The efficiency of lytic enzymes for *Ascaris* eggs inactivation

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DECLARATION BY STUDENT

I declare that this thesis, submitted for the award of Masters in Applied Sciences at the Durban University of Technology, is the original work of the author and has not been submitted for a degree at any other University. Where use is made of any author's work, it has been duly acknowledged.

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ABSTRACT

Faecal sludge (FS) contains organic matter that can enhance soil quality if pathogenic organisms, such as *Ascaris lumbricoides,* are reduced to safe levels. *A. lumbricoides* is a highly resistant nematode used to assess the efficiency of FS and wastewater treatment. The standard for reusing FS is to reduce *Ascaris* spp eggs to <1 egg/g for helminth reduction. However, in many developing countries, untreated FS is used as a soil enhancer without following proper guidelines. There are a number of FS treatment technologies in use, such as compositing, drying and the use of calcium carbonate. These techniques are able to reduce the pathogen concentration, especially *Ascaris* spp to accepted standard prior to application. Other alternative FS treatment technologies are under assessment and development.

The objective of this study was to evaluate the use of lytic enzymes to inactivate *Ascaris* spp eggs by targeting the egg shell of the parasite. *Ascaris* spp egg shells consist of protein, chitin, and a lipid layer. Therefore, it may be possible to disintegrate *Ascaris* spp by targeting these layers of the egg shell with enzymes such as protease, chitinase, and lysozyme, which are commonly produced by indigenous soil bacteria.

Ascaris spp eggs were detected in all FS samples collected from urine dehydration diversion toilets (UDDTs) in Durban, with an average concentration of 976-1118 helminth eggs/gram of FS. However, the viability of recovered eggs from FS was low and eggs were in different stages of development. This would negatively affect the experiments and produce inconsistent data. Therefore, for the lytic enzyme inactivation experiments, commercially bought eggs were used instead of the helminth eggs recovered from the UDDTs FS. Exposure of the eggs to commercial lytic enzymes was done following three different approaches. Firstly, *Ascaris* spp eggs were exposed to each of the enzymes individually, a second approach was employed where the eggs were consecutively exposed to the eggs with a rinse in between, and lastly, the eggs were exposed to a mixture of all three enzymes at once. Viability of the *Ascaris* spp eggs was determined via incubation, followed by microscopic examination of the eggs for visible motile larvae. For the single enzyme exposure, chitinase was the most detrimental enzyme resulting in a reduction of viability by 34% at room temperature. Exposure of the *Ascaris* spp eggs using the enzymes in series, achieved an egg viability reduction of up to 90 % at 37 °C, after 5 days exposure. Exposing the eggs to the mixed enzymes gave a reduced egg viability of 75 % at 37 °C, after 5 days exposure. Furthermore, the detection of microorganisms in the FS capable of producing lytic enzymes used was also confirmed. Enzymes were produced using selective media that resulted in the production of enzymes where the concentration (mg/L) and specific activity (U/mg) was determined to 0,68 mg/mL (0,08 U/mg) of protease from nutrient broth enriched with 2.5% milk, 3,17 mg/mL (0,006 U/mg) of chitinase produced from 1% colloidal chitin, and 7132 mg/mL (2600 U/mg) lysozyme from nutrient broth enriched with 1% *Micrococcus lysodeikticus* culture.

The lytic enzymes showed to have an antagonistic effect on the *Ascaris* spp eggs. This therefore serves as a proof of concept that lytic enzymes produced by microorganisms found in FS could potentially be used for the inactivation of *Ascaris* spp eggs. However, further work is required focusing on enhancing the enzyme production, testing of the inactivation potential of these enzymes in the presence of solids and other materials present in FS, and finally the technique for field application of such technology.

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DEDICATION

In loving memory

Of

My late mother

Thabisile P.J Mncube

And her mother,

Hilda Ntshingaba Mncube

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ABBREVIATIONS

BCA- Biological Control Agents

BSA – Bovine Serum Albumin

DALYs- Disability Adjusted Life Years

DEWATS – Decentralised Wastewater Treatment System

DTT - Dithiothreitol

EPA- Environmental Protection Agency

FF- Fresh Faeces

FLS- Flotation Solutions

FS- Faecal Sludge

FSM-Faecal Sludge Management

FSTP – Faecal Sludge Treatment Plant

GHF- Glycosyl Hydrolase Families

HEWL- Hen egg white lysozyme

HIV- Human Immunodefiency Virus

HO- Helminth Ova

KZN – KwaZulu-Natal

L1- 1st stage Larva

L2- 2nd stage Larva

L3- 3rd stage Larva

L4- 4th stage Larva

LMIC – Low and Middle Income Countries

LRV – Log Reduction Value

LPS- Lipopolysaccharide

MC – Moisture Content

MCA -Microbial Controlling Agents

MM- McMaster Method

MWe- Microwave Energy

MWI- Microwave Irradiation

NPK – Nitrogen, Phosphorous, Potassium

NAG – N-acetyl-D-glucosamine

NAM – N-acetylmuramic acid

NTD- Neglected Tropical Diseases

PCR- Polymerise Chain Reaction

PM – Plasma Membrane

qPDR- quantitative Paper-Based DNA Reader

RDP - Reconstruction and Development Programme

RFLP- Restriction Fragment Length Polymorphism

RPM – Revs per Minute

RT- Room Temperature

SCCA- Short Chain Carboxylic Acid

SCFA- Short Chain Fatty Acids

SG- Specific Gravity

STH- Soil Transmitted Helminth

TCA – Tri-chloro Acetic Acid

UDDTs- Urine Diversion Dehydration Toilets

VH – Viscous Heather

VIPs- Ventilated Improved Latrines

WASH- Water, Sanitation and Hygiene

WHO- World Health Organization

WW-Waste Water

WWTPs- Waste Water Treatment Plants

CHEMICALS

C-lysozyme	Chicken-type lysozyme
D-glutamyl-DAP	Meso-diaminopimelic acid
EDTA	Ethylenediaminetetraacetic Acid
Fn-III.	Fibronectin III
GluNAc	N-acetyl-D-glucosamine

GlcN	D-glucosamine
H_2SO_4	Sulphuric Acid
G-lysozyme	Goose type lysozyme
L-lysozyme	Invertebrate type lysozyme
NaCl	Sodium Chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NPK	Nitrogen, Phosphorus, Potassium
NH_3	Uncharged Ammonia
NH_4+	Ammonium
NH_4SO_2	Ammonium Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SspA or V8 protease	*S. aureus* virulence proteases include the serine glutamyl endopeptidase
ScpA/ StpA	Staphopain A
SspB/ StbB	Staphopain B
SG-1	SYBER Green 1
ZapA	*Listeria monocytogenes* and *Proteus mirabilis*
$ZnSO_4$	Zinc Sulphate

1 CHAPTER 1: INTRODUCTION

1.1 Background information

Soil-transmitted helminth (STH) are parasitic intestinal worms that have detrimental effect on human health by causing under-nourishment, growth retardation, tissue damage, malnutrition, diarrhoea and vomiting after ingestion and development in the intestines (Trönnberg et al., 2010; Freeman et al., 2017). The most common STHs, causing human infections, are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* or *Ancylostoma duodenale*) (Prasanphanich et al., 2013). STHs eggs are transmitted through contamination of soil or water, where faeces containing STHs eggs are passed into the environment and in turn contaminate the soil, food and water bodies (Sangwan and Motukupally, 2016; Karshima, 2018; Oliveira et al., 2020).

In this regard, open defecation and poor faecal sludge management (FSM) are dominating sources for STH infections, which are most prevalent in developing countries such as sub-Saharan Africa, Asia, the Middle East and Latin America where the provision of sanitation is poor (Freeman et al., 2017). The lack of sanitation or poor facilities in developing countries, force people to continue to practice open defecation or use unsafely managed sanitation systems (Amoah et al., 2016; WHO, 2017, 2021).

Even where basic sanitation exists, service providers and other stakeholders are faced with the burden of disposing human waste which is often dumped into water bodies and fields leading to unsafe re-use of faecal sludge (FS) (Okem et al., 2013; Tilley et al., 2014). This has a negative impact on the standard of living and the health of the people, and generally increases the transmission of diseases (Amoah et al., 2016; WHO, 2017, 2018).

The re-use of FS is partly promoted due to the presence of organics that can enhance the soil for agricultural purposes (Amoah et al., 2016). Organic matter is a source of valuable nutrients

such as nitrogen (N), phosphorous (P) and potassium (K) (NPK) (Harroff et al., 2017; Chaoua et al., 2018). The complex organic material found in FS is made up of polysaccharides, peptides, lignin and lipids (Karn and Kumar, 2019). This complex organic material contains microorganisms that produce extracellular lytic enzymes such as amylase, alkaline phosphatase, lipase and proteases that can break down complex organic molecules (Karn and Kumar, 2019). FS in this context is human excreta that is mixed with urine and solid waste (household waste) (Tilley et al., 2014).

FS may contain high amounts of pathogens if not treated (Jiménez, 2007; Amoah et al., 2017). Unsafe handling of FS during collection, composting, and consumption of contaminated food and water by community members presents a risks of pathogen transmissions (Schönning et al., 2007). The microbial health risks associated with the use of untreated FS is the driving force for the need of its treatment. Several methods in use aim at inactivating pathogens, using *A. lumbricoides* as the indicator organism. It has been estimated that the natural die off time span of *A. lumbricoides* eggs can take up to 600 days from excreted faeces and 150 days in soil (WHO, 2006).

Suggested FS treatment method for inactivating STHs is ammonium stabilisation (Nordin et al., 2009; Vu-Van et al., 2017). Vu-Van et al. (2017) achieved 85% reduction of viable *A. lumbricoides* eggs using 10% w/v lime in 111 days in an alkaline medium. However, conventional treatment methods for FS, including ammonium stabilization, composting, and sludge drying can sometimes be tedious, time consuming and non-specific (Al-Gheethi et al., 2018; Zewde et al., 2021).

The inert egg shell of *A. lumbricoides* is made up three distinctive layers that makes up the complex plasma membrane (PM) (Gortari and Hours, 2008). These authors further state that the outer layer of an *Ascaris* spp egg is primarily composed of proteins. The protein layer occurs as a protein-chitin matrix complex, which contributes to the mechanical strength of the

egg shell structure. The middle layer is made up of chitin which is the most abundant amino-polysaccharide in nature and forms a rigid structural component of many organisms. The inner layer of the *Ascaris* spp eggs is made up of lipoproteins and lipids. It is embedded and held together by peptidoglycan; the alternating sugars of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) (Salazar and Asenjo, 2007).

This three-layered impenetrable wall-structure makes the *Ascaris* spp egg the most resistant stage in the life cycle of this parasite, and gives it the ability to remain in the environment for extended periods and still have the potential to cause an infection once ingested by a suitable host (Gortari and Hours, 2008; Moodley et al., 2008). Even though the egg structure is the most resistant stage in the life cycle of helminths, the layers of the egg shell can be potentially broken down using lytic enzymes that can target each layer (Gortari and Hours, 2008).

Lytic enzymes can potentially target and rupture the egg shell, resulting in non-viable *Ascaris* spp eggs (Gortari and Hours, 2008; Prasanphanich et al., 2013). In the ecosystem, several microorganisms produce lytic enzymes such as protease, lysozyme and chitinase in order to break down cell walls of other bacterial cells, plants and insects (Salazar and Asenjo, 2007).This has been elaborated on by Salazar and Asenjo, (2007) as well as Gortari and Hours, (2008).

Protease is a hydrolase enzyme that is used to break down proteins into peptides and amino acids. Chitinase is used to break down chitin by microorganisms. Proteins and lipids are the components the inner lipid layer. Chitin as the second layer of *Ascaris* spp eggs is susceptible to chitinase degradation (Veliz et al., 2017). Lysozyme can cleave and disrupt the covalent bonds of carbohydrates and lipids (Salazar and Asenjo, 2007).

The ability of lytic enzymes to break STH egg shells has been demonstrated in DNA extraction studies by using lytic enzymes as a DNA extraction pre-treatment step (Leles et al., 2012). The use of these lytic enzymes was reported to increase permeability of the cell wall, improving the

yield of DNA during the DNA extraction process (Carlsgart et al., 2009; Leles et al., 2012). These authors also point out that chitinase has been recognised as an enzyme that can potentially increase the permeability of the egg shell by breaking open the middle chitin layer of STHs egg shell.

Therefore, enzymatic inactivation could possibly be an efficient approach in the inactivation of *Ascaris* spp eggs compared to conventional treatment methods alone. Lytic enzymes as biocontrol agents can therefore be considered as a possible environmentally friendly and a sustainable way of inactivating STH eggs in FS (Schönning et al., 2007; Okem et al., 2013). This is further investigated in this thesis.

1.2 Problem statement

Exposure to FS either directly or indirectly are a main route of transmission of STHs, leading to mortalities and morbidities globally, especially among children in developing countries, like South Africa. In addition to STHs, several other potential pathogens are present in FS, but the most difficult one to inactive are the STH eggs. *Ascaris* spp egg is the most resistant of these STHs, and is therefore used as an index pathogen for FS treatment. The current *Ascaris* spp egg inactivation methods have different drawbacks in their reduction abilities and may also be time consuming or expensive. Therefore this research aimed at looking at biological control agents such as lytic enzymes e.g protease, chitinase and lysozyme as potential inactivation agents for STHs in FS. This could be a safer, sustainable and effective method to treat FS and reduce faecal pathogens contamination to safe levels.

1.3 Aim

The main aim of the study was to determine the potential inactivation of *Ascaris* eggs using lytic enzymes produced by soil microorganisms enumerated from FS.

1.4 Objectives

To achieve the aim of this study, the following objectives were pursued;

1. To recover soil-transmitted helminths eggs from faecal sludge taken from urine dehydration diversion toilets.
2. To assess the impact of commercially available enzymes chitinase, lysozyme and protease on the viability of *Ascaris* spp eggs under laboratory conditions.
3. To analyse faecal sludge for chitinase, lysozyme and protease (lytic enzymes) producing microorganisms using selective media.
4. To identify and purify the lytic enzymes using biochemical techniques.

1.5 Thesis structure

This thesis is composed of 6 chapters, which includes an introduction, critical review of the literature, three technical chapters and a general conclusion and recommendations chapter. The technical chapters contain a brief introduction, methodology, results, discussion and conclusion. These technical chapters were prepared to capture each of the objectives, therefore objective 1 is covered by Chapter 3 (first technical chapter), and Objective 2 covered by Chapter 4 and Objectives 3 and 4 covered by Chapter 5.

2 CHAPTER 2: LITERATURE REVIEW

2.1 What are Soil-transmitted helminths?

Soil-transmitted helminths (STHs) are pathogenic multicellular intestinal parasitic worms that are excreted by humans or other hosts and have the ability to survive for extended time-periods in soil, mainly in warm and tropical regions (Bethony et al., 2006; Prasanphanich et al., 2013; Freeman et al., 2017; Karshima, 2018). STHs are transmitted through the faecal-oral route by ingestion of water, soil and foods contaminated with the infectious eggs of the respective parasite (Bethony et al., 2006; Prasanphanich et al., 2013). In some cases, STH infections occur through skin penetration by the larvae (Amoah et al., 2016). The eggs develop and completes the parasite's life cycle into larvae within a suitable host. Thereafter eventually the new generation of eggs are excreted with the faeces of the host (Bethony et al., 2006; Prasanphanich et al., 2013).

The most common human infectious species of intestinal parasites are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* and *Ancylostoma duodenale*) (Bethony et al., 2006; Roach et al., 2012; Gordon et al., 2017; Karshima, 2018). Other common STHs include for example *Strongyloides stercoralis, Enterobius vermicularis*, *Capillaria philippinensis* and *Trichostrongylus colubriform* (Jourdan et al., 2018; Bundy et al., 2020). The groups can be differentiated based on their shape, sex, body cavity, body layers etc (Table 2.1) (Bethony et al., 2006; Roach et al., 2012; Gordon et al., 2017; Karshima, 2018).

Table 2-1: Characteristics of Soil-transmitted helminths (Moodley et al., 2008; Jimenez, 2007).			
Morphologic	**Nematodes (roundworm)**	**Cestodes (tapeworms)**	**Trematodes (flukes)**
Shape	Cylindrical	Segmented plane	Unsegmented plane
Body cavity	Present	No	No
Body covering	Cuticle	Tegument	Tegument
Digestive tube	Ends in anus	No	Ends in cecum
Sex	Dioecious, monoecious and Parthenogenesis species.	Hermaphroditic	Hermaphroditic

Humans are the primary hosts for STHs, however, a single species has the ability to infect both animals and humans in order to complete its life cycle (Bethony et al., 2006; Melfi and Poyser, 2007; Jourdan et al., 2018; Bundy et al., 2020). STHs infections are now recognised among the most prominent neglected tropical diseases (NTDs) (Dold and Holland, 2011; Prüss-Ustün et al., 2019). STHs yearly infect around 2 billion people globally, with *A. lumbricoides* being the species of most concern to humans (Freeman et al., 2017; Gordon et al., 2017; WHO,2017; WHO, 2022). It is estimated that out of the 2 billion yearly infections, *A. lumbricoides* affects over 1.2 billion people globally, and caused an estimated 870 000 deaths in 2016 (Jourdan et al., 2018; Ranjan et al., 2015; WHO,2017; WHO, 2022).

One estimate of the global infections caused by STHs has been that it affects 48% of the 5 billion people globally living in developing countries where more than 10% were infected by two or more species (Bundy et al., 2020). Of the global infections recorded mainly in developing countries, 26% was due to *Ascaris* spp, 17% to *T. trichiura* and 15% to Hookworms (Roach et al., 2012). Intestinal helminths can be treated but infections may be fatal if neglected (Karshima, 2018).

2.2 Ascaris lumbricoides

The *Ascaris* spp egg is surrounded by an inert egg shell that provides protection in the environment and extend its survivability to be longer than other pathogens in faecal sludge (FS) and the environment (Gortari and Hours, 2008; Harroff et al., 2019). The egg shell consists of three layers: an outer protein layer, a middle chitinous layer and an inner lipoprotein layer (Figure 2.1) (Gortari and Hours, 2008; Rogers, 1956).

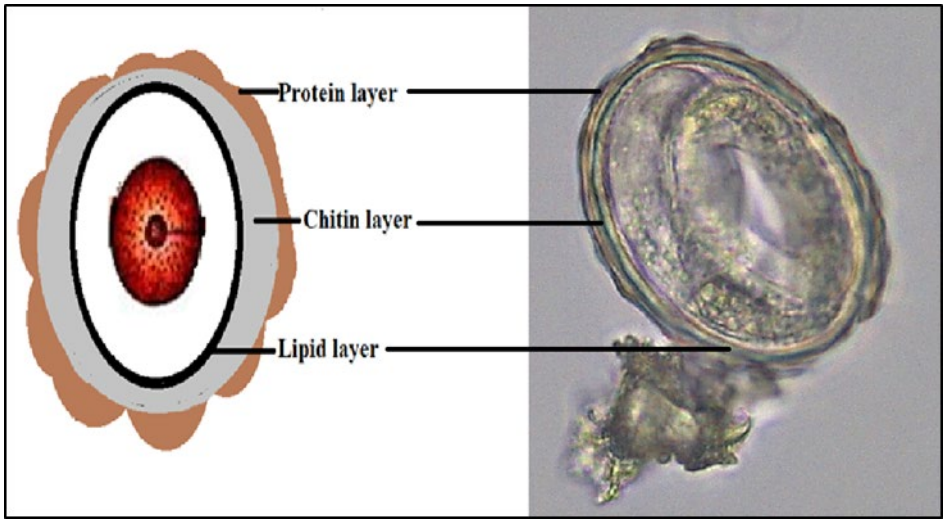

Figure 2-1: Illustrative diagram of the *Ascaris lumbricoides* egg shell with a nucleus on the left and a developed egg on the right (Illustration: Thabiso Zikalala).

The outer layer of *Ascaris* spp eggs is primarily composed of a protein-chitin matrix complex that contributes to the mechanical strength of the egg shell structure. The protein layer has an irregular slime appearance originally thought to protect the cell against desiccation but further believed to be obtained when the egg leaves the uterus after the lipid and chitinous layers have been formed (Rogers, 1956). According to Gortari and Hours (2008), the middle 3-4 µm layer of the *Ascaris* spp egg shell is made up of chitin and gives the egg its rigid and resilient characteristic. This layer is unique for *Ascaris* spp among the nematode eggs. The chitin layer is the most resistant structure of the *Ascaris* spp egg and is composed of N-acetyl-D-glucosamine (GluNAc) and D-glucosamine (GlcN). The inner lipid layer of the *Ascaris* egg is

made up of lipoproteins and lipids embedded and held together by peptidoglycan that allows an exchange of water, lipid solvents and ions into the egg's cytoplasm. During the developmental stage of *Ascaris* spp, the egg shells becomes stronger in order to protect the embryo until it is fully developed and hatches within the host.

A. lumbricoides was first discovered and described by Carl Linnaeus in 1758 as a homo-sapiens parasite (Gazzinelli-Guimarães et al., 2013; Leles et al., 2012). *A. lumbricoides* is the largest worm in the *Ascaris* genera and its eggs are brown to yellow (George, 2016). *Ascaris* spp male worms can grow up to 10-30 cm in length, 2-4 mm in width, and have a pointy coiled tail (Figure 2.2) (Moodley et al., 2008; Gan et al., 2014). *Ascaris* spp female worms can grow up to 20-35 cm in length, 4-6 mm in width, and have a straight tail (Gan et al., 2014). The womb takes up most of the female body compartments and it can contain more than 27 million eggs (George, 2016).

Figure 2-2: *A. lumbricoides* nematode fertilized egg (A), male (B) and female worm (C) (2008).

Ascariasis is an infection of the small intestine caused by *A. lumbricoides* (Sangwan and Motukupally, 2016; Dold and Holland, 2011). Ascariasis is most common amongst women and children. Children are most susceptible and are likely to suffer malnutrition, intellectual retardation and growth impairment, as well as growth retardation, gastrointestinal volvulus,

and interception and obstruction of the intestinal or binary tract if heavily infected (Bethony et al., 2006; Sangwan and Motukupally, 2016; Jourdan et al., 2018). Malnutrition is due to the absorbance of the nutrients by the worm in the gastrointestinal tract, alteration of the gastrointestinal microbiota while abdominal pain and obstruction may be due to tangling of the adult worms (Gazzinelli-Guimarães et al., 2013; Melo et al., 2016; Sangwan and Motukupally, 2016; Jourdan et al., 2018).

Upon infection, STHs can live in the gastrointestinal tract without the host showing any symptoms (Bethony et al., 2006; Prasanphanich et al., 2013; Freeman et al., 2017). Gastrointestinal symptoms and diseases are due to the presence of worms in the intestine (Gan et al., 2014). The movement of larvae causes either coughs or sneezing, allergic reactions, asthma, urticaria (hives, a skin rash), airway obstruction, cholangitis, anaemia, cholecystitis, inflammation of the gall bladder as well as eosinophilic pneumonitis (Sangwan and Motukupally, 2016; Gordon et al., 2017; Jourdan et al., 2018).

Other symptoms of ascariasis include, cysts in kidney, bladder, appendix and pancreas, vomiting, coughs, fever, dyspnoea (shortness of breath), and haemoptysis of the respiratory system (bloody coughs) (Sangwan and Motukupally, 2016). In rare cases of ascariasis, the larvae can accidentally reach the eye and become trapped in the eye's ocular tissue, leading to optic papillitis (inflammation and deterioration of the optic disk) (Sangwan and Motukupally, 2016). Infected individuals can excrete up to 10^2–10^4 helminth ova (HO) eggs/g of faeces daily (Amoah et al., 2018a).

2.3 Life cycle of *Ascaris lumbricoides*

Generally, female worms can produce two types of eggs, a fertilised inseminated egg or an infertile egg. Fertilised eggs are inseminated by mating with a male worm and are passed into the environment (Sangwan and Motukupally, 2016). In the environment, the eggs are embryonated and develop into infective larvae (Dold and Holland, 2011, Sangwan and

Motukupally, 2016). Embryonation is a developmental process of the larvae to their infective stage in the soil (Dold and Holland, 2011, Sangwan and Motukupally, 2016). A single fully developed egg at the larval stage is required to cause an infection once it is ingested (Hotez et al., 2005).

A. lumbricoides has a life cycle that includes the egg and four juvenile stages (Figure 2.3) (Gortari and Hours, 2008). These stages have been outlined in literature, including papers from Moodley et al. (2008), Medina et al., (2015), Sangwan and Motukupally, (2016), and Jourdan et al., (2018). The first larval embryo development stage (L1) occurs in the soil, outside the host's body, and is capable of causing an infection once ingested (Melo et al., 2016).

After being swallowed, it hatches in the duodenum and moult into the second stage larva (L2) which attach itself to the duodenum and penetrate the intestinal mucosa wall. Thereafter, it migrates into the liver and enter the blood stream (portal veins) into the right side of the heart and further to the lungs. Here in the lung, it moults to a third stage larva (L3) where it penetrates and migrate through the capillaries into the bronchial tree and to the trachea where they are coughed out and swallowed. The L3 larva enter the small intestine where it during, three weeks after ingesting the egg, moult into stage four larva (L4), which is an adult worm, in the caecum (Jourdan et al., 2018).

The adult female worm lays about 200 000 eggs per day which occurs about two to three months after ingesting the eggs and may continue for 1-2 years without the host showing symptoms of infection (Lamberton and Jourdan, 2015; Jourdan et al., 2018). Fertilised eggs passed through faeces into the environment require 9-14 days (called latency period) before they develop into the first larval stage, and the eggs can survive in the environment for years (Medina et al., 2015; Sangwan and Motukupally, 2016; Jourdan et al., 2018).

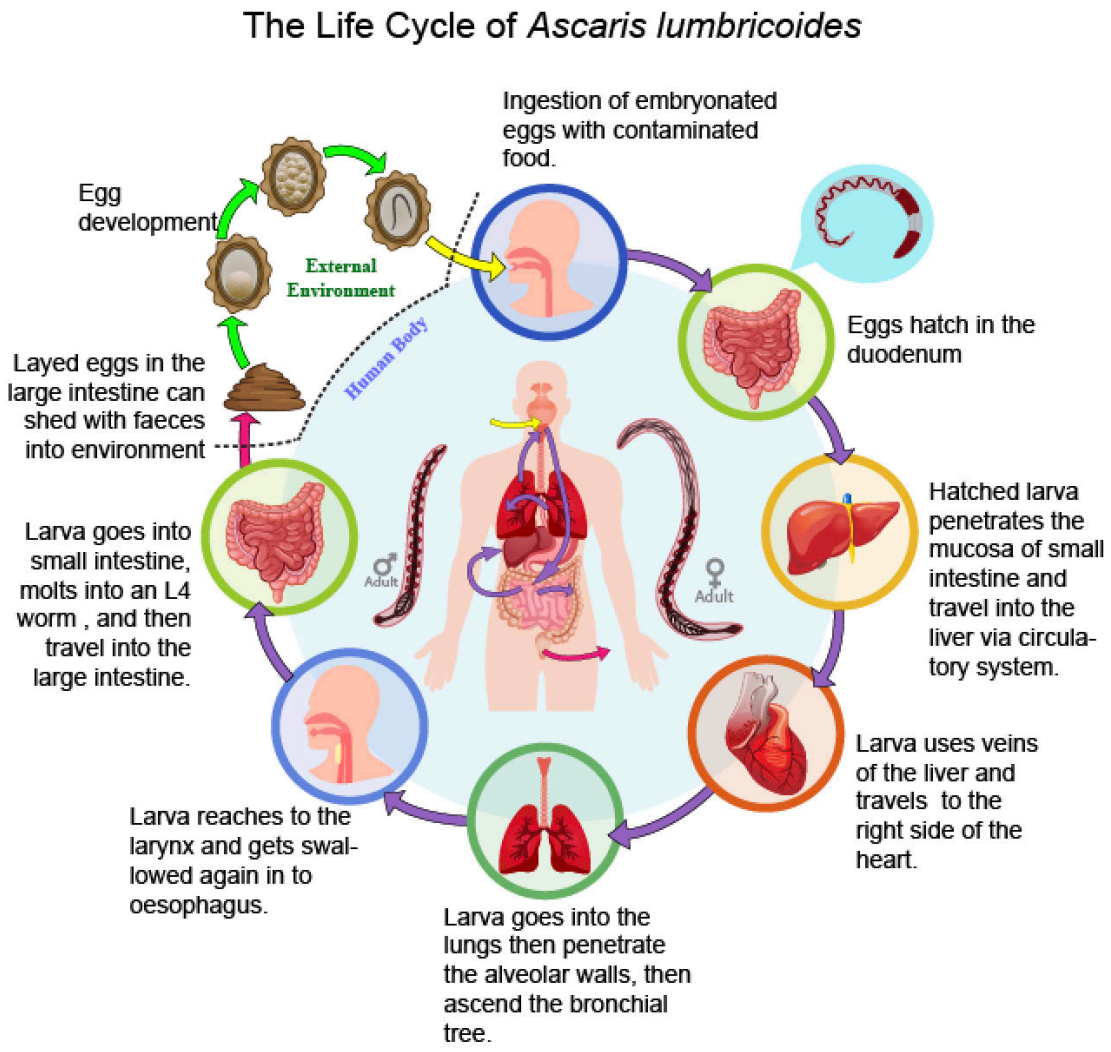

Figure 2-3: Life cycle of *A. lumbricoides* (Gordon et al., 2017).

2.4 Association between sanitation and exposure to faecal matter

Sanitation is defined as the provision and access to facilities that can be used for safe human waste separation, containment, transportation, treatment, disposal and in some cases, safe re-use (Freeman et al., 2017; WHO, 2018). Adequate sanitation is described as flush toilets connected to the sewer, septic tanks and dry sanitation systems like latrines that protects humans from direct exposure and contact with untreated faecal matter (Grimes et al., 2014; WHO, 2018). Inadequate sanitation and poor hygiene are the main causes of STHs transmissions (Chaoua et al., 2018; Karshima, 2018; Oliveira et al., 2020).

Geographic demarcations, densely overpopulated areas, poverty and low socio-economic standing and education are contributing factors to the spread of STHs in developing countries

and communities, mainly due to the increasing population in developing countries, high unemployment rates, poor access to inadequate or a complete lack of safe sanitation facilities and hygiene (Freeman et al., 2017; Jourdan et al., 2018; Harroff et al., 2019; Prüss-Ustün et al., 2019). The lack of proper sanitation in countries within sub-Saharan Africa and South East Asia (as shown in Table 2.2) could be the reason for the increased STH infections in these regions.

Table 2-2: Population percentage of exposure of low middle-income class to WASH services (Prüss-Ustün et al., 2019).

Region	Sanitation services	Piped water on premises	Hygiene (wash hands with soap)	Population % with > 75% WASH services
Sub Saharan Africa	30.8	25.5	8.4	13.3
America	85.1	58.3	36.2	75.8
Eastern Mediterranean	69.1	53.8	21.6	54.8
Europe	92.5	55.6	24.9	93.3
South East Asia	50.9	24.9	27.8	31.9
Western Pacific	75.1	28.5	17.1	63.2
Total LMICs	62.0	34.1	21.8	45.3

In 2017, the WHO estimated that 2.3 billion people lacked access to basic sanitation services (WHO, 2017). In 2020, 3.6 billion people were estimated to be without safely managed sanitation services, this included 1.9 billion people without safe basic sanitation services, 580 million people without access to basic sanitation services, 616 million people using dry sanitation technologies, and 494 million practicing open defecation (WHO, 2021).

2.5 Sanitation in the South African context

By the year 2000, after the cholera outbreak in South Africa, the government had provided 5.15 million households with ventilated improved pits (VIPs) toilets deemed as an acceptable and safe sanitation facility at the time (Tilley et al., 2014). It was estimated that a further 3.96 million households were left without provision of a safe toilet. In addition, the VIP facilities that have been previously provided to the households have become damaged due to the VIP latrines not being serviced, abandonment of superstructure, and used beyond its capacity (Department of Water and Sanitation, 2018).

According to the National Water and Sanitation Masterplan 2017 data, out of 17 million households in South Africa, 89% have access to safe drinking water, 64% has a reliable water supply, 80% has access to sanitation services, and 4 million households did not have access to safe sanitation. Approximately, a third of the South African population lived in rural areas without safe sanitation facilities and 14.1 million people still used sanitation facilities below the Reconstruction and Development Programme (RDP) standard in 2017 (Department of Water and sanitation, 2018).

Urbanisation continues to have a major impact on water supply and sanitation provision with many people moving to cities in search for jobs and better living conditions. The inadequate provision of sanitation facilities creates a critical health risk that leads to the transmission of diseases.

2.6 Prevalence of Ascariasis in South Africa

In South Africa, a mean concentration of 33 400 HO eggs/g was detected in the top faecal layer in a study that monitored 120 urine diversion dehydration toilets (UDDTs) (Trönnberg et al., 2010). In the Ugu district of KwaZulu-Natal, a study was done to determine the prevalence of *A. lumbricoides* infection among 1057 pupils tested across 18 schools. In 15 of the schools pupils were infected with both *A. lumbricoides* and other helminths, and two schools had pupils

only infected with one STH (Zulu et al., 2020). A total of 5 733 samples collected from adults from random public hospitals in KZN were screened for STHs and a 20.4% *A. lumbricoides* prevalence occurred in these samples (Kwitshana et al., 2008). Infections were most common in coastal regions (Kwitshana et al., 2008). In Eastern Cape, more than 65% of the stool samples collected tested positive for the presence of one or more STHs among 162 learners (85 boys and 77 girls) (Nxasana et al., 2013). More than 30% (47) stool samples collected tested positive for *A. lumbricoides* while other parasites were observed but had a lower prevalence than *A. lumbricoides* (Nxasana et al., 2013).

2.7 Faecal sludge treatment methods

As stated in Chapter 1, section 1.1, *A. lumbricoides* eggs are used as the indicator/index organism for FS treatment because they are considered as one of the most resistant pathogens that can withstand both WW and FS plant treatment processes (Harroff et al., 2019). *Ascaris* spp inactivation is therefore used to measure the efficiency of treatment methods to reduce pathogens in WW or FS (Schönning et al., 2007; Espinoza et al., 2012; Fidjeland et al., 2015; Harroff et al., 2019).

In 1992, regulations were set based on the importance of *Ascaris* spp eggs inactivation and its role in causing infectious diseases (Jiménez, 2007). The 2006, WHO WW and FS re-use guidelines stipulated an amount of HO that bio-solids should contain to less than 1 HO/g TS (WHO, 2006; Jiménez, 2007; Gordon et al., 2017). However, a high level of non-compliance with these guidelines occur. In Mexico, FS that is discharged into landfills contains up to 150 HO/g TS whereas in developed countries like the United States, France and Italy amounts most often are in the range of 1-2 HO/g TS (Méndez-Contreras et al., 2002). Several methods have been used for treating FS to eliminate these pathogens, as further exemplified in this section are some of these methods.

2.7.1 Conventional FS treatment methods

Composting (temperature), conventional drying, ammonium inactivation (alkaline stabilisation) and anaerobic digestion are conventional methods that are used to treat human excreta in wastewater treatment plants (WWTP) and FS treatment plants (FSTP) (Jiménez, 2007). *Ascaris* spp eggs have shown to be more resistant to UV light as well as various chemical agents and commercial detergents such as chlorine, phenol, cresol, potassium hydroxide, quaternary ammonium compounds, glutaraldehyde and paraformaldehyde (Kim et al., 2012; Oh et al., 2016).

A study by Oh et al. (2016) highlighted the following, home detergents, domestic and industrial chemical agents are ineffective in inactivating *Ascaris* spp eggs. *Ascaris* spp eggs exposed to different types of chemical agents developed into fully grown infective larvae without interruption of the embryo development stages. The only chemical agent able to achieve over a 90% reduction of *Ascaris* spp eggs after one minute was 10 % povidone iodine. Povidone iodine is used effectively against viruses, bacteria, fungi, protozoa and spores and is commonly used for skin disinfection, mucocutaneous trauma, chronic pharyngitis and oral ulcers. Complete inactivation of *Ascaris* spp eggs by 10% povidone was after six weeks of exposure. Detergents do not inactivate *Ascaris* spp eggs but delay their embryo development (Ki-Seok et al., 2016).

2.7.1.1 Wastewater treatment plants

STH eggs are present in WW as suspended solids and mainly removed by sedimentation with the addition of flocculants to trap or filter floating materials (Jiménez, 2007, Chaoua et al., 2018). The suspended solids are compacted and concentrated in sludge (Jiménez, 2007; Chaoua et al., 2018). *Ascaris* spp eggs cannot be completely removed or inactivated by filtration, sedimentation, flocculation, coagulation and sludge beds, and thus additional steps are required to treat the FS (Chaoua et al., 2018).

According to Al-Gheethi et al. (2018), there is a wide spectrum of pathogenic microorganisms detected in treated sewage that is discharged into the ocean and other natural water bodies. This practice is becoming unacceptable as it poses a risk to the human population. Additionally, the inability of the WWTP conventional disinfection processes in removing pathogens and pollutants have highlighted the need of employing advanced technologies that uses temperature phased anaerobic digestion, auto thermal aerobic digestion, ionised irradiation and chemical disinfectants in order to produce safer sewage effluents.

A study done in South Africa and Lesotho has shown that decentralised wastewater treatment systems (DEWATS) that serves a small region outside of the municipal grid, and a centralized conventional WWTP removed 95% and 67% of STH eggs respectively from wastewater (Amoah et al., 2018b). Treatment is based on technologies such as the anaerobic baffled reactors combined with vertical and horizontal gravel filters that serves as the final cleaning steps in removing pathogens and other impurities for the DEWATS systems (Amoah et al., 2018b).

A study done in Morocco also showed that STH eggs can be completely removed using a two-way treatment system that uses activated sludge and natural lagoons whilst conventional treatment plants removed up to 95% (Chaoua et al., 2018). This study had 100% of *A. lumbricoides* eggs removal from 153.49 eggs/L to 0 eggs g/L in Marrakech and 86,75 eggs/L to 5 eggs/L in Chichaoua (Chaoua et al., 2018).

2.7.1.2 Anaerobic digestion

Anaerobic digestion is a breakdown process of organic material by microorganisms in the absence of oxygen; the process generates biogas that can be used to maintain high temperatures of up to 78°C and facilitate in treating biosolids (Jiménez, 2007; Al-Gheethi, et al., 2018). Anaerobic digestion is considered to result in a high pathogen inactivation and to reduce organic matter by 20-30% and sludge mass by 40-80% to produce stable biosolids (Jiménez,

2007). However, it is an expensive and complicated process to operate; but produces methane that can be used as an energy power source (Jiménez, 2007).

After anaerobic digestion, FS becomes easier to dewater and is more stabilized (Jiménez, 2007). Brazil developed and demonstrated thermal drying of anaerobically digested FS, a process which uses methane produced from anaerobic digestion to heat and dry the digested sludge. A simulation of *A. lumbricoides* reduction using *A. suum* showed that anaerobic digestion can reduce viability by 35% at 35°C after 16 days while 95% reduction was achieved after 24 days (Manser et al., 2015). Thermophilic anaerobic sludge removed eggs down to 0.28 HO eggs/g TS in 20 days (Cabirol et al., 2002).

2.7.1.3 Composting

As mentioned in Tiley et al (2014), composting is storage of human excreta or FS for 2-4 months that can reach thermophilic temperatures of 55-60°C. It is further a biological degradation process by microorganisms that breaks down organic matter to earth-like brown material. The high temperature reduces microbial content to acceptable levels, allowing the compost to be safely used for agricultural purposes. Compost has soil conditioning properties and nutrients, mainly NPK. However, achieving high temperatures when composting is sometimes difficult, and if not achieved can lead to poor reduction of pathogenic microorganisms from human excreta, making the use of the compost unsafe (Tilley et al., 2014). Composting requires land for long-term treatment (Tilley et al., 2014; Mawioo et al., 2016b). Composting is one of the most applied methods to inactivate pathogens from human excreta and FS (Hawksworth et al., 2010; Naidoo and Foutch, 2017). Appiah-Effah et al. (2018), demonstrated an 80% inactivation of *Ascaris* eggs by composting FS in a rotatory drum for 84 days at up to 62°C (Appiah-Effah et al., 2018).

2.7.1.4 Ammonium inactivation

Ammonium inactivation is an alkaline stabilization process that uses uncharged ammonia to inactivate and kill pathogens; it is easy to operate and is applicable in large and small WWTP and onsite dry sanitation technologies (Jiménez, 2007). Uncharged ammonia has been shown to inactivate different organisms except for *Clostridium* spp, viruses, protozoa and STHs (Espinoza et al., 2012; Fidjeland et al., 2015). Ammonia as a gas has the advantage of penetrating the pocket matrices in treated material such as FS allowing it to affect more surface area of the material (Espinoza et al., 2012). Uncharged ammonia passes through the cell membrane of pathogens, increases pH levels inside the cell, and interrupts metabolic pathways (Fidjeland et al., 2015). Stored urine is converted to ammonia and bicarbonate which increases alkalinity and reaches pH levels above 9, which is detrimental to pathogens that can be reduced to safe levels after less than six months of exposure to stored urine (Tilley et al., 2014).

Ammonium inactivation does not stabilize organic compounds, which results in foul odour and will support the re-growth of bacteria after treatment (Jiménez, 2007). Additives such as lime or the use of temperature and land requirements also make the treatment procedure costly and unsustainable, as well as increasing the FS volume by 20-40% (Jiménez, 2007; Fidjeland et al., 2015) . Ammonia inactivation on its own is inadequate and requires being augmented with urea, lime (calcium hydroxide) or heating (temperature or composting) (Espinoza et al., 2012; Fidjeland et al., 2015).

Lime can be added to dewater FS and to increase pH levels to above 12 (Jiménez, 2007; Fidjeland, 2015). The addition of lime increases pH and converts ammonium (NH_4^+) into uncharged ammonia (NH_3) (Méndez-Contreras et al., 2002; Fidjeland et al., 2015). Espinoza et al. (2012), showed that 1% and 2 % w/w urea as an ammonium agent mixed with dry FS (30% moisture content) required 14 days, 3 days and 1 day to achieve 100% *Ascaris* spp eggs inactivation at 28°C, 35°C and 40°C respectively (Espinoza et al., 2012).

Espinoza et al. (2012) also showed that sterilization of FS can be achieved with addition of urea to FS, and the work is summarised below. Samples with a moisture content (MC) less than 28 % can be sterilized free of *Ascaris* spp eggs after 14 days, 3 days and 1 day at 28°C, 35-40°C and 45°C respectively mixed with 1% or 2% urea. Samples with a minimum concentration of 1350 NH_3 mg/L at 28-35°C and 750 NH_3 mg/L at 40°C achieved 100% *Ascaris* spp inactivation. Pecson et al. (2007) established that 2992 NH_3 mg/L can achieve 99% inactivation after 72 hours at 38°C. This is in agreement with Nordin et al., (2012) that showed that sterilization can be achieved after exposure to 1% urea after 10 days at 35°C, and 2% urea achieved inactivation after 4 days at 40°C.

2.7.2 Unconventional treatment methods and technologies

Innovative technologies are being developed and tested that can reduce or inactivate pathogens to safe levels (Naidoo and Foutch, 2017). Alternative treatment systems include microwave irradiation (MWI), acid treatment, dry sanitation treatment technologies and enzymatic digestion (Butkus et al., 2011; Mawioo et al., 2016a, b; Naidoo and Foutch, 2017). These include technologies such as the viscous heater and solar drying toilets (Butkus et al., 2011; Naidoo and Foutch, 2017; Mawioo et al., 2016a, b). New technologies that will offer new treatment methods and that can be coupled to conventional wastewater treatment plants or treat human waste independently is of importance and reduces the demand of conventional WWTP especially in rural areas where WWTP cannot be built (Harroff et al., 2017).

2.7.2.1 Microwave irradiation

Mawioo et al. (2016a, b) discusses Microwave irradiation (MWI) technology in detail with the following summary from the publications. Microwave irradiation (MWI) technology uses microwave energy (MW_e) with wavelengths between 1 mm and 1 m and frequencies between 30 MHz and 300 GHz to target and heat different materials through molecular motion. Heated

material results in disrupting the hydrogen bonds of the microbial cells, leading to the breakage and denaturing of the microbial cells. The dielectric constant is heat that is retarded on the surface of the material and is not absorbed whereas the dielectric loss factor is equivalent to the energy that is absorbed by the material as heat, the latter is more susceptible to MWI technology.

MWI efficiency has been shown by Hong et al. (2004, 2006) against bacterial microorganisms. Another study by Mawioo et al. 2016a tested MWI against helminth eggs. Mawioo et al. (2016a) demonstrated the ability of the MWI technology using 100 g and 200 g of black water FS. For 100 g FS exposed to MW at 1085 W and 1550 W in less than 3 minutes achieved 3 log reduction value (LRV) of viable STHs eggs at 78 °C and 83°C. For 200 g FS exposed to MW at 1085 W and 1550 W took longer than 3 minutes in order to produce FS with a 3 LRV of viable STHs eggs at 81 °C and 87°C. Mawioo et al., (2016a) removed above 90% of all bacteria and achieved 70% volume reduction by generating temperatures above 65°C using black water sludge. MWI has a high energy requirement compared to methods like composting and anaerobic digestion. This shows the potential that MWI technology possesses a rapid heating and treatment technology in a small-scale setting, however, it is energy intensive.

2.7.2.2 Acid treatment

Acid treatment using short chain fatty acids (SCFA) and carboxylic acids can be used to inactivate *Ascaris* spp eggs (Butkus et al., 2011; Harroff et al., 2017). SCFA have been reported to be toxic to bacteria, viruses, fungi, insects, nematodes and birds (Butkus et al., 2011; Harroff et al., 2017). Harroff et al. (2017) showed that under controlled conditions, below pH 4.8, carboxylic acids exist as uncharged hydrophobic molecules; this allows them to be able to cross the eggshell of nematodes. Uncharged 200 mM carboxylic acid inactivated *Ascaris* spp eggs at pH 2, 4 and 5, 37°C after 5 days exposure. Low concentrations of 30 mM hexanoic acid inactivated *Ascaris* spp eggs after 2 days exposure and 20 days for 10 mM hexanoic acid.

Furthermore, high concentrations of 500 mM uncharged butyric acid inactivated *Ascaris* spp eggs within 2 days, and 20 days for 100 mM uncharged butyric acid (Harroff et al., 2017). Work by Jiménez (2007) showed that a concentration of 500 ppm paracetic acid is required to inactivate helminth eggs in FS in 10 days in order to achieve a 2-3 log reduction. The advantage of acid treatment is that it does not increase sludge volume (Jiménez, 2007). However, this is expensive due to the requirements of the long chain fatty acid (Harroff et al., 2017).

2.7.2.3 Viscous heater

A viscous heater (VH) is a technology that generates high temperatures for the reduction of pathogens in FS. A VH uses shear stress between two cylinders with a stationary outer shell and a rotating inner shell that is separated by a millimetre gap (Naidoo and Foutch, 2017). The rotation causes shear stress that generates heat as FS is passed through, in the gap between the inner shell and outer shell. The shear stress can cause temperatures to rise and reaches a temperature of up to 95°C. The heat has the capacity to reduce bacterial load and inactivate *Ascaris* spp eggs, treatment of FS at 80°C for 4 seconds resulted in <1% viable HO eggs (Naidoo and Foutch, 2017).

2.8 Potential of lytic enzymes in faecal sludge

Lytic enzymes, like proteases, chitinase and lysozyme are increasingly being used as microbial controlling agents (MCA) to replace pesticides which are known to be harmful to the environment (Singh et al., 2013; Hasan and Anand, 2014; Zhang et al., 2017). For instance, the use of chemical pesticides has led to the development of pathogens resistant to conventional pesticides (Dong and Zhang, 2006; Yang et al., 2007; Gortari and Hours, 2008; Hasan and Anand, 2014; Zhang et al., 2017). These enzymes can potentially act as MCA whose mechanisms can include the production of antibiotics active against plant parasites and human pathogens (Dong and Zhang, 2006; Veliz et al., 2017; Karn and Kumar, 2019).

It is postulated that enzymatic inactivation could possibly be an environmentally friendly, sustainable and direct approach in the inactivation of *Ascaris* spp eggs compared to other inactivation methods that are currently being employed (Schönning et al., 2007; Okem et al., 2013). This will provide a FS management (FSM) system that will produce a safe soil enhancer. Several microorganisms naturally produce these types of lytic enzymes in order to break down cell walls of other microorganisms as well as, plants and insects (Salazar and Asenjo, 2007). However, the efficiency of the lytic enzymes in inactivating organisms are limited by abiotic factors (Gortari and Hours, 2008). Little is known about how to apply the already naturally occurring lytic enzymes for sustainable bioprocesses as efficient bio-controlling agents (Yan and Fong, 2015). Discovery of lytic enzymes that can work under stressful conditions caused by abiotic factors for degradation of polymers will be essential (Yan and Fong, 2015).

Enzymes are biocatalysts that speeds up the rate of reactions. These reactions would otherwise remain inactive without the speed, specificity and efficiency that resides within the enzyme's active site (Oyeleye and Normi, 2018; Chakraborty and Karmakar, 2020). The most commonly used lytic enzymes are bacteriolytic enzymes for biotechnological, industrial and medical purposes and whose production is enhanced by using recombinant proteins. Bacteriolytic enzymes are used to break down microorganisms for medical or antibacterial purposes (Salazar and Asenjo, 2007).

Proteases break protein peptide bonds to smaller peptides or amino acids, and are classified as intracellular or extracellular (Lebrun et al., 2009; Patil et al., 2015; Abrar, 2017). Intracellular proteases (endoproteases) are important for physiological functions that guide metabolic processes and establish infections while extracellular proteases (exoproteases) are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb nutrients (Hamza and Woldesenbet, 2017). *Bacillus* spp are a group of predominant producers of proteases and have shown significant stability at high pH and temperature (Singh, 2015, Patil

et al., 2015). Fungal proteases are active over a wide pH range of 4 to 11, and have a wide range of substrate per enzyme (Abrar, 2017). Both groups of organisms are abundant in soils and sludge but fungal protease's activity is low and not as stable and tolerant as bacterial proteases (Abrar, 2017).

Bacterial protease producers are mainly *Staphylococcus*, *Bacillus, Alcaligenes faecalis*, *Pseudomonas fluorescens*, *Helomonas*, *Arthrobacter* and many more (Rehbar and Batool, 2017; Alam et al., 2017; Chakraborty and Karmakar, 2020). Luang-In et al. (2019) identified nineteen bacterial protease producers from soil, which include *Bacillus*, *Enterobacter* and *Staphylococcus* spp. Palsaniya et al. (2017) enumerated *Pseudomonas fluorescens, Bacillus subtilus, E. coli and Serratia marscens* which produce proteases from soil samples, and identified *B. subtilus* to have the highest enzymatic activity. The *Bacillus* spp are major producers of extracellular proteases and are known to secrete large amounts of protease which is used for commercial purposes (Aramesh and Ajoudanifar, 2017; Palsaniya et al., 2017; Chakraborty and Karmakar, 2020). Bacterial proteases are advantageous due to their proteolytic activity, broad substrate specificity, rapid proliferation and stability, rapid expression rate, small space requirements for cultivation, simplified purification, genetic manipulation and wide range application (Dong and Zhang, 2006; Abrar, 2017; Alam et al., 2017; Palsaniya et al., 2017; Rehbar and Batool, 2017; Luang-In et al., 2019).

Chitinase is a hydrolytic enzyme that can breakdown structural cell walls which contain chitin (Singh et al., 2013, Oyeleye and Normi, 2018). Chitin is the second most abundant polymer after cellulose (Oyeleye and Normi, 2018; Veliz et al., 2017). Chitin is a polysaccharide, composed of a linear chain of N-acetyl-D-glucosamine (NAG) units that are linked to each by a β-(1-4) glycosidic bond within the cell wall of many different organisms except plants and vertebrates (Deeba et al., 2016; Veliz et al., 2017). Chitinase are glycosidases which hydrolyse the β-(1-4) glycosidic bonds between the NAG residues (Veliz et al., 2017). Chitinases are

classified as endochitinase and exochitinase (chitobiosidases and β-(1-4)-N-acetylglucosaminidase) (Hasan and Anand, 2014; Deeba et al., 2016; Veliz et al., 2017). Endochitinase cleaves internal chitin bonds to produce chitotetraose and chitotriose whilst exochitinase cleaves the non-reducing end of chitin to produce diacetylchitobioses. These products are further broken down into N-acetylglucosamine by β-(1-4)-N-acetylglucosaminidase (Hasan and Anand, 2014; Deeba et al., 2016; Veliz et al., 2017). Chitinase can potentially act as a biological control agent that can breakdown the cuticle and membrane of insects, fungal cell walls and the egg shell of nematodes (Gortari and Hours, 2008).

Primary chitinolytic bacteria are *Actinomycetes* (Veliz et al., 2017). *Actinomycetes* are antibiotic producers that can control soil borne pathogens; the most antagonistic species are the saprophytic *Streptomyces* spp (Dong and Zhang, 2006). *Streptomyces* spp are antagonistic against plant nematode eggs; *Streptomyces avermiitilus* produce anthelmintic abamectin and avermictins, which are macrocyclic lactones (Dong and Zhang, 2006; Han et al., 2008).

Other bacterial chitinase producers include *Serratia, Vibrio, Bacillus, Chromobacterium*, *Agrobacterium* and *Yersinia* species (Dong and Zhang, 2006; Han et al., 2008; Oyeleye and Normi, 2018). Fungal chitinase are important for accessing nutrients, morphogenesis (to break down their cell wall during cell division), competition, defence (against fungal disease) or parasitism (Deeba et al., 2016; Oyeleye and Normi, 2018). Fungal antagonistic enzymes can inhibit the growth of fungi, insects, plants and nematodes for mycoparasitic purposes (Gortari and Hours, 2008; Deeba et al., 2016) Chitinase from *Trichoderma, Penicillium* and *Aspergillus* species are the most characterised chitinase (Deeba et al., 2016; Oyeleye and Normi, 2018).

Lysozyme is a glycoside hydrolase enzyme that is also known as muramidase or N-acetylmuramide glycanhydrolase that is made up of 129 amino acids (Xue et al., 2004; Ercan and Demirci, 2016). The natural substrate for lysozyme as a glycosyl hydrolase is

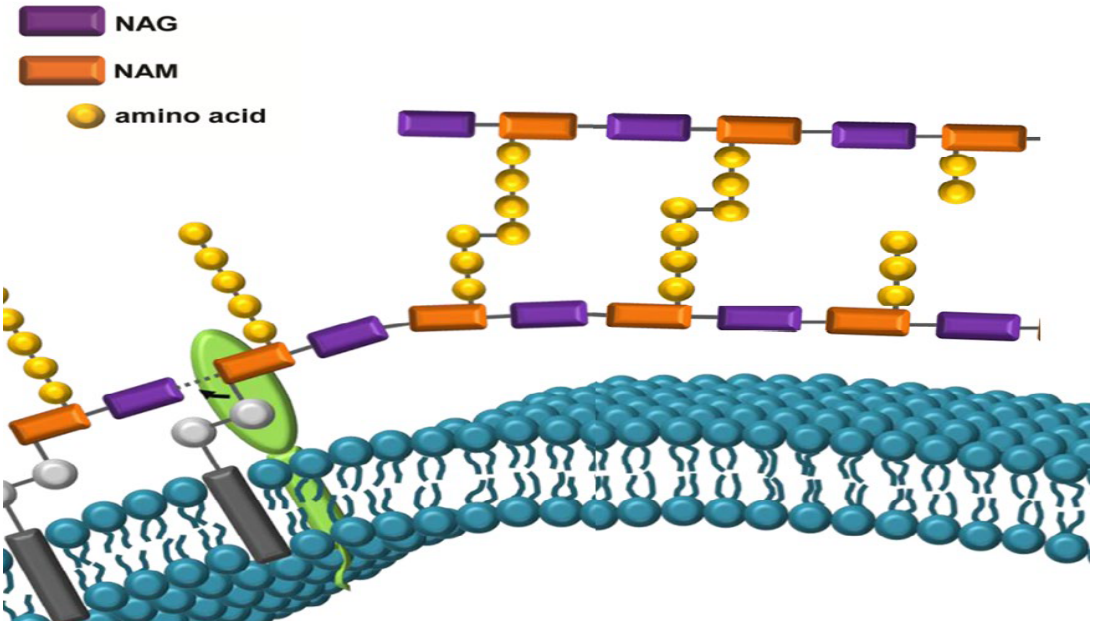

Figure 2-4: Structure of peptidoglycan showing the NAG and NAM linking to the phospholipid bilayer (Ragland and Criss, 2017).

peptidoglycan (murein), the bacterial cell wall (Figure 2.4) (Xue et al., 2004; Ercan and Demirci, 2016). Peptidoglycan is a polymer made up of β-(1-4) linkages that join alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid of bacterial cell walls (NAM) (Ragland and Criss, 2017; Veliz et al., 2017). Lysozyme (muramidases) functions in breaking down murein through hydrolysis of the β-(1-4) glycosidic linkages between the N-acetylglucosamine (NAG) and the N-acetylmuramic acid (NAM) adjacent to each other (Ragland and Criss, 2017).

Lysozyme has been reported in *Arthrobacter crystallopoites, B. subtilis, B. thuringensis, Clostridium acetobutyricum, Enterococcus hirae, Lactobacillus acidophilus, Pseudomonas aeruginosa, Streptomyces coelicolor, Streptomyces erythraeus, Streptomyces globisporus* and *Streptomyces griseus* (Jollès, 1996). Hawiger (1968) showed that lysozyme produced by *Staphylococcus aereus* was capable of lysing *Micrococcus lysodeikticus*. Bacterial cells with peptidoglycan are known to produce a variation of lysozyme (Jollès, 1996; Bilej, 2015). It is not clear if bacterial cells that produce lysozyme are harmed by the lysozyme or if it only

contributes to physiological functions: metabolism, cell growth and enlargement of the cell (Jollès, 1996).

2.8.1 Application of lytic enzymes

Lytic enzymes currently have a variety of industrial uses and functions, such as bacteriostasis, antivirus, cosmetics and immunity enhancement (Dai and Li, 2021). The current use and research of proteases, chitinases and lysozyme are outlined in Veliz et al. (2017), Hashemi et al. (2018), Razzaq et al. (2019), Pangcong and Liu et al. (2021). The information of the above-mentioned enzymes is summarised below.

Razzaq et al. (2019) outlines that proteases account for more than 30% of the enzyme market usage with applications in the food, detergent and medical fields. The genus *Bacillus* spp is vital for commercially important alkaline protease active at alkaline pH ranging between 9 and 11. In the food industry, proteases are used for their high nutritional value preparations of protein hydrolysates using microbial alkaline proteases, and storage life of all available sources of proteins. Proteases are incorporated into pharmaceutical drug formulations and as potential molecules under stress environmental conditions. The essential component or ingredient for detergents includes proteases and have been used for cleaning of household laundry, dentures, or contact lenses.

According to Veliz et al. (2017), *Actinobacteria* are known for the synthesis of chitinolytic enzymes, antibiotics and secondary metabolite production. *Streptomyces rimosus* isolated from agricultural soil in the centre of Poland was found to use chitosan concentrated in shrimp shells as a nutrient source. *Actinomycetes* isolates from soil samples in Jordan exhibited *in vitro* fungicidal activity against mycelial growth and sclerotia formation of *Sclerotinia sclerotiorum*, when they synthesized chitinase. *Streptomyces roseolus* showed inhibitory effect on fungal hyphal of *Rhizoctonia solani* responsible for sugar beet damping-off disease. Soil with

Streptomyces roseolus prevented sugar beet damping-off completely, and significantly improved seedling growth in both infected and uninfected conditions.

Paenibacillus illinoisensis isolated from coastal soil in Korea was reported to have strong in vitro chitinolytic activity when assayed on colloidal chitin. It also deformed and destroyed the eggshell of the root-knot nematode (*Meloidogyne incognita*). *Paenibacillus* spp that produces high amounts of chitinases could be used to control *Helicoverpa armigera*. The use of this strain resulted in a 40% mortality of the larvae, and is stable when mixed with a pesticide. *Serratia marcescens* endochitinase and chitobiase has been shown to inhibited *Botrytis cinerea* conidiospores and distorted germ tube development of the fungus. *S. marcescens* can also control the damping-off disease of cucumber, which is caused by *Phytophthora capsici*. *Bacillus* spp that produce chitinases have been used as postharvest biocontrol agents. *Bacillus subtilis* isolates with chitinase activity yielded up to 83% inhibition of *Fusarium oxysporum* and *Botryodiplodia theobromae* infection in yam after 36 hours.

Lysozyme can be used as a preservative for food and alcohol, and has been and is receiving considerable attention in the research field (Hashemi et al., 2018). Li et al. (2020) states that the addition of lysozyme to wine, beer and other alcoholic beverages can not only inhibit the growth and proliferation of acidogenic bacteria, but also has no effect on the flavour of wine, and is less affected by alcohol clarifier. Lysozyme has been shown to effectively inhibit or kill microorganisms on the surface of fruits and vegetables, and maintain its original flavour and nutritional value, which has a good preservation effect. Lysozyme combined with ice-water mixture could prolong the shelf life of spinach, effectively maintain its sensory quality, and slow down the formation of nitrite and oxalic acid in spinach.

Ding and Zhang (2019) studied that lysozyme enteric-coated tablets combined with Kouyanqing grains in the treatment of recurrent oral ulcer could significantly improve clinical symptoms, but also improve immune ability, shorten the course of disease, which had good

clinical application value. Lysozyme can be used as a bactericidal agent that is used as a food preservative when modified with hydrophobic peptides or an emulsifier when mixed with gum arabic. Gum Arabic is an anionic arabinogalactan-protein polysaccharide used in food and pharmaceutical industries, due to its unique emulsification, film forming, and encapsulation properties. However, because of its effect on the cell wall of the Gram-positive bacteria but not on the Gram-negatives, the industrial application of lysozyme is limited.

2.8.2 *Ascaris* spp inactivation by lytic enzymes

The properties of the lytic enzymes show that they have the ability to break down cell walls of bacteria, and some have shown this in other nematodes other than *Ascaris* spp. The potential of lytic enzymes in combination is intriguing, given that their diverse individual applications in detergents, emulsifiers, and other commercial products have demonstrated the ability to selectively target specific layers of the Ascaris spp egg shell. Of particular interest in this study are the lytic enzymes; protease, chitinase and lysozyme (Veliz et al., 2017). STHs eggs can be potentially targeted using MCA to suppress nematode production or egg hatching (Dong and Zhang, 2006). There is a lack of efficient FS treatment methods and real solution treatment technologies that can inactivate *Ascaris* spp eggs and reduce their viability to acceptable levels (Mawioo et al., 2016b). Solutions are needed to turn human waste into treated reusable safe products; the inactivation of pathogens is a hindrance for this process (Mawioo et al., 2016b). There is an increasing demand for developing and implementing self-sustaining and advance technologies in developing countries (Méndez-Contreras et al., 2002; Espinoza et al., 2012). The new technology should be able to merge into already existing conventional technologies in urban areas, reduce water consumption, reduce sludge production, save freshwater resources, recycle water and nutrients, and have the potential for producing safe biosolids (Méndez-Contreras et al., 2002; Espinoza et al., 2012; Fidjeland et al., 2015; Karn and Kumar, 2019).

3 CHAPTER 3: RECOVERY OF *ASCARIS* EGGS AND ENZYME INACTIVATION WITH COMMERCIAL ENZYMES

This chapter is primarily focused on the inactivation of *Ascaris* eggs using both single lytic enzymes and a combination of inactivation process such as exposure of the lytic enzymes individually in a consecutive manner or mixed together. Furthermore, to highlight the problem with *Ascaris* egg occurrence and persistence in partially treated faecal sludge from Durban, where an initial recovery from sludge was performed. The numbers reported here suggests that there is a higher occurrence of STH infections in communities.

3.1 Methodology

3.1.1 Urine diversion dehydration toilet faecal sludge sampling

Urine diversion dehydration toilet faecal sludge (UDDT FS) was obtained from a research facility at a wastewater treatment plant in Durban, Isipingo (-29.98266792731432, 30.90521497940144). UDDT pits were emptied by the eThekwini municipality as part of the maintenance of the UDDT toilets, and the FS was then taken to the research facility where it was kept in drying beds. Different samples from different pits were mixed together to make a composite sample and transferred into a 1 L container. The sample in the 1 L container was transported back to the laboratory in a cooler box with ice bricks and stored in a temperature-controlled cold-room at 4°C until analysis. The sampling was carried out over three (3) months (May, June, July), each sample was tested in triplicate, (n=9).

3.1.2 Recovery of *Ascaris* spp eggs from faecal sludge

Ascaris spp eggs were recovered from UDDT FS by weighing 20 g of FS sample into a beaker. Gradually, 0.1% Tween 80 (1 mL/999 mL of distilled water) (Sigma Aldrich, Germany) was added to the beaker up to the 50 mL mark and mixed with the weighed sample for 10 minutes,

at 1500 revs per minute (rpm), at room temperature. The mixture was poured unto a 100 µm sieve stacked on top of a 20 µm sieve and washed using tap water to prevent clogging of the sieve mash. The 100 µm sieve mesh content was discarded and the 20 µm sieve mesh content was divided into test tubes and centrifuged (Remi-Neya 8, China) at 10 000 rpm for 3 minutes. After centrifugation, the supernatant was discarded and the pellet was gradually mixed with 1.3 specific gravity (SG) zinc sulphate ($ZnSO_4$) (500 g/800 mL distilled water) (Radchem products, USA) in 50 mL centrifugation tubes with intermittent shaking. The $ZnSO_4$ mixture was centrifuged at 10 000 rpm for 2 minutes. After centrifugation, the supernatant was transferred on to a 100 µm sieve stacked on top of a 20 µm sieve and washed for a second time using tap water. The 20 µm sieve content was separated into test tubes and centrifuged at 10000 rpm for 3 minutes. After centrifugation, the supernatant was discarded, and the pellets in the tubes were diluted with distilled water up to the 50 mL mark and left to stand and settle overnight. The supernatant was discarded, and the remaining pellet was mixed with an addition of at least 4 mL of 0.1 N sulphuric acid (H_2SO_4) (1.4 mL/500 mL) to a final volume of 5 mL and kept in the cold room at 2-4°C until further processing. All samples were analysed in triplicates. See Annexure A, section 3.1.1-3.1.3 for complete list and preparations of all chemicals.

3.1.3 Assessment of viability of recovered *Ascaris* spp eggs

For an initial count, aliquots of 1 mL from the 5 mL tube were transferred on to micro-slides using pasteur pipettes and sealed with a cover slip. The 1 mL was microscopically examined under the light microscope DM 2000 (Leica microsystems, Germany) in drops of 2-4 until the whole 1 mL aliquot was completed. The different stages of the eggs were here identified and counted. The eggs are classified into the categories of dead, infertile, undeveloped, developing, immotile and motile (Figure 3.1). Aliquots of 1 mL of the eggs were incubated at 26°C for 30 days to determine the viability of the recovered eggs.

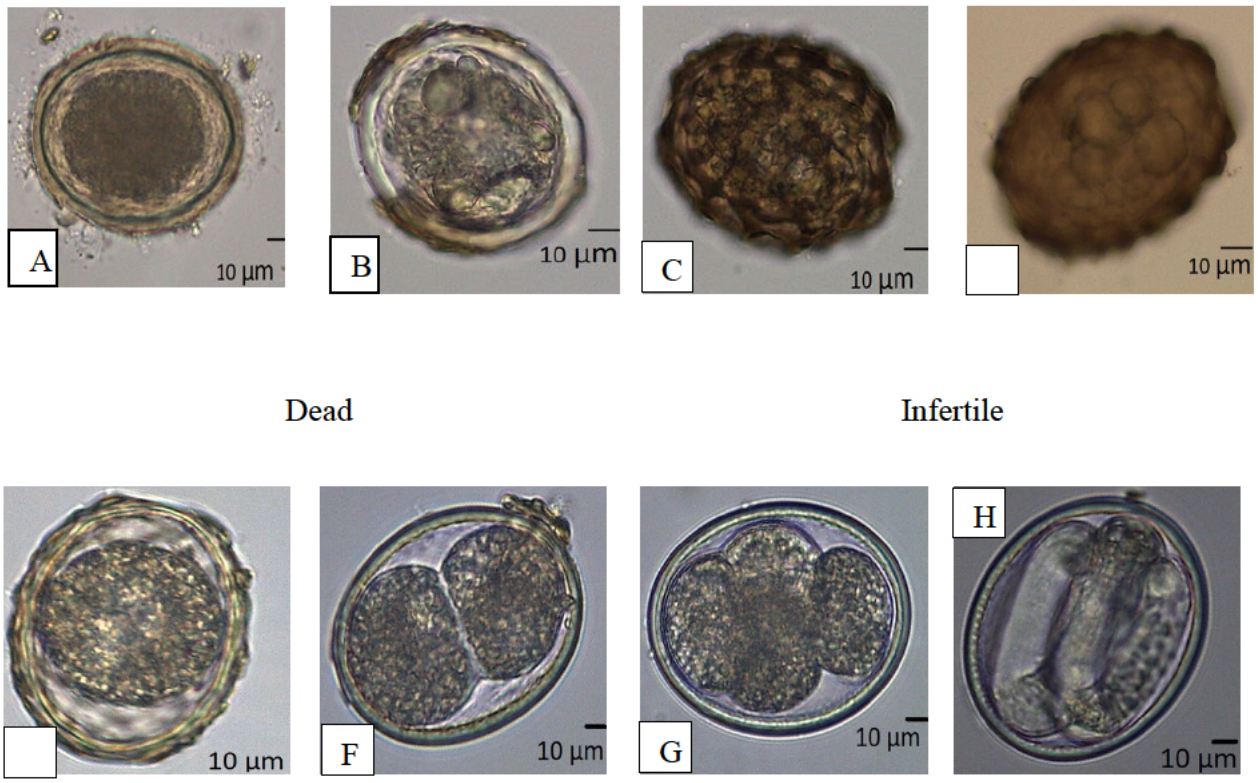

Figure 3-1: Developing stages of *Ascaris spp* eggs used for viability assessment including those in deceased (A, B), infertile (C, D), undeveloped (E, F), developing (F, G) and motile/immotile (G, H) stages (x100) (Source: Thabiso Zikalala, photos taken using Leica microscope LEICA LAS EZ v3.0).

After the 30 days of incubation at 26°C, viability was assessed by observing egg morphology. Dead eggs have a globular content, undeveloped eggs have a single stage embryo, developing eggs have a uniformly dividing embryo, immotile larva contains a clear fully developed larva, motile eggs have a moving larva and a potentially dying egg has an irregular non-motile deteriorating larva that has globules inside. All experiments were carried out in triplicate.

3.1.4 Assessment of enzyme inactivation of *Ascaris* spp eggs

3.1.4.1 Egg stock viability count and preparation

Due to the low concentration of viable *Ascaris* spp eggs recovered from FS, *Ascaris suum* eggs were purchased from Excelsior Sentinel Inc., (United States of America) for the inactivation experiments, Chapter 3, section 3.1.5.1 – 3.1.5.3. The eggs were kept and classified following the categories outlined in Chapter 3, section 3.1.3, Figure 3.1. After the classification count, a 1:2 dilution of the 50 mL of the stock egg solution was prepared using distilled water in order to get a countable number of eggs per millilitre. Aliquots of 1 mL of the eggs were incubated at 26°C for 30 days to determine the viability of the diluted egg stock solutions and viewed under the microscope for assessment as stated in section 3.1.3.

3.1.5 *Ascaris suum* eggs exposure to lytic enzymes

Determination of the impact of lytic enzymes on *A. suum* spp eggs was performed through three procedures;

(1) exposure to the individual enzymes

(2) consecutive exposure to the individual enzyme

(3) combined mixed exposure to the three enzymes.

These exposures were done at room temperature (RT) and also at 37°C (in a 34 L water bath (Model 132) (Vactech Instrulabs, South Africa)). Due to anticipated changes in viability over time, the eggs were kept at the respective temperature without exposure to enzyme as a control.

3.1.5.1 Exposure to individual lytic enzyme

For the exposure of *A. suum* eggs to individual lytic enzymes at RT, aliquots of 1 mL from the stock solution was transferred to 15 mL tubes, rinsed by centrifugation (REMI Lab World, India) at 3000 rpm for 10 minutes. Tubes were individually mixed with 1.0 mg/mL proteinase

(Sigma Aldrich, Germany), 1.0 mg/mL chitinase (Sigma Aldrich, Germany) and 1.0 mg/mL lysozyme (Amresco, United States of America) each for 1 day, 3 days and 5 days (n=9). The mass, concentration and activity of the enzymes was determined as stated in the Annexure, section 3.1.4, Table 3.1. *A. suum* spp eggs in distilled water were used as negative controls, where three sets of test tubes were incubated at room temperature for 1 day, 3 days and 5 days respectively (n=9). All experiments were carried out in triplicates.

3.1.5.2 Consecutive exposure of lytic enzymes in series

Exposure of *A. suum* eggs to individual lytic enzymes consecutively was performed by systematically exposing the same set of *A. suum* eggs to each individual enzymes. In brief, 1 mg/mL proteinase was mixed with the eggs and kept for 1 day. Thereafter the eggs were centrifuged at 3000 rpm for 10 minutes to remove the protease . The resulting pellet was then mixed with 1 mg/mL chitinase for a day and the eggs were similarly rinsed to remove chitinase by centrifugation at 3000 rpm for 10 minutes. Finally, the resulting pellet was then mixed with 1 mg/mL lysozyme, and the eggs were rinsed free of the lysozyme by centrifugation at 3000 rpm for 10 minutes. This experimental setup was repeated but with exposure to each enzyme for 3 or 5 days respectively in each step, with all experiments done in triplicates. *A.suum* spp eggs in distilled water were used as negative controls, with three sets of test tubes incubated at room temperature for 1 day, 3 days and 5 days (n=9).

3.1.5.3 Mixed lytic enzyme exposure

Exposure of the *A. suum* eggs to the mixed enzymes was made by mixing proteinase, chitinase and lysozyme, each at a concentration of 1.0 mg/mL. Exposure of the eggs to the mixed enzymes was set for 1 and 5 days. After each time interval, the eggs were rinsed free of the enzyme mixture by centrifugation at 3 000 rpm for 10 minutes. The pellet was re-suspended in 0,05 M sulphuric acid (H_2SO_4) (Sigma Aldrich Chemicals (Merck), Germany) and incubated for 30 days at 25°C along the controls as stated in section 3.1.5.1 and 3.1.5.2.

All *A. suum* eggs exposures to the lytic enzymes was carried out in triplicates. *A. suum* eggs in distilled water were used as negative controls with three sets of test tubes incubated at room temperature for 1 day, 3 days and 5 days (n=9). The same procedure was followed for the exposure of *A. suum* eggs to lytic enzymes at 37°C where the sample tubes were kept in a water bath (Vactech Instrulabs, South Africa).

After each incubation period for the test samples and the control, the set of tubes were centrifuged to rinse free the eggs of the lytic enzymes, to get a pellet. The pellet was re-suspended in 0.05 M sulphuric acid, and the tubes were incubated at 26 °C for 30 days inside an incubator. After 30 days, to determine the viability of the eggs after exposure to lytic enzymes, the eggs were viewed under the microscope for assessment as stated above, section 3.1.3.

3.1.5.4 Statistical analysis

Data was captured in Microsoft Excel (Microsoft Corporation, USA) and graphs plotted. Comparison of *Ascaris* spp egg concentrations over the three-month sampling period was performed using the Kruskal-Wallis Test, with Dunn's Multiple Comparison. The same statistical tests were used to ascertain the impact of exposure time in days for each of the lytic enzymes. Statistically significant differences in the concentration of viable eggs after exposure to the enzymes under the various conditions was determined using the Mann-Whitney Tests.

Prior to the statistical analysis, the percentage of eggs that remained viable after exposure to the enzymes was subtracted from the percentage viability determined in the control. For each experimental setup, such as single enzyme exposure, single consecutive enzyme exposure and mixed enzyme exposure, control experiments were ran in conjunction with the experiments where the eggs were not exposed to any enzymes.

3.2 Results

3.2.1 Concentration of total and viable *Ascaris* spp eggs recovered from faecal sludge

Ascaris spp eggs were detected in all FS samples (n=9) analysed, with varying concentrations between 976 and 1118 HO eggs/g over the three-month sampling period. Although the concentrations appear to be different (Figure 3.2) this was not statistical verified (Kruskal-Wallis Test). No significant difference thus appeared over the three month period (p value ≥ 0.05). Dunn's multiple comparison test also did not identify any statistically significant differences. Over the three months sampling period, the viable *Ascaris* spp egg numbers, confirmed via incubation, ranged between 107 and 123 HO eggs/g (Figure 3.2). This corresponded to a viability percentage of 11%. Similar to the total *Ascaris* spp recovered, there was no statistically significant difference in the concentration of viable eggs recovered (p value ≥ 0.05).

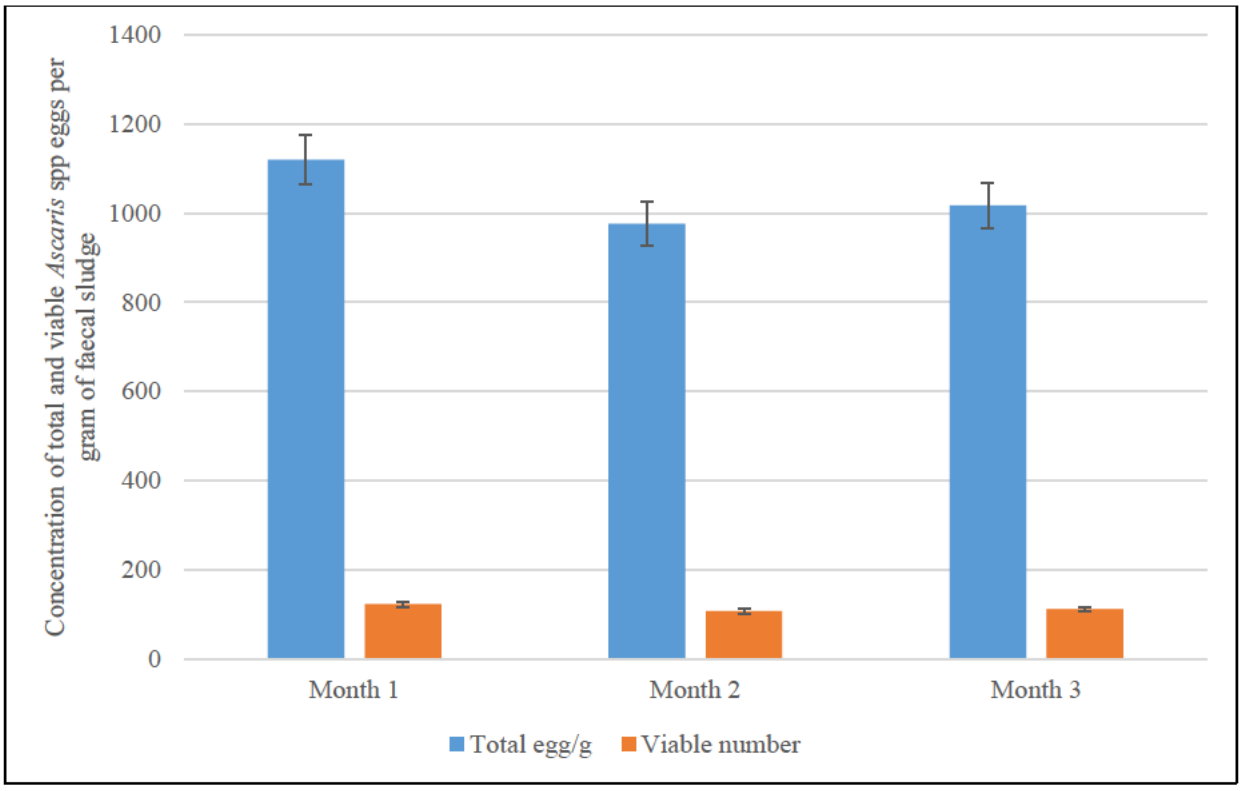

Figure 3-2: Total and viable *Ascaris spp* eggs in faecal sludge samples over a three-month period. Error bars, n=9.

3.2.2 Enzyme inactivation of *Ascaris* spp eggs

3.2.2.1 Viability assessment of commercial *Ascaris suum* eggs

The purchased (Excelsior Sentinel Inc., U S A) egg stock concentration was estimated to be 1300 HO eggs/mL in the50 mL egg stock solution, which sums up to 65 000 HO eggs/50 mL tube. The average viability of the bought egg stock was calculated to 52.1%, thus significantly higher than what was recovered from the faecal sludge. These purchased eggs were used in the experiments below in 3.2.2.2 - 3.2.2.4.

3.2.2.2 Single lytic enzyme exposure

The exposure to protease, chitinase and lysozyme was more effective at reduced viability of the *A. suum* eggs after 1 day of exposure. The eggs had the lowest viability of 35.0%, 34.9% and 40.8% after exposure to protease, chitinase and lysozyme respectively. After the experiment, the control setup for these experiment gave a final viability of 40.8-45.9% which is lower compared to the viability assessment performed for the stock egg solution (52.1%). The statistical analysis, conducted using the Kruskal-Wallis Test, revealed that the concentration of *A. suum* eggs that remained viable after exposure to the enzymes individually was significant, as indicated by a p-value of less than 0.05.

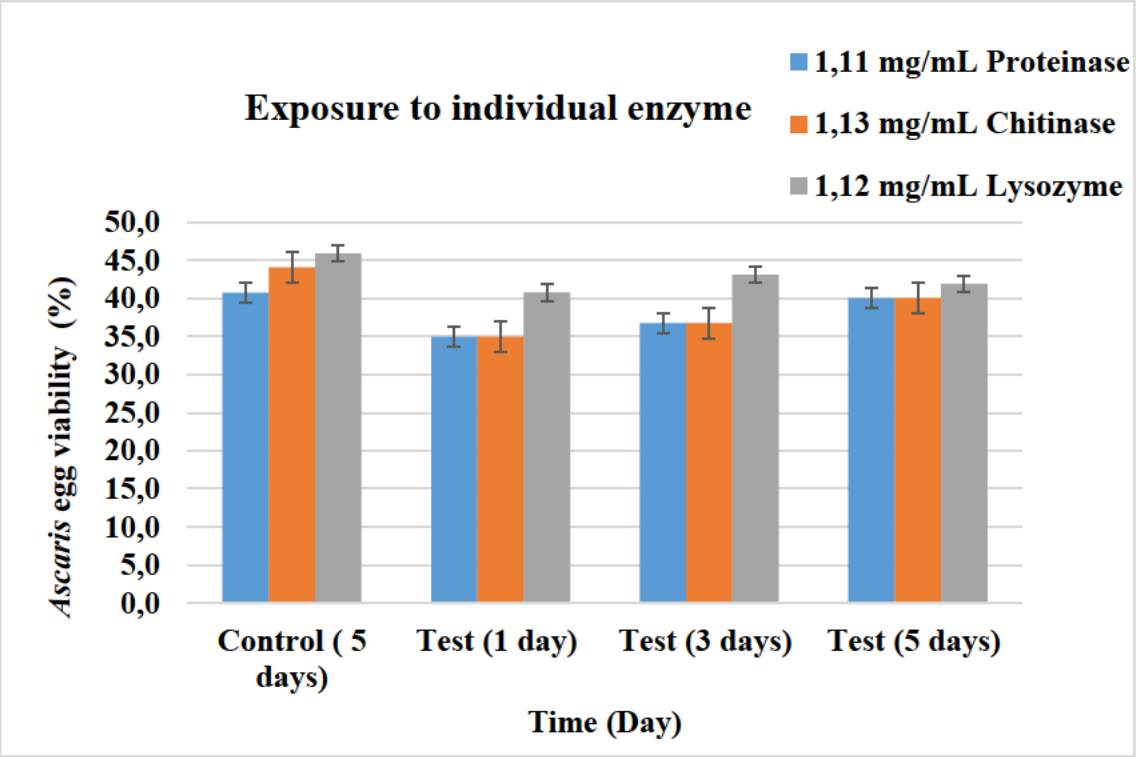

Figure 3-3: Percentage of *A. suum* eggs viable after exposure to 1 mg/L of protease, chitinase and lysozyme (Series 1 is for 1 day of exposure, series 2 and 3 for 3 and 5 days of exposure respectively). Error bars, (n=9).

3.2.2.3 Consecutive exposure to enzymes

The control set up for the consecutive exposure of the eggs to the enzymes at RT resulted in 38.5% and 37.8% viability after 1 and 5 days of exposure respectively. After consecutive exposure of the eggs to the enzymes at RT, the lowest viability at RT was observed to be 43.0% after 5 days of exposure. The control set up for the consecutive exposure of the eggs to the enzymes at 37°C resulted in 41.8% and 14.0% viability after 1 and 5 days of exposure respectively. The lowest viability at 37°C after consecutive exposure to the enzymes was 4.8% after 5 days of exposure. Exposure to the enzymes at 37°C resulted in further reduction of egg viability. However, the reduction in viability observed in the experiments where the eggs were exposed to the enzymes at room temperature (RT) was not statistically significant as indicated by the p-value (>0.05), when compared to the control experiments. Additionally, the reduction

in viability was found to be statistically larger at 37°C than at room temperature, as illustrated in Figure 3.4.

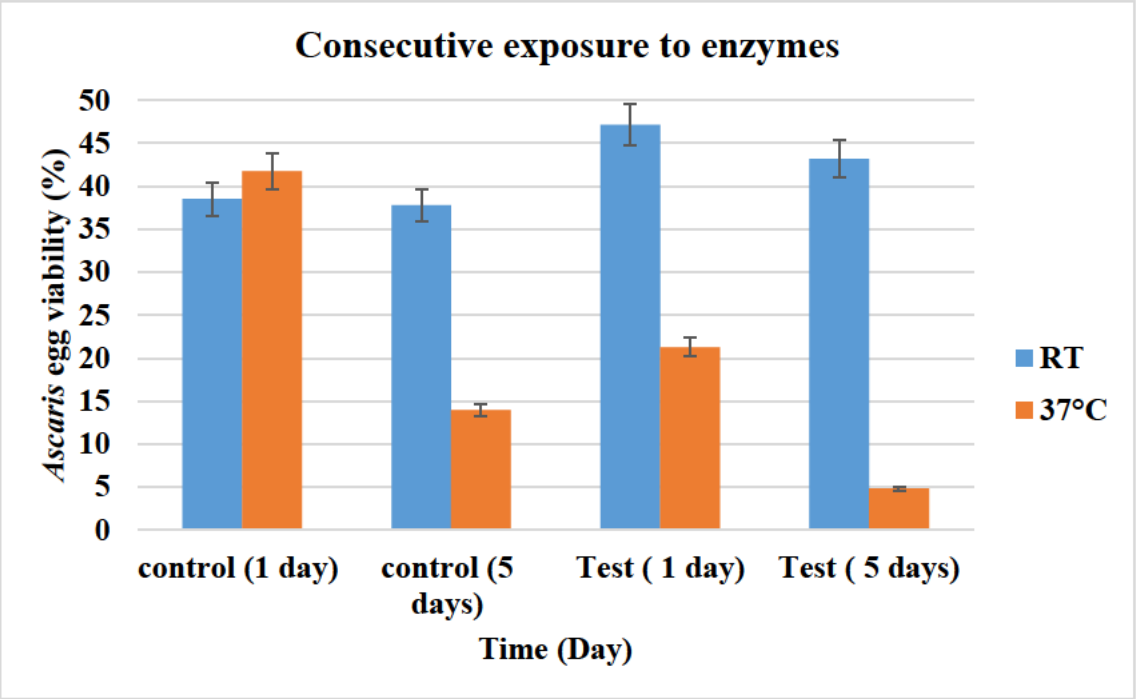

Figure 3-4: *A. suum* inactivation using single lytic enzyme 1 mg/L protease, chitinase and lysozyme in a consecutive manner at room temperature (RT) and controlled temperature (37°C). (Series 1 is for 1 day of exposure, series 2 for 5 days of exposure). Error bars, (n=9).

3.2.2.4 Mixed lytic enzyme exposure

The control set up for the mixed exposure to the enzymes at RT resulted in 34.3% and 29.5% viability after 1 and 5 days of exposure respectively. After mixed exposure of the eggs to the enzymes at RT, the lowest viability at RT was 27.6% after 5 days of exposure. The control set up for the mixed exposure to the enzymes at 37°C resulted in 21.8% and 18.8% viability after 1 and 5 days of exposure respectively. The control experiments for exposure at 37°C resulted in a lower viability percentages than at RT. The experimental eggs exposed to the mixture of enzymes at 37°C resulted in the lowest viability of 12.9% after 5 days of exposure.

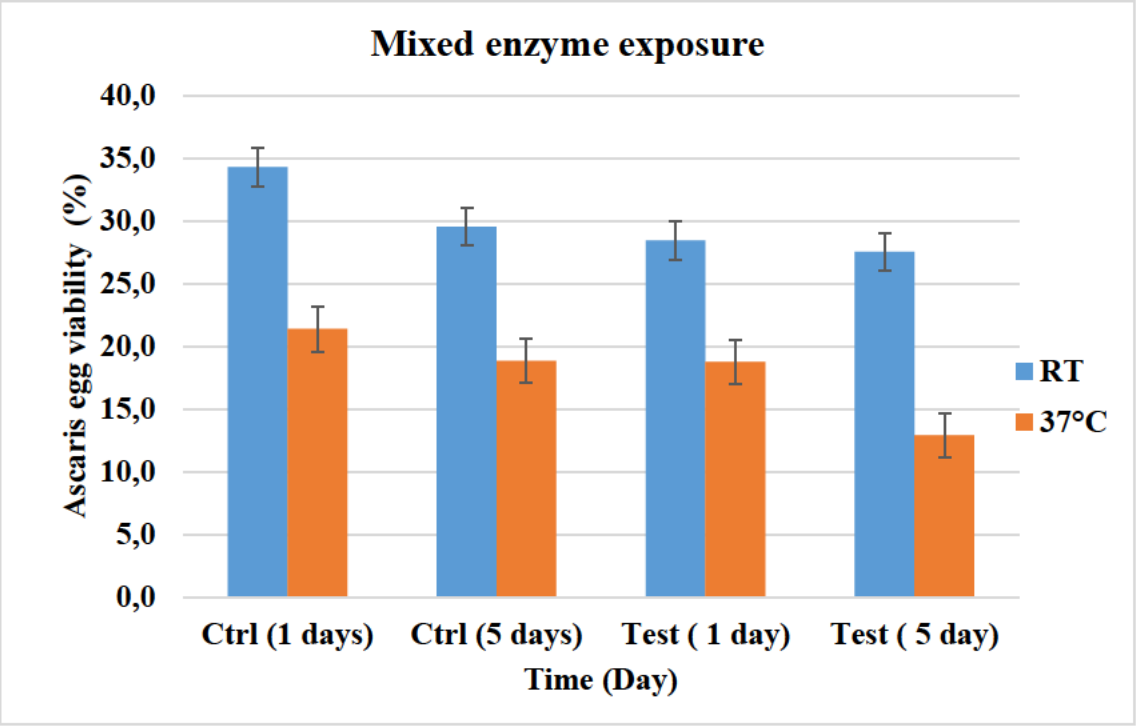

Figure 3-5: *A. suum* eggs inactivation using a mixture of lytic enzyme 1 mg/L protease, chitinase and lysozyme at room temperature (RT) and controlled temperature (37°C). (Series 1 is for 1 day of exposure, series 2 for 5 days of exposure). (Error bars, n=9).

3.3 Discussion

Since 1989, the WHO has set limits for the content of treated wastewater, sludge or any human excreta to be used for agricultural purposes to have a limited amount of STH eggs (Maya et al., 2012). In 2006, the guidelines stipulated that safely treated human excreta should contain less than 1 egg per gram of total dry solids (1 HO eggs/g TS) (Maya et al., 2012; WHO, 2006). Therefore, the partially treated UDDT sludge collected for this experiment contains a high amount of *Ascaris* spp eggs, above the set limit for FS in order for it to be used for agricultural purposes. This highlights the need for the FS to be treated further in order to reduce the pathogens to safe levels set by the WHO standard.

The viability in the eggs recovered from UDDT FS samples before incubation was low (around 11%). The observed concentration was still above 1 egg per gram of total dry solids (1 HO eggs/g TS), above the recommended guideline value for safe FS re-use by the WHO sludge guidelines (Maya et al., 2012). These eggs have been in the environment for variable periods

of time exposed to unfavourable conditions that include high temperature and low moisture content levels. Low moisture levels is also a factor in UDDT systems meant to desiccate human excreta in order to reduce pathogens that is passed out within faecal matter. An enhancement of the moisture reduction is achieved by adding dry material after each use (Okem et al., 2013, Tilley et al., 2014, Mkhize et al., 2017). UDDT do not completely inactivate pathogens and the top layer is expected to have a higher number of pathogens, including viable *Ascaris* spp eggs and other STH eggs.

In the enzyme inactivation experiments a homogenous population and higher concentration of viable eggs were needed, and therefore purchased from a commercial supplier. The purchased eggs had a higher viability of 52.0% compared to 11.0% of the recovered eggs from UDDT FS samples. It is worth noting that the low number of viable eggs observed was due to the sampling strategy used, samples were taken from a treatment facility instead of sampling from a top layer of FS from a UDDT toilet (Trönnberg et al., 2010). The condition of the bought eggs were further more uniform. In each experimental setup a control was also added, without addition of the enzymes, to facilitate the determination in viability attributable to the action of the enzymes. This further avoided variations based on the day of experiment and the batch variation of *A. suum* eggs.

The impact of the protease, chitinase and, lysozyme during different time periods aimed to assess the ability of each enzyme to break open the eggshell of the *A. suum* eggs, to increase the osmotic pressure and to affect the physiological properties of the egg that will halt its development to a fully developed viable infectious egg (Ragland and Criss, 2017). After considering the natural die off rate of the control samples, this was factored into calculating the reduction in viability due to the action of the enzymes alone. This is also considered for the rest of the experiments discussed below.

After exposure of the eggs to protease, a reduction in the viability to 35.0%, 36.8%, and 40.0% after 1, 3, and 5 days of exposure respectively. The protease used only reduced the viability slightly (between 0.3-4.0% in the different experiments). Exposure to protease thus reduced viability by 2.3%, 0.3% and 4.0% for 1, 3 and 5 days of exposure respectively. This was after subtracting the viability of the control experiments as there was a significant drop in the viability of the control samples as well. The impact of protease could have been limited by the *A. suum* eggshell structure. Protease can efficiently break open the outer protein-based layer without affecting the subsequent middle and inner layers (Gortari and Hours, 2008). The activity and specificity of the protease is not effective against the other layers of the egg shell, leaving them intact and allows further development of the *A. suum* egg into viable infectious eggs (Salazar and Asenjo, 2007).

The control experiment for chitinase had a 44.1% viability, the experiment set up had 34.9% 36.8% and 40.1% viable eggs after 1, 3 and 5 days exposure to chitinase. Chitinase achieved a slightly better reduction (4.0 -9.1%) in egg viability after exposure than protease. This means that chitinase reduced viability by 9.1%, 7.3% and 4.0% for 1, 3 and 5 days of exposure respectively.

Chitinase affects the middle chitin layer, which joins and holds the outer and inner layers but remnants of chitin are also found in the other layers and are pivotal in maintaining the integrity of the egg shell (Gortari and Hours, 2008). The chitin layer is the strongest structural component of the egg shell (Deeba et al., 2016; Veliz et al., 2017). This allows chitinase to disrupt the first layer, break the second layer and possibly and also account for impact on the inner layer (Butkus et al., 2011).

The reduction in egg viability was inversely proportional with time, as the number of days of exposure increases, the reduction in viability decreased. This could be attributed to a reduction in enzyme activity. It was observed that the control for the experiments had nematode trapping

fungal contamination which uses its structures to penetrate the eggshell and inactivate the development of nematodes (Hasan and Anand, 2014; Stoykov et al., 2015; Zhang et al., 2017).

Nematophagous fungi are most antagonistic towards nematode eggs, which are more susceptible to fungi during their development stage (Hasan and Anand, 2014; Stoykov et al., 2015; Zhang et al., 2017). Fungal hyphae weaken the egg shell and stop the developmental process, and this is observed on the eggs with unhealthy and deteriorating looking juvenile worms (Zhang et al., 2017; Hasan and Anand, 2014).

This may explain the unexpectedly high reduction of viable eggs in the control experiment groups compared to the 52% viability of the initial count of the egg stock solution. However, the fungal contamination was not observed on the chitinase samples (Salazar and Asenjo, 2007, Deeba et al., 2016, Veliz et al., 2017). This is because the fungal causing contamination in the control samples has hyphae that is made up of chitin and glucans. Fungal growth was inhibited and reduced in experimental samples due to the presence of enzymes.

The enzymes used for the experiments tentatively prevented fungal contamination in the test samples. Future experiments should therefore be concerned about a broad concentration range of chitinase. *Streptomyces rimosus* have been shown to have antifungal activity against *Fusarium solani* and *Alternaria alternate* (Veliz et al., 2017). *Streptomyces viridificans* efficiently lyse fungal cells of *Rhizoctonica, Colletotrichum, Aspergillus, Fusarium, Sclerotinia* and many others that contain chitin as part of their cell wall structure (Veliz et al., 2017).

Exposure of the eggs to lysozyme alone did not improve the inactivation of the eggs compared to protease and chitinase (Fig 3.3). Lysozyme also achieved the highest reduction of the egg by 5.1% after 1 day exposure, a similar trend. This appears as if the enzyme provides a protection for the eggs by killing off the fungal contamination that reduces the viability of the

control samples. The reduction of the control samples compared to the experiment samples is between 8-14%, slightly higher than the reduction observed for the experiments.

Lysozyme further requires a penetration of the two outer layers (Gortari and Hours, 2008). A conclusion may be that a single enzyme will not impact the other layers enough, thus leaving the egg shell intact and allowing the egg to continue to develop to its full growth phase (Khan et al., 2004; Khan et al., 2006; Gortari and Hours, 2008). As a comparison, lysozyme cannot break yeast cells that have β-(1-3) glucans and β-(1-6) glucan branches; thus the same rationale can be applied in these results for *Ascaris* eggs (Ercan and Demirci, 2016). The low reduction of viable eggs observed from the use of a single enzymes could also be attributed to the specificity of the enzymes (Singh et al., 2013; Oyeleye and Normi, 2018). Each enzyme has its own unique active site and specificity (Oyeleye and Normi, 2018). The enzyme could possibly show higher affinity and activity against other organisms like bacteria, plant cell walls, insect cuticles, or have a synergistic effect on the eggs (Salazar and Asenjo, 2007; Gortari and Hours, 2008). This hypothesis was further assessed in the consecutive exposure of eggs.

For the exposure of *A. suum* eggs to consecutive enzymes in series, the control experiments at room temperature had a higher reduction compared to the experimental samples after 1 day (12.6%) and 5 days (13.3%) exposure. The reduction in egg viability was statistically higher in the control experiments than the consecutive exposure experiments (p value < 0.05). The consecutive exposure of lytic enzymes to the eggs resulted in an increase of the eggs viability by 8.7% and 5.4% after 1 day and 5 days respectively. The control samples were observed to have a lower viability count of 38.5% and 37.8% after 1 day and 3 days respectively compared to the experiments that had a higher viability of 47.2% and 43.2% after 1 day and 5 days respectively. This means that the reduction in viability achieved by the enzymes was less than the reduction in the control experiment that occured as a result of external factors. This may falsly have been due to fungal hyphae contamination observed in the control samples. Because

fungal hyphae are susceptible to breakdown by lytic enzymes, mainly chitinase, this eliminated contamination on the test samples and allowed them to develop (Khan et al., 2004; Nagpure and Gupta, 2013).

Exposure of *A. suum* eggs to the consecutive lytic enzymes at 37°C resulted in a significantly higher reduction in viability of 2.6% and 5.9% after 1 and 5 days of exposure respectively, after the accounting for reduction achieved in the control experiments at 37°C. The eggs can survive over a year at 40°C (Naidoo and Foutch, 2017; Hawksworth et al., 2010). The enzymes will further have a documented catalytic activity at 37°C (Deeba et al., 2016). Lytic enzymes such as chitinase can functions at an optimum temperature of 30-40°C (Deeba et al., 2016).

For the exposure of *A. suum* eggs to the mixed enzyme (Figure 3.5), the control setup for this experiment achieved a viability of 34.3% and 29.5% after 1 and 5 days respectively at room temperature. Exposure of the eggs to the mixed enzymes at RT achieved a lower viability of *A. suum* eggs of 28.4% and 27.6% after 1 and 5 days respectively. Accounting for the reductions achieved in the control experiments, it can be deduced that exposure to the mixed enzymes achieved mean viability reductions of 5.8% and 2.0% after 1 day and 5 days at RT respectively. However, a reverse was observed at 37°C. Considering the reduction of the controls, it can be deduced that exposure to the mixed enzymes achieved mean viability reductions of 2.6% and 5.9% after 1 and 5 days respectively. Statistically, there was a significant difference in the reduction of viability achieved by exposure of the eggs to the mixed enzymes compared to the control setup at 37°C (p value < 0.05), which is in contrast to the experiments at room temperature. The results also show that the increased duration of exposure increased the inactivation of the eggs at 37°C.

3.4 Conclusion

Although the viability of *Ascaris* spp eggs recovered from the FS was low, their concentration was higher than recommended WHO sludge reuse guidelines. To further reduce their viability, lytic enzymes were hypothesized to be effective. Chitinase was the most effective single enzyme at reducing viability at room temperature. The experiments carried out at 37°C gave a higher reduction than those performed at room temperature for the single enzyme exposure. This could be due to an increased enzyme activity at 37°C as well as a slightly higher detrimental action at this temperature. The consecutive exposure to lytic enzymes at 37°C was more effective than the use of single enzymes, mixed enzymes at room temperature and at 37°C, supporting the conclusion that higher temperatures could lead to better inactivation.

4 CHAPTER 4: ENZYME PRODUCTION, PURIFICATION AND CHARACTERIZATION

4.1 Background information

This chapter demonstrates the culturing of proteinase, chitinase and lysozyme producing bacteria found in FS using selective media. The bacterial isolates are used for the production and extraction of extracellular lytic enzymes such as proteases, chitinase and lysozyme. Furthermore, the enzyme are characterised based on their functions and properties using assay techniques. FS has shown great potential as starting material for the generation of compost and energy, additionally FS can be used to enumerate microorganisms that can produce lytic enzymes (Karn and Kumar, 2019). The naturally occurring saprophytic population of soil microorganisms in FS makes it an ideal matrix for the enumeration of bacteria that can produce lytic enzymes (Veliz et al., 2017). These processes need to be explored on how the naturally produced lytic enzymes in soil or FS can be enhanced and used to inactivate *Ascaris* spp and other pathogens, generate other secondary products whilst reducing waste.

4.2 Methodology

4.2.1 Sampling of FS and dilution for enzyme production

UDDT FS samples were collected as described in Chapter 3. A stock solution of diluted UDDT FS was prepared by mixing 1 g of UDDT FS with 100 mL of distilled water. A serial dilution 1:10 dilution was then done by mixing 1 mL of the diluted UDDT FS with 9 mL of distilled water to make a series of final dilutions between $1x10^{-1}$ to $1x10^{-8}$.

4.2.2 Protein production

4.2.2.1 Culturing of protease-producing bacteria

The culturing of protease-producing bacteria was done using HiChrome media (Sigma Aldrich, Germany) inoculated with 0.5 mL of the diluted $1x10^{-8}$ UDDT FS and incubated at 37°C for 48 hours. After 48 hours, bacterial colonies from the HiChrome media agar plates were diluted to $1x10^{-8}$ as described in section 4.2.1. The highest serial dilution was then used to inoculate 2.5% milk selective agar and incubate at 37°C for 48 hours. Bacterial colonies on this agar with zones of clearance around them were transferred to 1.25% (v/v) casein broth (Sigma Aldrich, Germany) and incubated at 32°C for 3 days with continuous stirring at 140 rpm.

4.2.2.2 Culturing of chitinase-producing bacteria

A method outlined by Sato et al., (2009) was followed for the cultivation of chitinase producing bacteria and is as follows. Chitinase-producing bacteria was isolated by mixing approximately, 1 g of UDDT FS with 100 mL of 1% chitin enrichment media (Sigma Aldrich, Germany) and left at 0-4°C for 1 week (E_O). After 1 week, a serial dilution of E_O was made up to $1x10^{-8}$ where the highest dilution was inoculated to fresh 100 mL 1% chitin enrichment media labelled E_1, and E_1 was left at 0-4 ° C for another 1 week. The process was repeated until E_4. A serial dilution to 10^8 of each of the inoculated enrichment media were made, and 1 mL of the 10^{-4}-10^{-8} (E_0-E_4) dilutions were inoculated on 1% chitin agar (Sigma Aldrich, Germany) at 35°C for 48 hours. Bacterial colonies on 1% chitin agar with zones of clearance around them were transferred to 1% chitin broth and incubated at 35°C for 3 days with continuous stirring at 140 rpm.

4.2.2.3 Culturing of lysozyme-producing bacteria

Culturing of lysozyme-producing bacteria was done on Hichrome agar inoculated with diluted 0.5 mL UDDT FS and incubated at 32°C for 48 hours. After 48 hours, bacterial colonies were

serially diluted up to 10^{-8}. The highest serial dilution was then used to inoculate 0.05 % *M. lysodeikticus* nutrient agar (w/v) (Sigma Aldrich) and incubate at 32°C for 48 hours. Bacterial colonies on this agar with zones of clearance around them were transferred to 0.05% *M. lysodeikticus* (w/v) nutrient broth and incubated at 30°C for 3 days with continuous stirring at 140 rpm.

4.2.2.4 Ammonium sulphate precipitation of produced enzymes

The enzymes produced by the bacteria in 0.05% *M. lysodeikticus* (w/v) enriched nutrient broth (crude protein) were precipitated by sonicating the broth for 30 minutes at 25°C which was then centrifuged (REMI lab instruments, India) at 5000 rpm for 10 minutes. The resulting pellet and an aliquot of the supernatant were each mixed with an equal volume of dithiothreitol lysis buffer (50 mM Tris-HCl, 0.01mM Dithiothreitol) (Sigma Aldrich, Germany) and stored on ice. The remaining supernatant was measured and gradually mixed with NH_4SO_2 (ACE chemicals, South Africa) until it was completely dissolved at 0-4°C to bring the saturation to 20%. The 20% saturated supernatant was centrifuged at 5000 rpm for 10 minutes. The resulting pellet and an aliquot of the supernatant were each mixed with an equal volume of the lysis buffer and stored on ice. This procedure was repeated until a 60% saturation was achieved for all of the enzymes produced. Pellets and supernatant fractions with positive results with the exception of blanks are reported below.

4.2.3 Characterization of precipitated enzymes

4.2.3.1 Protein quantification

A six-point (0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/mL) calibration curve of bovine serum albumin (BSA) (Sigma Aldrich, Germany), a set of test samples and a blank were made for enzyme quantification. An aliquot of 1.5 mL was added to each tube, and incubated for 5 minutes at

room temperature and measured at 595 nm using a Prove 300 spectrophotometer. See Annexure B, section 4.5.1 for detailed results.

4.2.4 Enzyme activity assay

A selection of pellets and supernatant samples of partially purified enzymes using the NH_4SO_2 precipitation method that yielded positive results from the protein quantification method from Chapter 4, section 4.2.3.1 were further used for the enzyme activity assay. The activity assays for protease, chitinase and lysozyme are outlined below.

4.2.4.1 Protease activity assay

A set of two test tubes was required for the activity assay that involves a two-way process. The first set consists of 2.5% w/v casein that was mixed with the NH_4SO_2 purified enzymes. The second set of tubes contained a known amount of tyrosine. For the NH_4SO_2 purified enzymes, a volume of 5 mL 2.5% w/v casein was added to each tube containing the purified 1 mL protease. Distilled water (1 ml) was instead added to the blank and 1 mL of 1 mg/mL enzyme was used a positive control. Each purified enzyme was tested in triplicate. The tubes were sealed with parafilm and incubated at 37°C for 10 minutes with constant shaking using a benchtop shaker at RT. After 10 minutes, 5 mL of the 5% tri-chloro acetic acid (TCA) was added to each tube and centrifuged at 5000 rpm for 5 minutes. The supernatant was then collected. For the second process, a blank, a five-point calibration curve of the 2 mg/mL tyrosine standard (Sigma Aldrich, Germany) and the set of tubes containing the supernatant were used from this point (n=9). An aliquot of 2 mL from the supernatant and the five-point tyrosine standard were mixed with 5 mL 0.5% sodium carbonate (w/v) (Sigma Aldrich, Germany) and 1 mL phenol reagent (Sigma Aldrich, Germany). The tubes were then placed in the water bath at 37°C for 30 minutes. The tubes were centrifuged at 5 000 rpm for 5 minutes

at room temperature, and the absorbance of the supernatant was read at 660 nm using a Prove 300 spectrophotometer (Sigma Aldrich, Germany).

4.2.4.2 Chitinase activity assay

Sets of two test tubes was required for this assay. The first set consisted of colloidal chitin broken down by the NH_4SO_2 purified enzymes into varying concentrations of N-acetylglucosamine (NAG) (Sigma Aldrich, Germany). The second set of tubes contained known standard NAG concentration. Each purified enzyme was tested in triplicate. Approximately, 0.5 mL of the NH_4SO_2 purified chitinase was added to 2 mL of the colloidal chitin. For the blank, 0.5 mL of distilled water was added to 2 mL of the colloidal chitin 1 mL of 1 mg/mL enzyme was used a positive control.. The test tubes were sealed and then incubated at room temperature for 2 hours with constant shaking at 250 rpm using a benchtop shaker. After 2 hours, the test tubes were placed in boiling water for 5 minutes and then placed in cold water for another 5 minutes. After cooling, the test tubes were then centrifuged at 5000 rpm for 5 minutes to obtain a clear supernatant. An aliquot of 0.9 mL from the supernatant was withdrawn and mixed with 0.1 mL of N-acetylglucosaminidase (Sigma Aldrich, Germany) into each test tube. A set of tubes were prepared for the blank, a five-point calibration curve of the 2 mg/mL NAG standard with 0.1 mL of N-acetylglucosaminidase (n=9). The supernatant samples, the blank and the five-point 2 mg/mL NAG calibration tubes were placed in the water bath for 5 minutes at 37°C whereafter 2 mL of the stop solution was added to each tube and read at 405 nm using a Prove 300 spectrophotometer.

4.2.4.3 Lysozyme activity assay

For the lysozyme activity, 600 µL of 0.5 mg/mL *M. lysodeikticus* was added to 200 µL of 300 mM $NaCl_2$ (ACE chemicals, South Africa) and 400 µL NH_4SO_2 purified enzyme. The mixture was read at 540 nm every 10 seconds for 1 minute using a Prove 300 spectrophotometer. For the blank sample, distilled water was used instead of the enzyme.

Data was captured and plotted in Microsoft Excel (Microsoft Corporation, USA). Comparison of lytic enzymes concentration from the different media was performed using the Kruskal-Wallis Test, with Dunn's Multiple Comparison. The same statistical tests were used to measure the enzyme activity on the prescribed substrate for each of the lytic enzymes. Statistically significant differences of the of the enzyme activity of the different enzymes against each other was determined using the Mann-Whitney Tests.

4.3 Results

Samples from the production of protease, chitinase, and lysozyme were purified and fractionated to concentrate enzymes using the ammonium sulphate method (Krisna et al., 2014). This purification method is to concentrate separate enzymes from the media and isolate them as close as possible to their purest form. This purification method uses a high concentration of NH_4SO_2 (0-100%) to precipitate proteins that can be centrifuged out of the media solution. This generates a pellet and a supernatant with every purification step. The crude samples contain the media, substrate, bacteria and enzymes. The crude pellet and the crude supernatant refer to the crude fractionations that are obtained before processing the samples using the ammonium sulphate method, these are referred to as the 0% pellet or the 0% supernatant. The 20%-60% pellet and supernatant refers to the fractionations of each increments of the salt concentrations during the purification process. The purified enzymes are assessed for total mass, enzyme activity and specific activity as shown in Table 4.1- Table 4.3 below.

4.3.1 Concentration and activity of the produced protease enzyme

4.3.1.1 Calibration of enzyme concentration

Figure 4.1 is a standard curve that demonstrates the absorbance at 660 nm by tyrosine amino acids standard solutions with known concentrations. This standard curve is used to determine

the protein activity of the purified protease by measuring the amount of tyrosine amino acids released from casein as a substrate by the purified protease. The amino acids released from the unknown solutions are determined using the tyrosine standard curve.

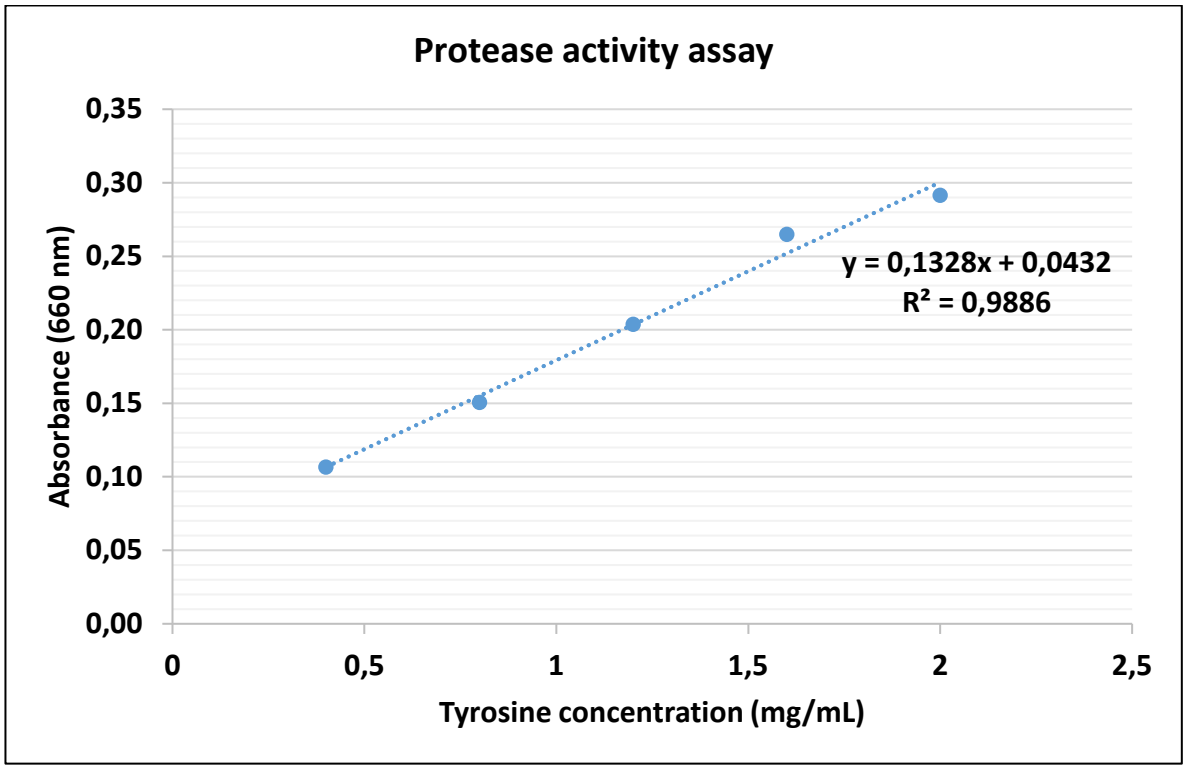

Figure 4-1: Standard curve used for the determination of tyrosine amino acid units.

4.3.1.2 Concentration and activity of the produced protease enzyme

The crude media had the highest protease concentration of 0.59 mg/mL, the protease activity was determined to be 0.05 U and the specific activity to be 0.08 U/mg. However, the highest specific activity of 0.09 U/mg was determined to be on the 20% NH_4SO_2 saturation pellet. The lowest protease concentration was produced by the 40% NH_4SO_2 pellet with 0.012 U and 0.03 U/mg.

Table 4-1: Characterization of the produced and partially purified protease enzyme samples.					
Ammonium Saturation point	Total protease (mg/mL)	Tyrosine (mg/mL)	Activity (U)	Specific act (U/mg)	Yield (%)
Blank	0,00	0,00	0,00	0,00	0,00
Positive control (Protease 1 mg/mL)	0,99	0,16	0,06	0,06	-
Crude	0,59	0,13	0,05	0,08	100
0% - P	0,68	0,10	0,04	0,05	71,91
0% - S	0,44	0,06	0,02	0,05	46,63
20% - P	0,45	0,12	0,04	0,09	85,96
20% - S	0,45	0,11	0,04	0,09	83,15
40% -P	0,38	0,03	0,01	0,03	24,16

4.3.2 Concentration and activity of the produced Chitinase enzymes

4.3.2.1 Calibration of enzyme concentration

Figure 4.2 is a standard curve that demonstrates the absorbance of NAG at 405 nm by a standard solution with known different concentrations. This standard curve is used to determine the enzyme activity of the purified chitinase by measuring the amount of NAG it releases from

chitin as a substrate. The unknown concentrations of NAG released by the produced and purified chitinase are determined using the NAG standard curve below.

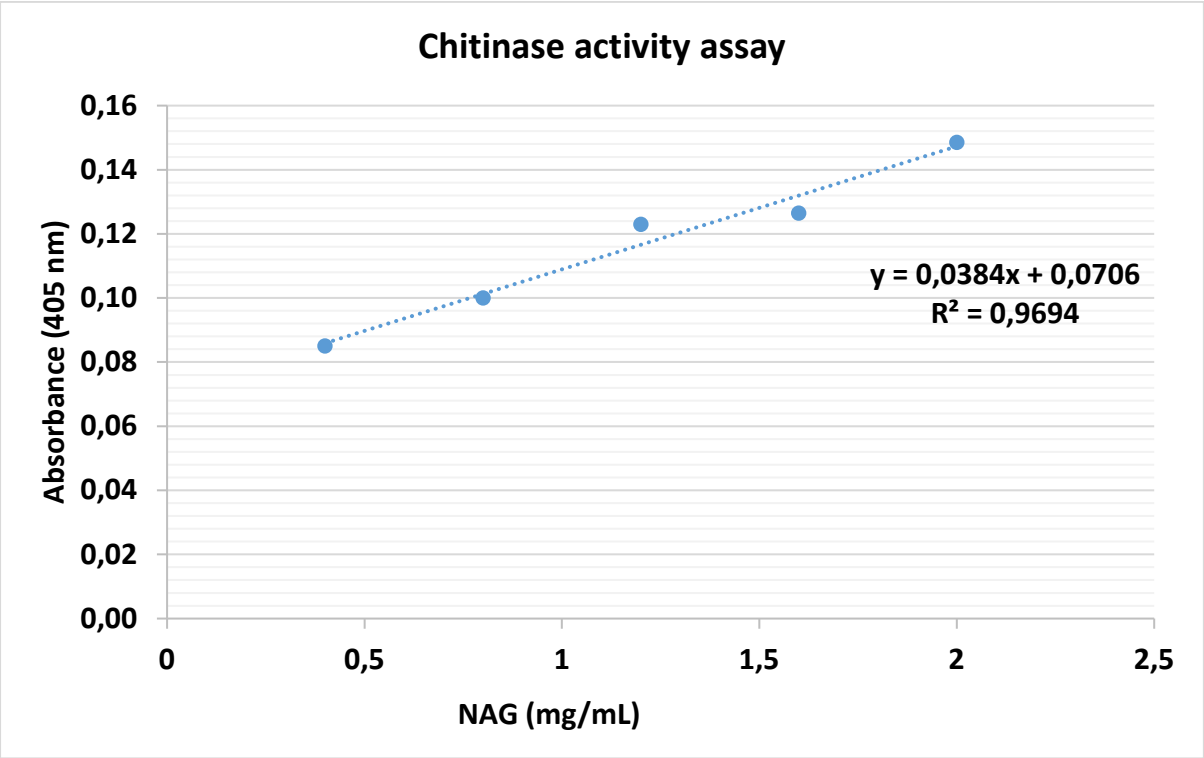

Figure 4-2: N-acetyl-Glucosamine (NAG) standard curve used for the determination of NAG units released by produced and partially purified chitinase samples.

4.3.2.2 Concentration and activity of the produced chitinase enzyme

The 0% crude pellet had the highest chitinase concentration of 3.17 mg/mL, the enzyme activity was determined to be 0.02 U with a specific activity of 0.01 U/mg. However, the highest specific activity was determined to be 0.23 U/mg on the crude extract. The lowest chitinase concentration was 0.22 mg/ml, produced by the 40% NH_4SO_2 pellet with 0.01 U and 0.04 U/mg.

Table 4-2: Characterization of the produced and partially purified chitinase enzyme samples.					
Saturation	Total chitinase (mg/mL)	NAG (mg/mL)	Activity (U)	Spe. Act (U/mg)	Yield (%)
Blank	0,01	0.00	0,00	0,00	
Positive control (Chitinase 1 mg/mL)	1,00	16,16	0,06	0,06	
Crude	0,37	3,42	0,09	0,23	100,0
0% - Pellet	3,17	24,72	0,02	0,01	21,19
20% - Pellet	0,54	7,57	0,03	0,06	36,54
40% - Pellet	0,22	0,49	0,01	0,04	10,34

4.3.3 Concentration and activity of the produced lysozyme

4.3.3.1 Calibration and concentration of lysozyme

Lysozyme activity assay measures the ability of lysozyme to digest the peptidoglykan in the cell wall of gram-positive *M. lysodeikticus* bacteria. The breakdown of the gram-positive cell bacteria causes a decrease in absorbance that is measured spectrophotometrically at 450 nm (Table 4.3). The measurements are taken every 10 seconds for 1 minute. The crude media had the highest lysozyme concentration of 2.74 mg/mL, the lysozyme enzyme activity was

determined to be 7132.5 U with a specific activity of 2600.2 U/mg. The lowest lysozyme concentration was 0.34 mg/mL from the 40% NH_4SO_2 pellet with 3365,9 U and 8925,2 U/mg. However, 8925.2 U/mg was the highest specific activity observed.

Table 4-3: Digestion of *M. lysodeikticus* cells for lysozyme activity assay

Time	10 sec	20 sec	30 sec	40 sec	50 sec	60 sec	Protein (mg/mL)	Enzyme activity (U)	Specific activity (U/mg)	Yield (%)
Blank	0,25	0,25	0,25	0,25	0,25	0,25	0.000			
Positive control (1 mg/mL lysozyme)	0,44	0,42	0,41	0,40	0,39	0,38	0.99	318,30	322.14	
Crude	3,12	3,11	3,11	3,11	3,11	3,10	2.74	7132,50	2600,15	100
0% Crude pellet	2,72	2,71	2,71	2,71	2,71	2,71	2.32	6140	2645,47	86.09
20% Pellet	2,40	2,39	2,39	2,38	2,38	2,37	1.84	5323,8	2885,76	74,64
40% Pellet	1, 60	1,59	1,59	1,60	1,60	1,60	0.38	3365,90	8925,19	47,19
60% Pellet	0.74	0,74	0,73	0,73	0,72	0,72	0.60	1195	2003.91	16.75

4.4 Discussion

Enzyme quantification, enzyme activity and specific assays are used to assess enzymes under set conditions (Scopes, 2002). Enzymes need to be purified before characterization, the NH_4SO_2 precipitation procedure provides one of the primary purification steps (Krisna et al., 2014). This allows the removal of unwanted proteins and increases the specificity of enzyme activity and specific activity. The concentrations of the different enzymes produced using the selective media described above are determined using the Bradford assay, Chapter 4, section 4.2.3.1. Enzyme activity is measured based on the amount of substrate used and the product formed based on the catalytic activity (Scopes, 2002).

Protease activity was assessed based on the ability of the purified proteases to break the peptide bonds that make up casein to tyrosine amino acids. Before enzyme purification, the crude extract, which is a mixture of cell culture and extracellular proteins after sonication, contained 0,6 mg/mL protease at 100% recovery. The enzyme activity and specific activity were 0.1 U and 0.1 U/mg respectively. The subsequent purification steps have decreasing amount of the enzyme to a point where no activity was detected after the 40% pellet saturation point. This reiterate that the assessed protease is an extracellular enzyme as it is found in the production medium (crude extract) before undertaking any purification processes (Alam et al., 2017; Chakraborty and Karmakar, 2020; Aramesh and Ajoudanifar, 2017, Palsaniya et al., 2017). However, the amount of protein in the pellet and supernatant shows that the separation using the NH_4SO_2 precipitation process were partly unsuccessful. There is no significant difference in the amount of the proteins in the pellet and supernatant for the following process of the 20% pellet and supernatant, as well as the 40% pellet NH_4SO_2 purification steps. The protein is soluble in the production medium and cannot be separated due to the ionic strength of the medium. The NH_4SO_2 present inhibits the protease to self-aggregate and precipitate. Proteins with positive and negative charges self-aggregate easily, low salt concentrations can inhibit

self-aggregation of proteins unless the concentration is increased to change the ionic strength significantly and restore the charges on the protein surface that allows it to self-aggregate (Krisna et al., 2014). For this protease to be precipitated out, this will require an exorbitant amount of NH_4SO_2.

The amount of enzyme is reduced with each purification step as the NH_4SO_2 saturation is increased. This is expected to occur until a pellet is no longer observed and has been precipitated out from the prior saturation steps with NH_4SO_2 (Krisna et al., 2014). This means that the highest protease activity was precipitated at 0% saturation before the addition of the NH_4SO_2 salt. The salting-out process using NH_4SO_2 is not crucial for the separation of this extracellular protein. However, it is expected that the specific activity should increase as the enzyme is purified, however, the enzyme can become denatured during the purification process and the activity is lost.

The 20% pellet NH_4SO_2 saturation point proved to be the optimum saturation point as it had the highest specific activity. This means that the crude media had the highest enzyme activity, the highest ability to form tyrosine monomers from casein. Also, despite that the highest protease (0.7 mg/ml>0.6 mg/ml) was from the 0% pellet, however, the purest form of the enzyme across all the fractionations was found on the 20% pellet NH_4SO_2 saturation point. This is shown by the higher specific activity of 0.1 U/mg compared to the enzyme activity of the crude and 0% pellet, as well as an increase in the enzyme yield itself.

This means that NH_4SO_2 used up the solution creating hydrophobic interactions thus forcing the protein to aggregate the most at 40% NH_4SO_2, yielding the highest purified form of the protein. After the 40% pellet NH_4SO_2 saturation purification step, no further enzymes were purified and detected. This shows that better techniques for enzyme purification needs to be

employed as the purification reaches a plateau after the 20% NH_4SO_2 saturation (Mothe and Sultanpuram, 2011).

Mothe and Sultanpuram (2016), reported production of protease produced by *Bacillus caseinilyticus* where 1.9 mg/mL and 1.2 mg/mL of protease was detected from the crude culture and the partially purified media respectively. A high production of 3 250 mg protease from the crude culture and 190 mg protease after the purification steps was detected by Hadjidj et al. (2018), much higher than in the current investigation, potentially due to multiple purification steps.

The characterization of this purified enzyme in the current study proves it to be of low quality compared to what is reported in the literature. Mothe and Sultanpuram, (2011), reported 5.9 U and 8.5 U for the crude extract and the NH_4SO_2 purified enzyme respectively. Mothe and Sultanpuram, (2011), also reported that this increased drastically to 89.2 U by employing other techniques such as ultrafiltration. The low protein quality of the protease purified could be attributed to the bacterium concentration, the enrichment component used on the agar plate and other parameters that needed to be controlled or determined such as pH, temperature, macronutrient concentrations and the lack of purification techniques employed to purify it (Khan et al., 2011).

Chitinase activity assay is based on the ability of chitinase to breakdown the chitin polymer to its monomer, N-acetyl-glucosamine. The released glucosamine reacts with 4-nitrophenyl-N-acetyl-β-D-glucosaminidase that releases p-nitrophenol in its reduced form, p-netrophylate. The reduced p-netrophylate turns the solution yellow and can be read at 405 nm using a spectrophotometer (Figure 4.2).

For chitinase, NH_4SO_2 purification was carried out up to 40% saturation (Table 4.2). Before enzyme purification, the crude extract contained 0.4 mg/mL chitinase at 100% recovery. The

crude extract had the highest enzyme activity (0.1 U) and highest specific activity (0.2 U/mg). The 0% crude pellet had the highest amount of enzyme concentration (3.2 mg/mL) but low enzyme activity (0.02 U) and specific activity (0.01 U/mg). It is expected that enzyme concentration will decrease and for specific activity to increase with every purification step. This trend is observed for the 20% pellet NH_4SO_2 saturation point. After precipitation with NH_4SO_2 salt at 20% NH_4SO_2 saturation, the amount of enzyme was decreased to 0.5 mg/mL and the specific activity increased to 0.06 U/mg. An average of 0.20 mg/mL chitinase was recovered for the pellet and supernatant at 40% NH_4SO_2 saturation point. Further precipitation did not yield different results and the analysis was stopped at this point. The 20% NH_4SO_2 was the peak where the chitinase was precipitated out based on the increase value of the specific activity and the yield of the enzyme decrease after that point. After this point, the salt may have neutralized the protein in a process known as salting-in , which contributed to the chitinase not being separated efficiently from the pellet and the supernatant (Krisna et al., 2014).

The enzyme activity and specific activity dropped for the 0% pellet NH_4SO_2 saturation. This could be attributed to the mixed components on the media before purification that limits the activity of the enzyme (Krisna et al., 2014). The chitinase enzyme activity and specific activity was increased for the 20% NH_4SO_2 saturation, from (0.02 U, 0.01 U/mg) at 0% NH_4SO_2 saturation to (0.03 U, 0.06 U/mg) at 20% NH_4SO_2 saturation. The 20% NH_4SO_2 saturation was the best salt concentration to precipitate chitinase as it yielded the highest amount of chitinase (34%) and the highest specific activity. This means that the NH_4SO_2 salt provided high ionic strength to separate the enzyme sufficiently from the production medium.

Literature suggests that purification steps can lead to significant enzyme loss; however, each purification step yields a form of the enzyme that is purer, characterized by reduced impurities (Kharthik et al., 2015, Mothe and Sultanpuram, et al., 2016, and Hadjidj et al., 2018). As observed, the yield decreases as the enzyme activity and specific activity increases. If the

quality of the enzyme is improved, the enzyme activity and specific activity will increase resulting in a more efficient enzyme. Kharthik et al. (2015), reported a crude extract with 4.18 mg/mL chitinase and a 60% NH_4SO_2 purified chitinase with a reduced concentration of 2.05 mg/mL chitinase that had a specific activity of 13.0 U/mg and 23.5 U/mg respectively (Kharthik et al., 2015). However, low concentrations and activity of the chitinase enzyme were produced with low activity in this study compared to that reported by Kharthik et al. (2015) (2.1 mg/ml, 48.2 U and 23.5 U/mg) and Esho (2018) reported (9.7 U, 37.3 U/mg). However, if the enzyme activity of the 20% NH_4SO_2 saturation pellet is optimised, it may be applicable in inactivating *Ascaris* eggs as demonstrated in Chapter 3, Figure 3.3.

The crude lysozyme extract contained the highest amount of lysozyme protein of 2.7 mg/mL lysozyme at 100% recovery. The enzyme activity and specific activity were 7132.5 U and 2637.2 U/mg respectively. This also shows that lysozyme produced by the bacterium using *Bacillus* spp selective media, HiCHrome agar, is an extracellular enzyme. The highest enzyme concentration was found in the production medium before undertaking any purification steps. After the NH_4SO_2 saturation purification steps, the specific enzyme activity of lysozyme increased, this means that a cleaner and more purified enzyme was obtained with each purification step. However, the subsequent purification steps were observed to reduce the amount of the enzyme to a point where no activity was detected in the supernatant. After precipitation with NH_4SO_2 salt at 20% - 60% NH_4SO_2 saturation, the characterisation of lysozyme was as follows at 20% saturation (1.8 mg/mL, 5323.0 U, 2 885 U/mg), 40% saturation (0.4 mg/mL, 3 365.9 U, 8925.2 U/mg) and 60% saturation (0.6 mg/L, 1 195.0 U, 2 003.9 U/mg). This decrease in enzyme concentration is expected due to the loss of enzyme with each purification step (Ercan and Demirci, 2016). At 40% NH_4SO_2 saturation, the enzyme activity increased from 2 885,8 U/mL that was recorded at 20% NH_4SO_2 saturation to 8925.2 U/mg. This is because protein solubility decreased when the concentration of the

NH_4SO_2 was increased, which changes the conformity of the proteins to be hydrophobic in a salting-out process and allows it to be precipitated out efficiently (Krisna et al., 2014). The specific activity decreased at 60% NH_4SO_2 to 2 003.9 U/mg. This confirms that the saturation at 40% saturation corresponds to the isoelectric point of lysozyme, allowing optimum separation at that point.

The enzyme concentration produced using selective media was statistically higher compared to that of the control used (p value >0.05). Control samples made up of commercial enzymes bought were tested as control for the protein quantification and the activity assays. This was done to compare the how these enzymes used as standards would compare to the partially purified enzymes produced using enriched media. The control enzymes namely protease, chitinase and lysozyme concentration using the Bradford essay was 0.59, 0.10 and 0.12 mg/mL respectively. However, this is in contrast for the produced protease that produced a lower concentration from the produced enzymes compared to the control (p value <0.05). It is worth mentioning that the high enzyme concentration of the crude extract and the 0% pellet might be attributed to the contaminants in the media before the purification processes. The interferences might be microbial cells, proteins, fungal growth and debris within the media that causes an interference and give high values of the analytes.

A higher statistical difference was observed for the activity of protease, chitinase, and lysozyme compared to the concentration of each of the enzyme observed from the Bradford assay (p value>0.05). The protein quantity determined through the Bradford assay exhibited an inverse relationship with enzyme activity. With increasing salt concentration, both the protein concentration and specific activity decreased. It is expected for specific activity to increase with every purification step, this pattern was observed for lysozyme but only observed either at 20% or 40% NH_4SO_2 for protease and chitinase. The high enzyme concentration and specific activity upstream created a big margin with the values of those parameters obtained

downstream of the purification process. This highlight that there is no set purification method for enzymes and other method should be explored for purification of these enzymes. The data and the irregular patterns from this study gives highlights but makes it difficult to draw conclusive ideas about the enzyme production and characterization of these enzymes. This highlights that casein, chitin and *M. lysodeikticus* can be used as substrates to enrich media for the isolation of lytic enzymes producing bacteria including and enzyme production, however, these process along the enzyme purification methods needs to be optimised.

4.5 Conclusion

Microorganisms in the FS produced lytic enzymes, including protease, chitinase, and lysozyme. To produce these enzymes, nutrient broth was enriched with milk, colloidal chitin, or *Micrococcus lysodeikticus* culture. The crude extracts had the highest protein/enzyme content, followed by the raw pellet. Increasing the amount of ammonium sulfate led to a decrease in protein precipitation, and centrifugal force was sufficient for separating the protein from the production medium. However, further purification and removal of ammonium sulphate residue is needed in order to obtain a pure enzyme. Lysozyme had the highest enzyme concentration, enzyme activity and specific activity of 2.7 mg/mL (crude), 7 132.5 U (crude) and 2 600.2 U/mg for the crude medium. The highest specific activity was observed for the 40% NH_4SO_2 pellet, 8 925.2 U/mg. This is an indication that the NH_4SO_2 was able to precipitate and separate the enzyme from the production medium until the 40% NH_4SO_2 saturation point. Additional purification methods, such as SDS-PAGE, dialysis, chromatography, Sephadex, may be necessary to reduce interferences during protein quantification and activity assays. Although the extracts were crude, they suggest that microorganisms in FS have the potential to produce lytic enzymes capable of inactivating STH eggs.

5 CHAPTER 5: POTENTIAL REDUCTION IN *ASCARI*S SPP EGG VIABILITY USING PRODUCED LYTIC ENZYMES

5.1 Background information

The concentration of *Ascaris* spp eggs recovered in FS from the study area as reported in Chapter 3, raises public health concern especially with sludge reuse. Concentrations of *Ascaris* spp detected from FS ranged from 976 HO eggs/g of FS to 1118 HO eggs/g, of which 114 HE/g were viable eggs. The work described so far in chapters 3 and 4 shows the potential ability of microorganisms in FS producing lytic enzymes with the ability to inactivate *Ascaris* spp eggs. This chapter aims at demonstrating the potential impact that the produced lytic enzymes would have when applied to the recovered *Ascaris* spp eggs from FS. The potential reduction in viability of the *Ascaris* spp eggs recovered from FS is calculated based on the viability reduction observed on purchased *Ascaris* spp eggs using commercial lytic enzyme from chapter 3 in different exposure scenarios. The chapter therefore addresses the potential reduction in viability and factors that could significantly impact on the action of these enzymes in faecal sludge.

5.2 Methodology

5.2.1 Analysis of solid and moisture content of the faecal sludge

To assess the impact of the lytic enzymes on the viability of *Ascaris* eggs in FS, the composition of the FS was determined. Some of the parameters measured were the total solids, moisture content and volatile solids. To measure total solids, a porcelain crucible was dried in the furnace

at 550°C for two hours. The crucible was transferred to the desiccator until it reached room temperature and weighed using an analytical balance (W_1). A sample mass of 20 g was transferred to the crucible, the mass of the sample was noted down (W_{sample}), the crucible was transferred to the oven at 103-105°C for 24 hours. After 24 hours, the crucible was removed from the oven and weighed (W_2) and transferred to the muffle furnace for 2 hours at 550°C (W_3).

Equation 5-1: Total solids in Wet Sample (g/g)

Total solids in Wet Sample (g/g) $= \frac{(W_2 - W_1)g}{W_{sample}\,(g)}$

Equation 5-2: Moisture Content (g)

Moisture Content (g) $= \boldsymbol{W_{sample}(g) - [(W_2 - W_1)]g}$

Equation 5-3: Volatile solids in wet sample

Volatile solids in wet sample (g/g)$= W_2(g) - W_3(g)/W_{sample}(g)$

Equation 5-4: Volatile Solids in dry sample

Volatile Solids in dry sample $(\frac{mg}{mg}) = \frac{W_3(g) - W_1(g)}{Total\ solids\ (wet\ sample)}$

5.2.2 Determination of potential reduction in egg viability

To determine the potential reduction of egg viability achievable with the enzyme concentration reported in Chapter 4, and the results discussed in Chapter 3, a viability reduction percentage was calculated. The following equation is used to determine the reduction in viability:

Equation 5-5: Number of viable Helminth ova

Number of viable Helminth ova $(HO\frac{egg}{mL}) = a - (a * b)$

Where, "*a*" is number of viable eggs /dry FS sample and "*b*" the reduction viability percentage

Approximately, 1 mL of the crude enzyme extracts were exposed to 1 mL of the stock egg concentrations following the same procedure described in Chapter 3, section 3.2. The viability of the eggs after was then determined as previously described.

5.3 Statistical analysis

Microsoft Excel (Microsoft Corporation, USA) was used to capture the results and for data visualization. Viable *Ascaris* spp eggs achieved after exposure to the enzymes individually was compared using the Kruskal-Wallis test, however comparison of the impact of the two temperature ranges used (room temperature and 37°C), viability after 1 and 5 days of exposure to mixed enzymes and serial exposure to the enzymes was performed using the Mann-Whitney test. All statistical analysis was done at a 95% confidence interval GraphPad Prism version 7 (GraphPad Software, CA, USA).

5.4 Results

5.4.1 Potential reduction in viability

The FS samples had an average total solid content of 0.25 (g/g wet sample), moisture content of 0.75 (g/g wet sample), volatile solids of 0.13 (g/g wet sample) and volatile solids of 0.51 (g/g dry sample). Concentration of viable *Ascaris* eggs prior to enzyme exposure was determined to be an average of 114 HO eggs/g, 453 HO eggs/g TS. The viability reduction achieved by the enzymes in Chapter 3, section 3.1.5.1-3.1.5.3 was theoretically applied to the average of viable eggs recovered from FS, Chapter 3, section 3.1.2. Varying concentrations of the viable eggs was determined after exposure to the three enzymes (Table 5.1). Briefly, after 1, 3 and 5 days of exposure to 1 mg/L protease, mean viable *Ascaris* spp eggs were 83, 87 and 79 HO eggs/g respectively. Statistically (Kruskal-Wallis Test), there were no significant differences in the viable egg concentrations between the three days of exposure. Additionally,

exposure to 1 mg/mL chitinase for the same 1, 3 and 5 days exposure resulted in mean viable *Ascaris* spp eggs of 75, 79 and 86 HO eggs/g respectively. Exposure of the eggs to the 1 mg/mL lysozyme resulted in mean viable *Ascaris* spp egg concentrations of 88, 93 and 90 HO eggs/g for 1, 3 and 5 days of exposure respectively. The reduction in viability as described above indicates lesser impact of lysozyme on egg viability compared to the other three lytic enzymes, supporting the results reported in Chapter 3. Thus, the reduction achieved by the single use of lytic enzymes was insignificant, as indicated by a p-value less than 0.05.

Table 5-1: Potential viable *Ascaris* spp eggs after exposure to lytic enzymes using reduction percentage calculated from the experiments in Chapter 3, section 3.1.5.1-3.1.5.3. See Annexure A, section 3.2.1-3.2.3.

Day	Protease	Chitinase	Lysozyme	Series -RT	Series- 37°C	Mixed - RT	Mixed - 37°C
0	114.2 HO eggs/g						
1	82,9	75,4	87,9	111,3	47,5	63,5	41,9
3	87,3	79,2	92,9				
5	79,4	86,3	90,4	96,1	10,7	61,5	28,8

Serial exposure of the *Ascaris* spp eggs in FS to the lytic enzymes at room temperature resulted in mean viable egg concentrations of 111 and 96 HO eggs/ g after 1 and 5 days of exposure respectively. Exposure at 37°C for 1 and 5 days yielded mean viable egg concentrations of 47.5 and 10.7 HO eggs/g respectively (Figure 5.2). The results of this study indicate that the mean viable concentrations determined for exposure at 37°C were statistically lower than those observed in the mixed enzyme exposure group (p-value >0.05). A similar trend was observed in terms of viable egg concentrations when the eggs were exposed to a mixture of enzymes. For example, at room temperature, the mean viable *Ascaris* spp egg concentrations were 64 and 62 HO eggs/g after 1 and 5 days of exposure. However, when the eggs were exposed at

37°C, the mean viable concentrations were found to be 42 and 29 HO eggs/g of FS. These results show a significant reduction in viability after exposure to the mixed enzymes at 37°C compared to room temperature (p value ≥ 0.05), similar to the results obtained in Chapter 3.

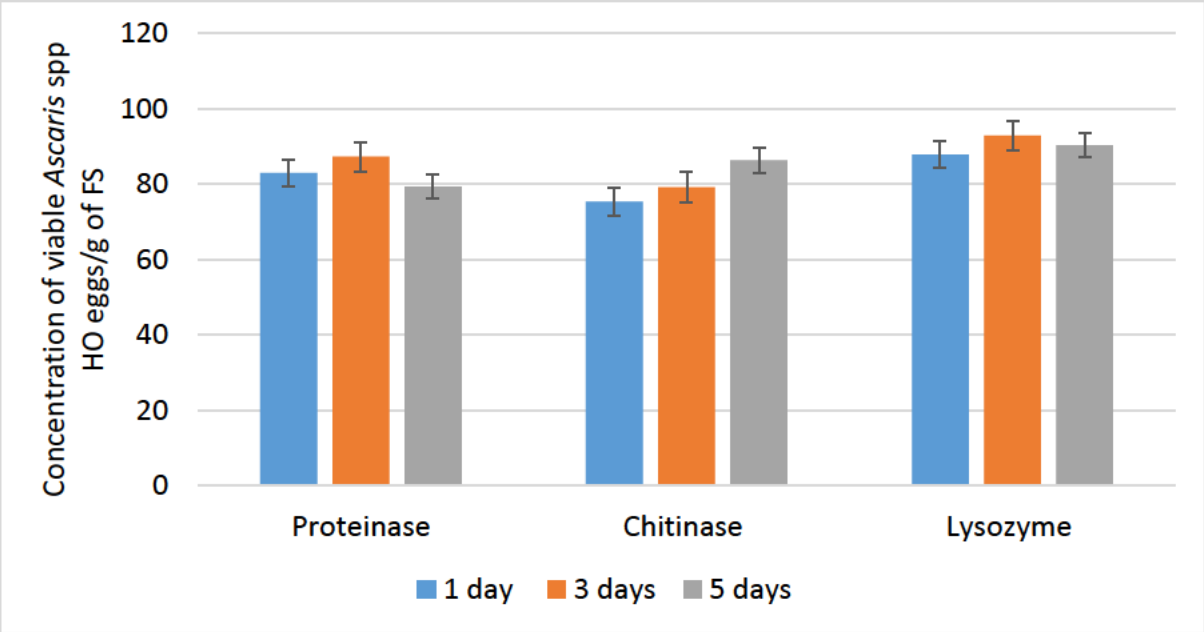

Figure 5-1: Concentration of viable *Ascaris* spp eggs after exposure to lytic enzymes in faecal sludge Error bars, n=9.

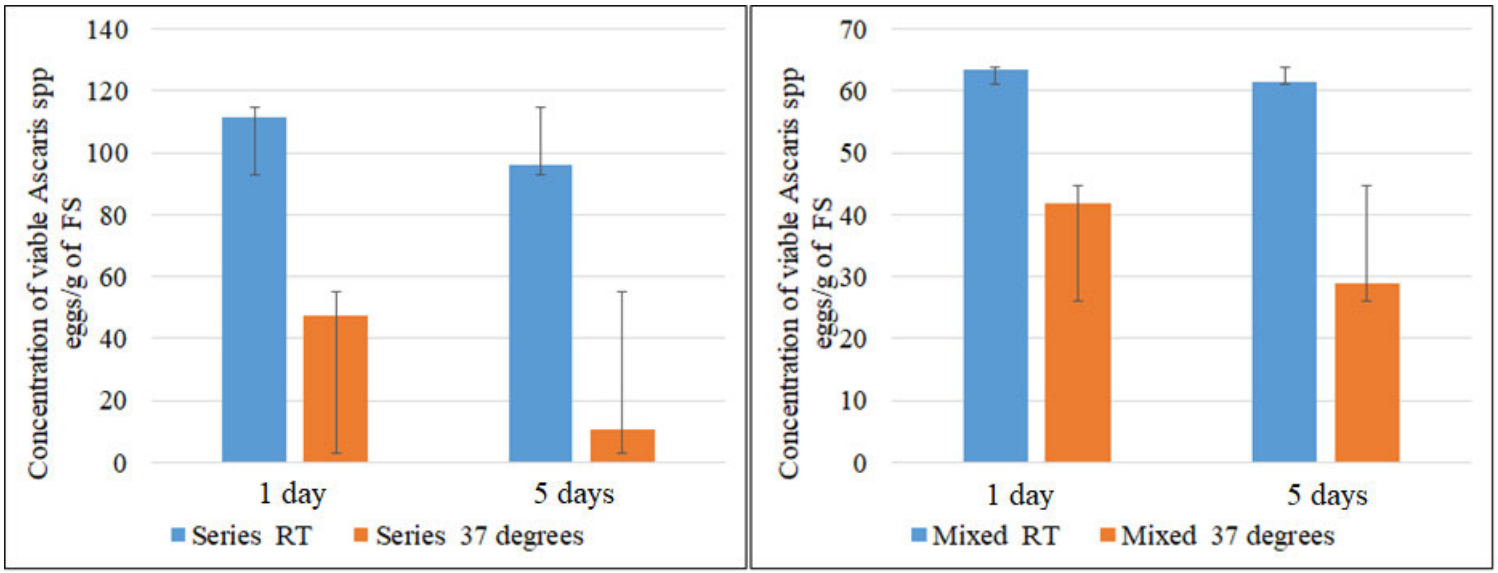

Figure 5-2: Concentration of viable *Ascaris* spp eggs after serial and mixed exposure to lytic enzymes at room temperature (RT) and 37°C. Error bars, n=9.

5.5 Discussion

The concentration of viable *Ascaris* spp eggs in the FS far exceeded the 2006 WHO sludge reuse guidelines, which recommend that treated human excreta should contain at most 1 egg per gram of FS (1 HE/g FS) (Maya et al., 2012; WHO, 2006). On the average, the FS had 114 viable HO eggs/g FS. The action of lytic enzymes as described in Chapter 3, shows a potential for the inaction of these viable eggs. The results presented in this chapter, Figure 5.1-5.2 is the reduction achieved by the enzymes in chapter 3 without accounting for the reduction of viability from the control samples. Exposure of the recovered eggs to chitinase alone was the most efficient in reducing the egg viability. Chitinase reduced viability from 114 HO eggs/g TS to 75 HO eggs/g TS. This is supported by the viability reduction of 350% reported in Chapter 3.

The reduction is improved by the consecutive use of single enzymes, and this is further facilitated by an increase in temperature. The use of consecutive lytic enzymes achieved a viability reduction percentage of 90.6% after exposure at 37°C and to a concentration of 11 HO eggs/g after 5 days but still above the standard recommended value of <1 HO eggs/g TS sample. The comparative number for mixed enzymes exposure were 29 viable egg/g TS after five days.

The concentration of the enzymes produced as discussed in Chapter 4 also played a role in the inactivation of the *Ascaris* spp eggs determined in this chapter. For instance, chitinase produced in Chapter 4 section 4.3.2 was found to have the highest inactivation potential and was produced at an average concentration of 3.4 mg/mL which is higher than the 1.0 mg/mL used for the inactivation experiments in Chapter 3. Chitinase (3.4 mg/mL) and lysozyme (2.7 mg/mL) produced and purified in Chapter 4 had concentration levels higher than 1 mg/mL that was used in Chapter 3 for the inactivation of *Ascaris* spp eggs. Only protease concentration produced by the microorganisms in the FS was lower than the concentration used for the earlier

inactivation experiments (0.8 mg/mL). It is therefore worth mentioning that in the environment, lower reduction in viability by protease should be expected due to the observed enzyme concentration produced under favourable controlled conditions. If less than 1 mg/mL is produced in the lab, lower concentrations will be produced in the environment. As seen in Chapter 3 section 3.3.2, 1 mg/mL is not effective at getting desirable viability reduction of the helminth eggs. High solid content has been reported to impact the action of enzymes. Previous research on cellulases has shown numerous probable pathways that contribute to the so-called solids effect, such as inhibition of product, lack of enzyme adsorption, restriction of enzyme and product diffusion, ineffective mixing, and lack of water availability (Li et al., 2020). Therefore, the solid content of the FS could have significantly reduced the enzyme action. It has been reported that pH of FS is normally in the range of 6.5-8.0, this could however be as high as 12.6 or as low as 1.5 (Padhi, 2016). These variations in pH could have significantly impacted the action of the lytic enzymes in this study. For instance, Parmar et al. (2001) found that at pH of 3-6 the action of cellulase, fungal protease and lipase was high in reduction of FS solid content. However, for alkaline protease the best pH was determined to be 9. This therefore shows that the pH of the FS in this study plays an important factor in the inactivation of the *Ascaris* spp by these enzymes.

Using the reduction percentage by exposure to single lytic enzymes, single lytic enzymes in series, mixed lytic enzymes, none of the enzymes reduced viability to less than 1 HO eggs/g dry weight. This is including the technique that achieved above 91% reduction, as reported in Chapter 3 as well. This shows that the technique to use the enzymes in series, by targeting a single layer at a time is the best method. For the single enzyme exposure in series and mixed enzymes, the most effective method was the exposure to lytic enzymes at 37°C. The impact of temperature on the enzyme action was discussed in Chapter 3.

The results presented in this chapter shows the potential of enzyme inactivation of *Ascaris* spp eggs on FS. However, it also highlights some challenges that need to be addressed in order to improve the activation of the enzymes, such as pH, temperature and impact of solids.

5.6 Conclusion

Enzyme inactivation of *Ascaris* spp eggs in FS could be achievable based on the results presented in this chapter. However, the enzyme treated FS cannot be used as a safe soil enhancer since more than 1 viable HE/g remains after exposure to the enzymes. The use of consecutive lytic enzymes at 37°C achieved the highest reduction of viable eggs/g. Heat must be applied with lytic enzymes to achieve maximum reduction. The production of lytic enzymes needs to be optimised to achieve reduction at RT and to produce a sufficient amount to use in VIP and UD toilets where other factors might hinder or reduce the effectiveness of lytic enzymes. However, some challenges that could significantly impact the field application of these enzymes were identified. These include the solids in the FS and the pH. Other factors such as the temperature and concentration of enzymes was already determined in the previous chapters.

6 CHAPTER 6: GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 General Conclusion

This study was to demonstrate the ability of lytic enzymes to inactivate the pathogenic nematode, *Ascaris* spp by targeting their egg shell during its developmental phase into an infectious worm. This is a proof of concept study that was developed after the demonstration of the lytic enzyme's ability to improve the DNA extraction from nematode eggs (Leles et al., 2009). The inert *Ascaris* spp eggshell allows it to survive harsh environmental conditions, due to the chitin middle layer that serves as the most inert structural component (Gortari et al., 2008). A combination of FS treatment methods are not able to destroy the eggshell and therefore viable eggs are always recovered from FS post treatment.

Ascaris spp eggs were recovered from FS of UDDT toilets, the FS that has a low moisture content due to the addition of dry additives after each use (Tilley et al., 2014, Espinoza et al., 2012). This is to reduce the moisture, and in turn, reduce the number of pathogens contained within the dry FS facility. Additionally, the FS is treated in a WWTP facility and dried in drying beds as secondary treatment. An amount of 976-1 118 HO eggs/g was recovered from the treated FS.

The amount of eggs recovered from the FS is more than that stipulated by the USEPA guidelines for FS re-use as a soil enhancer (Roach et al., 2012). The amount of helminth eggs for FS reuse should be 1 HO eggs/4 g of TS (Roach et al., 2012). This is a huge problem as 65-100% of households still use onsite sanitation that generates an excess amount of FS infested with pathogens (Maffo et al., 2019).

This brings the need for the development of new technologies and strategies to treat FS in order to meet the set limits to reuse FS. The lytic enzymes showed varying ability in inactivating *A.*

suum eggs. For the single use of commercial lytic enzymes, 1 mg/mL chitinase was the best enzyme to reduce the viability of *Ascaris spp* eggs by 34%. For the commercial lytic enzymes used in series and as a mixture, viability was reduced efficiently after 5 days at 37°C up to 90% and 74% respectively.

Lytic enzymes were then produced from microorganisms from the FS collected from UDDTs. The lytic enzymes produced by the microorganism were mostly detected in the crude media, this is before the partial purification steps using the ammonium sulphate (NH_4SO_2) precipitation procedure. This is in agreement with literature regarding the lytic enzymes produced by *Bacillus spp* were extracellular as *Bacillus* selective agar was used to grow the lytic enzymes producers (Zhang et al., 2014).

Protease produced was quantified to be 0.45-0.68 mg/mL with enzyme and specific activity last detected at the 40% NH_4SO_2 saturation. Chitinase produced was quantified to be 0.22-3.17 mg/mL with enzyme activity and specific activity last detected at the 40% NH_4SO_2 saturation. Optimum precipitation was observed at the 20% NH_4SO_2 saturation point due to the peak of its specific activity (0.057 U/mg). Lysozyme produced was quantified to be 0.38-2.74 mg/mL with enzyme activity and specific activity last detected at the 60% NH_4SO_2 saturation (1 195 U/mg). Optimum precipitation was observed at the 40% NH_4SO_2 saturation point (8 925.2 U/mg) due to its increase in the specific activity.

This shows that the microorganisms have the potential of producing adequate amount (concentration) of lytic enzymes that can be used to inactivate *Ascaris spp* eggs at crude level. The quantified amount of the lytic enzymes produced by the microorganisms at crude medium level is higher than the 1 mg/ml of the commercial lytic enzymes that were used for the inactivation of the *Ascaris spp* eggs described in Chapter 3, section .3.1.51-3.1.5.3. Due to the limitations of this study, the proteins could not be purified using other techniques to obtain a

higher purity. This reduced the enzyme and specific activity, especially of protease and chitinase.

The reduction in viability by commercial lytic enzymes on the purchased *Ascaris* spp eggs was applied theoretically to the *Ascaris* spp eggs recovered from the UDDT FS in Chapter 3. An assimilation exercise of the reduction in viability of *Ascaris* spp eggs in their natural environment using produced enzymes from Chapter 4 was demonstrated. The highest reduction of 90% by the use of enzyme in series at 37℃ still had >1 HE/g. This shows that with abiotic factors within FS will have a hindrance on the enzymes and reduce their effectiveness as well.

As a proof-of-concept, this study demonstrated the technique and conditions for the use of lytic enzymes for *Ascaris* spp eggs inactivation. More work and investigations need to be carried out to determine how to optimise enzyme production and the best application method of these lytic enzymes.

6.2 Recommendations

To advance the use of lytic enzymes for inactivation of STH eggs, further work is required. The following recommendations are therefore made for future research in the area;

1. ***Impact of fungal hyphae on STH:***

   Fungal hyphae is known to have different mechanisms that can penetrate the egg shell of nematodes and stop the development of larvae. The control samples used in this study, for the exposure of *Ascaris* spp eggs to single enzymes in series were observed to have fungal contamination. This led to the *Ascaris* spp eggs viability being reduced by more than 20%, this was higher than the experimental samples that had a reduction lower than 20. The fungal contamination reduced viability of the control samples at a higher rate compared to that of the experiment samples with enzymes. This study could be used to compare the ability and efficiency of fungal hyphae compared to purified or

commercial lytic enzymes in controlling the development of *Ascaris* spp eggs to infective larvae.

2. ***Higher concentration of enzymes should be tested:***

   The reduction in viability of *Ascaris* spp eggs using 1 mg/mL at RT is below 50%, except for the mixture of lytic enzymes incubated for 5 days at 37℃. A higher concentration of lytic enzymes is recommended to obtain a higher reduction in viability. This is further demonstrated by the enzyme activity and specific activity assays of commercial lytic enzymes in Chapter 4, section 4.3.1-3. The results of the commercial lytic enzyme (1 mg/mL) used for enzyme activity and specific activity assays are similar to those of the partially purified enzymes produced from the FS cultured microorganisms. The commercial lytic enzymes were expected to show higher enzyme activity by liberating amino acids at a faster rate. However, the results are similar. This suggests that a higher concentration of lytic enzymes, greater than 1 mg/mL should have been used for the inactivation of *A. suum* eggs in Chapter 3 to achieve better results in reducing viability of the *A. suum* eggs. Other purified techniques should be employed for the purification of enzymes such as dialysis and chromatography in addition to salting out.

3. ***Quantification of enzyme producing microorganisms in the FS:***

   Soil microorganisms were cultured but not quantified. Future studies could also include the identification and quantification of the bacteria present in FS that can produce lytic enzymes. Microbial cultures, especially those of fungus, should be investigated at their ability and efficiency of reducing *Ascaris* spp eggs viability. This will identify bacterial species that are much more efficient at producing lytic enzymes.

4. ***Testing of Ascaris spp egg inactivation from exposure to produced enzymes:***

   Enzymes were partially purified using the ammonium sulphate protein purification step. The produced enzyme needs to be further purified using ultrafiltration, dialysis, Ion exchange chromatography and other methods. The further purified enzymes should then be used on the *Ascaris* spp eggs to see if they can reduce their viability. The ultra-purified enzymes can then be compared to the commercial lytic enzymes. This will also help in coming up with solutions on what can be done to optimise the production of enzymes and if they can be further used in inactivating STH eggs alone.

5. ***In-situ application of lytic enzyme inactivation of STH eggs:***

   Abiotic factors hinder the activity of enzymes and shield the eggs in the environment. The FS microbial produced and purified enzymes need to be tested on FS with *Ascaris* spp eggs and other nematodes in a complex sample matrix. This will show if the impact of concentrated enzymes diminishes or is efficient. This will indicate if lytic enzymes are suited to be applied directly in non-sewered sanitation systems, WWTP treated FS or compost.

6. ***Use of fresh faecal for recovery of Ascaris spp eggs***

   The sampling methodology employed in this study is the reason for the low viability counts for the recovered *Ascaris* spp eggs from UDDT toilets. Sampling should be done on individual UDDT toilets from the top layer in the actual pits obtained high viability of *Ascaris* spp eggs.

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ANNEXURE A

Chapter 3

3.1 Materials

3.1.1 0.1% Tween 80 preparation

The detergent solution 0.1% tween 80 (Sigma Aldrich, Germany) was prepared by adding 1 mL in 999 mL of distilled water.

3.1.2 1.3 SG Zinc sulphate preparation

Zinc sulphate ($ZnSO_4$) (Radchem Products, United States of America) was prepared by dissolving 500.23 g of $ZnSO_4$ in 800 mL of distilled water. The solution's specific gravity (SG) was checked using a hydrometer and adjusted by adding more water until the SG reached 1.3.

3.1.3 Sulphuric acid preparation

0.1N sulphuric acid (H_2SO_4) (Sigma Aldrich, Germany) was prepared by adding 1.4 mL H_2SO_4 in a half filled 500 mL volumetric flask and topped up to the mark using distilled water.

3.1.4 Assessment of enzyme inactivation of *Ascaris* spp eggs

For the exposure of *Ascaris* eggs to individual enzymes at room temperature (RT), protease, chitinase (Sigma Aldrich Chemicals (Merck), Germany) and lysozyme (Amresco, USA) were dissolved in distilled water.

- To make 1 mg/mL of each enzyme, 0.010 g of each enzyme was separately dissolved in 10 mL distilled water.
- To make 1 mg/mL of proteinase-k, 0.020 g protease was dissolved in 15 mL PBS (8 g/L sodium chloride (ACE chemicals, South Africa), 0.2 g/L potassium chloride, 1.15 g/L disodium hydrogen phosphate, 0.2 g/L potassium dihydrogen phosphate).

- To make 1 mg/mL chitinase, 0.016 g chitinase was dissolved in 14 mL 2mM magnesium chloride ($MgCl_2$) (Sigma Aldrich Chemicals, Germany).
- To make 1 mg/mL lysozyme, 0.030 g lysozyme was dissolved in 30 mL 0.1 M EDTA (Ace Chemicals, South Africa). For the exposure of *Ascaris* eggs to a mixture of lytic enzymes at RT, 3 mg/mL of each of the enzymes was prepared in a buffer solution and mixed together to make a final concentration of 1 mg/mL.
- To make 3 mg/mL of each enzyme, 0.090 g protease was dissolved in 3 mL PBS (8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L disodium hydrogen phosphate, 0.2 g/L potassium dihydrogen phosphate).
- To make 3 mg/mL chitinase, 0.012 g chitinase was dissolved in 3 mL 2 mM $MgCl_2$. To make 3 mg/mL lysozyme, 0.037 g lysozyme was dissolved in 9 mL 0.1 M EDTA. This makes an initial concentration of 3 mg/mL proteinase, 4 mg/mL chitinase and 4.1 mg/mL lysozyme.
- To make 1 mg/mL of each enzyme in the mixed solution, 3 mL aliquot of each enzyme were mixed together to a final volume of 9 mL.

Table 3.1: Summary of concentrations used for the exposure of *Ascaris* eggs to enzymes.

Exposure 1: Exposure of *Ascaris* eggs to individual enzymes at RT.

Enzyme	Mass (g)	Final volume (mL)	Concentration (mg/mL)	Initial activity (units/mg)	Final activity (units/mg)
Proteinase-k	0.0111	10	1.11	23500	26085
Chitinase	0.0102	9	1.13	0.2000	0.2260
Lysozyme	0.0101	9	1.12	3.0000	3.3600

Exposure 2: For the series exposure of *Ascaris* eggs to individual enzymes at RT.

Enzyme	Mass (g)	Final volume (mL)	Concentration (mg/mL)	Initial activity (units/mg)	Final activity (units/mg)
Proteinase-k	0.019	15	1.3	23500	3055
Chitinase	0.016	14	1.1	0.2000	0.024
Lysozyme	0.030	30	1.0	3.0000	0.540

Exposure 3: For the series exposure of *Ascaris* eggs to individual enzymes at 37°C.

Enzyme	Mass (g)	Final volume (mL)	Concentration (mg/mL)	Initial activity (units/mg)	Final activity (units/mg)
Proteinase-k	0.0153	15	1.02	23500	23970
Chitinase	0.0103	10	1.03	0.2000	0.2060
Lysozyme	0.0154	12	1.03	3.0000	3.0900
Exposure 4: Exposure of *Ascaris* eggs to mixed enzymes at RT					
Enzyme	Mass (g)	Final volume (mL)	Concentration (mg/mL)	Initial activity (units/mg)	Final activity (units/mg)
Proteinase-k	0.0090	3	3.0	23500	70500
Chitinase	0.0012	3	4.0	0.2000	0.800
Lysozyme	0.0037	9	4.1	3.0000	12.300
Combined	Mass	Final volume	Concentration	Initial activity	Final activity
Proteinase-k	-	-	1.0	23500	23500
Chitinase	-	-	1.3	0.2000	0.2630
Lysozyme	-	-	1.4	3.0000	4.200
Exposure 5: Exposure of *Ascaris* eggs to mixed enzymes at 37°C.					

Enzyme	Mass (g)	Final volume (mL)	Concentration (mg/mL)	Initial activity (units/mg)	Final activity (units/mg)
Proteinase-k	0.0250	6	4.1	23500	96350
Chitinase	0.0140	3	4.6	0.2000	0.920
Lysozyme	0.0380	9	4.2	3.0000	12.600
Combined	Mass	Final volume	Concentration	Initial activity	Final activity
Proteinase-k	-	-	1.370	23500	32195
Chitinase	-	-	1.533	0.2000	0.307
Lysozyme	-	-	1.407	3.0000	4.221

3.2 Results

3.2.1 Single lytic enzyme exposure: protease

Table 3.2*: Ascaris suum* reduction from exposure to 1.11 mg/L protease

Percentage viability after exposure		Viability percentage converted		Viability reduction after exposure (%)	
1,11 mg/mL Protease-k		1,11 mg/mL Protease		1,11 mg/mL Protease	
Control	52,96	**Control**	100	**Control**	0

Control (5 days)	40,77	**Control (5 days)**	76,98	**Control (5 days)**	23,02
Test (1 day)	38,47	**Test (1 day)**	72,64	**Test (1 day)**	27,36
Test (3 days)	40,49	**Test (3 days)**	76,46	**Test (3 days)**	23,54
Test (5 days)	36,82	**Test (5 days)**	69,52	**Test (5 days)**	30,48

3.2.2 Single lytic enzyme exposure: chitinase

Table 3.3: *Ascaris suum* reduction from exposure to 1.13 mg/L chitinase

Percentage viability after exposure		Viability percentage converted		Viability reduction after exposure (%)	
1,13 mg/mL Chitinase		1,13 mg/mL Chitinase		1,13 mg/mL Chitinase	
Control	52,96	**Control**	100	**Control**	0
Control (5 days)	40,77	**Control (5 days)**	76,98	**Control (5 days)**	23,02
Test (1 day)	34,96	**Test (1 day)**	66,01	**Test (1 day)**	33,99

Test (3 days)	36,75	**Test (3 days)**	69,39	**Test (3 days)**	30,61
Test (5 days)	40,05	**Test (5 days)**	75,62	**Test (5 days)**	24,38

3.2.3 Single lytic enzyme exposure: lysozyme

Table 3.4: *Ascaris suum* reduction from exposure to 1.12 mg/L lysozyme

Percentage viability after exposure		Viability percentage converted		Viability reduction after exposure (%)	
1,12 mg/mL Lysozyme		1,12 mg/mL Lysozyme		1,12 mg/mL Lysozyme	
Control	52,96	**Control**	100	**Control**	0
Control (5 days)	42,05	**Control (5 days)**	100	**Control (5 days)**	20,61
Test (1 day)	40,78	**Test (1 day)**	76,98	**Test (1 day)**	23,00
Test (3 days)	43,10	**Test (3 days)**	66,01	**Test (3 days)**	18,63
Test (5 days)	41,92	**Test (5 days)**	69,39	**Test (5 days)**	20,84

$$Viability\ percentage\ converted = \left(\frac{Testviability}{controlviability}\right) * 100$$

Viability reduction after exposure

$$= \text{viability percentage converted} - \text{Percentage viability after exposure}$$

The equations above are used for calculations for the exposure in series and for the mixed exposure at RT and at controlled temperature,

3.2.4 Consecutive lytic enzymes exposure in series

Table 3.5: *Ascaris suum* reduction from exposure to 1 mg/L lytic enzymes in series

Viability			Viable %			% reduction		
	RT	37°C		RT	37°C		RT	37°C
Control (0 days)	51,15		**Ctrl (0 days)**	100		**Control (0 days)**	0	
control (1 day)	38,51	29,51	**Control (1 day)**	75,28	57,69	**Control (1 day)**	24,72	42,31
Control (5 days)	37,82	9,66	**Control (5 days)**	73,93	18,89	**Control (5 days)**	26,07	81,11
Test (1 day)	49,88	21,26	**Test (1 day)**	97,52	41,56	**Test (1 day)**	2,48	58,44

Test (5 days)	43,04	4,79	**Test (5 days)**	84,14	9,37	**Test (5 days)**	15,86	90,63

3.2.5 Mixed lytic enzyme exposure

Table 3.6: *Ascaris suum* reduction from exposure to 1 mg/L mixed lytic enzymes

Viability			Viable %			% reduction		
Mixed 0,1 mg/mL enzymes			Mixed 0,1 mg/mL enzymes			Mixed 0,1 mg/mL enzymes		
	RT	37°C		RT	37°C		RT	37°C
Control (0 days)	51,15		**Control (0 days)**	100,00		**Control (0 days)**	0	
Control (1 days)	25,72	21-39	**Control (5 days)**	50,28	41,82	**Control (1 days)**	49,72	58,18
Control (5 days)	24,53	18,85	**Test (1 day)**	47,95	36,85	**Control (5 days)**	52,05	63,15
Test (1 day)	28,43	18,76	**Test (3 day)**	55,59	36,68	**Test (1 day)**	44,41	63,32
Test (5 day)	27,55	12,91	**Test (5 day)**	53,86	25,23	**Test (5 day)**	46,14	74,77

Annexure B

Chapter 4

4.1 Media preparation

4.1.1 Protease bacteria: HiChrome media

Hichrome agar (Sigma Aldrich, Germany) was prepared by dissolving 25 g in 250 mL distilled water and diluted up to 500 mL final volume. The pH of the dissolved agar powder was adjusted from pH 7.46 to pH 7.20 using 0.1N sulphuric acid (Sigma Aldrich, Germany). The solution was then boiled, left to cool at room temperature and then transferred to petri dishes to solidify.

4..1.2 2.5% (v/v) selective protease milk agar

Protease selective media was prepared by dissolving 8.5 g of plate count agar powder in 500 mL distilled water. The pH of the dissolved agar powder was adjusted from pH 7.38 to pH 7.20 using 0.1N sulphuric acid (Sigma Aldrich, Germany) and autoclaved at 120 psi, for 15 minutes. An aliquot of 12.5 mL of milk 2.5 % (v/v) was added to the autoclaved dissolved agar to make 2.5% (v/v) of milk agar. The selective media was cooled at room temperature, transferred to petri dishes and stored at 0-4°C until needed.

4.1.3 1.25% (v/v) casein broth

Protease milk broth was prepared by dissolving 8 g of nutrient broth powder in 500 mL distilled water. To make 1.25% of casein media (w/v), 6 g of casein and 5 g of sodium chloride were added to the dissolved nutrient broth powder. The pH of the dissolved agar powder was adjusted from pH 6.86 to pH 6.99 using 0.1N NaOH (Sigma Aldrich, Germany) and autoclaved at 120 psi, for 15 minutes. An aliquot of 1 mL of milk 0.2 % (v/v) was added to the autoclaved

1.25% casein broth (v/v). The 1.25% casein broth (v/v) solution was cooled at room temperature and then transferred to a 250 mL conical flask before use.

4.1.4 Chitinase bacteria: Colloidal chitin

Colloidal chitin was prepared by gradually adding 20 g chitin powder (Sigma aldrich, Germany) to 300 mL of concentrated hydrochloric acid (ACE chemicals, South Africa). Chitin powder was dissolved by heating the mixture at 55°C for 30 minutes and then 30°C for the next 30 minutes with continuous stirring. After one hour, cold water was added (0-4°C) to the chitin mixture and chilled at 0-4°C for 24 hours. The mixture was centrifuged after 24 hours at 5 000 rpm for 10 minutes and re-suspended in cold water. The pH was measured and the wash process was repeated until the mixture reached pH 5.

4.1.5 1% (v/v) chitin enrichment media

1% chitin enrichment media was prepared by mixing 7 g potassium dihydrogen phosphate (Radchem (PTY) LTD, South Africa), 3 g dipotassium hydrogen phosphate (ACE chemicals, South Africa), 5 g magnesium sulphate (Sigma aldrich, Germany), 0.2 g ammonium sulphate (ACE chemicals, South Africa), 10 mL colloidal chitin, 2 mg ferrous sulphate (Ace chemicals, South Africa) and 1 mg zinc sulphate (Radchem (PTY) LTD, South Africa).

4.1.6 1% chitin agar (v/v)

250 mL of the enrichment media was mixed with 4 g of plate count agar and autoclaved at 120 psi, for 15 minutes. The selective agar was cooled at room temperature and then transferred to petri dishes and stored at 0-4°C until needed.

4.1.7 1% chitin broth (v/v)

250 mL of the 1% enrichment media was mixed with 4 g of broth powder and autoclaved at 120 psi, for 15 minutes. The 1% (v/v) chitin broth solution was cooled at room temperature and then transferred to a 250 mL conical flask and stored at 0-4°C until needed.

4.1.8 Lysozyme bacteria: Hichrome agar

To prepare Hichrome agar, 50 g of HiChrome agar was dissolved in 1 L distilled and boiled; after boiling, the agar was transferred to petri dishes. The petri dishes were stored at 0-4°C until needed.

4.1.9 0.05 % Micrococcus lysodeikticus (w/v) nutrient agar

To prepare 0.05 % *Micrococcus lysodeikticus* (w/v) nutrient agar, 17 g of plate count agar was dissolved in 500 mL distilled water. A mass of 0.5 g of *M.lysodeikticus* culture was added to the dissolved nutrient agar and autoclaved. The PCA was stored at 0-4°C until needed.

4.1.10 0.05 % *Micrococcus lysodeikticus* (w/v) nutrient broth

To prepare 0.05 % *Micrococcus lysodeikticus* (w/v) nutrient broth, 17 g of plate count agar was dissolved in 1 L distilled water. A mass of 0.5 g of *M.lysodeikticus* culture was added to the dissolved PCA and autoclaved. Nutrient broth was stored at 0-4°C until needed.

4.2 Ammonium sulphate precipitation

An ammonium sulphate (NH_4SO_2) protein precipitation lysis buffer was prepared by dissolving 6.05 g of tris-amino-hydrochloride (Tris-HCl) (ACE chemicals, South Africa) to make 50 mM Tris-HCl solution. Dithiothreitol (DTT) was prepared by dissolving 15.4 mg in distilled water to make 0.01 mM DTT. An aliquot of 10 mL was added to 500 mL Tris-HCl to make the lysis buffer. The NH_4SO_2 (RADCHEM (PTY) LTD, South Africa) was used in a dry powder form.

4.3 Enzyme assay and quantification

4.3.1 Protein quantification

Bovine serum albumin (BSA) (Sigma Aldrich, Germany) stock solution was prepared by diluting 60 mg in 30 mL distilled water; 2 mg/mL BSA is diluted to a five calibration point of (0.4, 0.8, 01.2, 1.6 and 2.0) mg/mL.

4.4Assay activity

4.4.1 Protease

For the protease activity assay, 2.5% (w/v) casein hydrolysis (Sigma Aldrich) was prepared by dissolving 12.5 g casein in 500 mL distilled water. 5% Trichloroacetic acid (TCA) (w/v) was prepared by dissolving 25 g TCA in 500 mL distilled water. A solution of 1 mg/mL protease was prepared by dissolving 15 mg protease in 15 mL 10mM sodium acetate (NaAc) (ACE chemicals, South Africa). 0.5% sodium carbonate solution (w/v) (Sigma Aldrich) was prepared by dissolving g0.5 g of sodium carbonate in 100 mL distilled water. A solution of 2 mg/mL tyrosine standard (Sigma Aldrich, Germany) was prepared by dissolving 0.1 g of the tyrosine standard in 50 mL distilled water.

4.4.2 Chitinase

For the chitinase activity assay, to make 200 mM potassium phosphate monobasic (KH_2PO_4) (Sigma Aldrich, Germany) and 2 mM calcium chloride ($CaCl_2$) (Minema chemicals, South Africa), 2 g of KH_2PO_4 and 22 mg $CaCl_2$ respectively were dissolved in in 100 mL distilled water and stored on ice. To make 1M potassium hydroxide (KOH), 5.5 g of KOH was dissolved in distilled water. To make 1.25% chitin (w/v), 0.6 g of colloidal chitin was mixed with distilled water. To make 2 mM sodium hydroxide (NaOH) (Radchem (PTY) LTD), 0.7 g NaOH was dissolved in distilled water. A mass of 12 g sodium potassium tartrate was dissolved in 2 mM NaOH. A mass of 0.4 g 3-5 dinitrosalicylic acid (Sigma Aldrich, Germany) was dissolved in 20 mL of distilled water and mixed with 2mM NaOH and then diluted up to 40 mL. A diluted

1 unit/mL of chitinase was prepared by dissolving 2 mg of chitinase in the KH_2PO and $CaCl_2$ mixture and kept on ice. B-N-Acetylglucosaminidase (Sigma Aldrich, Germany) was prepared by diluting 50 µL of the enzyme up to 50 mL. To make 2 mg/mL 4-Nitrophenyl N-acetyl-β-D-glucosamine (NAG) (Sigma Aldrich, Germany), 20 mg of NAG was dissolved in 10 mL distilled water. The enzymatic stop solution (Sigma Aldrich, Germany) was prepared by diluting the aliquot powder sachet in 118 mL distilled water.

4.4.3 Lysozyme

For the assay activity, 1 mg/mL lysozyme was dissolved in 15 mL 0.1M ethylenediaminetetraacetic acid (EDTA) (0.0558 g/15 mL) (ACE chemicals, South Africa). 0.5 mg *Micrococcus lysodeikticus* (Sigma Aldrich, Germany) culture was prepared by dissolving 1 mg in 20 mL in phosphate buffered saline. A solution of 300 mg sodium chloride (NaCl) was prepared by dissolving 90 mg NaCl in distilled water.

4.5 Results

4.5.1 Protein quantification

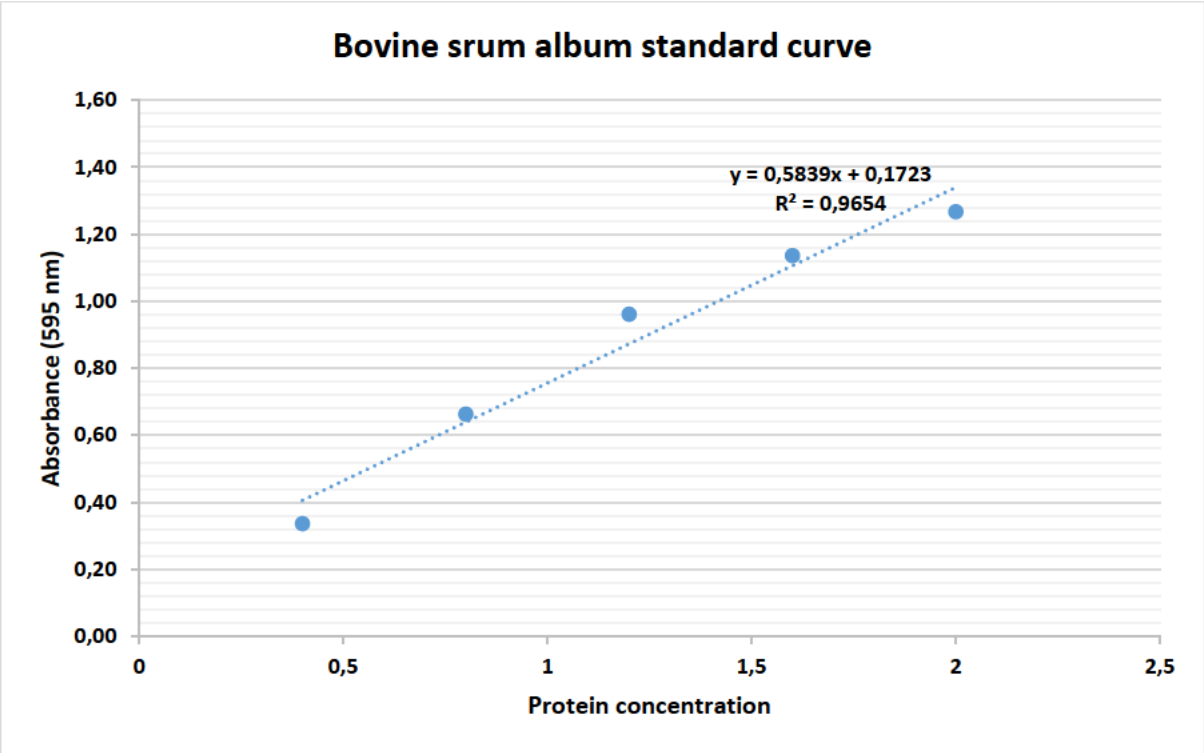

Figure 4.1: Bovine serum albumin concentrations determined using Bradford assay for the determination of protein concentrations.

4.5.2Protein quantification

Table 4.1: Quantification of enzymes using Bradford assay

Saturation	Fractionation	Protease (mg/mL)	Chitinase (mg/mL)	Lysozyme (mg/mL)
Blank	Blank	0.000	0.000	0.000
Positive control	Positive control	1.000	1.000	1.000
Crude	Crude	0,591	0,372	2,743107
0%	Pellet	0,680	3,166	2,320945
0%	Supernatant	00,438	-0,010	-0,18548
20%	Pellet	0,448	0,542	1,844836
20%	Supernatant	0,446	-0,037	0,27625

40%	Pellet	0,376	0,220	0,377119
40%	Supernatant	-0,292	-0,064	0,19147
60%	Pellet	-	-	0.596
60%	Supernatant	-	-	0.204

Chapter 5

5.1 Potential reduction in viability

The following equation was used to calculate the potential reduction of viable helminth eggs from FS using the viable reduction percentage obtained from Chapter 3, section 3.2.2-3.2.4.

$$viable\ eggs\ \left(HO\frac{g}{g}TS\right) = viable\ eggs * (reduction\ percentage)$$