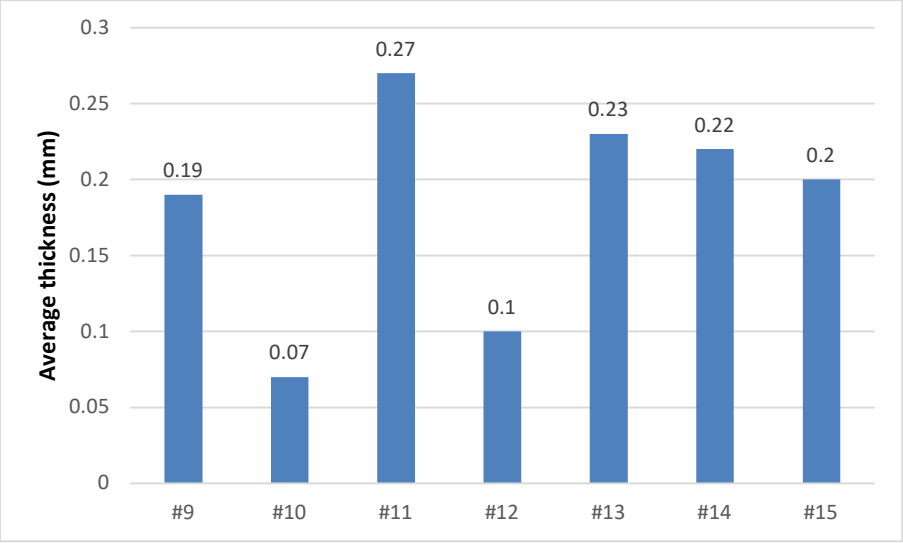


Figure 4.12 Average thickness (mm) of CBFPs. Thickness values were measured at four different sites of the films.

4.3.4 Solvent and solids content

The polymer after initial casting contained a portion of the solvent varying from 11.63% (#13) to 42.50% (#12). CBFP #12 had the highest solvent content and lowest solids content (Figure 4.13). #10 and #15 had similar solvent content with values of 14.82% and 14.28%, respectively, a 0.54% difference. CBFP #9 had a solvent content percentage of 16.43% which was 2.58-fold less than that of #12. #11 and #14 had solvent values of 13.22% and 15.37%, respectively. Solids contents ranged from 57.50% (CBFP #12) to 88.37% (CBFP #13). The relationship between solids content and solvent content was inversely proportional, as combined they represent 100% of the sample. #10 (85.18%) and #15 (85.72%) had close solids percentage values with a difference of 0.54%. #9 had a solids content of 83.57% which was 45% more than #12. #11 and #14 had a solids content of 86.78% and 84.63%, respectively. Six of the seven (#9, #10, #11, #13, #14, #15) prototypes vary from each other within an ±6% margin for both solids content and solvent content percentages.

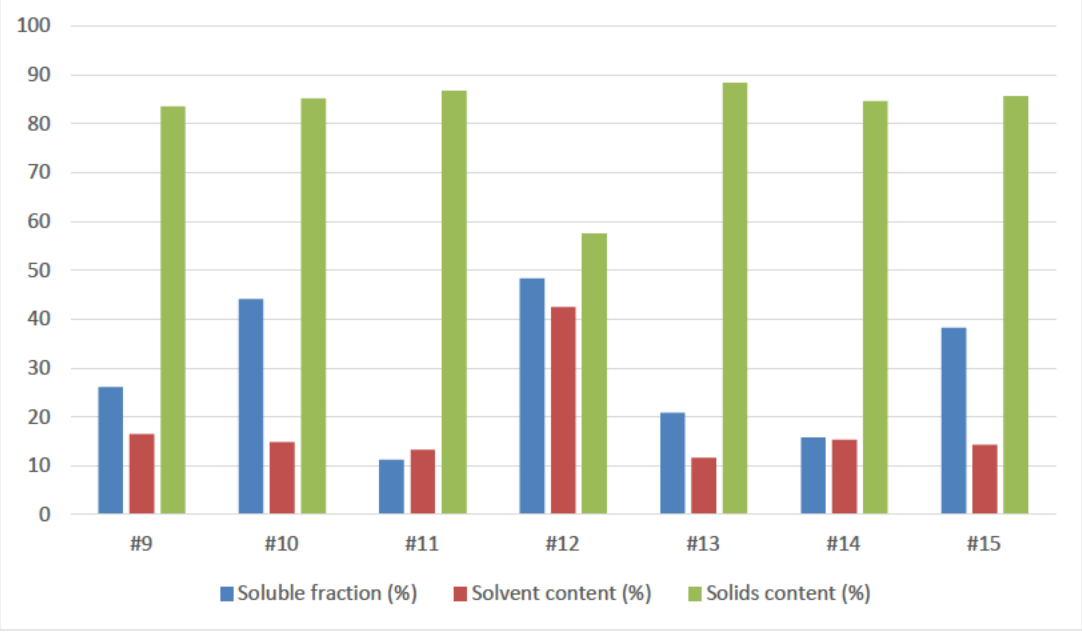


Figure 4.13 Soluble fraction (%), Solvent content (%), and Solids content (%) of CBFPs.

4.3.5 Film solubility in deionized water

The soluble fractions of the CBFPs show a degree of proportionality to the solvent content and composition (Figure 4.13). Soluble fractions ranged from 11.21% (CBFP #11) to 48.34% (CBFP #12). For #10 the soluble fraction was 44.09%. When compared to #9 (26.11%) this represented

a 17.98% increase. CBFP #11 possessed the lowest soluble fraction across all the tested prototypes despite not having the highest solids content, with only #13 possessing a higher solids content. CBFP #11 and #14 showed the most direct relationship between the soluble fraction and solvent content which are directly proportional. Their soluble fractions (SF) were 11.21% and 15.82% and the solvent contents (SC) were 11.63% and 15.37%, respectively. #11 was the only CBFP where SC was higher than the SF. CBFPs #9, #10, #11 and #13 (20.87% SF), and CBFPs #14 and #15 (38.18% SF) had relatively consistent values for solids content and solvent content, while the soluble fraction (%) contrast starkly as it shows the impact of the solvent, additives and plasticizer used in the polymer matrix. CBFP #12 was the exception to this trend.

Table 4.4. Summary of physico-chemical properties of CBFPs

Sample	The average thickness of the film (mm)	Soluble fraction (%)	Density (mg/mm^3)	Solvent content (%)	Solids content (%)
#9	0.189	26.11	1.3633	16.43	83.57
#10	0.071	44.09	1.0876	14.82	85.18
#11	0.274	11.21	1.3585	13.22	86.78
#12	0.097	48.34	0.7789	42.50	57.50
#13	0.228	20.87	1.4586	11.63	88.37
#14	0.221	15.82	1.0141	15.37	84.63
#15	0.201	38.18	1.1538	14.28	85.72

4.3.6 Scanning electron microscopy (SEM)

The SEM micrographs show that changes in the composition appear as distinct visual differences across the variants viewed (Figure 4.14). CBFP #11 had minor tears, probably due to the shearing upon removal of the Petri plates because of the rigidity of this variant (Figure 4.14.C). Some difficulty was encountered in the removal of certain films from the casting vessels. CBFP #13 had the most different appearance in contrast to the other CBFPs. This was most likely due to the higher concentrations of the additives (CELL and MMT) (Figure 4.14.E).

CBFP #12 had undulating crests and troughs throughout the surface of the film (Figure 4.14.D). CBFP #14 was noticeably smoother in appearance, and this was likely due to the higher concentration of chitosan present in the matrix in contrast to CBFP #9 which exhibits “veins” present throughout the cast polymer (Figure 4.14.F and 4.14.A).

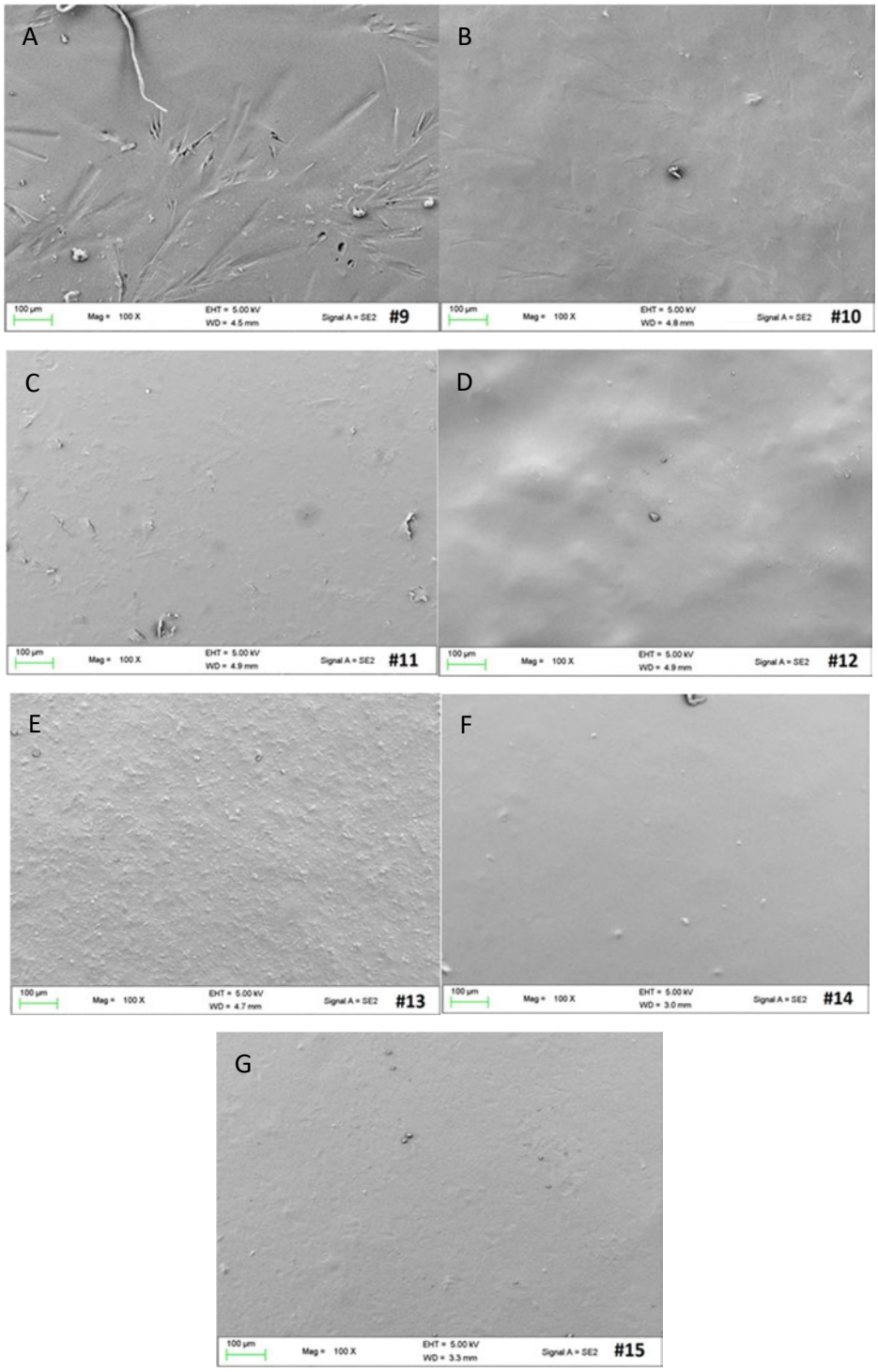


Figure 4.14 SEM Images of CBFPs (A) #9, (B) #10, (C) #11, (D) #12, (E) #13, (F) #14, (G) #15, all samples are at 100× magnification.

The presence of the veins could be due to the evaporation of solvent during the casting process and polymer deforming, stretching, and contorting to accommodate a greater reduction in volume. CBFP #10 presented with less distinct “veins” than CBFP #9 and had a more uniform distribution of morphological features (Figure 4.14.B). CBFP #15 showed slight pitting and undulations similar to that of CBFP #11. No effects of surface shearing were observed in this sample (Figure 4.14.G).

4.4 Discussion

In this chapter, chitosan was used as the primary biopolymer formulated with solvents and additives for the development of filmogenic materials. Subsequent characterization of the prototype biopolymer films was accomplished through dynamic mechanical analysis, film solubility testing, physico-chemical properties analyses, SEM analysis and thermogravimetric analysis. The compositional differences are responsible for the inherent chitosan film prototype properties. When comparing film properties, valid differences can only be determined with samples which have undergone testing using the same parameters. The polymers' results depend on the film-forming materials, film formulation fabrication methods, operating conditions, testing conditions, and testing equipment (Hernandez-Izquierdo and Krochta, 2008).

The main reason for the failure of multiple CBFPs is, in part, due to the limitations of the methods as well as the intercompatibility of chitosan, the additives and solvents at varying concentrations, given that certain ratios are required for successful monodisperse film layer formation. Formic acid solvent variants impart rigidity, but at ratios greater than 60% of total solvent, flat films are no longer possible upon drying as the polymer matrix contorts into a solid 3D shell structure. In certain variants, the plasticizing agents or additives reduce the integrity of the film to a point where the film shears and tears upon removal from the casting vessels, usually glass petri plates. This was observed in this study with 1% chitosan in conjunction with glycerol at concentrations higher than 0.5% (w/v) and SB greater than 0.1% (w/v). The utilization of glycerol as a plasticizer, to enhance flexibility, reduced the integrity of the polymer films, even when used in conjunction with formic acid. Films with 0.5% (w/v) or greater MMT and CELL concentrations had a powdery feel. This was most likely due to the excess additives and incomplete solubilization and partially complexed polymer from the saturated polymer matrix. Certain organic acids, due to their innate properties such as pKa, molecular size and 3D orientation will complex molecules differently, as is the case with lactic acid where 0.5% (w/v) of MMT and CELL reached saturation point, but with acetic acid and formic acid it was completely incorporated into the film of the polymer matrix. CBFPs prepared with butyric acid and propionic acid had comparable properties to that of the formic acid variants, where the CBFP would contort during the drying process. The larger molecular weight acids with a linear carbon chain also exhibited longer time periods for the casting of the polymers. Furthermore,

the release of unpleasant volatiles necessitated that these solvent systems were not used. There have been similarities and differences in the characteristics of the CBFPs with varying compositions, inferring that certain formulations can produce identical characteristics with completely different compositions.

The incorporation of sodium benzoate at low/below recommended concentrations did increase the longevity of the produced films in particular films which had single-component solvent systems. It was noted that CBFPs without the SB developed microbial growth on the films after months of storage at room temperature. The main function of the SB as an additive is to impart antimicrobial properties. Even though this was not directly assessed in this study, it has improved the storage life of the other reported prototypes (Falguera et al., 2011). CBFP #9 is the most commonly prepared composition for chitosan polymer films. Acetic acid-based chitosan polymers are the most commonly used formulations in multiple applications and are the preferred solvent for chitosan (Tanuma et al., 2010; Moura et al., 2011).

The higher tensile strength of CBFP #14 was probably due to the higher concentration of chitosan and having fewer additives, along with the use of only acetic acid. This solvent has been proven favourable in previous work in producing higher tensile strength values (Velasquez-Cock et al., 2014; Pavoni et al., 2019). This is likely due to the complexing of uniform repetitive units of the two-carbon carboxylic acid in line with the chitosan structure. In contrast to CBFP #10, the addition of ethanol adds to the functional groups present. During the casting of the polymer the arrangement and distribution of the ethanol in the polymer matrix changes as the ethanol evaporates. These changes contribute to the differing DMA measurements obtained, compared to CBFP #9 in which ethanol is absent. The extra carbonyl group on the acid affects how tightly the molecules are bound to one another. Van der Waals forces would also be greater due to the higher carbon content. CBFP #12 with a different matrix composition yielded better DMA values, in contrast with CBFP #9, which is the most commonly prepared composition of chitosan polymer films. Samples which did not fracture (CBFPs #11, #13 and #15) exhibited distinct differences in their properties even though there were identical solvent systems for CBFPs #11 and #15. With the upper limits of the load cell of 18 N being reached with CBFPs #15, #13 and #11, further stress testing at higher loads is recommended to determine the exact static forces for those samples that did not break. CBFPs #9 and #15 had

similar compositions with the difference being the acids and incorporation of SB. This produced extremely different mechanical properties, but similar thickness. CBFP #11 was the toughest and most durable variant produced, reflected in the DMA values and the physico-chemical properties. This was due to the use of a multi-acid solvent system and the use of the additives at their highest concentrations.

The thickness of the film had a proportional relationship with the chitosan content and additive loading as can be seen with their composition (Table 4.1) and physico-chemical properties (Table 4.4). Changes in the composition and concentration of constituents affected the dispersion rate due to the change in viscosity, which has been found in previous unpublished studies. CBFP #9 and #14 physical data showed that an increase in thickness did not necessarily imply an increase in density (Table 4.4). CBFPs #14 and #11 with the less additive and higher chitosan content samples had thinner films. The greater the proportion of additives incorporated did not scale with a linear relationship with CBFPs properties. The entire formulation needed to be adjusted with every additive introduced into the matrix. Certain components produced non-desirable characteristics at any concentration and others enhanced only at an appropriate concentration. The films with the highest densities, CBFPs #9, #13 and #11, yielded no noticeable improvement in terms of density versus the static force when compared with #15 (Figure 4.11). Generally, a material with greater density corresponds to greater forces it can withstand. The density of the prototype polymers was within the range of petropolymers like HDPE (0.93 g/cm^3) (Adhikary et al., 2008) and thickness similar to PET polymers. PFA has a density of 2.15 g/cm^3 (Leivo et al., 2004), double what was obtained with the prototypes. CBFP #12 had the highest solvent content and lowest solids content. This was most likely due to the high ethanol concentration of the matrix, most of which would have evaporated during the casting and solubility determination procedure. Physical characteristics influenced the mechanical properties of the films. CBFPs #12 and #10, with ethanol present in the matrix, were the thinnest films and shared a similar TGA degradation pattern. CBFP #15 which was the base for CBFP #11 features the same multi-acid solvent system for matrix preparation but due to the absence of MMT and CELL the soluble fraction was substantially larger at 38.18% compared to 11.21% for CBFP #11 (Figure 4.13). CBFP #12's approximately 50% soluble fraction was likely due to the abundance of hydroxyl groups present. The same occurrence was true for CBFP #10 for which the matrix contained 10% ethanol, which increased

soluble fraction to 44.09% from 26.11% (CBFP #9). Bonilla et al. (2011) reported that with an increase in chitosan concentration in the polymer matrix, the crystallinity also increased which hinders the solvents' ability to penetrate and dissolve the film.

TGA of chitosan films showed the typical degradation pattern of chitosan, with additives altering thermograms by 5 to 20%. The predominantly chitosan-based biopolymers containing various additive loading of up to 50%, were within the same 5 to 20% margin of variance reported by Thanpitcha et al. (2006). The variations of the thermogram overlay (Figure 4.10) indicate potential for further optimization of chitosan polymers, given that the compositional manipulation of the matrix yielded similar but distinct degradation patterns. The weight-loss gradient below 100°C varied due to the solvent content of the polymer, for example with prototypes #10 and #12. This was likely due to the ethanol content present in the matrix. The ideal maximum operating temperatures of most of the variants was up to 250°C, after which 80% of integrity and mass deteriorated. There were two weight-loss peaks. The first, at approximately 100°C, corresponded to the evaporation of water and the second, at 284°C corresponded to the degradation of chitosan polymer chains. CBFP #10 and #12 had much steeper gradients in the 50 to 100°C range, due to higher un-complexed solvent content in the film matrix. The residual masses post-TGA showed a minimum of 30%, unlike petropolymers which yield far less. Therefore, pyrolysis as a means of disposal would not be viable for polymers with significant proportions of chitosan. Temperature values at 20% and 50% weight-loss points indicated which prototypes will maintain integrity at an elevated temperature. The constituents of CBFPs #11, #13 and #14 imparted better thermal characteristics than those of #9, #10 and #12. The higher loading of chitosan (2.5%) in CBFP #14 performed best at 245°C, with only 20% mass loss, as well as having the highest 50% mass loss temperature of 435°C. CBFP #12 had a higher 50% mass loss temperature (50PWLT) of 450°C but a lower 20% mass loss temperature (20PWLT), therefore it would not be as good a candidate as CBFP #14 for applications that require thermal stability. CBFP #13 had a 10°C lower 20PWLT than CBFP #14 but had a 50PWLT 100°C lower than that of CBFP #14. This difference was due to the higher loading of cellulose and nanoclay in the polymer matrix. This trend was also apparent in CBFP #11. Both the 20PWLT (230°C) and 50PWLT (330°C) for CBFP #11 differed by only 5°C when compared to CBFP #13 (235°C & 335°C), respectively. Due to CBFP #14 having a higher chitosan loading as well as higher 20PWLT and 50PWLT, the solvent appeared to contribute to the matrix

formation, intercalation and constituent arrangement affecting the thermal degradation peaks (Lavorgna et al., 2010).

The film-forming ability of the casted prototype biopolymers within the parameters of the method used was the criterion used for the selection of prototypes. Specifically, the formation of a monodisperse film layer was the desired outcome for a successful prototype. All CBFPs which did not meet this criterion were not assessed further. Since only successful films were viewed under SEM, differences in failed films may have presented vastly different appearances. SEM images of prototype CBFP #13 had a coarse sandpaper-like appearance which was likely because of the poor intercalation of the chitosan chains and the nanoclay. These phenomena are due to hydrogen bonding between the constituents, whereby components are held in a defined formation as opposed to a random arrangement (Lavorgna et al., 2010). This can be prevented by the use of a plasticizer such as glycerol or increasing the chitosan content or decreasing the nanoclay/nanofiller loading.

CBFP #14 would be best suited for tensile strength-based applications as indicated by the forces exerted on the film during the tensile tests. The measurements obtained were similar to blended HDPE petro-plastic variants, with additives producing values similar to CBFP #14, which is within the range of 50-100% HDPE polymers (Şirin et al., 2013). The melting point of chitosan is in a similar range to that of the fluoropolymers perfluoroalkoxy alkanes (PFA) (Leivo et al., 2004). Therefore, applications which do not require as robust mechanical properties of PFA but require high thermal tolerance could use chitosan or chitosan-PFA hybrid polymers as an alternative. In applications which allow for minimal deformation under 100°C, CBFPs #11, #13, #14 and #15 would be viable options, depending on the desired mechanical properties. The use of an epoxy-chitosan resin would allow an alternative to pure epoxy-based resins used in traditional carbon fibre autoclave production processes (Nasri-Nasrabadi et al., 2019). Chitosan has already been considered an adhesive which is the main function of epoxy (Mati-Baouche et al., 2014). The chitosan-epoxy composite resin would have the advantages of both, as well as being a bio-blend resin rather than being wholly petroleum-derived (Stanzione and La Scala, 2016).

To summarize, the use of additives and solvent systems in the polymer matrix does make substantial differences in the properties of the produced films. The main difficulty for the viability of chitosan-based polymers is the cost of the polymer. The cost-effective Production of chitosan would enable chitosan polymers to have a broader range of low-cost applications. The current situation where the production cost is expensive, allows only niche biomedical applications where the biocompatible, apo-allergenic and antimicrobial properties of chitosan are advantageous. This an area where fossil fuel-based plastics are unable to compete given their toxicity. The many disadvantages of petropolymers are the primary reason why biopolymers have been proposed as alternatives for general polymer applications. The two main criteria are the sustainable nature of biopolymers in contrast to finite fossil fuel deposits and the environmental impacts of petroleum and related derivatives' production. In contrast, the production processes of biopolymers are much more environmentally friendly, with little to no toxic waste being produced.

The library of prototypes, developed with varying formulations and properties, will allow an application-based subset of prototypes to be adopted and utilized as viable alternatives to petroplastics. Further alterations to the unassessed CBFPs may lead to the discovery of properties required for other niche applications. The successful prototypes produced have unique characteristics based on the specific subset of components. Further optimization would be solely application-based, on prototypes in which further formulation changes can be implemented to further improve desired characteristics. Based on the properties observed in this study, there is potential for the application of chitosan-based biopolymers as alternatives to traditional petroleum-based plastics, since the mechanical characteristics are equally as good and even better in certain prototypes.

CHAPTER 5 : GENERAL DISCUSSION

The need to reduce the impact of petropolymers has never been greater when looking at the notorious legacy of the fossil fuel industry and the level of pollution petropolymers have wrought on the planet (Andrady, 2011; Van Cauwenberghe et al., 2013; Danso et al., 2019). The goal of keeping the planet healthy and habitable for future generations will only be realized by minimizing our adverse impact. Biopolymers provide an opportunity to assist this process and reduce any possible further damage to the global ecosystem.

Commercial chitosan is still largely of crustacean origin, which is seasonal and production on demand will not be met if the supply is limited. Fungi, however, can be grown in bioreactors/ fermenters for the generation of chitosan. Due to the lower yield of fungal chitosan, in contrast to crustacean chitosan, it can be used as a supplement and substitute rather than as an alternative. The increasing number of studies focusing on the applications of chitin, chitosan and their derivatives ranging from weight-loss and dietary supplements to cancer therapy, will require an increase in chitosan generation to successfully produce the needed supply for the growing demand (Jiang et al., 2019; Huang et al., 2020; Ahn et al., 2021; Tufan and Arslan, 2021). Most fungal chitosan research aims to increase chitosan yield from the fungal biomass by utilizing fermentation optimization, better chitin extraction methods, and genetic modification of the fungi strains. Manipulation of chitin production pathways selective screening for variants of higher chitin/chitosan content in cell walls and cell wall composition manipulation.

The amount of organically derived waste present on the planet is vast. Processes which use a waste product are creating business opportunities and reducing the adverse impact of the waste on the environment, i.e., contamination and pollution (Abu Yazid et al., 2017). The use of waste substrates as fermentation media will require continuous evaluation, due to differences in origin. Batch-to-batch variances also infer the need for quality control to measure composition. If the characteristics differ too much, they may not serve as viable options for the process and produce less than optimal yield. In the instance, that the batches' properties are toxic/inhibitory towards the culture, little to no biomass may be produced. This was the case with DBW in this study. It proved a difficult medium to cultivate *M. circinelloides*, as a

component of a fermentation medium and as well as a sole fermentation medium. Further pre-treatment along with detoxification and removal of potential inhibitors is necessary for future use of this waste for fungal fermentation. Alternatively, certain waste substrates may not be suitable as a complete fermentation medium, as it may only be a good source of a single type of macronutrient, required for biomass growth. Examples are MOL, with high sugar and carbohydrate content, and CSL with high protein and mineral content. A multi-waste substrate medium which is suitable for fermentation with an optimized composition, with little to no additives would be ideal for high-yielding biomass fermentations. The validated predictions of the biomass production optimization showed that fermentations could achieve high biomass yields with solely waste substrates (77.87 g/L).

Very few of the commercially important microbial biopolymers currently use waste substrates for their production (Table 2.5) (Kreyenschulte et al., 2014). Selection of substrates for fermentations should be done after studying the desired fermenting microorganism. Screening of cultures which can grow in the available substrates is a necessary prerequisite. With chitosan extraction generally yielding approximately 10% of the fungal biomass, chitosan yield per litre of validated waste substrate fermentation medium would be ±7-8 g/L. Chitosan in this study was produced from waste substrates obtained at no cost. However, waste substrates in larger volumes may incur a cost but at considerably less cost than that of commercial fungal fermentation laboratory media. Nutritional deficits of the waste substrate can be addressed by supplementation during fermentation, additives, or blending of various waste substrates to achieve an optimum growth medium. Waste substrates require extensive analyses to evaluate compositional and nutritional value along with trialling and optimization to maximize biomass/bioproduct yield. The composition of MOL whereby total sugar content is high, is apt for use as a substrate for producing many biopolymers. Waste substrates therefore represent a valuable valorization opportunity and continued studies on the use of fermentation additives to overcome the deficits present in waste substrates for high biomass/bioproduct yield are necessary (Liu et al., 2016; Safaei et al., 2016).

Co-production of bioproducts is an efficient and cost-effective method given the niche applications and the high production cost of low-volume naturally occurring bioproducts of biological processes/systems and/or organisms (Flores-Albino et al., 2012; Zininga et al., 2019).

Multiple fermentations in successive bioreactors can facilitate the production of multiple bioproducts from each stream of waste substrate, considering the affinity for certain microbes to be cultivated in each substrate to maximize the output of the fermentations.

The waste substrates successfully used in this study had no adverse effects on the chitosan polymer properties, since bleaching and proper washing steps were conducted. Minimal differences were present in the FTIR profiles of the fungal chitosan when compared to the commercial crustacean chitosan and pure substrate monomer. The results also showed that CBFPs suffered no detracting properties due to the source of the chitosan. Fungal chitosan is therefore identified as a viable alternative for use in any given applications that crustacean chitosan be utilized.

There are various applications of biopolymers, while only certain biopolymers are appropriate as alternatives to petropolymers. It is unlikely for typical plastics to function as biopolymers would (Kreyenschulte et al., 2014). The use of biopolymers as traditional petro-plastic substitutes still has many hurdles to overcome. They are hindered mainly by the production cost and technology required, along with the infrastructure and equipment needed to produce these biopolymers. There are stark differences in biopolymer production processes in contrast to typical petro-plastic production processes. The differences between the mechanical properties of biopolymers and petropolymers can be minimized by altering formulations of biopolymers. The key components for formulation manipulation used in this study were the solvents, cellulose and MMT. These provided the most notable changes in the polymers produced. The CBFP #14 has similar properties when compared to HDPE. The CBFP was the denser of two, #14 at 1.0141 g/cm^3 compared to 0.93 g/cm^3. #14 had a tensile strength of 23.53 MPa while HDPE with 21.4 MPa (Adhikary et al., 2008).

Chitosan polymers as a coating or thin film have been widely investigated. Fluoropolymers are the petropolymers of choice in the field of coatings. Despite the more robust properties of fluoropolymers, chitosan has properties which allow applications in more biologically inclined niches. A broad range of analytical techniques used to characterize prototype biopolymers, will result in a fuller comprehension of their potential. This may lead to applications which were

not initially considered. This is especially relevant for modified prototypes as these characterizations will enable comparison to the current polymers used for specific applications.

The automotive industry uses many petropolymers in manufacturing vehicle components, many of which will aim to introduce biopolymers either in pure form or as blends. Typical internal combustion engines have operating temperatures of 60 to 100°C. Polymer components near the engine compartment need to withstand these temperatures. Electric vehicles, which have even lower operating temperatures, would be ideal candidates for use of biopolymers in manufacturing of components given their thermal stability as seen in the TGA of the prototypes developed in this study (Figure 4.10). Specifically, engines and motors that are liquid-cooled have coolant operating temperatures of 80 to 90°C and use fuel and components that are fossil fuel derived. The concept of reducing the carbon footprint and opting for "entirely green" alternatives is an ever-increasing current trend. Sustainability is another driving factor for being environmentally friendly. The filamentous fungus used in this study could be used as both a fuel and component manufacture precursor, that is biodiesel and chitosan, respectively (Zininga et al., 2019).

Biopolymer/petropolymer blends which are not innately compatible by typical means of processing can use an intermediary component. These additives will permit future hybrid polymers which can be used to create blends with varying concentrations of the copolymers within the limits of compatibility of the intermediary component with the formulation. With SB imparting its original use of antimicrobial properties and also being a nucleating agent for HDPE, chitosan's petropolymers competitor. SB is compatible with the chitosan biopolymers matrix, it may be used to enable the production of hybrid polymers (Seven et al., 2016).

Method optimizations are always being conducted in research as newer methods are being developed. The newer methods may reduce the energy, labour and cost required to produce the bioproduct while potentially increasing the total amount of bioproduct produced (Zamani et al., 2010; Mahdy Samar et al., 2013; He et al., 2016b). This will in turn make entry into the applications of bioproducts more market accessible. Industries may be tempted to consider using chitosan for both new applications based on its unique properties and existing petropolymer dominated applications.

In this study, *Mucor circinelloides* ZSKP was grown on waste substrates (MOL and CSL). Biomass production was maximized using the Plackett-Burman Design and Response Surface Methodology. The validated optimal waste substrate fermentation medium yielded 77.87 g/L of biomass. The fungal biomass underwent chitin extraction and deacetylation to produce fungal-derived chitosan. The chitosan was used to develop varying biopolymer prototypes. The prototypes were assessed using physico-chemical, physical, and thermal techniques including SEM. Prototype CBFP #11 displayed the most improved properties when compared to the most typically prepared composition of the chitosan biopolymer, with a 3-fold improvement in tensile strength. A reduction in the production cost of chitosan should enable greater access to chitosan. Cost-effective chitosan will allow far more applications to be developed. Chitosan produced from fungal biomass grown on waste substrates is an avenue to achieve significantly cheaper chitosan. Fungal-derived chitosan does not have any adverse impacts on biopolymer-based applications. Formulations using varying solvent systems, additives as well as methods of preparation are the key modulators in generating considerable improvements in chitosan-based biopolymers.

As recommendations for future studies, the following areas were identified. Firstly, mass spectrophotometry in conjunction with NMR analysis and rheological analysis of the films from solution to final cast at every stage as well as post-analysis done would give a better understanding of the conformational interactions of the subcomponents during preparation as well in the simulated testing environments in efforts to maximize optimization potential of the polymer. Secondly, a statistically optimized method of film production whereby each component of the film matrix is evaluated, and every significant concentration and formulation adjusted to optimum values, would yield much more robust and application-derived polymers. Statistical optimization is the main recommendation for the enhancement of composition based on a specific characteristic or required characteristics based on a specific application. Thirdly, the use of a Teflon coating or wax applied to glass petri plates would be beneficial as rigid films with both higher chitosan content and higher additive loading are difficult to remove post-casting. Fourthly, low-temperature testing is advisable to correlate better with operating temperatures in real-world applications. Fifthly, higher magnification SEM micrographs at nanometre scale for both surface and cross-section of the biopolymer films to observe individual macro-molecular interactions between the additives and the primary biopolymer in

the film matrix are required. Finally, the use of chitooligosaccharides as a copolymer additive may produce a more cohesive matrix using varying sizes to enhance Van der Vaal's forces and intermolecular hydrogen bonding.

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