Production of chitosan and lipids from a newly isolated *Mucor circinelloides*

Submitted in complete fulfillment for the Degree of Master of Applied Science in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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REFERENCE DECLARATION

I, Mr. J.T. Zininga – 21143318 and Prof Kugen Permaul 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 carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of Prof Kugen Permaul and Prof Suren Singh.

Student's signature

DEDICATION

This work is dedicated to my late father Aaron Zininga who died on the 9th of March 2006 at the age of 42.

“The graveyard is the richest place on earth, because it is here that you will find all the hopes and dreams that were never fulfilled, the books that were never written, the songs that were never sung, the inventions that were never shared, the cures that were never discovered….”

Les Brown, motivational speaker and author

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ABSTRACT

Filamentous fungi are well-known sources of a wide variety of industrially-useful biomolecules. This study demonstrates the applicability of a newly isolated oleaginous fungi *Mucor circinelloides* ZSKP for lipid and chitosan production. Parameters affecting co-production were identified and were statistically optimized, which resulted in a 3–fold improvement in lipid production. The lipid profile showed a high content of unsaturated fatty acids including oleic, linolenic and linoleic acids, while palmitic acid was the major saturated fatty acid (21%). A comparative study to evaluate the efficacy of enzymatic and chemical treatments for biodiesel production from fungal lipids and sunflower oil revealed slightly enhanced production of biodiesel from fungal lipids, using a commercial lipase. The biodiesel synthesized using lipids from *M. circinelloides* ZSKP satisfied standard specifications and had a higher cetane number (56), lower kinematic viscosity (4.6 mm^2/s) and lower acid number (0.03) compared to sunflower oil. Upon optimizing chitosan production and extraction processes the chitosan production was improved 2-fold. The fungal chitosan showed antimicrobial properties and was more effective against *Aspergillus niger* A chitosan spray was developed which was able to increase the shelf life of fresh fruit produce. These results indicate that *Mucor circinelloides* ZSKP is a promising candidate for concurrent production of lipids and the versatile bio-polymer chitosan.

LIST OF ABBREVIATIONS

ACP	Acyl Carrier Proteins
AIM	Alkaline Insoluble Material
AMP	Adenosine Monophosphate
ASTM	American Standard Methods
CoA	Coenzyme A
DNA	Deoxyribose Nucleic Acid
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography-Mass Spectrometry
GlcNAc	D-Glucosamine
HCI	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulphuric acid
MIC	Minimum Inhibitory Concentration
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
PBD	Plackett-Burman Design
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RSM	Response Surface Methodology
SDA	Sabouraud Dextrose Agar
TDS	Transdermal Drug Delivery System
UDP	Uridine Diphosphate

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CHAPTER 1: INTRODUCTION

There is need to focus more research on sustainable use of biological waste in the production of valuable products (Taylor 2008). Thus, in this regard there has been tremendous progress made in the production of chitin, chitosan and chitooligomers from shrimp shell wastes. When shrimp is being prepared for human consumption more than 40% of the biomass is waste and from this waste as much as 40% can be recovered as chitin (Younes *et al.* 2012). These wastes are usually dumped into the sea, where they accumulate or get washed onto beaches which is one of the main pollutants in coastal areas (Viarsagh *et al.* 2009), Some are, however, used in poultry feed but these are in negligible quantities. The further processing of crustacean waste to produce valuable products has the benefits of not only reducing environmental pollution but can assume the role of a sustainable and viable waste disposal system. These shell wastes are made up primarily of calcium carbonate (30-50%), proteins (30-40%) and chitin (20-30%) (Younes *et al.* 2012). To extract chitin from shrimp shells, strong alkaline solutions (sodium hydroxide) are used for deproteination and strong acidic solutions of hydrochloric acid are used for removal of the mineral and protein constituents of the shells and these normally end up being released into the environment to its detriment (Alishahi and Aïder 2011). Therefore, finding alternative processes for chitin production and extraction is necessary. The other challenges associated with using crustaceans as a sole source of chitin for chitosan production are seasonal and limited supply, confined production locations and product variability, which leads to inconsistency of the chitin produced. Chitosan generated from the deacetylation of chitin extracted from crustacean wastes exhibits properties which are inconsistent, because of the strong acids and bases used in the isolation process. Chitosan from crustacean material exhibits inconsistent physico-chemical characteristics due to factors that include raw material batch, degree of deacetylation and protein contamination variations (Cauchie 2002). The source from which chitin is derived also determines its characteristics which includes: its purity; crystal structure; and arrangement of the polymer chain (Rinaudo 2006b). These and other problems have led to a search for other sources of chitin and one major possibility that has received much attention is its recovery from fungal cell walls.

Turning crustacean waste, which would otherwise be dumped into the environment to decompose creating a potential environmental pollution hazard, into products of high economic value like chitin, chitosan and their oligosaccharides epitomizes a perfect economic model. However, financial viability is threatened by challenge of availability of crustacean materials

which is seasonally dependent. Thus, the pursuit to find a suitable, feasible and sustainable alternative to crustacean waste is ongoing.

1.1. Chitosan production from fungi

Fungi are known to accumulate chitin and chitosan in their cell walls, presenting a possible alternative source for the biopolymers. The quantity of chitosan in the cell wall is dependent on the fungal grouping, strain, growth conditions and fermentation techniques (Akila 2014). Zygomycetes have the most abundant chitosan and most strains are known chitin deacetylase producers (Chatterjee *et al.* 2005). Fungi offers an advantage in that growth conditions can be controlled to favour optimal yield. Fungal chitosan yield considerations are critical in feasibility analysis as an alternative to crustacean waste material. The process of chitosan extraction from fungi is less rigorous as compared to crustacean materials thus it uses less quantities of strong bases and acids which would need to be responsibly discarded to minimize potential environmental damage. The major setbacks are that fungal chitosan yield is less compared to crustacean material and the process of accumulating fungal biomass is costlier. Thus, fungal chitosan production focuses on increasing yield since the yield that has been reported is still lower than that of crustacean wastes like shrimp shells. There is therefore a need to ensure that all the components of the fungal biomass are utilized to augment chitosan extraction in order to enhance economic feasibility of the process. This process would need to operate as a biorefinery. There are various biochemical metabolic products that can be commercially produced together with chitosan. These includes enzymes, amino acids and other macro-molecules like proteins and lipids. Production of these potential co-products have already been extensively reported in fungi. What remains is to harness these processes and integrate production parameters to favour optimal concurrent output.

The use of fungi as a feed stock also offers the advantage that it does not include the use of arable land as with the case with the energy crops, lower water consumption rates and herbicides are not needed. Fungi are broadly biodiverse and have been used for many decades as an important source of specific metabolites, such as enzymes, amino acids, antioxidants and polysaccharides. Thus, the presence of by-products provides an opportunity to set up an efficient fungal-based biorefinery system (Demirbas 2009b) which will reduce the pricing pressure on biodiesel. This has shifted the focus to microorganisms that accumulate more than 20% of lipids as part of their total biomass. Besides the advantage that these do not compete for arable land, they also offer the advantage that they have potential lipids with the desired fatty acid structure, high

productivity and growth rate and once they store these lipids there is minimal reutilization. This study focuses on the use of fungal biomass to produce valuable products like chitosan and lipids along the principles of the biorefinery concept. The aim is to concurrently produce chitosan with a coproduct in partial implementation of the biorefinery concept, through isolation of a high chitosan accumulating strain and then identifying the co-product to co-produce with the chitosan. The production matrix would then be ultimately optimised

CHAPTER 2: LITERATURE REVIEW

2.1. The biorefinery concept

A biorefinery is the culmination of the sustainable processing of biomass into a spectrum of marketable products and energy (Demirbas 2009b). The application of petroleum refinery methods to biomass brings about the biorefinery concept. This would entail producing bio-based chemicals as well as biofuels. Bio-based heat and power can be generated too (Taylor 2008). It has been strongly suggested that the ideal biorefinery scenario is one where a bio-fuel is produced along with a bio-based chemical bioproduct. The efficiency of these biorefineries can be enhanced when the heat and energy released during the generation of one product is used for another co-product as well as the further development of the co-product (Ferreira *et al.* 2016). The setting up of biorefinery industries are likely to result in further development, especially to rural areas. These biorefineries have the advantage of flexibility in terms of size unlike petroleum-based refineries which are always large. The different sizes also mean different biorefinery installations can combine in terms of material flow, thus completely utilizing the

biomaterial which may be locally available in most rural settings. The production of biofuels for the transportation sectors is expected to drive increased investment in biorefineries. This will be due to the high demand for fuel. That means the success of biorefineries is hinged on the feasible production of biofuels, which is still a major issue of concern as the feasibility of numerous bio-based chemicals has already been proven (Ferreira *et al.* 2013).

2.2. The use of fungal biomass

Fungi which are filamentous microorganisms generates particular interest due to their ability to grow on a wide range of substrates including agro-based and lignocellulosic waste, with different kinds of fermentations being used. These can then be subsequently scaled-up in a bioreactor. Zygomycota, Ascomycota, Chytridiomycota, Basidiomycota and Deuteromycota make up the fungal kingdom. Two fungal classes have been extensively used in various fermentation processes and these are zygomycetes and ascomycetes (Ferreira *et al.* 2013). Zygomycetes have been reported to be the highest chitosan accumulators (Nyman *et al.* 2013; Vaingankar and Juvekar 2014). Some species which belong to the zygomycetes like *Mucor* sp. have also been reported to be oleaginous. Zygomycetes are mainly saprophytes which are fungal strains growing on dead organic matter. Some have also shown pathogenicity in plants and animals, however, suitably selected strains have been used in the food industry. They are able to grow on different kinds of carbon sources from glucose to complex polymers like cellulose. Zygomycetes biomass contains chitosan, chitin, amino acids, lipids and proteins which can be present in substantial quantities. This makes it suitable as a feed for both humans and animals. They demonstrate versatility by growing in different culture media and conditions. It is important to have inexpensive unspecific substrate for biomass production to ensure that the process is economically viable. The range of carbon and nitrogen sources in which they can grow is wide, including complex sugars and lignocellulosic material. The ascomycetes are a bigger group when compared to zygomycetes. Consequently, they have a wider range of applications. The production of antibiotics by *Penicillium chrysogenum* has made this species one of the most studied industrial strains together with *Aspergillus griseus*. *Aspergillus* sp have been widely used in fermentations to produce organic acids (Ferreira *et al.* 2016). They produce enzymes that break down recalcitrant industrial residues and lignocellulosic wastes. Filamentous fungal growth allows easier biomass harvesting as opposed to single cell cultures. Non-filamentous and unicellular members of the phylum ascomycota have also been found to be producers of fermentation products like organic acids, amino acids, fatty acids, lipids etc. Therefore, the use of fungal biomass in biorefineries is a critical, relevant and strategic development to the

endeavour of establishing feasible biorefineries, in that, fungal biomass offers a different and ever-growing spectrum of products. The production of organic acids, amino acids, enzymes and antibiotics has already been reported using different fungal fermentation techniques (Jeong-Hoon *et al.* 2010; Liu, Liao and Liu 2016) .

2.3. Biochemical synthesis of chitin and chitosan in fungi

Glucose-6-phosphate is converted to fructose-6-phosphate and the reaction is catalyzed by the enzyme phosphoglucoisomerase. The fructose-6-phosphate is then combined with glutamine to give glucosamine-6-phosphate and glutamate. Glutamine is made from the combination of alpha ketoglutarate which comes from the Krebbs cycle and ammonia which would have been absorbed from growth medium. This forms L-glutamic acid which is then converted to glutamine, in the presence of the enzyme glutamine synthetase and ammonia. The glucosamine-6-phoshate is then combined with acetyl coenzyme A to form N-acetyl glucosamine-6-phosphate and coenzyme A. This is further combined with one adenosine triphosphate molecule (ATP) in the presence of the enzyme phosphoacetyl glucosamine mutase to form N-acetylglucosamine-1-phosphate. A further addition of uridine triphosphate and the enzymes pyrophosphorylase and transferase yields uridine diphosphate-N-acetylglucosamine and a free phosphate molecule. This is subsequently added to poly-[N-acetyl glucosamine]n by the enzyme chitin synthetase and magnesium cations to form chitin, which is then deacetylated by the enzyme chitin deacetylase to form chitosan (Mcmurrough, Flores-Carreon and Bartnicki-Garcia 1971; Paul *et al.* 2004; Nwe, Furuike and Tamura 2011).

2.4. Chitin

Chitin is a bio-polymer that has structural similarities with cellulose. The difference is that the second carbon atom has an acetylated amine group, thus chitin is made up of acetyl-glucose amine monomers as opposed to cellulose which is made up of glucose monomers. Chitin and cellulose are considered to be almost equally abundant (Rinaudo 2006a). The monomers are linked by an α-(1-4) glycosidic bond (Roy, Mondal and Gupta 2003). The similarity to cellulose includes linearity, inertness and insolubility in water. It also plays a structural role just like cellulose (Nitschke *et al.* 2011). The insoluble nature and rigidity of this polymer makes it very difficult to break down and this causes major pollution problems in coastal areas. The solubility of chitin is hindered by the presence of intermolecular hydrogen bonding, however, solubility can be achieved in its derivatives. These can take different shapes due to various chemical

interactions in products like hydrogels, fibres and membranes (Cauchie 2002; Domard 2011). The amine group and the acetyl group allow chitin to be both a nitrogen and carbon source, unlike cellulose. It is a white, hard, insoluble and inelastic nitrogenous polysaccharide found in the exoskeleton and internal structures of invertebrates, which are widespread in nature. This has resulted in extensive studies into possible applications of chitin and its derivatives, which has yielded positive results in the medical, agricultural, biotechnological, food, waste water treatment and industrial sectors. Chitosan is one such derivative of chitin which has found the most use.

2.4.1 Deacetylation

The removal of the acetyl groups from chitin is called deacetylation, resulting in chitosan. The process of deacetylation produces a chemical with a high degree of deacetylated amine groups, allowing for more reactive properties (Figure 2.2). The deacetylation can be achieved chemically using a strong acid at high temperatures or enzymatically using chitin deacetylase. Chitanases found in various microorganisms especially fungi can break down chitin and chitosan into monomers and oligomers.

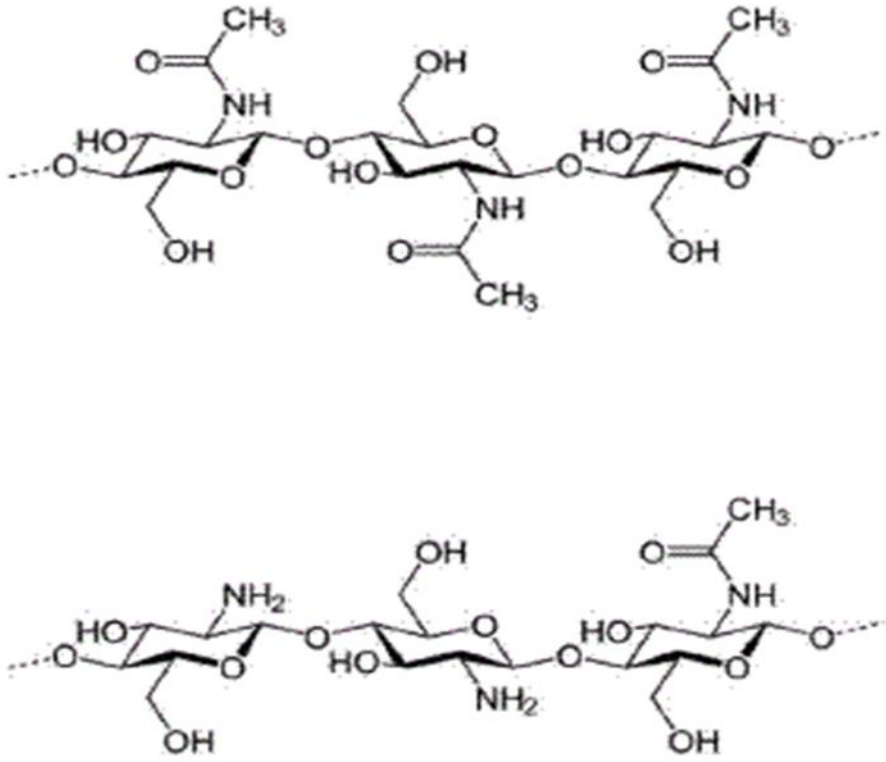


Figure. 2. 1. Partial deacetylation of chitin to form chitosan. Acetyl groups are removed from chitin (top) to produce chitosan (below) which has a mixture of glucosamine and N-acetylglucosamine residues (Rinaudo 2006b).

Chitosan may be considered as such when the degree of deacetylation is greater than 60 percent and the extent depends on the source as well as the treatment methods. Fungal chitosan generally exhibits a high degree of deacetylation (Akila 2014). Commercially available shrimp shell derived-chitosan has a degree of deacetylation of 75% and above. The degree of deacetylation has a direct influence on the physicochemical properties and this directs the kind of applications the chitosan can be utilized for. Biodegradability as well as immunological activity are also prone to the degree of deacetylation (Zhang *et al.* 2010). It is therefore imperative to give attention to the factors that affect deacetylation and these include: duration of deacetylation; the temperatures applied; the concentrations of the strong acids or bases applied; and the particle size and density of the chitin (Toan, Hanh and Thien 2013). It becomes critical to ensure that the deacetylation conditions are optimized so as to have chitosan with the desired characteristics, especially regarding solubility (Pillai, Paul and Sharma 2009). However, care has to be taken that the chitosan is not completely hydrolyzed. Various instruments can be used to analyze the deacetylation products and these include chromatography, infrared, ultraviolet and nuclear magnetic resonance spectroscopy. Thermal analysis as well as titration schemes can also be applied. In this study, the use of a more recent and highly accurate form of IR spectroscopy known as Fourier Transform Infrared (FTIR) spectroscopy was used.

2.5. Chitosan

Chitosan is the partially N-deacetylated derivative of chitin in which the deacetylation is never complete (Majeti and Kumar 2000). The distinction between chitin and chitosan has no absolute definition and many views have been debated. Some describe chitosan as simply chitin that has undergone more than 50% deacetylation whereas others define it based on its solubility in 1% acetic acid which is absent for chitin. A conclusive nomenclature has not been agreed upon with regards to the distinction of the extent of N-deacetylation between chitin and chitosan (Montgomeryl, Welschmeyer and Kirchman 1990). However, chitosan can be safely described as a polymer made up of β-1, 4 D-glucosamine (GlcNAc) monomers, derived from the deacetylation of chitin. The extent of deacetylation varies which means there are some N-acetylglucosamine residues in the polymeric chain (Wu *et al.* 2005; Rinaudo 2006a).

2.6. Structure and properties of chitosan

Chitosan is a polymer that has no toxicity, is biodegradable and can be of high or low molecular weight depending on the source of the chitin and the conditions of deacetylation. Just like chitin, it is similar in structure to cellulose as shown in Figure 2. 1.

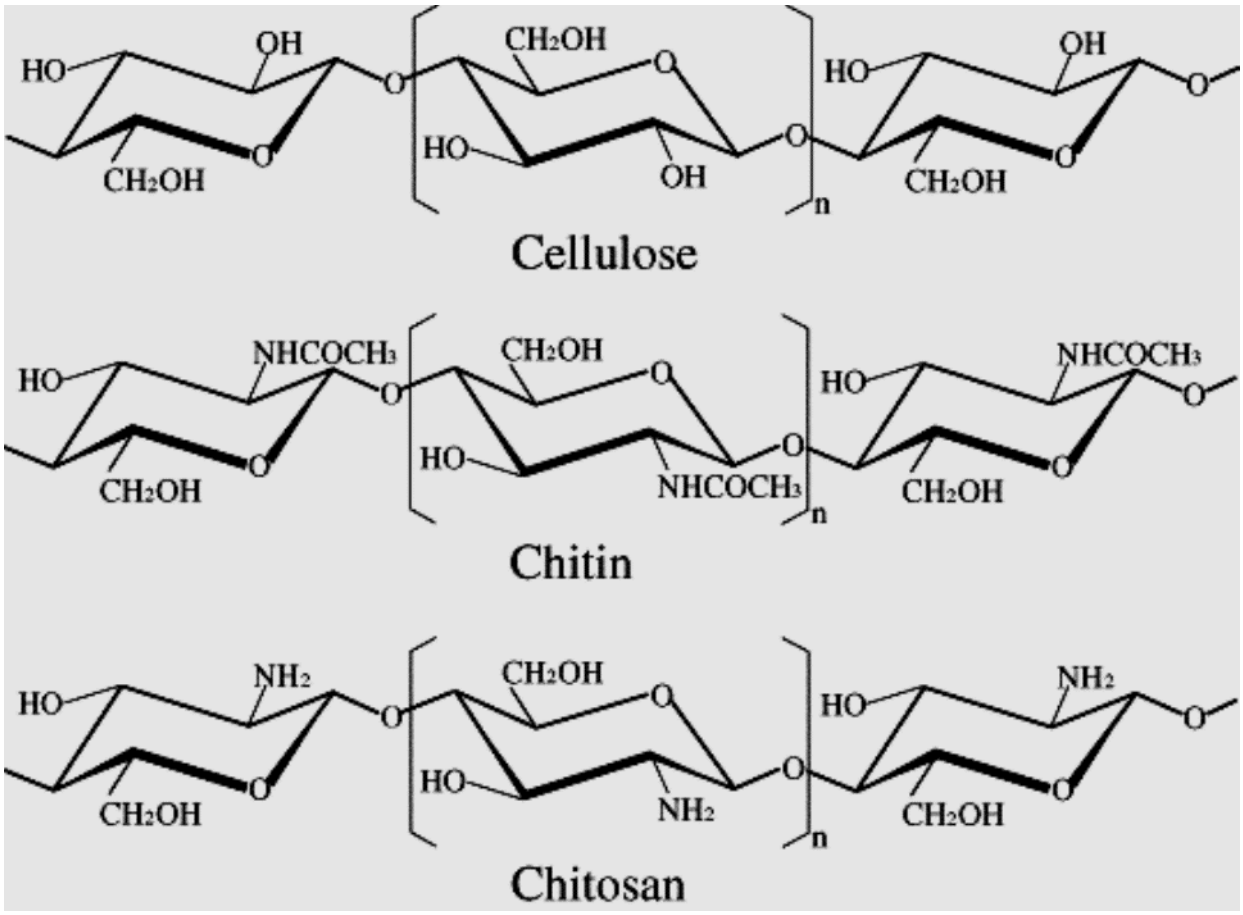


Figure. 2. 2. Structural similarity between cellulose, chitin and chitosan (Pillai, Paul and Sharma 2009).

As the above diagram shows, the only differences between chitosan, cellulose and chitin is the carbon atom number 2 which has an amine group for chitosan instead of the hydroxyl group in cellulose and the N-acetyl group in chitin. Unlike cellulose, chitosan (and chitin) possesses positive ionic charges as a result of the protonation of the amine group in solution and can interact chemically with negatively-charged molecules (Chatterjee *et al.* 2005; Wu *et al.* 2005). It is because of this seemingly small difference that chitin and chitosan have become commercially important as suitable resource materials with useful properties including ability to

form edible biodegradable films, antimicrobial activity and to chelate metal ions (Majeti and Kumar 2000; Toan, Hanh and Thien 2013).

2.6.1. Physical properties

Chitosan in its solid state shows great variability in terms of physical and chemical properties. It is whitish in color with a semi crystalline structure. Single crystals can be produced from low molecular chitin after complete deacetylation (Zhao, Park and Muzzarelli 2010). The molecular weight of extracted chitosan is influenced by its source. Fungal-sourced chitosan is generally of low molecular weight while crustacean waste material-derived chitin is of higher molecular weight, ranging between 100000 to 1200000 Da (Nwe, Furuike and Tamura 2009; Zamani and Taherzadeh 2010). The viscosity as well as the rate of degradation is directly dependent on the molecular weight and this can be demonstrated using various techniques including viscometry, gel permeation chromatography and light scattering (Nitschke *et al.* 2011).

Chitosan unlike chitin is soluble in organic acids at a pH below 6. This is because of the dissociation constant of the amine group for protonation. Chitosan with protonated amine groups acts as a strong base. This therefore means the solubility of chitosan is strongly dependent on pH (Qin *et al.* 2006) . As the pH increases to above 6 the amine group is deprotonated and the net charge becomes zero, reducing interactions and making the chitosan insoluble. Since solubility is affected by whether the amine group is protonated or not, the degree of deacetylation is important as it determines the amount of amine groups that are subject to the pH induced protonation and deprotonation (Zamani and Taherzadeh 2010).

2.7. Characterization of chitosan

Infrared (IR) spectroscopy has been extensively used by scientists for chitosan and chitin analysis. It is premised on the principle of atomic vibrations in molecule upon the application of infrared energy. When infrared radiation is directed towards a sample that has dipole, whether induced or permanent, a spectrum is achieved and different fractions of the radiation are absorbed in at a corresponding energy particular to that fraction (Corazzari *et al.* 2015). The atomic vibrations are uniquely specific to molecular bonding between atoms and the energy released is reflected in peaks which can therefore be used to qualitatively identify bonding or functional groups unique to a sample. In order to improve the quality of the obtained spectrum

and to reduce the overall time needed to generate the data, an interference process is added to the analysis of the spectrum and conversion made into wavenumber or frequency. This is the principle behind the use of a Fourier-transform infrared spectroscopy (FTIR) (Duarte *et al.* 2002; Beil *et al.* 2012).

2.8. Fungi as a chitosan source

The search for an alternative source of chitin raw material has not yielded much. The major disadvantage of crustacean wastes is the challenge of seasonal availability and thus an alternative has to tick all the other boxes that crustacean wastes tick plus the main benefit of availability not dependent on seasons (Younes *et al.* 2012). Progress made in fermentation technology has opened up an opportunity to culture an organism that contains chitin or chitosan. Fungi are suited for this as there has been reports of chitosan accumulation in the fungal cell wall (Vaingankar and Juvekar 2014). The chitosan can be produced via fungal growth and the process can be optimised to increase productivity. The production of fungal biomass can be done throughout the year using fermentation technology (Paul *et al.* 2004). The controlled fermentation conditions can be adjusted to allow rapid production over short periods of time and this can be done continuously or in batches. The fact that fungal mycelia does not show drastic variation in terms of composition (Streit *et al.* 2009) is critical and an even better property is that it does not contain the high levels of inorganic metal ions hereby eliminating the acid and temperature intensive demineralization step used for crustacean extractions. It has been reported that the physical properties of chitosan are prone to manipulation through the adjustment of factors that make up the growth conditions as well as the downstream chitosan extraction techniques (Vaingankar and Juvekar 2014). Thus, the mycelia of various fungi including Ascomycetes, Zygomycetes, Basidiomycetes and Deuteromycetes, are potentially a viable alternative source of chitosan to crustacean waste.

2.9. Applications of chitosan

Chitosan has applications in various fields which include cosmetics, food processing, industrial, medical, agricultural and biotechnological uses. The use of chitosan in these fields spans a period of over 30 years (Domard 2011). Application of chitin is hindered by poor solubility; hence chitosan has found more extensive use due to the improved solubility in organic acids.

2.9.1. Industrial applications

Chitosan has biofilm-forming abilities which has shown great resistance to abrasions. This together with the fact that it hast good optical characteristics makes it applicable in photography. Silver complexes are not strongly attached to chitosan and thus through diffusion are readily penetrated from one film layer to another (Dutta *et al.* 2009).

Chitosan embedded in paper reinforces its wet strength. Despite the availability of this polymer in large quantities it does not make commercial significance for this application due to its inertness However, chitin attached with hydroxymethyl groups as well as its water-soluble derivatives make it applicable in the manufacturing of paper. It therefore has the potential to be utilized especially to improve the properties of manufactured paper by giving it a better finish (Rinaudo 2006a).

Chitosan does not dissolve in water. This presents a challenge when used in solid state proton conducting batteries as the chitosan has to act as the proton donor and this can only be possible if there is water present to interact with the chitosan. Since the proton conductor is a polymer which is vital for solid state battery applications it means chitosan whilst being a potential proton donor polymer cannot be used alone because of its water insolubility. When chitosan is dissolved in acetic acid, it does provide proton conductivity (Qin *et al.* 2006). This is as a result of the partial dissociation of the acid in solution. There are micro voids in the chitosan polymer which acts as transport channels perhaps because of the dielectric constants being shown to be small. However, to make a better battery there has to be better electrode material selection (Akila 2014).

Chitosan can be used for soil repellent and antistatic properties in clothing and this is applied in the textile industries. The structure of chitosan is such that it allows it to bond with various classes of dyes that includes reactive, sulphur, disperse, acid, naphthol and direct dyes. It has a high affinity for these dyes. Consequently, it can also be used in the treatment of wastewater to remove dyes from effluent released after dye processing. Chitosan has also been used synthetically in the manufacture of medical related fibres, threads and sutures (Krajewska 2004; Wan Ngah, Teong and Hanafiah 2011).

Chitosan finds a wide variety of applications in chromatographic separations. The free hydroxyl and amine groups on the chitosan polymer, renders it as a suitable chromatographic support. It has been used in thin layer chromatography, for separation of nucleic acids or as sorbent material in solid phase extraction of phenol and chlorophenols using HPLC. In more advanced technology, dyes containing chitosan gels have been used as potential components in lasers and other light-emitting devises. This process is called doping and it utilizes dyes that resemble heme groups in blood (Rashidova *et al.* 2004).

2.9.2. Biomedical applications

Chitosan has all the properties critical for the production of an ideal contact lens. These properties include mechanical stability, gas permeability, oxygen permeability and immunological compatibility. Spin casting technology is applied in the manufacturing of contact lenses from purified chitosan that has been partially depolymerized. The contact lenses exhibit desirable physical properties that include modulus, elongation, permeability to water and oxygen as well as tear and tensile strength. The antimicrobial properties that chitosan possesses allows for wound healing hence the chitosan biofilms are relevant in the development of ocular bandage lenses (ElShaer *et al.* 2016).

Permeation-controlled transdermal drug delivery systems (TDS) using chitosan has been reported (Bhattarai, Gunn and Zhang 2010). Propanolol hydrochloride (prop-HCl) chitosan-based drug delivery systems uses various crosslinked chitosan membranes and these membranes may have different densities. The membranes act as a drug release control mechanism whilst the chitosan gel binds the drug. The physical and chemical properties of the membranes have been established with reports being made regarding permeability of the membranes to hydrophilic and lipophilic drugs. When a drug release profile was obtained *in vivo,* it showed that the drug prop-HCl was released in a manner that is reliable and reproducible. When chitosan membranes which are cross linked were used as way to regulate drug release, the release of prop-HCl was reduced drastically. The rate of release of the drug was influenced by the density of the cross links within the membranes (Thacharodi and Rao 1995).

The development of cells, tissues and organs *in vitro* summarizes the field of tissue engineering. This is done to revive or replace parts of the body that might be damaged. Some research has focused on the exploration of chitosan application in tissue engineering where it has been seen

to accelerate the processes owing to its polycationic nature. The degree of this depends on the level of deacetylation of the chitosan (Croisier and Jérôme 2013).

Serious burns have always been a medical problem and their treatment is often a lengthy process which is plagued by infections and chronic pain for patients. Chitosan does form strong, water sorbent, biocompatible films which can be layered directly onto the burn area as dissolved chitosan acetate solution. This treatment by chitosan solution enhances oxygen permeability and prevents oxygen deprivation of injured tissue. In addition, the chitosan film is naturally absorbed by the body as the wound heals which eliminates the risk of further damage to the burn site when dressings are removed (Jayakumar *et al.* 2011).

For wound therapy, nowadays, artificial skin is available, but it is very expensive so biopolymers such as chitosan which are cheaper and readily available are of great importance. The design of artificial skin which is applicable for long term use, focuses on manufacture of a non-antigenic biodegradable membrane for the synthesis of neodermal tissue. Chitosan polysaccharides can be considered for development of such a membrane (Jayakumar *et al.* 2011).

There has been development of surgical dressings that are composed of complexes made up of chitosan and gelatin. These dressings, when compared to the commonly used biological dressing showed better subcutaneous fat adhesion. The subsequent acceleration of wound healing as a result has been attributed to chitosan degradation by tissue enzymes which would release oligomers and these are vital in skin tissue regeneration which would complete wound healing (Ueno, Mori and Fujinaga 2001).

Chitin and its derived chitosan sulphates can act as blood anticoagulant as well as activate the release of lipoprotein lipase. For chitin 3,6-sulphate anticoagulant activity has been shown to double in its presence together with a 0.1-fold increase in the release of lipoprotein lipase when compared to the effect of heparin. Thus these derivatives can find potential application as heparinoids especially in blood dialysis (Vongchan *et al.* 2002).

Chitosan has also attracted some attention from pharmaceutical industries due to its reported antitumor activities. Tests have been done in mice and these have shown that chitosan and its

oligomers can prevent the proliferation of cancerous cell growth and indications are that this is done by enhancing the effects of the immune system (Westerink *et al.* 2002).

2.9.3. Biotechnological applications

The activity of extracellular lysozyme was increased in vitro mammalian cell cultures after addition of chitosan. This meant that the formation of connective tissue was initiated and the mechanism by which cells defend themselves against microbial infection was enhanced. These results have formed the premise upon which the commercial production of chitosan-based dressing materials for wound treatments in both humans and animals, is based (Wei *et al.* 2011).

Chitosan and other modified forms of this compound have been incorporated in the manufacture of antibacterial compounds. Chitosan has antibacterial activity against *E. coli* at concentrations as low as 0.025%. Chitosan has also been reported to inhibit the growth of *Fusarium*, *Alternaria* and *Helminthosporium*. The antibacterial activity seem to be related to the cell wall structure as the cationic amino groups of chitosan probably bind to anionic groups on the cell walls of these microorganisms, resulting in growth inhibition (Goy, Britto and Assis 2009).

Haemostaticity and thrombogenicity were confirmed for chitosan fibres in an in vitro test with chitosan fibres, N-hexanoyl and N-octanoyl groups were however anti-thrombogenic. Thus these chitosan fibres can be applied as haemostatic material; with N-octanoyl and hexanoyl fibres finding application as anti-thrombogenic materials (Badr *et al.* 2014).

Chitosan can also be used for weight loss. This application of chitosan as a dietary aid was introduced in 1997 and it has been known as a fat blocker (Yuan *et al*., 2006). This happens in the stomach upon ingestion when the chitosan attaches to the fats forming a complex that cannot be absorbed into the blood stream for metabolic processes. The resultant fat-chitosan complex can only be eliminated out of the body through the process of egestion. The uniqueness of chitosan fibre is in the ionic charge that it possesses as a result of protonation of the amine group and this gives it the ability to form ionic interactions with negative ionic charge ends of physiological molecules like fats, bile salts etc (Yuan, Li and Yuan 2006).

2.9.4. Food processing applications

Chitosan has been applied with great success in the food industry and this is mainly due to non-toxicity to humans. A good example is its use as a thickening agent in soups and ice-cream or as a food stabilizer. The fact that it cannot be digested by humans means it is also used as dietary fibre in baked foods. It can be used as a carrier for highly concentrated ingredients like food colourants. In India, it has been successfully incorporated into poultry feed where it decreases food consumption whilst at the same time increasing the weight of the animals (Aider 2010).

Due to its partial positive charge, chitosan is widely used in the recovery of important by-products like proteins which can be utilized in other areas like production of feed stock. Proteins and other peptides have also been removed from whey cheese using chitosan (Di Pierro *et al.* 2011).

In the fruit juice industry, various chemicals like gelatin and bentonite are employed to add clarity and aesthetic appeal to the product. Chitosan salts have also been shown to be effective as clarifying agents, as well being used to control acidity in fruit juices. Chitosan has found acid-controlling use in fruit juices like in grape juice, where addition of chitosan at a concentration of 0.015 g/mL resulted in a total acid content reduction of 52.6% (Chatterjee *et al.* 2004; Rungsardthong *et al.* 2006; Domingues *et al.* 2012).

Due its antibacterial and fungistatic nature, chitosan has been used as an edible protective coating for fruits (Jianglian 2013). Studies have shown that signs of infection in chitosan-coated fruits appeared days later compared to non-treated fruits when the fruits are left at the same storage temperatures. After more than two weeks of storage, chitosan-coating also reduced decay of strawberries by more than 60% without causing phytotoxicity in any of the treated fruits (Chien, Sheu and Yang 2007) .

2.9.5. Cosmetic applications

Organic acids are the most common solvent used in cosmetic products. Chitosan is suitable in these applications as it is readily soluble in organic acids. Chitosan also has the unique and very useful characteristic of becoming viscous when neutralized with acid. This makes it compatible with common skin and hair products. It also adds fungicidal and antibacterial nature to the products, increasing their functionality. Chitosan and hair carry opposite charges hence,

chitosan-containing hair products readily form a film on the hair making chitosan useful as an additive to shampoo, hair colourants and styling lotions. Chitosan is also useful in skincare products due to its positive electrical charge and high molecular weight which prevents chitosan from penetrating the skin. Thus, it can function as a low-cost moisturizer. Chitosan is also used in toothpaste, mouthwash and chewing gum because it aids in freshening the breath and prevention of plaque formation and tooth decay (Jimtaisong and Saewan 2014) .

2.9.6. Water engineering applications

Chitosan has a polycationic nature and high sorption capacity which means it can be used as a good flocculating agent in the purification of water and countries like the USA are already making use of this technology. It also has the ability to chelate and trap heavy metals dissolved in water. This has become very important as we have come to understand the dangers posed by heavy metals and pesticides if they accumulate in the food chain (Al-Manhel, Al-Hilphy and Niamah 2016; Rocha *et al.* 2016) . Its flocculating capability is also utilized to recover important ingredients from food processing waste

Chitosan has also been used as a sorbent to remove colour from dye house effluents and this has seen chitosan and its derivatives being globally used in water treatments. It has been used to decontaminate plutonium and methyl-mercury acetate containing water which is a major pollutant from the production of acetaldehyde. Other pollutants removed by chitosan flocculation and chelation are petroleum and arsenic. Regenerated chitosan could also be used for major processes like osmosis, membrane filtration, desalination, dialysis and haemodialysis (Crini and Badot 2008; Shen *et al.* 2011; Wan Ngah, Teong and Hanafiah 2011).

2.9.7. Agricultural applications

To prevent considerable economic losses, many studies have been developed for crop protection, such as seed coating technology as a form of protection against pests and diseases. It has been noted that addition of chitosan to soil results in reduction in pest infestation and also suppresses the development of fungal pathogens (Kashyap, Xiang and Heiden 2015). When seeds of wheat and other crops are pre-treated with chitosan they are found to exhibit significant acceleration in growth. Maize seeds pre-treated with chitosan showed reduction in germination time and increased shoot height and root length. Chitosan can also be used as a coating on delicate plants for frost protection (Sharp 2013).

Chitosan is often used in conjunction with chitin in agriculture where it has been reported to enhance growth and development by increasing key enzymes activities of nitrogen metabolism (nitrate reductase, glutamine synthetase and protease) and improving transportation of nitrogen in the leaves (Ohta *et al.* 1999).

Chitosan has been described as a "plant defense booster" with a mode of action that stimulates natural defense response systems in treated plants. It causes a wide range of physiological, biochemical and structural changes to take place within the target plant, suggesting possible alterations in the allocation of plant resources (Sharp 2013).

Chitosan has been applied as a soil amendment where it has inhibited growth of many fungal pathogens including the prevention of infection by *Fusarium acuminatum* and *Cylindrocladium floridanum* in forestry nurseries. Chitosan has also been used to increase the production of secondary metabolites when plants are grown *in vitro*. When *Brugmansia candida* was supplemented with chitosan at certain concentrations, it increased the content of root scopolamine and hyoscyamine, which are both valuable anticholinergic drugs used in the treatment of motion sickness (Kamari, Pulford and Hargreaves 2011; Sivanandhan *et al.* 2012).

2.10. Antimicrobial properties of chitosan

One of the most important properties of chitosan is its ability to inhibit the growth of microorganisms. This property is dependent on whether the chitosan is modified or natively derived, the source of the chitosan, the procedures of extraction, the environmental conditions and the extent of polymerization. This quality is the driving force behind many of its applications in different industries. It has been suggested that chitosan antimicrobial properties are subject to its molecular weight and this is evident when fungal and shrimp shell chitosan show antimicrobial activity at different concentrations against the same microorganisms (Wu *et al.* 2005; Qin *et al.* 2006; Toan, Hanh and Thien 2013).

The propagation of viruses and viroids was inhibited in plants with the addition of chitosan which acted by enhancing the host's hypersensitive response to infection. Studies have discovered that

a wide variety of important viruses like potato virus X, tobacco mosaic virus and necrosis viruses, which show susceptibility to chitosan (Chandra *et al.* 2017).

A wide range of bacteria like *Escherichia coli*, *Staphylococcus aureus*, some *Bacillus* sp, and several bacteria that infect fish are known to be inhibited by chitosan and its derivatives. The minimal growth-inhibiting concentration varies among species from 10–1,000 ppm. Of particular note are quaternary ammonium salts of chitosan, such as N,N,N-trimethylchitosan which has proven to be highly effective against strains of *E. coli*. The mechanism underlying the inhibition of bacterial growth is based on the interaction between the positively-charged amino-group on the chitosan backbone and the anionic components like N-acetylmuramic acid found on the cell surface. This may disrupt bacterial growth by impairing the exchanges with the eternal medium and inhibiting enzymes (Chatterjee *et al.* 2005; Domard 2011).

When used at pH 6, chitosan has shown great fungicidal activity against many fungi and oomycetes involved in plant pathogenesis. Its mode of action is to stimulate natural defense response systems of the plants via poly-D-glucosamine which binds to fungal receptor sites and mimics an attack by fungal spores. This sends signals to the nuclei of the plant and triggers signals causing genetic and biological responses, like the production of phytoalexins (anti-microbial compounds produced in plants), to inhibit infections. Studies show that 24 new derivatives of chitosan in the sub-groups of N-alkyl and N-benzyl chitosan have excellent anti-fungal properties which are much higher than native chitosan (Li *et al.* 2016; Tan *et al.* 2017).

The increase in chitosan derivatives has also led to the discovery of novel uses, as is the case with new chitosan-based insecticides whose activities are being reported using an oral larva feeding bioassay. The most active chitosan derivative is N-(2-chloro-6-fluorobenzyl) chitosan, which causes 100% mortality of insect larvae (Sajomsang *et al.* 2009; Prabhakar *et al.* 2016).

2.11. Fungal lipid accumulation

The ability of oleaginous fungi to efficiently accumulate intracellular neutral tryacyl glycerates renders them potential feedstock for biodiesel production (Karatay and Dönmez 2011). The use of microorganisms as feedstock for biodiesel production is affected by the high costs which competes unfavourably with plant oils. There is therefore a need to optimize the lipid yield as well as find cheap carbon and nitrogen sources. There is also a need to strike a balance between

lipid accumulation and biomass yield, which are not necessarily complementary. Thus, screening for novel oleaginous microorganisms focuses on isolates that accumulates lipids extensively even when growing on cheap sources of nutrients. The use of recombinant DNA technology and metabolic engineering techniques can be used to modify microbial strains towards high lipid accumulation, since genetic variation is also influential in the variation in lipid content amongst different strains and isolates even of the same species. Certain strains of microalgae, fungi and yeast have been reported to be oleaginous (Carvalho *et al.,* 2015). There has been special attention given to endophytic microorganisms as a source for various metabolites and lipid accumulation has also been investigated with positive results. It is important for fungi to possess inherent genetical traits to accumulate lipids without catabolic reutilization, especially when growing on cheap substrates as these two qualities would go a long way in enhancing feasibility in commercial utilization of fungal lipids. Lipids are important as a source for energy storage in some lipid accumulating microorganisms and this storage can be as high as 70% of dry matter. It appears that an excess of carbon source together with limitation of the nitrogen source triggers lipid accumulation. It has even been suggested that a carbon to nitrogen ratio of 100:1 and above would trigger lipid accumulation. In some instances the carbon to nitrogen ratio has been increased to as high as 140 with corresponding lipid yield increase (Dey, Banerjee and Maiti 2011; Galafassi *et al.* 2012; Carvalho *et al.* 2015) .

2.11.1. Lipid biosynthesis in fungi

The first step towards lipid accumulation is the synthesis of fatty acids and this takes place in the cytosol, in a reaction that is catalyzed by the enzyme Fatty Acid Synthetase (FAS). FAS needs pantothenate as a coenzyme, which activates the Acyl Carrier Protein (ACP). FAS is made up of six alpha and six beta subunits with each subunit having four functional domains. The six alpha subunits are encoded with f*as*1 whilst the six beta subunits are encoded with *fas*2. FAS is therefore a complex made up of the respective subunits with a size of 2.6 MDa. There are 48 functional centers to catalyze all the fatty acid synthesis reactions which are in a series of multistep reactions (Subhash and Mohan, 2014). Acetyl Coenzyme A (ACA) is first added to the Beta-Hydroxyacyl-Acyl Carrier Protein Synthase (BHACPS). This then initiates a cyclical chain of reactions that involves the enzymes β-Ketoacyl- Acyl Carrier Protein Reductase (BKACPR), β-Hydroxyacyl- Acyl Carrier Protein Dehydratase (BHACPD) and Enoyl- Acyl Carrier Protein Reductase (EACPR) sequential activity. ACA has to be constantly supplied for the initiation whilst Malonyl Coenzyme A (MCA) is needed for the elongation. The accumulation of lipids is subject to the mechanisms of biosynthetic pathways with the supply of

precursors like ACA, glycerol-3-phosphate and NADPH as a cofactor. Palmitic acid or myristic acid are the main products of fatty acid synthesis but this depends on the strain. The endoplasmic reticulum is the place for further elongation. Elongases catalyses the elongation and this requires MCA. Double bonds are inserted by desaturases. Esterification with glycerol or sterols on the FAS produced in the initial synthesis yields triacyl-glycerol and steryl esters and these are kept within lipid bodies (Ratledge 2004; Subhash and Mohan 2014; Carvalho *et al.* 2015)

2.12. Fungal lipid composition

Lipids in fungi accumulate abundantly serving various functions like storage in lipid bodies especially with regards to neutral lipids and also has a structural role in phospholipids. The composition of the lipids in the cell is determined by the size and complexity of the fungal cell. The quantity of lipids, however, is a result of various factors which include the age of the cell and growth conditions, thus, this can be manipulated to favour higher lipid content. Triacylglycerate, considering their storage role have the biggest contribution. They are necessary for energy-yielding catabolic and macromolecule-yielding anabolic processes. The other group that is quite significant is the sterols which serves the role of enhancing intracell permeabity of the lipids, especially acyl lipids as they have a liquifying effect on. They are also necessary in the making of steroids hormones that are active in sexual reproduction as they act as precursors. Phospholipids are also quite significant due to their structural role. The major fatty acids present in fungi are palmitic acid, oleic acid and linoleic acid. Palmitoleic acid, linolenic acid and stearic acid are also present though in lesser quantitites. gamma-linoleic acid is found uniquely in Mucorales. Other fatty acids found are docosahexanoic acid, eicosapentaenoic acid arachidonic acid (Pannkuk *et al.* 2014; Niu *et al.* 2017).

2.13. Fungal fermentation

Submerged fermentation has the uniform and reliable culture homogeneity which allows more effective transfer of heat and mass than solid state fermentation. This would enable flexibility and reproducibility. It is also easier to monitor. The way fungal mycelia grows within the media can result in different rheological properties (Vieira *et al.,* 2008). Filaments can develop into dense mycelia which would result in a viscous medium that would not allow effective oxygen and mass transfer. It is better when the filaments wrap around each other to form knots which

would result in a less viscous medium. Aeration is key in the optimal growth of fungi in bioreactors (Vieira *et al.* 2008). Thus, an effective oxygen transfer rate is a priority. However, this would also mean a greater demand for energy in terms of power input especially in widely used stirred tank reactors where the stirring rate would have to be increased. Bubble columns are an option that can be pursued as an alternative to the stirred tank reactors and does not contain any mechanical parts inside, thus eliminating the issue of fungal filament attachment. The airlift reactor therefore has better oxygen and mass distribution. The biggest advantage of the airlift bioreactor is that has a lower energy demand and more than 50% of energy is saved when compared to using a stirred tank reactor (Cui, Lans and Luyben 1997; Cui, Lans and Luyben 1998; Vieira *et al.* 2008).

Microorganisms can grow on material that is solid and with little moisture to give for example chitosan and lipid accumulation that is significant in solid state fermentation (Singhania *et al.*, 2009). Solid state fermentation offers advantages in that it is simple without the need of much energy use. Easier downstream processing which would also significantly lower the costs. Solid state fermentation can be modified by increasing the moisture content in a process called semi solid-state fermentation system. This would ensure the effective growth of single cell organisms and fungi in that it offers improved availability of sugars necessary for growth. There is however major limitations with the above two methods and in order to optimally attain high fermentation yield, submerged fermentation is preferable (Pandey, Soccol and Mitchell 2000; Pandey 2003; Singhania *et al.* 2009).

2.14. Nutrients in media for fungal growth and lipid accumulation

Nitrogen and phosphorous are critical nutrients needed by microorganisms to grow. They also need micronutrients like silica, calcium, magnesium, cobalt, potassium, zinc, iron, manganese, sulphur and copper. The above-mentioned elements and compounds are essential for growth and need to be added into the media. Nitrogen is needed by the cell for protein and nucleic acids synthesis. It is an essential component especially in the initial growth phase of the fungi. An absence of nitrogen means an absence of enzymes and nucleotides and this results in retarded growth. Nitrogen, therefore, has to be readily available to the fungi in sufficient quantities as well as in a form which is easily accessible. Its utilization has to be enhanced and unhindered for faster growth rate and hence higher productivity and a higher biomass yield. Nitrogen is normally available to microorganisms as ammonia, urea and nitrates. However, the continuous presence of nitrogen in the media whilst giving a higher biomass yield does not result in a higher yield for

lipids, as the excess carbohydrates available are converted to proteins when nitrogen is present (Carvalho *et al.,* 2015). Thus, there is need to modulate a balance in biomass and lipid yield in the context of nitrogen availability and induced deprivation, if possible. Limitation of nitrogen at the later stage in the log phase will result in more triglycerides accumulating in the fungi as there will be excess carbohydrates with little free nitrogen available for protein synthesis hence the carbohydrates are channeled into lipid synthesis biochemical pathways. Whilst total deprivation of nitrogen seems to increase lipid content in cells more than limitation of nitrogen, it however lowers the productivity so much that higher lipid yield is recorded from nitrogen-sufficient cultivation than in nitrogen-deficient cultivation (Ratledge, 2004; Karatay and Dönmez, 2010). Phosphorous is important for the formation of phospholipids, which are used when making membranes for the cell. It is also critical in the initial phases of the growth cycle. A total deprivation of phosphorous at the later stages of the log phase will increase the amount of neutral lipids which are more desirable as feedstock for biodiesel production Oleaginous yeasts react to a nitrogen deficiency by activating adenosine monophosphate deaminase which triggers an ammonium supply to the cell. The use of AMP means there is a decrease of its concentration in the mitochondria which affects the enzyme isocitrate dehydrogenase. This causes the isocitrate in the Krebbs cycle to accumulate. The excess isocitrate is exported out the mitochondrion where the enzyme ATP-citrate lyase (ACL) acts on the isocitrate resulting in oxaloacetate and acetyl-CoA products (Ratledge 2004; Karatay and Dönmez 2010; Galafassi *et al.* 2012; Akpinar-Bayizit 2014; Subhash and Mohan 2014; Carvalho *et al.* 2015).

Nitrogen is a major component in proteins and nucleic acids while carbon molecules are indispensable for energy and anabolic metabolic pathways. Carbon is present in carbohydrates, proteins, nucleic acids and lipids. The limitation of nitrogen slows down growth with the subsequent cessation of protein and nucleic acid synthesis. In some species, which are not oleaginous excess carbon is stored in storage polysaccharides as opposed to oleaginous species which will convert the excess carbon into storage triacylglycerates domained in intracellular lipid bodies. Lipid accumulation is therefore triggered by an environment that has excess glucose with the other nutrients especially nitrogen and phosphorous in limited quantities and availability (Laoteng, Čertík and Cheevadhanark 2011; Garay, Boundy-Mills and German 2014)

2.15.1. Biodiesel production

Since the 1970's there has been growing interests in using vegetable oils as an alternative as a matter of necessity considering the non-renewable and polluting nature of petroleum biodiesel.

There have been modifications to the diesel engines as a result of technological and operational advancement that brings the suitability of vegetable oils as an alternative due to high viscosity and low volatility. These would result in a fuel spray (poorly atomized) resulting in coking of injectors and valves. Thus, it became important to refine the vegetable oils to make them suitable using various attempts and these include pyrolysis blending and microemulsion. This, however did not solve the challenge of coking due to carbon deposits, hence transesterification became favourable. Transesterification involves the hydrolysis of the triacylglycerate into monoesters. Transesterification solves the viscosity problem as a result of the ester exchange yet the heat values are maintained. The cetane number is increased. Glycerol, a useful by product is produced at a ratio of 1 kg for every 9 kg of biodiesel produced (Amin 2009; Basha, Gopal and Jebaraj 2009; Atadashi *et al.* 2012).

Fungal lipids are quite suitable for the making of biodiesel which differs from petroleum diesel in that the source is biological and hence renewable. Some of the benefits include non-toxicity and biodegradability. It is a monoalkyl ester of free fatty acids found in renewable feed stocks in the form of plant, microalgal, animal and fungal oils. Biodiesel is suitable for compression ignition engines. Biodiesel has characteristics similar to petroleum diesel fuel. Animal fats have the disadvantage that they are not readily available and when they are available they are not in quantities which are sustainable. Vegetable oils raise concerns of arable land being used to grow them. Crop-based oil is suitable for biodiesel production from a chemical standpoint; however, the environmental impacts and sustainability are of major concern. Microalgal oils which have generated much interest in that they have shown to be suitable feedstocks for biodiesel whilst being renewable and potentially sustainable. Fungal and microalgal oils (single cell oils) are predominantly unsaturated and thus the viscosity problems are minimal. However, the challenge with these oils is the formation of trumpet and coke on injectors which prevents the proper occurrence of fuel atomization. In worst case scenarios, that would include carbon deposits, oil ring sticking and fuel atomization is prevented altogether (Karatay and Dönmez, 2011; Carvalho *et al.,* 2015). Other problems are encountered during storage as the double bonds in the unsaturated lipids are susceptible to attack by oxygen thereby causing rancidity. However, despite this, microalgal oils are more viable considering the large quantities required. Other advantages are liquid nature portability, heat content (80% of diesel fuel), ready availability and renewability.

Biodiesel has proven to be a viable renewable fuel that is compatible with petroleum diesel-powered engines (Maa and Hanna, 1999). When compared to petroleum diesel, it has the advantages of being biodegradable, less toxic and a cleaner emission profile. However, the use of biodiesel has its own disadvantages. Chief among them is the high costs involved in the production process and the major contributing factor being vegetable oils which costs 70-90% less (Chisti, 2007; Basha *et al*., 2009;. The use of plant oil mean that biodiesel production has to compete with the food industry for oil crops, a scenario that is untenable and unsustainable . This led to the pursuit for non-food crops like jatropha as an oil source but still those crops would have to compete with food crops for arable land and water. It has been calculated that huge tracts of arable land would have to be cultivated to meet some of the current biodiesel demands (Maa and Hanna 1999; Chisti 2007; Basha, Gopal and Jebaraj 2009; Leung, Wu and Leung 2010; Halim *et al.* 2011; Karatay and Dönmez 2011; Carvalho *et al.* 2015)

First generation feedstock are food crops like cereals, sugar crops and oil seeds. Examples would include maize, soya beans and sunflower. Concerns on food security with regards to the use of food crops led to the pursuance of feedstocks that does not exert a demand on the available food supply. This brought about second generation feedstock, which are non-edible crops like jatropha (*Jatropha curcas*), karanja (*Pongamia pinnata*), mahua (*Madhuca indica*) and tobacco (*Nicotiana tabacum*). Their oils are characteristically high in free fatty acids and they are more convenient for transesterification. These do not exert a pressure on the food supply and demand chain, however huge tracts of arable lands are needed to grow these plants as well as fertilizers and herbicides (Carriquiry *et al*., 2011; Havlık *et al.*, 2011). In pursuance of the sustainability and viability of biodiesel production, researchers have explored the possibility of using fungal oils as feedstock for the production of biodiesel due to higher biomass production and faster growth compared to energy crops. These together with other oleaginous microorganisms like microalgae form the third-generation feedstock and work on which is currently underway (Chisti 2007; Demirbas 2009a; Escobar *et al.* 2009; Hoekman 2009; Carriquiry, Du and Timilsina 2011; Havlık *et al.* 2011).

CHAPTER 3: CONCOMITANT PRODUCTION OF CHITOSAN AND LIPIDS FROM A NEWLY ISOLATED *Mucor circinelloides* ZSKP FOR BIODIESEL PRODUCTION

3.1. INTRODUCTION

Biodiesel is a monoalkyl ester synthesized from free fatty acids present in renewable feed stocks like plant, microalgal, animal and fungal oils. Biodiesel is a suitable fuel for compression ignition engines and has characteristics similar to petroleum diesel fuel. Animal fats have the disadvantage in that they are not readily available and when they are available, they are not present in quantities which are sustainably sufficient. Saturated fatty acids are the major component in animal fats unlike vegetable, microalgal and fungal oils. This contributes to the undesirable biodiesel properties of high viscosity and high melting point and affects the quality of the biodiesel as high viscosities cause clogging problems in the engines. Also, lower volatilities cause the formation of deposits in engines due to incomplete combustion and incorrect vaporization (Basha, Gopal and Jebaraj 2009).

Vegetable oils are produced on arable land and this can have negative impacts on food security. Microalgal oils have generated much interest in that they have been shown to be suitable feedstocks for biodiesel whilst being renewable and potentially sustainable. There are however, concerns over low productivity and the generally high costs of production (Brennan and Owende 2010). Fungal lipids have a higher productivity and a higher content of shorter chain unsaturated fatty acids. The major drawback is that fungi are heterotrophic whilst microalgae are autotrophic therefore the costs of adding a carbon source would have to be factored in.

Fungi which are filamentous microorganisms are interesting due to their ability to grow on a wide range of substrates including agro-based and lignocellulosic waste with different kinds of fermentations. These can then be scaled up in a bioreactor. Filamentous growth allows easier biomass harvesting as opposed to single cell cultures (Vieira *et al.* 2008; Ferreira *et al.* 2016). Two fungal classes have been extensively used in various fermentation processes and these are zygomycetes and ascomycetes (Ferreira *et al.* 2013). Zygomycetes have been reported to be the highest chitosan accumulators (Nyman *et al.* 2013; Vaingankar and Juvekar 2014). Some species such as *Mucor* sp and *M. circinelloides* have been reported to be oleaginous as well (Laoteng, Čertík and Cheevadhanark 2011; Wei *et al.* 2013). Zygomycetes can be found as saprophytes growing on dead organic matter. Some have also shown pathogenicity against plants and animals; however, suitably selected strains have been used in the food industry. They are able to grow on different kinds of carbon sources from glucose to complex polymers like cellulose. Besides chitosan and lipids, zygomycetes biomass is also a source of amino acids and proteins which are present in substantial quantities. This makes it suitable as feed for both humans and animals (Ferreira *et al.* 2013). Ascomycetes are a larger group than the zygomycetes and they have a wider range of applications including the production of antibiotics by *Penicillium chrysogenum*. *Aspergillus* sp have been reported in fermentations to produce organic acids (Ferreira *et al.* 2016). Biomass from ascomycetes is also a source of fatty acids and amino acids but there have not been many reports of chitosan production.

Fungi especially zygomycetes are known to accumulate chitin and chitosan in their cell walls and are an alternative source for the biopolymers. The quantity of chitosan in the cell wall is dependent on the fungal grouping, strain, growth conditions and fermentation techniques (Akila 2014). Zygomycetes have the most abundant chitosan and most strains are chitin deacetylase producers (Chatterjee *et al.* 2005). Chitosan has applications in various fields which include cosmetics, food processing, industrial, medical, agricultural and biotechnological. The use of chitosan in these fields spans a period of over 30 years (Domard 2011).

The ability of oleaginous zygomycetes to efficiently accumulate intracellular neutral triacyl glycerates renders them not only a potential feedstock for biodiesel production (Karatay and Dönmez 2011) but also a source for concurrent production of multiple products, as in a biorefinery. Fungal biomass offers different and an ever-growing spectrum of products. The

production of organic acids, amino acids, enzymes, antibiotics, chitosan and lipids has already been reported using different fungal fermentation techniques (Jeong-Hoon *et al.* 2010; Liu, Liao and Liu 2016). This chapter focusses on the isolation and identification of an oleaginous chitosan producing fungal strain, which was used for concurrent chitosan and lipid production, with the lipids produced being used for biodiesel production.

3.2. MATERIALS AND METHODS

3.2.1. Isolation and identification of a chitosan and lipid-co-producing fungal strain

Kitchen vegetable waste (50 g) was mixed with dried shrimp-shell waste (500 g) and left for composting in an open space for two weeks. The compost sample (1 g) was used for the isolation of fungal strains on potato dextrose agar (PDA) plates by incubating at 30˚C for 5 days. The 6 isolates obtained were further inoculated in 50 ml potato dextrose broth (PDB) and incubated at 30˚C for 5 days. The biomass was harvested by centrifugation at 3000*g* and freeze dried for subsequent production of chitosan and lipid. The isolate showing the highest chitosan and lipid production was selected and identified. The 18S and internal transcribed spacer (ITS) sequence was deposited in to the NCBI GenBank nucleotide sequence database. The fungal isolate was preserved in glycerol at -80˚C and as agar slants at 4˚C.

3.2.2. Extraction and estimation of chitosan and lipid

Lipid extraction was carried out using a modified method by (Venkata Subhash and Venkata Mohan 2011). Dried biomass was crushed using a mortar and pestle and added to centrifuge tubes containing 4 ml chloroform–methanol (2:1 v/v) solution for every 0.25 g of biomass. The biomass was sonicated for 15 minutes at 20 MHz (Misonix Ultrasonic Liquid processor XL 2000) and then centrifuged at 9000*g* for 15 min at 4˚C. The supernatant was transferred into centrifuge tubes, and sonication process was repeated. Ultrapure water (4 ml) was added to the samples and shaken for 5 min, followed by centrifugation, separation and filtering of the lipid fraction. The samples were evaporated in an incubator (Labcon, RSA) at 60˚C for 24 h to remove solvent. The weight of the crude lipid obtained from each sample was measured using an analytical balance (Mettler Toledo, USA). The final pellet obtained after centrifugation was washed four times and used for chitosan extraction, while the pooled supernatant was used for separation of lipids. Chitosan was extracted using sulphuric acid according to a modified method of (Zamani and Taherzadeh 2010) Briefly, the sonicated pellet containing disrupted biomass was mixed with 1 M sodium hydroxide (50 ml sodium hydroxide/g fungal biomass) and the mixture was homogenized. The mixture was then autoclaved at 121˚C for 20 minutes. The alkaline insoluble material (AIM) was then separated by centrifugation at 2500*g* for 25 min and washed several times to a neutral pH. The neutral AIM was then treated with 72 mM sulphuric acid (100ml per 1g) at room temperature for 10 minutes followed by centrifugation. The pellet was then further treated with 72 mM sulphuric acid (100 ml per 1 g) at 120˚C for 45 minutes in an oil bath. The chitosan-soluble solution was separated by using a 75 µm sieve at the high

temperature whilst simultaneously increasing the pH to between 8-10 using hot 150 mM sodium hydroxide. It was important to maintain the temperature above 120˚C during the process. The precipitated chitosan was harvested by centrifugation at 5000*g* for 15 minutes. The chitosan was freeze dried (CHRIST, Alpha 2-4 LDplus) and the weight of the dried chitosan was measured using an analytical balance.

3.2.3. Identification of significant parameters by Plackett-Burman Design

The variables that significantly affected lipid and chitosan production were predicted based on results of Plackett-Burman Design (PBD) using the statistical software package 'Design Expert' (Version 6.0, Stat-Ease Inc., Minneapolis, USA). A total of 12 experimental runs were performed in triplicates with 10 variables and 1 unassigned variable, which included a variety of nutritional and physical parameters (Table 3.1). The principal effect [E(Xi)] of each variable was determined by the following equation:

$$E(X_i)=2(\Sigma Ci^{+}-\Sigma Ci^{-})/N$$

where Ci^{+} and Ci^{-} are the production levels from the trials where the variable (X_1) under study was present at high and low concentrations, respectively and N is the number of experiments. The order of the experiments was fully randomized with the design being run in a single block. The significance of each variable (p-value) was determined via Student's t-test.

3.2.4. Enhancement of lipid production using Response Surface Methodology

The significant parameters affecting lipid production were identified using PBD and these independent variables were further optimized by Response Surface Methodology (RSM). Each factor was studied at five different levels ($-\alpha, -1, 0, +1, +\alpha$), with a total of 30 experiments. The range of variables investigated and the experimental design used for the study is shown in Table 3.3. The behavior of the system was explained by the following second order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

where Y is the predicted value of response, β_0 is the intercept, β_1, β_2, β_3, β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} are squared coefficients, β_{12}, β_{13}, β_{23}, β_{24}, β_{34} are interaction coefficients and A, B, C, D are independent variables.

The conditions predicted by the statistical model were validated with respect to lipid production in 250 ml– 2000 ml shake-flasks, and also in 5 l (Minifors) laboratory fermenter (Infors, Bottmingen-Basel, Switzerland)

3.2.5. FAME analysis

Fatty acid composition analysis was done by GC-MS. This was done using a PerkinElmer Clarus 600C GC-MS. This was done using an Elite-5MS, 30m x 0.25 mm x 0.25 µm column. The injector temperature was 220˚C.The oven was set at 35˚C hold for 10 minutes and then increased at 10˚C per minute until a temperature of 200˚C to hold for 10 minutes was reached. Helium was used as a carrier gas. The molecular weight of lipid was calculated from the lipid profile obtained from the GC using the equation ((Komers *et al.* 2001). Sunflower oil was used for comparison for all lipid and biodiesel work.

3.2.6. Enzymatic and chemical catalysis of lipids for biodiesel production

Enzymatic transesterification included the addition of methanol to the fungal lipids feedstock at a ratio 1:12 (w/w). The enzyme (Novozym 435) was added at a concentration of 10% total reaction mixture weight to give an initial specific enzyme activity of 1.042 U/g. The reaction was then performed at 60℃ for 72 hours with vigorous stirring at 500 rpm. In chemical transesterification methanol was added to the fungal lipid feedstock at a ratio 1:60. The sulphuric acid catalyst was added at 8% w/w) relative to the total reaction mixture. The reaction was performed at 60˚C for 72 hours with stirring at 500 rpm. The comparison of the two methods was done in terms of the biodiesel yield and characteristics**.**

3.2.7. Determination of biodiesel yield

The yield of biodiesel was determined by measuring the mass of the purified biodiesel and the mass of the microbial oil using the formula:

%Yield = (mass of biodiesel/mass of oil used) *100

Sunflower oil was used for comparison for all the lipid and biodiesel work.

3.2.8. Biodiesel characterization

The biodiesel was characterized in terms of the American Standard Methods (ASTM). Biodiesel blend analysis by FT-IR (ASTM D7371), flash point (ASTM D3941), kinematic viscosity (ASTM D445), ash content (ASTM D482), acid number (ASTM D974), cloud point (ASTM D2500), cetane number (ASTM D4737), sulphur content (ASTM D5453), iodine value (ASTM D5768;), water content (ASTM D6304), ester and glycerol (ASTM D6584)

3. 3. RESULTS AND DISCUSSION

3.3.1. Concurrent lipid and chitosan extraction

The isolated chitosan producer was identified as *Mucor circinelloides* ZSKP as shown in the phylogenetic tree (Fig.3.1). This species has been reported to be oleaginous (Xia *et al.* 2011; Wei *et al.* 2013) thus, the possibility of concurrently producing chitosan and lipids was explored. The concurrent yield attained from 1g of fungal biomass after extraction was 11.2% chitosan and 21.4% lipids. *M. circinelloides* has been reported to produce various metabolites (Chatterjee *et al.* 2005; Ferreira *et al.* 2013). The extraction methods for both products proved that they could be harmonized and make the lipids co-product with chitosan. This would be in line with considerations on the use of single cell oils as an alternative to the use of plant oils which would unsustainably need arable land to be produced (Vicente *et al.* 2009). The biochemical pathways towards the accumulation of lipids and chitosan in fungi do not directly share enzymes or intermediates, however both products are heavily dependent on the carbon source and thus the accumulation may not be directly proportional. Both are critical for the integrity of the cell wall even though chitosan is more abundant than phospholipids due to its structural role. Chitosan synthesis in fungi starts with fructose-6-phosphate which is generated in the investment stage of glycolysis and this shows how critical this is. Acetyl coenzyme A which is critical for lipid production is only involved later on, in initiating the citric acid cycle (Mcmurrough, Flores-Carreon and Bartnicki-Garcia 1971; Laoteng, Čertík and Cheevadhanark 2011; Carvalho *et al.* 2015). Lipid accumulation in oleaginous fungi like *M. circinelloides* strains leans towards neutral lipids like triacylglycerates and these are useful for biodiesel production. These are used as an energy storage and carbon skeletons during growth and are not reutilized once the cell is grown. Thus, co-production can be pursued up to optimization of conditions.

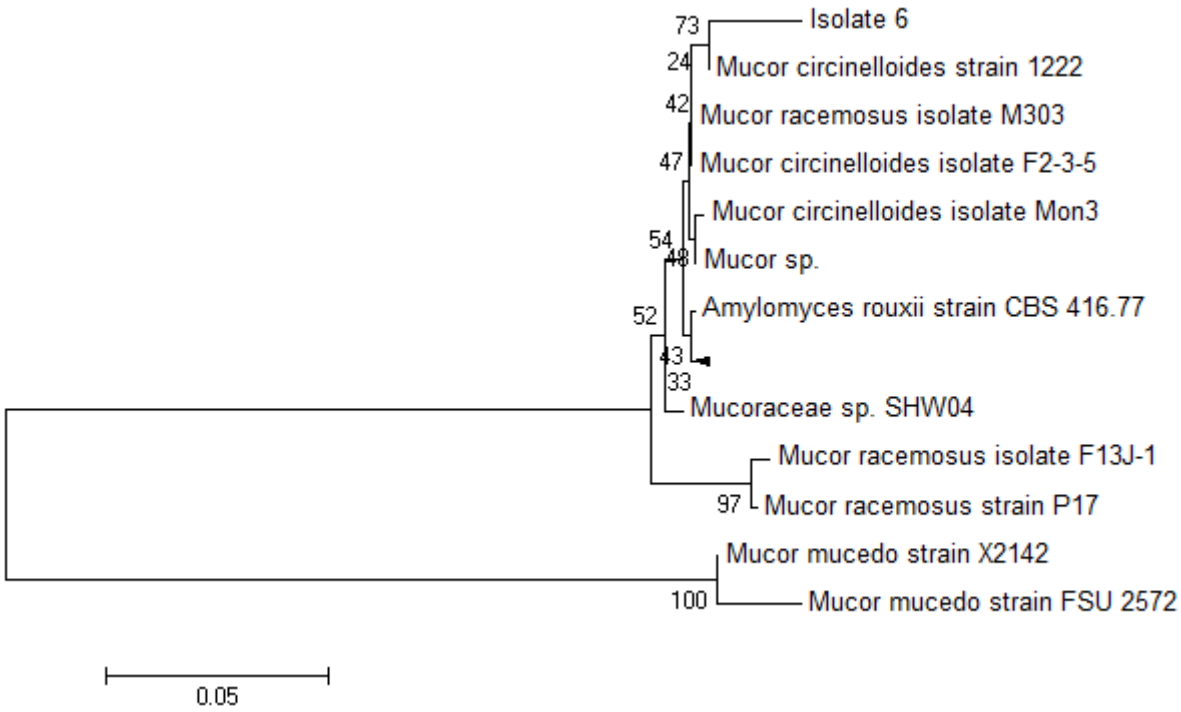


Figure 3.1. Phylogenetic tree showing close relatedness of *Mucor circinelloides* ZSKP (Isolate 6) with other *Mucor* isolates and the closely related *Mucor racemosus.* Bootstrap values are expressed as percentages of 1,000 replications and shown at the branch points. Scale bar represents the number of nucleotide substitutions per position

3.3.2. Plackett-Burman Design

Optimization of the chitosan and lipid yield is imperative as this is key in establishing a feasible process. The effects of various parameters on lipid and chitosan accumulation were investigated (Table 3.1). A Plackett-Burman Design was implemented with each parameter taking a high level and a low level. Ten parameters in total were considered. Run 4 gave the highest chitosan production of 3.60 g/l. Run 8 gave the highest lipid yield of 3.8 g/l. The analysis of variance was carried out on the Placket-Burman design model. The model had a F-value of 621.89 which confirms significance. The R-squared value was 0.9998 and an adequate precision value of 0.9982. The adjusted and predicted R-Squared was 0.9982 and 0.9768 respectively. Four factors were found to be significant to lipid production (Table 3.2) and these are glucose, yeast extract, pH and temperature. Glucose was the most significant and pH the least.

Table 3.1. Plackett-Burman Design showing concurrent chitosan and lipid extraction

Run no.	Variables†												
	A	B	C	D	E	F	G	H	J	K	Biomass yield	Chitosan Yield	Lipid Yield
1	5.00	1.00	0.50	0.50	8.00	25.00	300.00	72.00	10^5	72	12.70±0.99	1.18±0.09	3.07±0.29
2	1.00	1.00	0.50	0.05	3.00	37.00	300.00	120.00	10^5	168	4.30±0.28	0.15±0.00	1.56±0.11
3	1.00	0.10	0.50	0.05	3.00	25.00	100.00	72.00	10^5	72	3.90±0.33	0.74±0.04	0.98±0.06
4	**5.00**	**0.10**	**50.00**	**0.05**	**3.00**	**25.00**	**300.00**	**120.00**	**10^9**	**72**	**16.50±1.23**	**3.06±0.14**	**2.08±0.01**
5	5.00	1.00	50.00	0.05	8.00	37.00	100.00	120.00	10^5	72	14.60±1.39	1.40±0.10	3.30±0.19
6	1.00	0.10	50.00	0.50	8.00	25.00	300.00	120.00	10^5	168	4.86±0.33	0.23±0.00	1.50±0.09
7	1.00	1.00	50.00	0.50	3.00	37.00	300.00	72.00	10^9	72	6.82±0.59	1.14±0.09	2.10±0.09
8	**5.00**	**0.10**	**0.50**	**0.05**	**8.00**	**37.00**	**300.00**	**72.00**	**10^9**	**168**	**8.90±0.69**	**1.03±0.09**	**3.80±0.13**
9	1.00	0.10	0.50	0.50	8.00	37.00	100.00	120.00	10^9	72	6.7±0.39	0.63±0.06	1.70±0.09
10	5.00	0.10	5.00	0.50	3.00	37.00	100.00	72.00	10^5	168	7.90±0.46	0.51±0.00	2.80±0.1
11	1.00	1.00	5.00	0.05	8.00	25.00	100.00	72.00	10^9	168	9.53±0.88	1.67±0.08	2.20±0.09
12	5.00	1.00	0.50	0.50	3.00	25.00	100.00	120.00	10^9	168	9.80±0.91	2.70±0.11	2.60±0.12

†A, glucose (%); B, yeast extract (%); C, peptone (%); D, mineral salts (%); E, pH; F, temperature (˚C); G, rpm; H, inoculum age (h); J, inoculum size (spores/ml); K, fermentation time (h)

Table 3.2. The significant factors and their P-values

Variables	p-value
Glucose	0.0108
Yeast extract	0.0271
pH	0.0273
Temperature	0.0206

The two runs 4 and 8 which gave the highest yields of chitosan and lipids respectively differ in some conditions remarkably. Higher biomass yields seem to favour a higher yield for chitosan but not necessarily for lipids. Fungal lipids have been reported to highly accumulate in conditions which might be considered stressful for the organism (Niu *et al.* 2017). The common conditions in run 4 and 8 are the glucose concentration, agitation, yeast extract concentration and inoculum size. The conditions which differ are critically temperature, pH, incubation period and the concentration of peptone. This means investigation for the effect of the interactions of the significant factors for both chitosan and lipids would have to be carried out separately. Some of the factors were significant for both products however differing in the degree of influence. Thus, once the conditions are optimized individually there has to be a reconciliation for those conditions that differ towards optimum co-production.

3.3.3. Paretograph showing the effects of all the factors on lipid yield

The significant factors for lipid production which are glucose, yeast extract, pH and temperature were all significant at a higher level with glucose showing the highest significance (Fig.3.2).

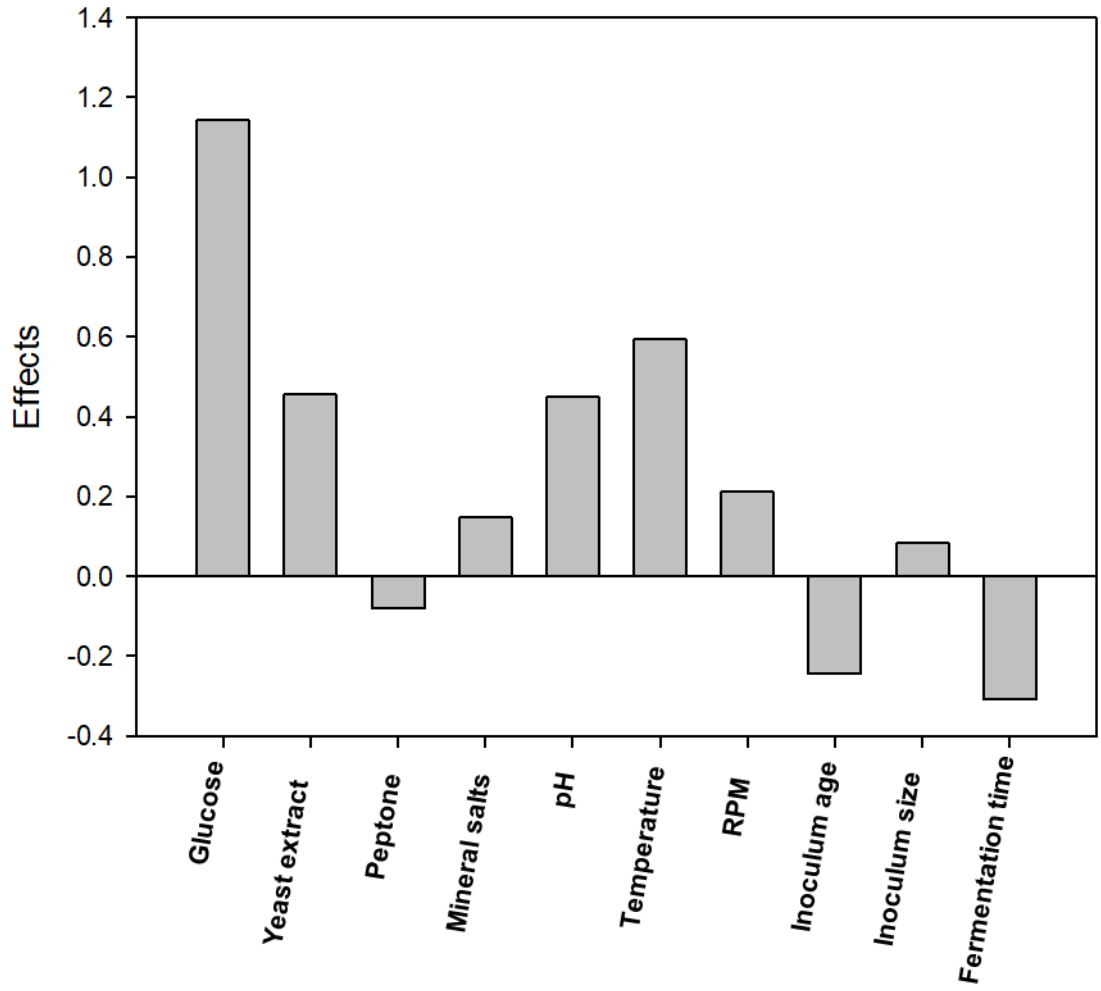


Figure 3.2. Paretograph for lipid production showing glucose, yeast extract, pH and temperature as the most significant factors at a higher level.

The significance of glucose and yeast extract is consistent with what has been reported in literature regarding fungal lipid accumulation (Subhash and Mohan 2014). Lipids tend to accumulate in stressful conditions that are characterized by an excess of carbon source. The carbon source can be varied to include polysaccharides and alkanes especially when considering the high costs of using monosaccharides (Akpinar-Bayizit 2014). Lipid accumulation in some fungal strains has been reported to be enhanced by growing on N-alkanes when compared to growth on other non-hydrocarbons. The higher levels of yeast extract *vis a vis* the glucose concentration would ensure balanced high growth rate which would result in depletion of nutrients especially the nitrogen source and the accumulation of lipids (Xia *et al.* 2011; Akpinar-Bayizit 2014). Thus, the rate of lipid synthesis is relative to the rate at which other cellular constituents are synthesized. Temperature and pH are also critical factors in lipid accumulation. Considering that the low level for temperature was 25˚C, as the temperature increases the growth

rate increases. However, the fact that temperature was significant at a higher level of 37˚C could be more to do with stressful conditions than high growth rate. Various fungi have been reported to grow optimally at 30˚C. The same considerations can be deduced for pH of which the lower level was 3. Fungi have been reported to grow optimally at a pH of around 6. It has been reported that a high growth rate would favour fungal lipid accumulation (Meng *et al.* 2009) but it seems that is only possible if stressful conditions occur as the fermentation progresses.

3.3.4. The effect of the interactions of the significant factors

Response Surface Methodology (RSM) using the central composite design was used to give the optima levels of the interactions of the four significant factors. The differences in the significance of factors for lipid and chitosan production meant that the investigation of the effects of the interactions of the significant factors for lipid and chitosan production had to be carried out separately. For lipid production 30 experimental runs were performed using four of the most significant factors obtained from the Plackett Burman (PB) experiment. The effect on lipid production of the interaction of the four significant factors was investigated and an optimized lipid yield of 6.89 g/L was achieved from a biomass of 14.08 g/L which represents a lipid accumulation of 48.9% (run 19). This is shown in Table 3.3. The analysis of variance shows that the lipid production is best described by the following polynomial equation:

$$\text{Lipid production (g/l)} = +5.36+0.38A-0.52B-1.13D-0.018A^2+0.16B^2-0.31C^2-0.61D^2-0.11AB+0.10AC+0.10AD+0.17BC-0.19BD+0.28CD$$

All four linear coefficients (A, B, C and D) and the quadric terms (A2, B2, C2 and D2) were significant for lipid production. The interaction of A with C and D, and D with B and C were significant model terms. The model had an F value of 621.89 and 'P>F value' of < 0.0001 which shows that the model was significant. An adequate precision value of 87.958 shows that the model signals are not susceptible to noise as any value greater than 4 is acceptable. An R-value of 0.9752 shows the applicability and accuracy of the design for lipid yield optimization. R-squared value of 0.9511 suggests that the model could explain 95% of the data variability.

Table 3.3. RSM showing the interactions of the four significant factors for optimal lipid production

Std	Glucose (g/l)	Yeast extract (g/l)	pH	Temperature (°C)	Biomass yield (g/l)	Lipid yield (g/l)
1	42.50	0.56	4.88	32.50	13.80±1.11	5.90±0.21
2	57.50	0.56	4.88	32.50	18.24±1.79	6.69±0.32
3	42.50	1.19	4.88	32.50	16.01±1.89	5.60±0.10
4	57.50	1.19	4.88	32.50	14.41±1.07	5.90±0.07
5	42.50	0.56	7.63	32.50	12.80±1.79	5.27±0.12
6	57.50	0.56	7.63	32.50	14.50±1.11	5.90±0.09
7	42.50	1.19	7.63	32.50	12.16±1.04	4.40±0.15
8	57.50	1.19	7.63	32.50	16.35±1.91	5.12±0.21
9	42.50	0.56	4.88	37.50	10.60±0.79	4.08±0.11
10	57.50	0.56	4.88	37.50	13.44±1.22	5.12±0.16
11	42.50	1.19	4.88	37.50	14.70±1.56	2.20±0.03
12	57.50	1.19	4.88	37.50	10.30±1.23	2.50±0.15
13	42.50	0.56	7.63	37.50	10.55±1.98	3.26±0.23
14	57.50	0.56	7.63	37.50	10.34±0.78	4.90±0.36
15	42.50	1.19	7.63	37.50	11.94±1.87	2.90±0.03
16	57.50	1.19	7.63	37.50	14.23±1.90	3.98±0.45
17	35.00	0.88	6.25	35.00	9.40±1.01	4.60±0.32
19	**50.00**	**0.25**	**6.25**	**35.00**	**14.08±1.56**	**6.89±0.24**
20	50.00	1.50	6.25	35.00	13.73±1.24	4.96±0.13
21	50.00	0.88	3.50	35.00	14.09±1.65	5.20±0.34
22	50.00	0.88	9.00	35.00	10.75±0.98	2.94±0.18
23	50.00	0.88	6.25	30.00	13.58±1.11	5.65±0.34
24	50.00	0.88	6.25	40.00	0.98±0.01	0.05±0.00
25	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26
26	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26
27	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26
28	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26
29	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26
30	50.00	0.88	6.25	35.00	13.78±1.78	5.36±0.26

The highest lipid production of 6.89 g/l represents a three-fold increase over the unoptimized medium. The glucose to yeast extract ratio in the run is 200:1 and it is the highest of all the runs. The temperature of 35˚C is high considering the optimal temperature of 30˚C for most fungal strains including previous reports on this particular strain (Carvalho *et al.* 2015). This could be

the cause of a relatively lower biomass yield. This brings into question the notion that a higher growth rate would result in higher lipid accumulation. A higher growth rate would mean a citric acid cycle that is constantly running to completion due to the high energy demand, thus affecting the amount of acetyl coenzyme A available for lipid synthesis (Ratledge 2004). It seems there is a need to create a balance between energy demand against an abundant supply of nutrients and that is where a higher temperature may have actually slowed down the growth rate. This lipid accumulation can be described in terms of the enzymes involved. Three enzymes are critical in lipid anabolism and these are ATP-citrate lyase which catalyzes the formation of acetyl CoA from citrate acetyl CoA, carboxylase which catalyzes the carboxylation of acetyl CoA to give malonyl CoA and malic enzyme which generates NADPH which is utilized by fatty acid synthase. Six isoforms of malic enzyme have been isolated and identified in *M. circinelloides* and these were expressed under different conditions which suggests that the enzymes have multiple metabolic functions and that different conditions affect storage lipids accumulation. However, malic enzyme activity or lack of it has been reported not to affect the synthesis of metabolic lipids (Laoteng, Čertík and Cheevadhanark 2011; Niu *et al.* 2017).

The RSM contour plot shown in Fig. 3.3 shows the interactions between temperature and pH. The plot was generated as representation of the interaction of the two variables with the other variable being kept fixed at '0' level. Optimal lipid production of 6.14 g/l was possible at a pH of 7 and a temperature of 33.8˚C (as obtained from the software analysis) and is reflected by the apex point illustration on the contour (Fig. 3.3). Any further increase in these two variables had negative implications to lipid product

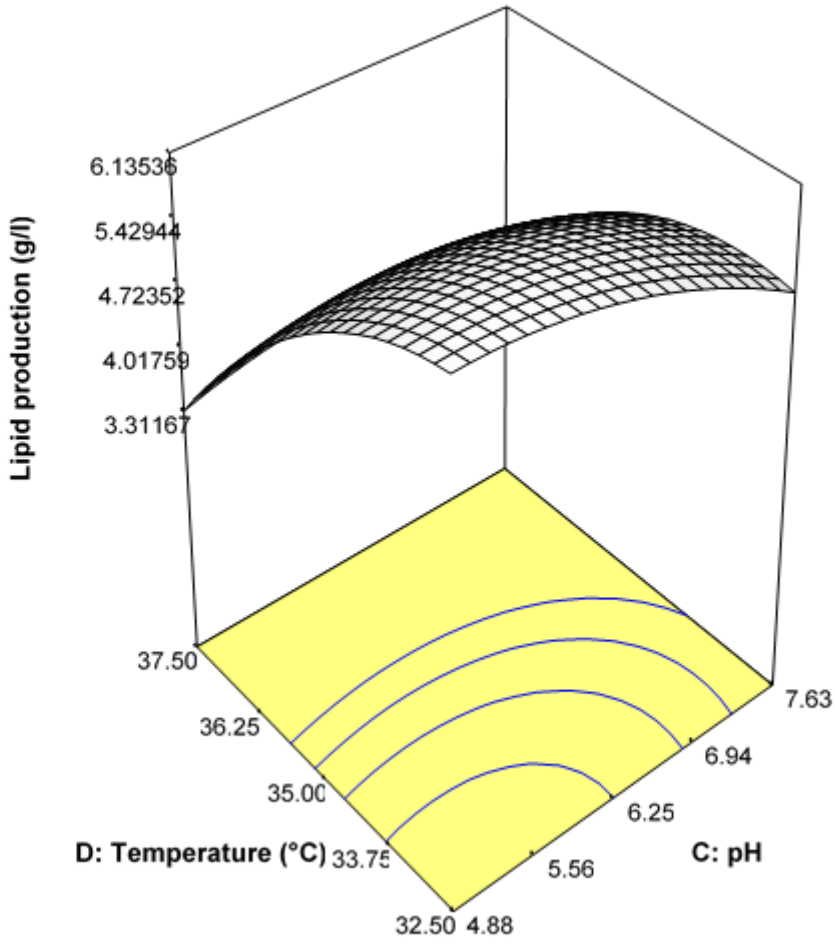


Figure 3.3. The three-dimensional response surface graph showing interaction between pH and temperature on lipid production.

The model was validated on the basis of its predictions against the actual experimental yield. This was done for all the four-significant variable in three random sets of experiments generated using the Design Expert software. The predicted response for lipid production was in good agreement with predicted and run two gave 98.9% accuracy (Table 3.4)

Table 3.4. Validation of RSM

	Glucose	Yeast extract	pH	Temperature	Lipid production (g/l)	
					Predicted	Observed
1	54.21	0.56	5.07	32.51	5.42	5.32 ± 0.36
2	57.46	0.58	5.51	33.19	5.75	5.69 ± 0.27
3	55.96	0.60	4.90	32.56	5.59	5.43 ± 0.32

Production was scaled-up in 500 ml and 1 litre flasks as well as a in a 5 litre bioreactor. The yield decreased slightly in the larger volume flasks, partly due to physical differences in the flasks used. The yield increased in the bioreactor to a maximum lipid production yield of 6.4 g/l (Table 3.5). The higher yield in the fermenter could be due to more efficient control of fermentation parameters as the conditions were better controlled

Table 3.5. Scaled up of production of lipids using optimized conditions

Volume (ml)	Volume of medium used (ml)	Lipid production (g/l)
Shake flasks		
250	50	5.69± 0.19
500	100	5.51 ± 0.46
1000	200	5.10 ± 0.24
Fermenter		
5 l	3000	6.4± 0.34

3.3.5. FAME analysis

The *M. circinelloides* ZSKP lipids produced were characterized in terms of fatty acid composition with a strict bias towards saponifiable lipids, which are necessary for biodiesel synthesis (Table 3.6). The chain length of the constituent fatty acids ranged from 14 carbon atoms to 24 carbon atoms. The most abundant fatty acids were oleic acid, palmitic acid, linolenic acid and linoleic acid. These are all 18-carbon acids long, except palmitic acid (16 carbon atoms) which is the second-most abundant constituent after oleic acid. The fungal lipids had a higher molecular weight and viscosity when compared to sunflower oil. About 26% of the fatty acids are saturated when compared to about 10% saturated fatty acids in sunflower oil. The fungal lipids have a high number of saponifiable lipids compared to the sunflower oil.

Table 3.6. Fatty acid composition, acid value and molecular weight of *M. circinelloides* ZSKP oil and sunflower oil

Fatty acid	Number of Carbon atoms: number of double bonds	*M. circinelloides* ZSKP oil (wt%)	Sunflower oil (wt%)
Myristic acid	C14:0	0.8	-
Palmitic acid	C16:0	21	5.8
Palmitoleic acid	C16:1	2.4	-
Stearic acid	C18:0	2	2
Oleic acid	C18:1	37	15.9
Linoleic acid	C18:2	14.3	71
Linolenic acid	C18:3	18.5	0.7
Arachidic acid	C20:0	0.2	0.6
Gadoleic acid	C20:1	0.6	-
Behenic acid	C22:0	0.5	0.8
Lignoceric acid	C24:0	1.2	-
Other	-	1.5	3.2
Saponifiable value	-	203	190
Acid value	-	38.8	0.2
Molecular weight	-	1024	980

Methyl esters obtained from the chemical or enzymatic hydrolysis of triacylgycerates in a transesterification reaction that includes methanol is referred to as biodiesel. The fungal lipids have a relatively high distribution of unsaturated fatty acids. This would enhance fluidity and plasticity, however, there is a drawback in that this can reduce oxidative stability as the double bonds are more susceptible to oxidative attack. Linoleic acid has been reported to be the source of the oxidation of oils and this then spreads to the other unsaturated fatty acids (Kumar 2017).The fungal lipids have much less linoleic acid (14.3%) when compared to sunflower oil (71%). The higher oleic and palmitic acids in the *M. circinelloides* ZSKP lipids as opposed to the high linoleic in sunflower oil, ensures the biodiesel obtained has better fluidity properties as well as enhanced oxidative and thermal oxidative stability. The high acid value shows that there is a higher content of free fatty acids in the lipid sample and this can be problematic when carrying out transesterification of glycerides with methanol. This would mean more refining for the lipids to reduce the moisture and free fatty acid content and careful selection of the catalyst for transesterification to avoid saponification. One of the major challenges in biodiesel production is viscosity and the fungal lipids produced have the advantage in that there is a wide distribution of 18-carbon and 16-carbon fatty acids thereby balancing the viscosity concerns.

3.3.6. Biodiesel yield

Biodiesel production was carried out with both enzymatic and chemical catalysis. The enzyme used was lipase and it gave the highest yield of 94%, compared to sulphuric acid catalysis which gave a yield of 92% (Table 3.7). The same pattern was observed with the sunflower oil, with a slightly higher yield of 94% from chemical catalysis and 96% from enzyme catalysis.

Table 3.7. Biodiesel yield after sulphuric acid and lipase catalysis of fungal and sunflower oil

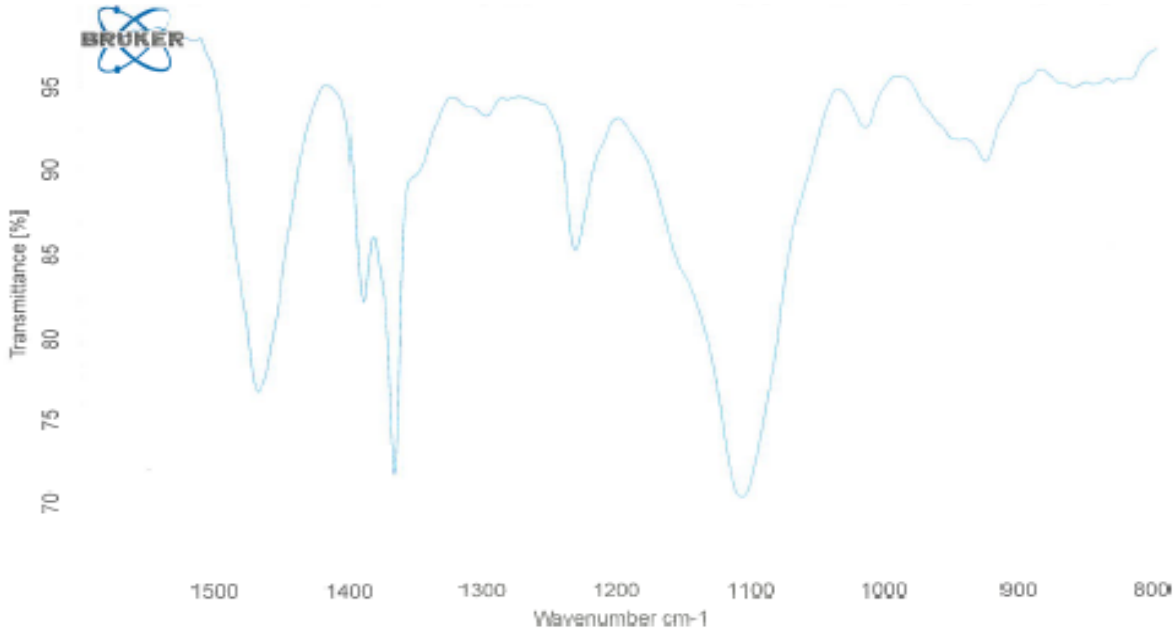
Type of catalyst	*M. circinelloides* ZSKP biodiesel yield	Sunflower oil biodiesel
Lipase	94%	96%
Sulphuric acid	92%	94%

The higher biodiesel yields from the sunflower oil is due to the quality of the oil in terms of lower free fatty acid and moisture content as it has gone through a refinement process. The type of catalysts used in the transesterification was influenced by the quality of the feedstock. The fungal lipids produced have a relatively high content of free fatty acids, thus an alkaline catalyst like sodium hydroxide would result in saponification which would in turn affect the quality of the biodiesel produced. An acidic catalyst like sulphuric acid is therefore suitable as long as the free fatty acid content is within standard specifications and there is low moisture content. Whenever an acid catalyst is used, the transesterification product has to go through various post-production steps that includes neutralization of the acid, removal of residues like pigments and deodorization. These steps would increase the costs of the process. The ratio of feedstock to methanol in the sulphuric acid method was 1:60 and the amount of methanol consumed was 5 times less when lipase was used. Lipase catalysis gave a higher biodiesel yield than the sulphuric acid method from the same feedstock. The fungal oil when catalyzed with lipase gave the same biodiesel yield of 94% as the chemically-derived biodiesel from the sunflower oil, regardless of its low quality. Lipases have been reported to be able to catalyze esterification reactions meaning that free fatty acids can potentially be utilized as well. However, the major drawback to lipase catalysis is that the costs of the enzyme itself is still very high and immobilizing the enzyme therefore enabling reusability can go a long way in addressing such cost issues (Antczak *et al.* 2009; Carvalho *et al.* 2015).

3.3.7. Characterization of the biodiesel

The biodiesel produced was analyzed by FTIR spectroscopy and compared to a commercial biodiesel standard. There is a general similarity between the spectra of the biodiesel from *M. circinelloides* ZSKP and the commercial biodiesel standard (Fig 3.4). The peaks at 1800-1700 cm^{-1} confirm C=O vibrations. The biodiesel fingerprint region is the range 1500-900 cm^{-1}. The peak at 1446 cm^{-1} is due to CH_3 which stretches asymmetrically and is characteristic of biodiesel. The peak at 1377 cm^{-1} confirms the presence of O-CH_2. The O-CH_3 stretch confirmed by a peak at 1196 cm^{-1} is also characteristic of biodiesel. Another region O–CH_2–Ce–CH_2–OH is identified at 1075-1100 cm^{-1}

M. circinelloides ZSKP biodiesel



Commercial biodiesel

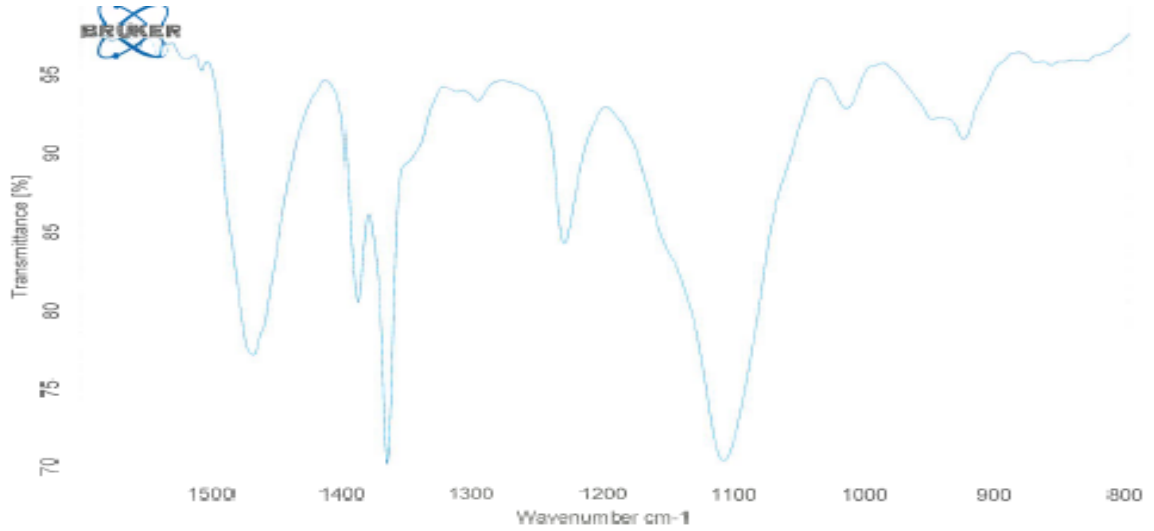


Figure 3.4. FTIR spectra showing similarity of the *M. circinelloides* ZSKP-derived biodiesel to a commercial biodiesel standard.

The properties of the fungal oil and sunflower oil biodiesel produced by chemical catalysis were analyzed and compared to ASTM specifications (Table 3.8). The kinematic viscosity of the *M. circinelloides* ZSKP oil-derived biodiesel was 4.3 mm/s^2 which is lower than sunflower oil-derived biodiesel (5.2 mm/s^2) and was well within the desired specifications. The flash point of the fungal oil derived biodiesel was 130˚C which was lower than the sunflower oil derived biodiesel at 136˚C. The acid number was also significantly lower in the fungal oil biodiesel at 0.02 mg KOH/g compared to 0.1 mg KOH/g for the sunflower biodiesel. There was less water and ash content in the fungal biodiesel at 0.01 and 0.012, respectively. All the other parameters investigated were within the specified limits.

Table 3.8. Properties of fungal and sunflower oil biodiesel obtained by chemical catalysis

Test	Specification	*M. circinelloides* ZSKP biodiesel	Sunflower oil biodiesel
Cetane Number	1-100	56	46
Density @ 15 ˚C, kg/m_3	860-900	879	880
Kinematic Viscosity at 40 ˚C, mm^2/s	3.5-5.5	4.3	5.2
Sulphur Content, ppm	10 max	<1	<1
Water Content, %m/m	0.05 max	0.01	0.025
Ash Content, %m/m	0.02 max	0.012	0.015
Acid Number, mg KOH/g	0.5 max	0.02	0.10
Flash Point, ˚C	120 min	130	136
Ester, %m/m	95 min	96.1	98.0
Glycerol, %m/m	0.25 max	0.003	0.2

The use of sulphuric acid as a chemical catalyst resulted in biodiesel with slight differences in some properties with the enzymatically catalyzed biodiesel which is shown in Table 3.9. However, all the parameters investigated are within the specifications. The viscosity increased slightly from 4.3 mm^2/s to 4.6 mm^2/s. The acid number also increased slightly from 0.02 to 0.03 mg KOH/g. The flashpoint was reduced to 128 ˚C which was well below the flash point of 140˚C for the sunflower oil biodiesel.

Table 3.9. Properties of fungal and sunflower oil biodiesel obtained from enzymatic catalysis

Test	Specification	*M. circinelloides* ZSKP biodiesel	Sunflower oil biodiesel
Cetane Number	1-100	56	46
Density @ 15 ˚C, kg/m_3	860-900	874	886
Kinematic Viscosity at 40 ˚C, mm^2/s	3.5-5.5	4.6	5.4
Sulphur Content, ppm	10 max	<1	<1
Water Content, % m/m	0.05 max	0.01	0.03
Ash Content, %m/m	0.02 max	0.013	0.017
Acid Number, mg KOH/g	0.5 max	0.03	0.36
Flash Point, ˚C	120 min	128	140
Ester, %m/m	95 min	95.2	96.4
Glycerol, %m/m	0.25 max	0.10	0.25

The biodiesel produced from both feedstocks using chemical and enzymatic methods met or exceeded the standard specifications. The major improvement of the biodiesel from the fungal lipids as compared to the sunflower oil derived biodiesel was the cetane number which was 56 as compared to 46. The cetane number is a critical indicator of the quality of the biodiesel and premium diesel fuels generally have a high cetane number. It is a reflection of the time it takes between injection of the biodiesel into the cylinder and the start of ignition. A higher cetane number means it takes a shorter combustion time and this kind of biodiesel is ideal for high speed and high-performance biodiesel engines. A higher cetane number is also correlated with a reduction in NO_X exhaust emissions which would address some environmental concerns. The fungal lipids derived biodiesel has a lower viscosity which can be attributed to the presence of shorter chain fatty acid esters. Higher viscosity can result in fuel system clogging. The acid number is also significantly reduced in the fungal oil biodiesel. This is important as a higher acid number would make the fuel corrosive to the engine. This could be due to the higher moisture contamination in the sunflower oil derived biodiesel. However, a lower flash point raises storage concerns as this would mean that the product is highly flammable. The biodiesel production from enzymatic catalysis showed a significant increase in glycerol as a byproduct and this can be attributed to the higher degree of successful transesterification. Glycerol can contaminate the biodiesel product however, advances in technology have seen glycerol having various uses and can be used as a substrate for lipid accumulation in some oleaginous fungi (Mitra *et al.* 2012)

CHAPTER 4: CHITOSAN PRODUCTION FROM A NEWLY ISOLATED *Mucor circinelloides* ZSKP AND ITS APPLICATION ON FRESH FRUIT PRODUCE

4.1. INTRODUCTION

Chitosan is a polymer made up of β-1, 4 D-glucosamine monomers. It has no toxicity, it is biodegradable and can be of high or low molecular weight depending on the source. It has applications in various fields which include cosmetics, food processing, medical, agricultural and biotechnological industries (Crini and Badot 2008; Aider 2010; Alishahi and Aïder 2011). One of the most important properties of chitosan is its ability to inhibit the growth of microorganisms. This property is dependent on whether the chitosan is modified or natively derived, the source of the chitosan, the procedures of extraction, the environmental conditions and the extent of polymerization. This quality is the driving force behind many of its applications in different industries (Duarte *et al.* 2002).

To prevent considerable economic losses, many studies have been developed for chitosan crop protection, such as seed coating technology against pests and diseases. It has been noted that addition of chitosan to soil results in reduction in pest infestation and also suppresses the development of plant pathogens (Kashyap, Xiang and Heiden 2015). When seeds of wheat and other crops are pre-treated with chitosan they are found to exhibit significant acceleration in growth. Maize seeds pre-treated with chitosan showed reduction in germination time and increased shoot height and root length. Chitosan can also be used as a coating on delicate plants for frost protection (Sharp 2013).

There has been an increase in the demand for fresh farm produce which includes fruits and vegetables because of a market that is becoming more health conscious. These fruits and vegetables play a major role in human diet as a source of various nutrients. The consumption of fresh farm produce has been linked with a significant decrease in life threatening conditions like cardiovascular diseases and cancers. It therefore makes it imperative that these produces are safe and of the highest quality. This is even further necessitated by a demand for the fruits and vegetables in freshly-cut servings. It is estimated that at least 20% of all fresh farm produce is lost each year due to spoilage. In addition there is also the possibility that these fruits and vegetables are a source for food borne pathogens (Barth *et al.* 2009; Kumar and Mritunjay 2015; Qadri *et al.* 2015).

For continuous industrial production of chitosan, it is important to find an alternative source for chitosan to complement crustacean wastes, especially when crustaceans are out of season. Crustacean shell wastes are ideal for chitosan production as they offer a high yield, are cheap and the extraction process rids the environment of material that would otherwise have been discarded causing environmental pollution. The major disadvantage of crustacean waste as a source of chitosan, however, is that availability is seasonal and thus a suitable alternative has to be available independent of season over and above matching most of the advantages that come with the use of crustacean wastes (Younes *et al.* 2012). Fungi are known to accumulate chitin and chitosan in their cell walls, thus presenting a possible source for the biopolymers. The quantity of chitosan in the cell wall is dependent on the fungal grouping, strain, growth conditions and fermentation techniques (Akila 2014).

The use of fungi for chitosan production has an advantage in that growth conditions can be controlled to favour optimal yield. The process of chitosan extraction from fungi is less rigorous compared to crustacean materials. Lower quantities of strong bases and acids are required which need to be carefully discarded to alleviate potential environmental damage. The major disadvantage is that fungal chitosan yield is less compared to crustacean material and the process of accumulating fungal biomass is costlier but with higher productivity. Thus, fungal chitosan production focuses on increasing biomass yield (Kim *et al.* 2001; El-Hefian *et al.* 2010; Domard 2011).

This chapter focuses on optimization of the production of chitosan from the recently isolated *M. circinelloides* ZSKP, characterization and application of the chitosan in improving the shelf life of fresh fruit produce.

4.2. MATERIALS AND METHODS

4.2.1. This isolate was identified as described in section 3.2.1.

4.2.2. Chitosan extraction

Two acids (sulphuric acid and acetic acid) were used to extract the chitosan and the results compared. Fungal biomass was mixed with 1 M sodium hydroxide solution (1 g fungal biomass per 50 ml sodium hydroxide) and the mixture homogenized using a vortex. The content was autoclaved at 121˚C for 20 minutes. The alkaline insoluble material was separated by centrifugation as the pellet at 4000 *g* for 25 min and washed several times to neutral pH. For the acetic acid extraction, the neutral AIM was treated with 2% acetic acid for 8 hours at 95˚C at a ratio of 1:30 (w/v). The supernatant containing soluble chitosan was separated from the acetic acid-insoluble material by centrifugation. The pH of the supernatant was adjusted to between 8-10 to precipitate the chitosan. The precipitated chitosan was then separated from the solution by centrifugation, washed to neutrality and subsequently dried in an oven at 40˚C overnight. The sulphuric acid extraction was as described in section 3.2.2

4.2.3. Identification of significant parameters

The method to identify significant parameters for chitosan is as described in the Plackett-Burman method described in section 3.2.3.

4.2.4. Enhancement of chitosan production using Response Surface Methodology

The significant parameters affecting chitosan production were identified using PBD and the independent variables were further optimized by Response Surface Methodology (RSM) as described in section 3.2.4. The conditions predicted by the statistical model was validated with respect to lipid production in 250,500 and 100 ml shake-flasks, and also in 5 l (Minifors) laboratory fermenters (Infors, Bottmingen-Basel, Switzerland)

4.2.5. FTIR analysis

The samples of chitosan produced were characterized in KBr pellets by using an infrared spectrophotometer, namely Fourier-transform infrared (FTIR) spectroscopy in the range of 400

to 4000 cm^{-1} (Tanya et al., 2001). This involved 0.8 mg of each sample being mixed with 200 mg of pure grade KBr (Merck) powder and the mix was compacted into thin pellets of 13 mm diameter. The transmission spectra of acetic and sulphuric acid extracted chitosan were then compared to the spectrum produced by commercial chitosan extracted from crab shells (Sigma).

4.2.5.1. Calculation of the degree of deacetylation

The following equation was used to calculate the degree of deacetylation

$$DDA=100-[(A_{1655}/A_{3450}) *100/1.33]$$

A_{1655} represents the absorbance of 1655 cm^{-1} which results in amide-l band, quantifying the content of N-acetyl group. A_{3450}, quantifies the hydroxyl band at absorbance 3450 cm^{-1}. The theoretical value for the A_{1655}/A_{3450} ratio for fully deacetylated chitosan was presumed to be zero and that there is a rectilinear relationship between the N-acetyl group content and the amide-l absorbance band

4.2.6. Determination of minimum lethal concentration for fungal chitosan

A 1 g of chitosan was dissolved in 1% acetic acid to make up 1% (m/v) chitosan solution. The chitosan solution was further diluted using 0.9% physiological water to make diluted concentrations of 0.1 %, 0.08 %, 0.06 %, 0.04 %, 0.02 % (w/v). Two controls were used: one with 1% acetic acid only without and with water. The pH for all the solutions was adjusted to 5.5 using 2M sodium hydroxide. After autoclaving, 10 ml solutions were prepared and inoculated with 0.05ml of the freshly prepared microbial cultures followed by mixing using a vortex. The solution cultures were incubated at 37˚C for 24 hours. The viable cells were enumerated by spread plating on nutrient agar plates (for bacterial) and potato dextrose agar (for fungi) then incubating at 37˚C for 48 hours. The minimum lethal concentration was defined as the minimum concentration of the chitosan that kills all the microbial cells and this was confirmed by observing no growth on the plates.

4.2.7. Application of the chitosan spray in fresh fruit produce

Apples and tomatoes, purchased from the local market, were the subjects of the fruit testing models. Apples and tomatoes were first washed with tap water, then with distilled water, and were sprayed with 70% alcohol and left to dry naturally at ambient temperature. Three groups of fruits were involved in each experiment. The first one involved a chitosan spray 0.5% (m/v) dissolved in 1% acetic acid which was applied to the fresh fruits. The control group involved

using a 1% acetic acid spray without the chitosan which was applied to the fruits. The second control group consisted of the fruits with only water applied on them. Presence of spoilage was then observed

4.3. RESULTS AND DISCUSSION

4.3.1. Selection of the best chitosan producer

Six unidentified fungal isolates were grown in the same media under similar conditions. The chitosan was extracted from the dried alkaline-insoluble material (AIM) with isolate 6 giving the highest chitosan yield of 54.29 mg/g AIM as shown in Table 4.1. This is the isolate that was selected for all the subsequent experiments. This isolate was identified as *M. circinelloides* and named *M. circinelloides* ZSKP

Table 4.1. Chitosan yield from the six selected isolates

Fungal isolate	Chitosan yield g/litre
Isolate 1	0.031
Isolate 2	0.012
Isolate 3	0.037
Isolate 4	0.036
Isolate 5	0.041
Isolate 6	**0.120**

The accumulation of chitosan in fungi is dependent on various factors which include nutritional, environmental and genetic combination (Akila 2014). The genetic variation in composition is expressed in fungal classification. Fungi of the family Zygomycota have been reported to show a generally high accumulation of chitosan in the cell wall (Streit *et al.* 2009; Vaingankar and Juvekar 2014). The same media and culture conditions were applied to select the isolate that has highest accumulation of chitosan. This would be grown in different growth and environmental conditions for optimization experiments.

4.3.2. Comparison of the acetic acid and sulphuric acid chitosan extraction methods

Two methods of chitosan extraction were compared on the *M. circinelloides* ZSKP strain with the intention to select the better method. The methods were acetic acid chitosan extraction and sulphuric acid chitosan extraction. The chitosan obtained from both methods showed a high degree of deacetylation. The extraction process using sulphuric acid takes only 45 minutes which is significantly less than the same process using acetic acid (10 hours). However, higher temperatures have to be applied (121˚C) and this requires specialized equipment as compared to 100˚C where a water bath would suffice. The most important consideration was chitosan yield

and the sulphuric acid method gave a yield of 156.85 mg/g AIM almost three times higher as compared to the 54.29 mg/g AIM acetic acid extraction chitosan yield (Table 4.2). The major drawback to the sulphuric acid extraction method was the potential formation of chitosan sulphate and chitosan phosphate complexes which resulted in the chitosan being insoluble in acetic acid. This setback could, however, be circumvented by adjusting the method. The sulphuric acid chitosan extraction method was thus selected for the rest of the chitosan extraction in the project.

Table 4.2. Comparison of the acetic acid and sulphuric acid methods

Acid	Chitosan yield/g AIM	Degree of deacetylation	Duration of extraction	Complex formation	Temperatures applied
Acetic acid	54.29 mg/g	>90%	10 hours	-	100˚C
Sulphuric acid	156.85 mg/g	>90%	45 minutes	chitosan sulphate, chitosan phosphate	121˚C

The method used for extraction of chitosan is critical for any process that intends to obtain high yield of chitosan from fungal biomass. The chitosan accumulates in fungal cell wall where it exists both freely and as a complex with glucan. It has been reported that fungal cell walls contain 80-90% polysaccharides (Latge 2007; Nwe, Furuike and Tamura 2011). Thus, an effective extraction method would have to involve breaking not only the cell wall but chitosan glucan complexes as well. Chitosan extraction using acetic acid is the more commonly used technique. Acetic acid being a weak acid, would make the process more environmentally-friendly. However, this process takes more than eight hours and gives comparatively lower yields which outweighs the above advantage. The sulphuric acid method takes significantly less time giving a significantly higher yield. Concerns about sulphuric acid being a strong acid are allayed by the relatively low concentrations (72 mM) used. The major concern however is the formation of chitosan sulphate and chitosan phosphate complexes. The chitosan sulphate complex is formed during extraction at 120˚C. As the temperature drops, the chitosan becomes insoluble as a chitosan sulphate complex because of the protonated amine group due to the low pH which would form a bond with the negatively charged sulphate anions. The low pH means the amine group on the chitosan glucosamine monomers is protonated, hence the formation of the complex. The protonated amine group can also form a complex with phosphates released from the cell wall (Zamani *et al.* 2010). The implications of the complexes are that the chitosan becomes insoluble in acetic acid, thereby rendering it inapplicable for future processes. However, getting

rid of the phosphates before extraction by soaking in 72 mM sulphuric acid for 10 minutes and then centrifuging would get rid of the phosphates through discarding the supernatant. Increasing the pH to between 8-10 using sodium hydroxide simultaneously with the temperature shift filtration using 150 mM sodium hydroxide have been shown to significantly improve solubility. This is because the instantaneous increase in pH simultaneous with the decrease in temperature, would deprotonate the amine group of the precipitating chitosan.

4.3.3. FTIR analysis of the chitosan

To confirm the production of the chitosan as well as calculate the degree of deacetylation, FTIR analysis was carried out. This was done for both the acetic acid and sulphuric acid-derived chitosan with commercial shrimp shell chitosan as a reference. The peaks at 3417 cm^{-1} corresponds to the OH functional group and the absorption band at 3417 cm^{-1} is characteristic of amino groups vibrations. The methylene stretching CH is identified by the presence of the 2877 cm^{-1}peak. The deformation of the NH amino group is highlighted by the peak at1600 cm^{-1}. Peaks at1631 and 1747 cm^{-1} represents amide carbonyl and ester carbonyl group stretch vibrations respectively. The glucose amine ring is shown by the highlighting of the COC stretch vibrations through the presence of the 1072 cm^{-1} peak. All these functional groups are used to identify chitosan

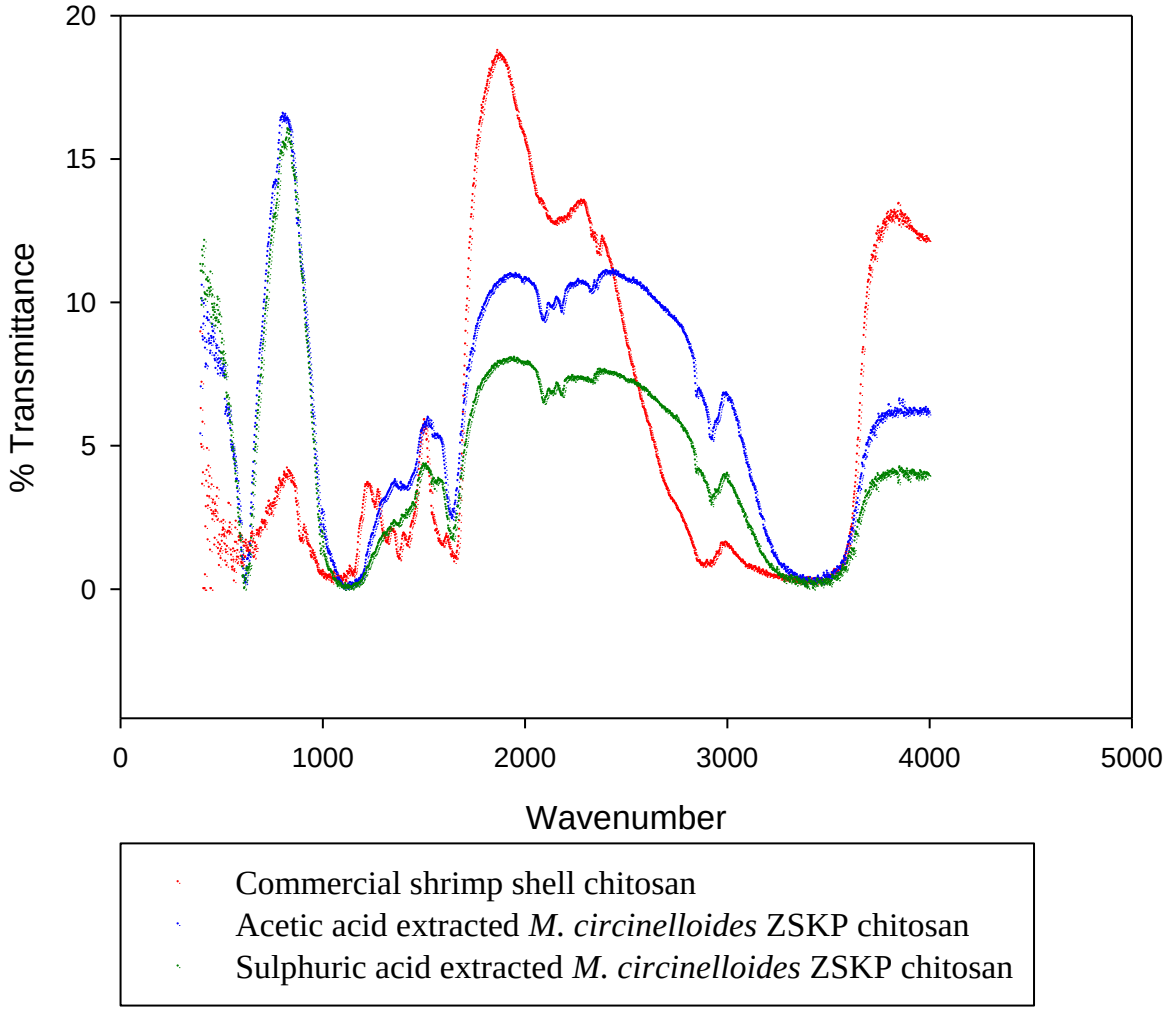


Figure 4.1. FTIR spectrum of chitosan produced by acetic acid and sulphuric acid extraction against that of commercial shrimp shell chitosan.

FTIR analysis confirms that it is chitosan produced through both the sulphuric acid and acetic acid methods. The slight differences in the shape of the peaks is because of the differences in moisture content as well as some residual sodium hydroxide which might still be detected despite washing to neutrality

4.3.4. Chitosan production in different media

Four different media derived from literature (Chatterjee *et al.* 2005) were identified and were used as chitosan production media using the *M. circinelloides* ZSKP strain. Medium 3 which was composed of glucose, peptone and yeast extract gave the highest chitosan yield 0f 1.94 g/L as shown in (Table 4.3). This is despite medium 2 giving the highest biomass yield of 11.17 g/L

Medium 4 had the least biomass and chitosan yields. This medium was then selected and used for the optimization of chitosan experiments.

Table 4.3. Comparison of chitosan yield in four different media

Medium	Biomass yield (g/L)	AIM yield (g/L)	Chitosan yield (g/L)
Medium 1	6.8	3.44	0.86
Medium 2	11.17	5.86	1.88
Medium 3	10.68	5.96	1.94
Medium 4	3.75	1.44	0.44

Medium 1- Sabouraud Dextrose Broth, Medium 2- Yeast extract Tryptone glucose, Medium 3- Yeast Extract Peptone Glucose, Medium 4- Sucrose, Sodium nitrate, Mineral salts

Growth conditions influence the accumulation of chitosan in the fungal cell wall (Kim *et al.* 2001; Kannan *et al.* 2010). Whilst higher biomass yield will likely give a higher yield of chitosan as is evident in Table 4.3, however, medium 2 which had the highest biomass yield did not automatically correspond to the highest chitosan yield. The yield of the alkaline insoluble material which comes after the deproteinization step hints towards greater protein accumulation in the biomass from medium 2 when compared to the biomass in medium 3. The only difference in these two media is the protein source. Medium 2 contains tryptone and medium 3 contains peptone. Between the two it seems tryptone is utilized better. Peptone is a protein source that is partially hydrolyzed by acid hydrolysis, whilst tryptone is an example of a peptone that is specifically hydrolyzed by the enzyme trypsin. Peptone has a lower quantity of nitrogen but has a higher carbohydrate content this could also have contributed to a higher yield of chitosan. Once a medium for chitosan production had been selected, the next step involved identification of the most significant factors towards chitosan accumulation in the medium as well as the interactions of these factors towards optimal chitosan yields.

4.3.5. Selection of the most significant factors for chitosan production

Glucose, pH and temperature were found to be the most significant factors for chitosan production, with inoculum size being the most significant factor for chitosan production. Inoculum size was the most significant with a P-value of 0.0034 as shown in (Table 4.4). The model was significant with an F-value of 10602.56, R-Squared value of 1, Adj R-Squared 0.9999, Pred R-Squared 0.9986 and Adeq Precision of 329.024

Table 4.4. The factors most significant for chitosan production and their corresponding p-values

Variable	p-value
Glucose	0.0076
pH	0.0038
Temperature	0.0043
Inoculum size	0.0034

The pareto chart shows glucose and inoculum size which are significant factors for chitosan production showing significance at a higher level. The other two significant factors where significant at a low level. The rest of the non-significant factors where at a higher level

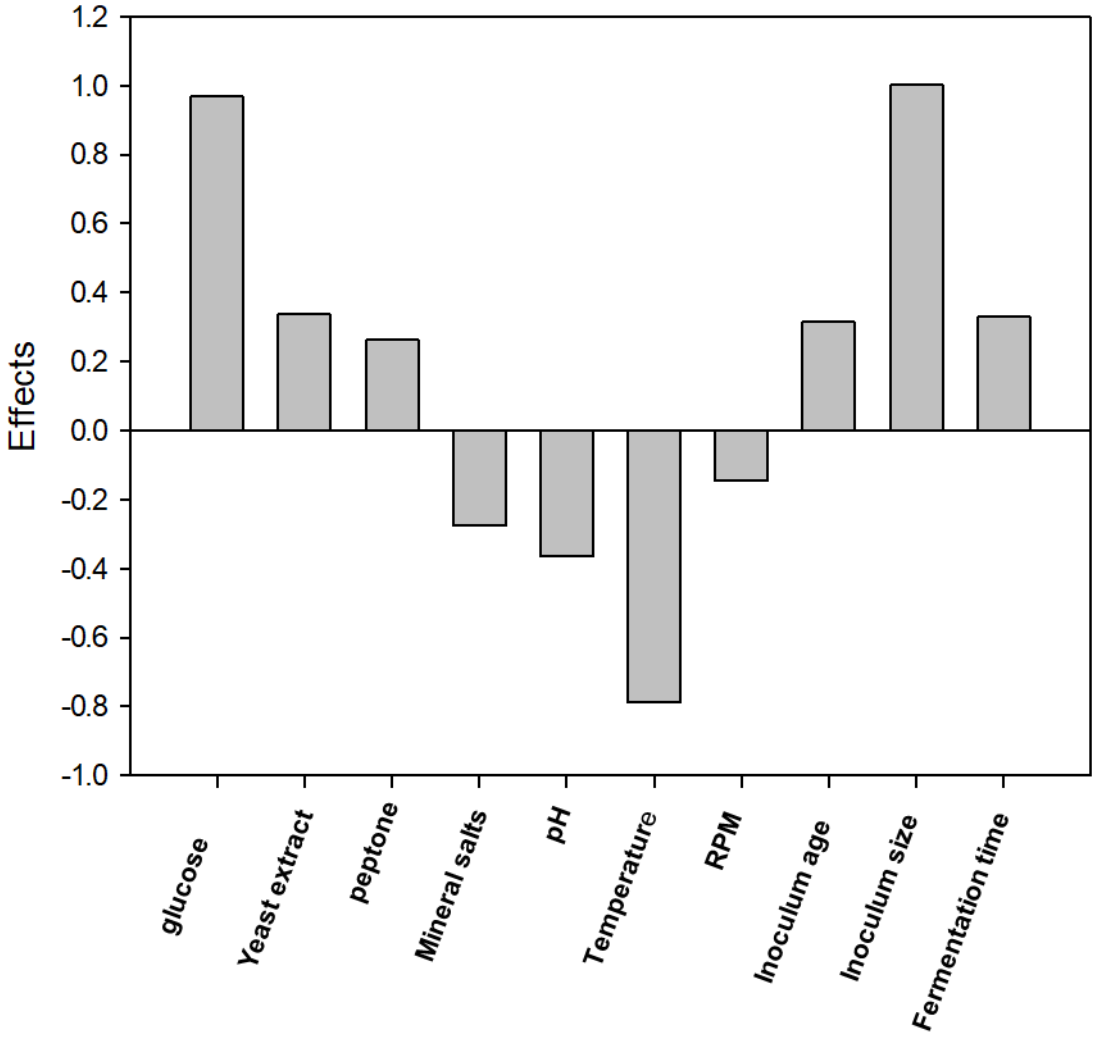


Figure 4.2. Paretograph for chitosan production identifying glucose and inoculum size as major significant factors.

4.3.6. The effects of the interactions of the significant factors on chitosan production

Optimal chitosan production was recorded in run 22 at 4.12 g/litre (Table 4.5) and the conditions in this run are: glucose at 6%; temperature of 30˚C, inoculum size of 10^{12} (log cfu/ml) and pH of 4 (Table 4.5). The predicted value for the model for optimal chitosan yield was 4.21 g/litre and the experimental chitosan production of 4.18 g/litre by *M. circinelloides* ZSKP was achieved. The experimental results are approximately 99% of the predicted value. This validates the model as it gives an accurate prediction of chitosan production. The analysis of variance shows that chitosan production can be explained in the following polynomial equation;

$$\text{Chitosan production (g/l)} = +3.92+0.35A-0.17B+0.19C+0.23D-0.23A^2-0.56B^2-0.13C^2-0.32D^2+0.033A\ B+0.12AC-0.12AD+0.068BC-0.20BD-0.10\ C$$

A 98% correlation between the predicted and observed results reflected by an R-value of 0.9873 shows the applicability and accuracy of the design for chitosan optimization. The model has an R-squared value of 0.9747 which suggests that the model could explain 97% of the data variability. The adequate precision value of 20.92 shows that the model signals are not susceptible to noise as any value greater than 4 is acceptable.

Table 4.5. RSM showing the interactions of the four significant factors for optimal chitosan yield

Std	A: Glucose (g/l)	B: Temperature (°C)	C: Inoculum size (log CFU/ml)	D: pH	Biomass yield (g/l)	Chitosan yield (g/ l)
1	40.00	27.50	7.50	3.50	16.93±1.23	1.91±0.11
2	80.00	27.50	7.50	3.50	18.96±1.11	2.42±0.13
3	40.00	32.50	7.50	3.50	17.50±1.38	1.68±0.15
4	80.00	32.50	7.50	3.50	19.60±0.89	2.51±0.09
5	40.00	27.50	10.50	3.50	16.34±0.84	1.99±0.11
6	80.00	27.50	10.50	3.50	20.34±1.9	2.96±0.13
7	40.00	32.50	10.50	3.50	17.03±1.69	2.11±0.07
8	80.00	32.50	10.50	3.50	22.69±1.98	3.38±0.23
9	40.00	27.50	7.50	5.50	18.12±1.76	3.19±0.19
10	80.00	27.50	7.50	5.50	19.87±0.99	3.3±0.29
11	40.00	32.50	7.50	5.50	16.79±0.98	2.33±0.20
12	80.00	32.50	7.50	5.50	16.89±0.79	2.52±0.16
13	40.00	27.50	10.50	5.50	18.34±1.79	2.85±0.26
14	80.00	27.50	10.50	5.50	21.59±2.01	3.58±0.31
15	40.00	32.50	10.50	5.50	14.68±0.98	2.32±0.09
16	80.00	32.50	10.50	5.50	16.9±1.20	2.88±0.13
17	20.00	30.00	9.00	4.50	15.23±0.89	2.33±0.22
18	10.00	30.00	9.00	4.50	23.89±1.9	3.89±0.17
19	60.00	25.00	9.00	4.50	16.9±0.69	2.22±0.19
20	60.00	35.00	9.00	4.50	17.8±1.19	1.36±0.05
21	60.00	30.00	6.00	4.50	17.9±0.89	2.94±0.13
22	**60.00**	**30.00**	**12.00**	**4.50**	**25.45±1.67**	**4.12±0.19**
23	60.00	30.00	9.00	2.50	15.56±0.79	2.36±0.17
24	60.00	30.00	9.00	6.50	17.89±1.37	3.16±0.12
25	60.00	30.00	9.00	4.50	18.89±1.01	3.91±0.29
26	60.00	30.00	9.00	4.50	18.78±0.89	3.88±0.32
27	60.00	30.00	9.00	4.50	19.12±0.98	4.01±0.36
28	60.00	30.00	9.00	4.50	18.98±1.61	3.76±0.33
29	60.00	30.00	9.00	4.50	19.69±1.32	4.08±0.39
30	60.00	30.00	9.00	4.50	19.45±1.23	3.88±0.29

Fig. 4.3 and Fig. 4.4 are the response surface contour plots for chitosan production. The chitosan yield varies with changing values of pH, temperature inoculum size and glucose. The optimum yield of the interactions of the variables is shown on the summit of the three-dimensional plot. The interaction between pH and temperature significantly gives a theoretical chitosan optimal yield of 3.99117 g/litre (Fig. 4.3). The interaction between inoculum size and glucose significantly gives a theoretical optimal chitosan yield of 4.22 g/litre

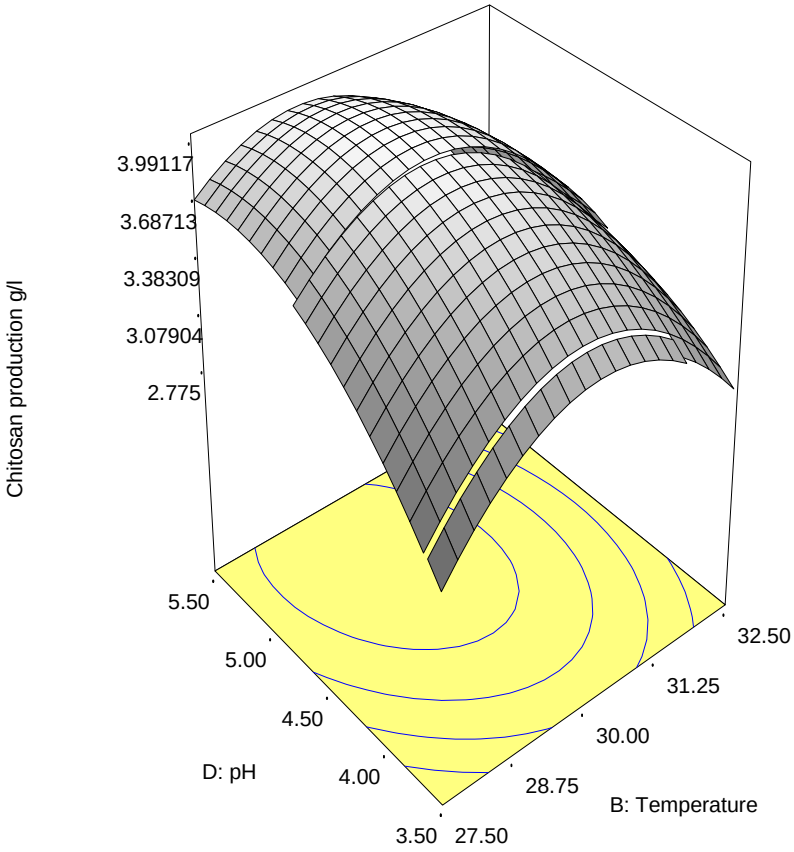


Figure 4.3. The three-dimensional response surface graph showing interaction between pH and temperature.

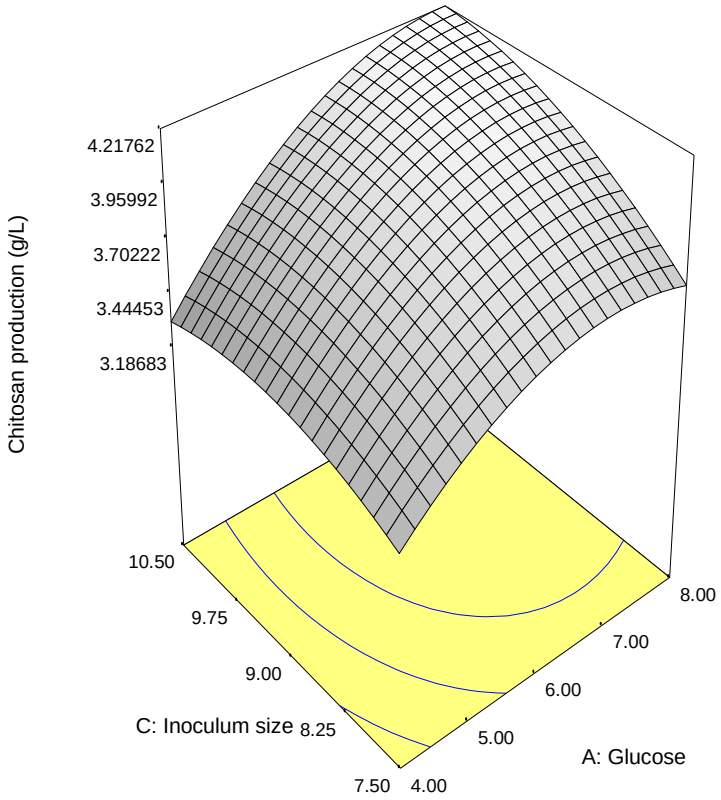


Figure 4.4. The three-dimensional response surface graph showing interaction between glucose and inoculum size.

To confirm the validity and accuracy of the model predictions three runs of confirmation experiments were carried out at optimal factor levels (Table 4.6). This was done in triplicate. Run 3 which had a predicted chitosan yield of 4.20 g/litre gave an actual yield of 4.19 g/litre which was more than 99% accurate.

4.3.7. Validation of the optimised chitosan production model

Table 4.6. Validation of RSM model

Run number	Glucose	Temperature	Inoculum size	pH		Chitosan production (g/l)	
						Predicted	Observed
1	70.29	29.44	9.50	4.55	1	4.13	4.03±0.39
2	70.20	29.29	10.40	4.94	2	4.15	4.09± 0.26
3	**70.61**	**29.69**	**10.20**	**4.51**	**3**	**4.20**	**4.19± 0.18**

The biochemical pathway for chitosan synthesis begins with the isomerised glucose molecule from the investment stage of glycolysis. This should explain why glucose is one of the most significant factors in chitosan production. Inoculum size is the most significant factor and this can be explained by the need for more cells to utilize the abundant glucose for growth, otherwise if the levels of glucose become excessive the cell metabolism will shift towards lipid accumulation. The appropriate temperatures (29.6˚C) and pH (4.51) for optimal chitosan accumulation are critical for the enzymes involved in chitosan biosynthesis and these includes: glutamine fructose-6-phosphate amidotransferase which is responsible for the conversion of fructose-6-phosphate to glucosamine-6-phosphate; glucoseamine-6-phosphate acetyltransferase which is responsible for the conversion of glucosamine-6-phosphate into N-acetylglucosamine-6-phosphate; chitin synthase regulatory proteins which are responsible for the conversion of UDP-N-acetylglucosamine into chitin and chitin deacetylase which converts chitin into chitosan.

The experiment was also scaled up in 500 ml, 1000 ml Erlenmeyer flasks and a 5 l bioreactor. Chitosan production decreased to 4.06 g/litre as the bigger flasks (500 ml and 1000 ml) were used when compared to the smaller ones (250 ml). However, chitosan production was drastically increased to 5.02 g/l when a 5l bioreactor was used (Table 4.7). This could be due to the fact that conditions are better controlled in a bioreactor.

Table 4.7. Scale up for chitosan production

Volume (ml)	Volume of medium used (ml)	Chitosan production (g/l)
Shake flasks		
250	50	4.21±0.25
500	100	4.11 ±0.38
1000	200	4.06 ±0.31
Fermenter		
5 l	3000	5.02 ±0.27

4.3.8. Antimicrobial properties of chitosan

The antimicrobial properties of the fungal chitosan were investigated and these were compared to the activity of the commercial shrimp shell chitosan. This was done on three bacterial strains and one fungal strain. The three bacterial strains were *E. coli*, *S. aureus* and *B. subtilis* and the fungal strain was *A. niger*. The extracted chitosan shows minimum lethal concentrations on the four microbial strains from a minimum concentration of 600 ppm to 1100 ppm, whilst the commercial shrimp shell chitosan shows activity from 300 ppm to 2000 ppm (Table 4.8). For *E. coli* and *S. aureus* the MLC values for fungal chitosan were higher and for *B. subtilis* the MLC values for both were identical. The fungal chitosan is more effective against *A. niger* with an MLC of 1100 ppm compared to 2000 ppm for commercial chitosan.

Table 4.8. Minimum lethal concentrations of fungal and commercial crustacean chitosan against *E. coli, S. aureus, B. subtilis* and *A. niger*

Microorganism	MLC for fungal chitosan	MLC for commercial crustacean chitosan
E. coli	900 ppm	500 ppm
S. aureus	600 ppm	300 ppm
B. subtilis	800 ppm	800 ppm
A. niger	1100 ppm	2000 ppm

Initially biochemical, biological, microbiological and molecular analysis have been performed on chitosan to investigate antimicrobial properties. These have pointed towards factors affecting the efficacy of chitosan antimicrobial properties having both intrinsic and extrinsic origins. The extrinsic factors would be environmental conditions and the cellular morphology. The intrinsic properties would include the chitosan properties that includes molecular weight, degree of deacetylation (which influences solubility) and complex ion formation (Goy, Britto and Assis 2009; Kong *et al.* 2010). One of the major differences between fungal-derived chitosan (LMW)

and crustacean shell-derived chitosan (HMW) is molecular weight. There have been conflicting reports on the actual effects of low and high molecular weight chitosan with some reporting increased antimicrobial activity with increased molecular weight whilst others report an increase in antimicrobial activity with decreasing molecular weight. The explanation to linking molecular weight to antimicrobial activity involves the chitosan forming interactions with cell receptor molecules on the cell wall forming a barrier that would prevent nutrient intake. Another mechanism is the lower molecular weight chitosan pervasively entering the cell thereby disrupting the physiological activity of the cell. It seems from the results obtained from this study that whilst molecular weight of the chitosan may be important for antimicrobial effect, this is also dependent on the nature of the cells targeted. The fungal chitosan was less effective in killing *E. coli* and *S. aureus* but had the same lethality on *B. subtilis* as crustacean chitosan. The fungal chitosan was more effective against *A. niger*. It is also possible that increasing the purity of the fungal chitosan to the same levels as the commercial chitosan may increase the potency of the fungal chitosan

4.3.9. Application of the chitosan in increasing the shelf life of fresh fruit produce

Considering the antimicrobial effects of the fungal chitosan against the bacterial and fungal strains chosen in this study which ranges from 900-1200 ppm A chitosan spray was developed using 0.5% (5000 ppm) chitosan being dissolved in 1% acetic acid and the spray was used on fresh fruits purchased from a local fresh produce store. Unsprayed fruits and those sprayed with 1% acetic acid were used as controls. The chitosan spray preserved the tomatoes and apples longer when compared with the controls. The chitosan sprayed apples and tomatoes and were still preserved even after 18 and 14 days of storage at room temperature respectively when compared to the controls (Figs. 4.5-4.7)

The tomatoes in Fig. 4.5 a and Fig. 4.5 b showed evidence of fungal contamination and decay around the areas where the pedicels attach. After 14 days, chitosan protected tomato (Fig. 4.5 c) still retained overall shape. However, there was evidence of slight dehydration on all the three fruits.

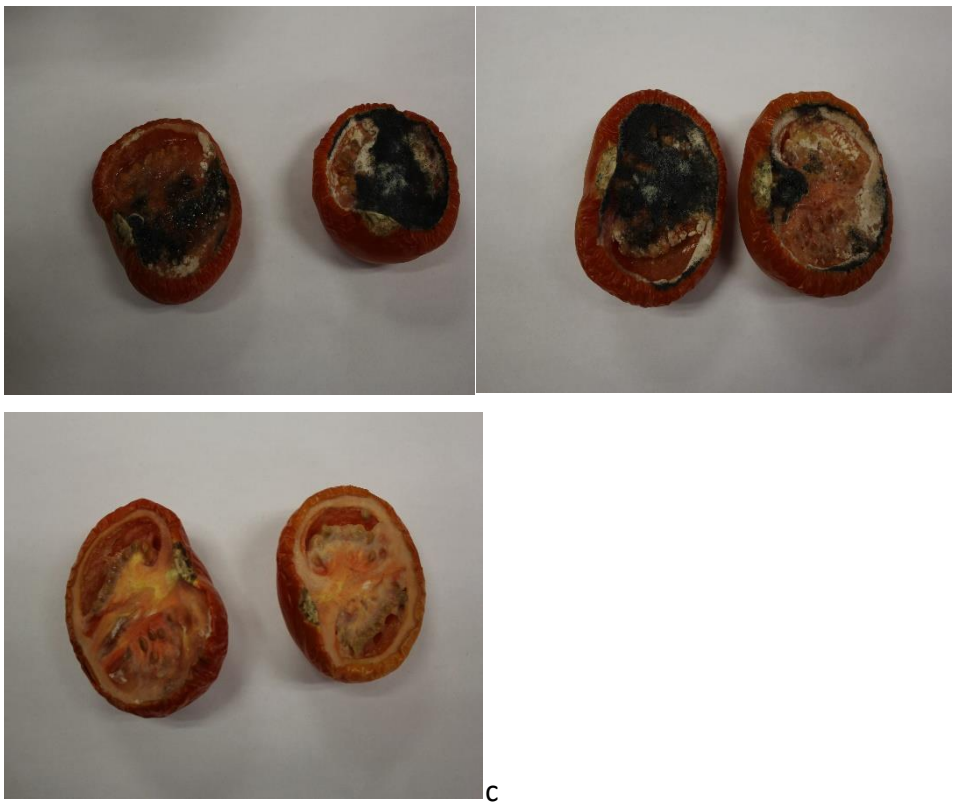
a b

c

Figure 4.5. The effect on chitosan spray on tomatoes after 14 days of storage at room temperature (a) tomato without any spray (b) tomato sprayed with 1% acetic acid (c) tomato sprayed with 1% chitosan.

The cut tomatoes in Fig. 4.6 a and Fig. 4.6 b shows severe infection by fungi and bacteria after four days. The cut tomato in Fig. 4.6 c shows they are largely free of microbial growth. There is also evidence of drying and size reduction as expected.

a b



c

Figure 4.6. The effect of chitosan spray on cut tomatoes after four days (a) without any spray (b) sprayed with 1% acetic acid (c) sprayed with 1% chitosan.

There is evidence of bacterial rot in on the apples in Figs 4.7 a and 4.7 b after eighteen days. In the same period the apple in Fig 4.7c appears normal and there was no sign of infections. The size and shape looked normal and visually appear to be fine to eat.

a b

c

Figure 4.7. The effect of chitosan spray on apples after eighteen days (a) without any spray (b) sprayed with 1% acetic acid (c) sprayed with 1% chitosan.

Chitosan can play a role in addressing the challenge of fresh produce being lost to spoilage when in cooperated in rapid post-harvest surface sanitation. The fungal chitosan has an advantage when making a chitosan spray for surface sanitation, because of the lower viscosity. This will allow higher concentrations of the chitosan to be applied. It is difficult to make a chitosan spray from crustacean chitosan especially at concentrations of 0.5% and above as the solution becomes too viscous. Fungal chitosan does not have allergenic proteins that come with the use of crustacean-derived chitosan thus enhancing its suitability in food preservation (Kannan *et al.* 2010). The chitosan spray was also applied on cut tomatoes. Currently there is an increasing demand for cut fruits and any method that increases the shelf life of the produce will be beneficial. In tomato production, an exposed fruit in the form of breakage can be the source of

spoilage for the entire batch including the intact ones. The advantage of using a chitosan spray to prolong the shelf-life is that it is edible and not detrimental to human health. The antimicrobial properties of the chitosan spray could reduce the risk of fresh produce-borne pathogens. There is currently an ongoing food safety issue in South Africa with the recent incidences of listeriosis which was said to be the largest ever by the World Health Organization (WHO 2017) thus the role of fungal chitosan in preserving as well as sanitizing food is more relevant than ever

CHAPTER 5: GENERAL DISCUSSION

Most of the commercially available chitosan is derived from crustacean wastes which would otherwise be discarded into the environment causing pollution. However, this would mean that the geographical location of the fisheries as well as the seasonal availability of the crustaceans are limiting factors to production of the chitosan. Fungi can easily be grown as has already been shown in various industrial fermentations under controlled conditions at any given time, to produce high yields of biomass which is an alternative source for chitosan. The fungal biomass unlike crustacean wastes contains lower levels of inorganic minerals thus removing the need for demineralization. Controlling the conditions for fungal growth have been shown to result in chitosan that is consistent in properties unlike the batch variation effects evident in the crustacean-derived crustacean. There has been only one Japanese company producing chitosan from fungi and it is called Kitozyme. This is, however, not through submerged fermentation of mycelial fungal biomass but through mushroom.

The reason why there has not been commercial chitosan production from fungal biomass using submerged or solid-state fermentations is because of the high costs that would make the final selling price exorbitant. This project initiates the process of applying the biorefinery concept in commercial submerged fermentation fungal chitosan production to make the entire process feasible. Concurrent production of chitosan and lipids was successfully carried out from the isolated chitosan producing strain. The process was optimized to give the highest chitosan yield of 5.02 g/l recorded to date. The two products were used in applications in line with the value addition approach.

According to a recent Sigma price list as at 27/10/2017 shrimp-shell derived practical grade chitosan with a degree of deacetylation of at least 75% costs R9090 per 500 g. However, lower molecular weight chitosan costs even more as it would involve further hydrolysis of the higher molecular weight chitosan. Low molecular weight chitosan has special uses but for the sake of feasibility considerations in this present study the price of the more commercially used high molecular weight chitosan will be considered.

To produce 500 g of the fungal chitosan would require 100 litres of fermentation medium. That would need at least 5 kg of glucose at a cost of R2140. If the other components of the media are

factored in such as peptone (1 kg needed at a cost of R4660), yeast extract (100 g needed at a cost of R500, mineral salts (5 g needed at cost of R500) as well as costs for energy consumption in the fermentation and extraction processes and the chemicals used. This will clearly show that fungal chitosan is still more expensive to produce than crustacean chitosan under these conditions.

In any applications where, low molecular weight chitosan is required then the use of fungal chitosan become more economically viable. Another way to get better returns is through value-addition to the chitosan. Medical-grade and food-grade chitosan fetches even higher prices which may outweigh further purification costs. It can therefore be deduced that the endeavor towards the feasible production of fungal chitosan should involve among other things, the replacement of medium components like glucose and peptone with cheaper alternatives. Such alternatives can be agro-based wastes and residues. Agro-based wastes containing complex carbohydrates can induce extracellular enzyme production from the fungi thereby increasing the economic potential of the fermentation broth. One such enzyme whose production can potentially be feasibly pursued is amylase and *M. circinelloides* isolates have been previously reported to produce this enzyme. This would mean agro-based residues rich in starch would be preferred.

The biomass residue that remains after chitosan and lipid extraction potentially still contains a significant amount of nutrients like amino acids as a result of protein hydrolysis during chitosan extraction and thus can be used as feed for fish and poultry. The high temperatures also break up the chitosan glucan complex hence glucan can be a potential supplementary carbon source in the feed.

At maximum chitosan production of 5.02 g/litre at least 2 g/litre of lipids are produced and these would translate to 80 ml of biodiesel when considering the biodiesel density of 0.88 g/cm^3. That also means whenever 500 g of chitosan is produced 200 g of lipids are also produced which would translate to 8 litres of biodiesel. The benefit of producing the biodiesel may be enhanced if the option of producing electricity to power the fermentation is pursued, especially considering the energy-supply deficit South Africa is currently facing. It has been suggested that in order to establish a global bio-based economy, the following three pillars should make up the foundation: bioenergy; biofuels and bio-based products (Taylor 2008). This means biodiesel in this instance is a very critical component as a biofuel. A biofuel would enable the establishment of the biorefinery without exerting demand on the national energy grid. The fact that agro-based

residues can be potentially be used as carbon and nitrogen sources enables placing the biorefinery in a rural setting where such materials are readily available.

The conditions that favor optimal production of both chitosan and lipids would have to be re-done, while accounting for costs considerations too. Whilst both products need high amounts of glucose to be optimally produced, it is the difference in the amounts of peptone required that can be modified, considering the high costs of the raw material. Optimal chitosan production utilized 5% peptone whilst optimal lipid production utilized only 0.1%. This means the amount of peptone to be used for co-production can be reduced without compromising the yield significantly.

In conclusion, a high chitosan-accumulating fungal strain was successfully isolated from compost. It was identified and given the designation *Mucor circinelloides* ZSKP. Co-production of chitosan and lipids from the fungal strain was successfully demonstrated. Production parameters were optimized using Plackett-Burman Design and Response Surface Methodology. Optimal chitosan yield was 5.02 g/l and optimal lipid yield was 6.4 g/l in a 5 litre bioreactor. The lipids produced were used to synthesize biodiesel via both enzymatic and chemical catalysis. Enzymatic catalysis gave the highest yield (96%). The biodiesel produced, met and surpassed all the prescribed ASTM standards. The chitosan produced demonstrated biological activity a was used to make a chitosan spray which was applied to fresh fruits to increase the shelf life of the fruits. Upon optimisation of production of other metabolites such as amylase and its efficient extraction from the fermentation broth, this fungal isolate has potential to be used in a biorefinery process.

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