PRODUCTION, PURIFICATION, AND CHARACTERISATION OF PROTEASES FROM AN ERICOID MYCORRHIZAL FUNGUS, *OIDIODENDRON MAIUS*

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MASTER OF SCIENCE IN BIOCHEMISTRY

At

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By

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FEBRUARY 2018

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science at Rhodes University. It has not been submitted before fore any degree or examination at any other university.

_____

Signed: Colleen Varaidzo Manyumwa

On this day _____ of _____

ABSTRACT

The aim of this study was to produce, purify and characterise proteases from the ericoid mycorrhizal fungus, *Oidiodendron maius* (CafRU082b/KP119480), as well as to explore their potential application in the recovery of silver from X-ray film.

Firstly, the growth of the ericoid mycorrhizal fungus, *Oidiodendron maius* (CafRU082b), was studied, and its ability to produce proteolytic enzymes was investigated. *O. maius* proved to grow well in the dark, submerged in Modified Melin Norkran's liquid medium at a pH of 5 and at 25°C. Pure cultures of the fungus were maintained on Potato Dextrose Agar (PDA). The fungus grew on PDA plates containing different substrates including haemoglobin, casein, gelatin as well as azocasein. Zones of clearance, however, were only observed on plates containing gelatin after treatment with mercuric chloride, $HgCl_2$. Proteases were successfully produced after 14 days when gelatin was incorporated into the growth medium.

After production of the proteases, purification and characterisation of the enzymes was performed. Purification of the enzymes was performed by acetone precipitation followed by ultrafiltration with 50 kDa and 30 kDa cut off membrane filters. A final purification fold of approximately 37.6 was achieved. Unusual yields of above 100% were observed after each purification step with the final yield achieved being 196% with a final specific activity of 2707 U/mg. SDS-PAGE revealed a protease band of 35 kDa which was also visible on the zymogram at approximately 36 kDa. The zymogram showed clear hydrolysis bands against a blue background after staining with Coomassie Brilliant Blue.

Physico-chemical characterisation of the protease revealed its pH optimum to be pH 3.0 and its temperature optimum 68°C. Another peak was observed on the pH profile at pH 7.0. The protease exhibited high thermostability at temperatures 37°C, 80°C as well as 100°C with the enzyme retaining close to 50% of its initial activity after 4 h of exposure to all three temperatures. All ions tested for their effects on the proteases, except Ca^{2+}, enhanced protease activity. Ca^{2+} did not exhibit any significant effect on the enzyme's activity while Zn^{2+} had the highest effect, enhancing enzyme activity by 305%. The proteases, however, were not significantly inhibited by EDTA, a metal chelating agent and a known metalloprotease inhibitor. The enzyme was classified as an aspartic protease due to complete inhibition by 25 µM of pepstatin A, coupled to its low pH optimum of 3.0. Addition of *trans*-Epoxysuccinyl-

L-leucylamido-(4-guanidino)butane (E-64), a cysteine protease inhibitor, and 2-mercaptoethanol increased protease activity.

The proteases exhibited a narrow substrate specificity towards gelatin and no other substrate. Substrate kinetics values were plotted on a Michaelis-Menten Graph and showed that the enzyme had a V_{max} of 55.25 U/ml and a K_m of 2.7 mg/ml gelatin. A low K_m indicated that the protease had a high affinity for gelatin.

Silver recovery studies from X-ray film revealed the proteases' capability to remove silver from X-ray film, leaving the film intact. The recovery of silver was perceived visually, by film observation, as well as by scan electron microscopy (SEM) images, where clearance of the film was observed after incubation with the enzyme. Energy dispersive X-ray spectroscopy (EDS) profiles also confirmed removal of silver from the film, with a Ag peak showing on the profile of the film before treatment with the proteases and no peak after treatment. The crude protease sample was, however, catalytically more efficient compared to the partially purified sample.

TABLE OF CONTENTS

DECLARATION…… i
ABSTRACT.....ii
TABLE OF CONTENTS.....iv
LIST OF FIGURES vi
LIST OF TABLES..... viii
LIST OF ABBREVIATIONS.....ix
DEDICATION.....xi
ACKNOWLEDGEMENTS.....xii
CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW.....1
1.1 MYCORRHIZAS.....1
1.1.1 ERICOID MYCORRHIZAS.....2
1.2 *OIDIODENDRON MAIUS*.....3
1.3 ENZYMES.....3
1.4 PROTEASES 4
1.4.1 CLASSIFICATION OF PROTEASES 6
1.5 RECOVERY OF SILVER FROM X-RAY FILMS USING PROTEASES.....15
1.6 RESEARCH HYPOTHESIS AND OBJECTIVES 16
CHAPTER 2 PRODUCTION OF PROTEASES BY *OIDIODENDRON MAIUS* 18
2.1 INTRODUCTION.....18
2.2 MATERIALS AND METHODS.....19
2.2.1 MATERIALS 19
2.2.2 SUBSTRATE HYDROLYSIS ON PLATES.....19
2.2.3 CULTURING OF *O. MAIUS* 20
2.2.4 ENZYME EXTRACTION 20
2.2.5 PROTEASE ACTIVITY ASSAY 20
2.2.6 PROTEIN DETERMINATION.....21
2.2.7 GROWTH KINETICS OF *O. MAIUS*.....21
2.3 RESULTS.....22
2.4 DISCUSSION 24
2.5 CONCLUSION.....26
CHAPTER 3 PARTIAL PURIFICATION OF PROTEASES FROM *O. MAIUS*.....27
3.1 INTRODUCTION.....27
3.2 MATERIALS AND METHODS.....28

3.2.1 ACETONE PRECIPITATION 28
3.2.2 SIZE EXCLUSION BY FILTRATION 29
3.2.4 SDS-PAGE 29
3.2.5 ZYMOGRAMS 29
3.3 RESULTS 32
3.4 DISCUSSION 34
3.5 CONCLUSION 36
CHAPTER 4 CHARACTERISATION OF PARTIALLY PURIFIED PROTEASES FROM *O. MAIUS* 37
4.1 INTRODUCTION 37
4.2 MATERIALS AND METHODS 39
4.2.1 PHYSICO-CHEMICAL CHARACTERISATION 39
4.2.2 SUBSTRATE SPECIFICITY 40
4.3 RESULTS 41
4.4 DISCUSSION 49
4.5 CONCLUSION 52
CHAPTER 5 RECOVERY OF SILVER FROM X-RAY FILM USING PARTIALLY PURIFIED PROTEASES FROM *O. MAIUS* 53
5.1 INTRODUCTION 53
5.2 MATERIALS AND METHODS 54
5.2.1 RECOVERY OF SILVER 54
5.2.2 SCAN ELECTION MICROSCOPY 54
5.3 RESULTS 55
5.4 DISCUSSION 59
5.5 CONCLUSION 60
CHAPTER 6 GENERAL DISCUSSION, CONCLUSION, AND FUTURE RECOMMENDATIONS 61
REFERENCES 67
APPENDICES 87

LIST OF FIGURES

Figure 1.1	Serine protease trypsin from *Streptomyces erythrea*	7
Figure 1.2	Catalytic triad in the active site of a serine protease	8
Figure 1.3	A 3-D structure of a pepsin-like protease, endothiapepsin	10
Figure 1.4	Mechanistic action of aspartic proteases	11
Figure 1.5	Structure of papain	12
Figure 1.6	Image of thermolysin isolated from *Bacillus thermoproteolyticus*	14
Figure 1.7	Structure of X-ray film	15
Figure 2.1	Zone of substrate clearance on a gelatin agar plate	22
Figure 2.2	Casein agar plates inoculated with *O. maius*	22
Figure 2.3	Bovine haemoglobin agar plates inoculated with *O. maius*	23
Figure 2.4	*O. maius* in liquid medium after growth for 2 weeks	23
Figure 2.5	Growth curve of *O. maius* and protein production over time	24
Figure 3.1	Purification steps for the partial purification of the proteases from the crude extract of *O. maius*	31
Figure 3.2	SDS-PAGE gel of partially purified enzymes with silver staining	33
Figure 3.3	Gelatin zymogram of protease fractions from *O. maius*	34
Figure 4.1	Effect of temperature on *O. maius* protease activity	42
Figure 4.2	Effect of pH on protease activity	42
Figure 4.3	Thermostability of the proteases at 37°C	43
Figure 4.4	Thermostability of the proteases at 80°C	44
Figure 4.5	Thermostability of the proteases at 100°C	44
Figure 4.6	Effect of metal ions on protease activity	45

Figure 4.7	Effect of EDTA, PMSF and 2-Mercaptoethanol on protease activity	46
Figure 4.8	Effect of pepstatin A and E-64 on protease activity	47
Figure 4.9	Michaelis-Menten Graph showing effect of substrate concentration on protease activity	48
Figure 4.10	Lineweaver-Burk plot	49
Figure 5.1	Protein concentration of silver recovery supernatant (crude samples)	55
Figure 5.2	Images of the X-ray film before and after incubation with the enzyme and after incubation with the buffer only	56
Figure 5.3	Protein concentration of silver recovery supernatant (Partially Purified Enzyme – PPE)	56
Figure 5.4	Images of the X-ray film after incubation with the partially purified enzyme fraction and after incubation with the buffer only	57
Figure 5.5	EDS profile of the X-ray film before incubation with the protease	57
Figure 5.6	EDS profile of the X-ray film after incubation with the protease	58
Figure 5.7	SEM images of the X-ray film before and after incubation with the enzyme	59
Figure II A.	Bradford standard curve for protein concentration determination	89
Figure II B.	Tyrosine standard curve for calculation of protease activity	89

LIST OF TABLES

Table 1.1	Different protease producers and examples of proteases produced	4
Table 3.1	Purification table for partially purified fractions of the protease	32
Table 4.1	Biochemical characteristics of some fungal proteases	38

LIST OF ABBREVIATIONS

°C	Degree(s) Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
BSA	Bovine serum albumin
E-64	*Trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane
ERM	Ericoid mycorrhizal
EDS	Energy dispersive X-ray spectrometry
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	Hour
kDa	Kilo Daltons
L	Litre
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MMN	Modified Melin Norkran
PDA	Potato Dextrose Agar
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM	Scanning electron microscopy

DEDICATION

I would like to dedicate this thesis to my parents, Dr C. Manyumwa and Dr E. Manyumwa. I would like to thank them for their love and unwavering moral, emotional and financial support.

Thank you for setting the pace for me mom and dad, I am following in your footsteps.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 MYCORRHIZAS

Mycorrhizal associations are mutualistic relationships between fungi and plants and are regarded as one of the oldest and most common types of plant-fungal symbiosis (Finlay, 2008; Turk et al., 2006). These interactions between plants and fungal species in the soil are highly significant and are controlled by ecological factors as well as features of the host plant and mycorrhizal fungus (Brundett, 1991). The significance of these associations is implied by the large percentage of plants that form these symbiotic relationships with fungal species (Behie and Bidochka, 2014). Mycorrhizas play an important part in facilitating the transfer of nutrients to the host plant and also assist in the removal of toxic substances from the rhizosphere (Usuki et al., 2003; Zhang et al., 2009). This symbiotic relationship is regarded as mutualistic, where the fungus breaks down organic material to provide its host with nutrients, which include nitrogen and phosphate.

Mycorrhizas are divided into two categories, which are ectomycorrhizas and endomycorrhizas. Ectomycorrhizas surround plant roots and cells whereas endomycorrhizas penetrate the root cells of the plant. The success of endophytes in their mycorrhizal associations is due to several properties, including the number of hyphae they can produce during and after root colonization, rate of hyphal growth, as well as functional hyphal features which control nutrient absorption and translocation (Brundett, 1991). After germination of its spores, the endomycorrhizal fungal hyphae develop in the direction of a host root and penetrate its root cortical cells. The coils it forms in the cortex are the site of nutrient exchange (Denison and Kiers, 2011). In exchange for enhancing the host's nutrient uptake, the fungus is provided with a carbon source from its host plant for growth (Bűcking et al., 2012). Endomycorrhizas are further divided into arbuscular, ericoid, and orchidaceous mycorrhizas (Mitchell and Gibson, 2006).

1.1.1 ERICOID MYCORRHIZAS

Approximately 1% of all plant species are known to form ericoid mycorrhizal associations (Brundett, 2009). Ericoid mycorrhizas are formed by the association between fungi and plants of the family Ericaceae which occur in a number of vegetative habitats and are naturally found in areas that are low in nutrients. Therefore, the need for ericoid mycorrhizal (ERM) fungi for growth in such environments is very significant (Finlay, 2008; Usuki et al., 2003).

The ERM association is the dominant type in plant communities at high latitude and altitude and these areas are subjugated by dwarf-shrub heath vegetation on swampy soils that are nitrogen deficient (Bardgett, 2005). The harsh environmental conditions that typify these regions hinder decomposition of plant litter, resulting in soils that are both acidic and rich in recalcitrant organic matter (raw humus) but deficient in available nutrients such as nitrogen and phosphorus (Cairney and Burke, 1998). ERM associations predominantly inhabit acidic soils that have low nutrient content but are rich in recalcitrant organic matter (Smith and Read, 2008).

ERM fungi improve growth of their host species and give them a competitive advantage over other plants by boosting their uptake of nutrients in the soil (Vohnik et al., 2005). The organic matter that builds up in the soil contains plant matter from the ericaceous hosts that is recalcitrant but compliant to degradation by ERM fungi (Wurzburger et al., 2012). These fungi are extremely effective when it comes to degrading organic sources for their hosts' nutrient supply and this is enabled by the biochemical traits they possess (Read et al., 2004). They produce an extensive range of extracellular enzymes in pure culture that could possibly contribute to plant litter degradation and nutrient procurement for the host (Wurzburger et al., 2012). They have been known to possess the capacity to take up dissolved organic nitrogen from the soil in the form of amino acids, pure protein and even highly recalcitrant proteins that are co-precipitated with tannins (Bardgett, 2005). A range of enzymes is also produced to ease hyphal penetration during root colonization (Perotto et al., 1995)

Typical ericoid fungal species include *Hymenoscyphus ericae* and *Oidiodendron* species (Zhang et al., 2009). Both *H. ericae* and some *Oidiodendron* species produce proteolytic enzymes which hydrolyse proteins into simpler amino acids which can be absorbed by the ERM fungi without the need for further deamination for the host plant (Leake and Read, 1990; Mitchell and Gibson, 2006; Wei et al, 2016).

1.2 *OIDIODENDRON MAIUS*

Oidiodendron maius Barron is an ascomycete from the class Leotiomycetes that can establish endomycorrhizal symbioses with the roots of ericaceous plants. This relationship is facultative for the fungi, because they also persist as free-living saprobes in the same habitat. Several species of *Oidiodendron*, an anamorph of the ascomycete family Myxothricacea, have been confirmed to form mycorrhizas with ericaceous plants in synthesis experiments (Vrålstad et al., 2002; Zhang et al., 2009). Among them, *Oidiodendron maius* has been frequently detected in roots of ericaceous species (Vohnik et al., 2005). *O. maius* was first identified by Barron in 1962 from collections of peat soil in Canada, and then later isolated from cultivated *Rhododendron* by Douglas et al. (1989). Subsequently, *O. maius* has been recorded as ERM endophytes of several taxa in the Ericaceae (Wei et al., 2016). It has been described as a typical ericoid mycorrhizal species in many parts of the world thus its distribution is considered to be worldwide (Wei et al., 2016; Zhang et al., 2009). Vallino et al., (2005) suggested that *O. maius* developed ways of regulating heavy metal uptake and accumulation, as well as detoxification, and this reduces metal toxicity to the host plant.

Most of the research on *O. maius* has focused on the mycorrhizal status of various species and on the ecological significance of this association (Douglas et al., 1989; Rice and Currah, 2005; Vallino et al., 2005). *O. maius* has been revealed to be capable of utilising different carbon, phosphate and nitrogen sources (Bizabani and Dames, 2016). This implies the production of enzymes such as cellulases, phosphatases and proteases. Enzymatic properties of *O. maius* have also been reported in various strains (Rice and Currah, 2005; Sigler and Gibas, 2005; Wei et al., 2016)

1.3 ENZYMES

Enzymes are described as natural catalysts that are able to facilitate reactions that occur within and outside cells (Puskas et al., 2009). A catalyst is a substance that accelerates a chemical reaction without undergoing a permanent change itself. Without enzymes, these reactions would be unable to match the pace of metabolism. James B. Sumner isolated the first enzyme in pure form in 1926, for which he won the 1947 Nobel Prize (Gurung et al., 2013).

It is crucial for organisms to be able to adjust to a shift in environmental conditions for their survival. At the metabolic level, this process of adaptation is linked to the enzyme's capacity

to develop advantageous functions in a biochemically changing environment (Cuesta et al., 2015).

Enzymes are highly specific and can be recognized and classified according to their catalytic functions - unlike functional proteins which require direct detection (Bisswanger, 2014). Their use in industry is environmentally friendly as it reduces the amount of pollution and waste produced (Jegannathan and Nielsen, 2013).

1.4 PROTEASES

Proteases are enzymes that hydrolyse peptide bonds. They perform functions that are either regulatory or non-specific within the cellular environment. Regulatory proteases are involved in selective degradation of proteins; for instance during apoptosis or cell recognition, and non-specific proteases perform general proteolysis (Cuervo et al., 2008). Proteases are regarded as one of the most important groups of enzymes as they account for approximately two-thirds of the world's total enzyme sales (Souza et al., 2015). They are produced by a variety of sources as shown in the Table 1.1.

Table 1.1 Different protease producers and examples of proteases produced

Protease source	**Example**	**Reference**
Animal	Trypsin, a main intestinal digestive enzyme.	Rao et al. (1998)
Plant	Bromelain, from pineapple stem and juice.	Lopez-Garcia et al. (2012)
Virus	Human immunodeficiency virus type 1 (HIV-1) protease which is a retroviral aspartyl protease necessary for viral replication.	Zhang et al. (2008)
Bacteria	Thermolysin from *Bacillus thermoproteolyticus.*	Krimmer et al. (2014)
Fungi	Endothiapepsin, produced by an ascomycete fungus, *Cryphonectria parasitica.*	Choi et al. (1993)

Micro-organisms such as bacteria and fungi are recognised as protease producers. They have become the preferred source of proteases for use in industry because of their ability to grow quickly and they also possess easily genetic manipulated properties that are required for their application (Rao et al., 1998; Sawant and Nagendran, 2014).

Proteases have a vast number of applications in various industries which include the leather, pharmaceutical and food industries. They are able to degrade non-collagenous constituents of the skin and are used in the dehairing of leather (Jisha et al., 2013; Singh et al., 2016). Processing of leather has normally involved the use of toxic chemicals like sodium sulphide, causing pollution. The use of proteases in leather processing reduces soaking time and the amount of waste that is generated (Jisha et al., 2013; Singh et al., 2016). Proteases for leather industry applications have been isolated from bacteria such as *Bacillus subtilis* (Hammeed et al., 1996), *Pseudomonas aeruginosa MCM B327* (Zambare et al., 2013), as well as fungi such as *Aspergillus tamarii* (Dayandaan et al., 2003).

Given their roles in many cell functions, proteases are considered as potential therapeutic drug targets or biomarkers for a number of diseases. These cell functions include apoptosis, inflammation, DNA replication and transcription, to name a few (Lopez-Otin and Bond, 2008). High levels of proteases potentially lead to a range of physiological processes eventually resulting in disease states. In such cases, the discovery of compounds or inhibitors that are capable of restoring the standard protease levels in the body are regarded as prospects for drug development (Palmer et al., 1995). Proteases are thus studied as diagnostic biomarkers or drug targets in diseases such as cancer; AIDS and cardiovascular diseases (Lopez-Otin and Bond, 2008; Turk, 2006).

Alkaline proteases are primarily used as detergent additives in the production of environmentally friendly detergents (Jisha et al., 2013). The application of these enzymes in detergents is due to their ability to hydrolyse stains from food, blood and other body secretions. In the absence of the proteases, the protein-natured dirt coagulates on the fabric (Kumar et al., 2008; Sawant and Nagendran, 2014). Alkaline proteases therefore, improve washing performance in domestic laundering. Alkaline proteases are also used in the food industry for various purposes such baking, preparation of soya hydrolysates, meat tenderization as well as in tonics as digestive aids (Jisha et al., 2013).

Acid proteases, also known as aspartic proteases, are used as milk clotting agents in the cheese manufacturing industry (Claverie-Martin and Vega-Hernandez, 2007; Theron and Divol,

2014). They have also shown potential use in the winemaking industry, by removing the heat-induced haze-forming proteins that are produced during storage or transportation (Reid et al., 2012; Van Sluyter et al., 2013).

Feathers make up to as much as 8% of a bird's body weight and are considered to contain more than 90% protein in the form of keratin (Jisha et al., 2013; Roh et al., 2012). In the poultry industry they are considered an agricultural waste product, but they can be hydrolysed using proteases releasing ample protein hydrolysate which could be used as a feed additive or in the production of foils as well as encapsulates (Jisha et al., 2013; Mokrejs et al., 2011; Saravanan, 2012). Degradation of keratin in feathers has been reported (Eslahi et al., 2014; Mazotto et al., 2013). Proteolytic enzymes are also applied in the degradation of gelatin on X-ray films for the recovery of silver (Cavello et al., 2013; Shankar et al., 2011).

Some proteases display high specificity toward an exclusive peptide bond of a particular protein. However, most proteases are rather non-specific for substrates, and some are blatantly promiscuous and hydrolyse numerous substrates in an unselective manner (e.g. proteinase K) (Lopez-Otin and Bond, 2008).

1.4.1 CLASSIFICATION OF PROTEASES

Proteases are divided into two major groups: exopeptidases and endopeptidases, depending on their site of action. Exopeptidases hydrolyse the peptide bonds closest to the amino or carboxyl termini of the substrate, whereas endopeptidases cleave peptide bonds distant from termini of the substrate (Ire et al., 2011; Rao et al., 1998). All viral encoded proteases are known to be endopeptidases. Proteases are further divided into four groups on the basis of the functional groups present at the active site. These groups are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Ire et al., 2011; Polgar, 1989; Rao et al., 1998).

1.4.1.1 Serine Proteases

Serine proteases are the most abundant and functionally diverse group of proteases, consisting of more than 33% of all known proteolytic enzymes. They are classified into 13 clans and 40 families and are normally endoproteases (Di Cera, 2009). Their name arises from the nucleophilic serine residue contained in the active site which reacts with organophosphorus compounds (Di Cera, 2009; Hartley 1960). Serine proteases include chymotrypsin, trypsin as well as elastase.

Trypsin-like serine proteases require activation of their inactive zymogen pre-cursor by peptide hydrolysis. Proteolytic processing of the pre-cursor occurs at the identical position in all known members of the family (Di Cera, 2009; Hedstrom, 2002). Chymotrypsin-like proteases take part in many physiological processes including apoptosis and digestion (Hedstrom, 2002).

Fig 1.1 shows an example of a serine protease, trypsin that was isolated from the bacterium *Streptomyces erythrea.*

Figure 1.1 Serine protease trypsin from *Streptomyces erythrea* (PDB 4m7g) (Blankenship et al., 2014).

Serine proteases take part in fungal pathogenesis by evading the host's immune system. They do this by degrading chitinases that target the fungal cell wall (Muszewska et al., 2017). The specific catalytic mechanism of serine proteases is dependent on the tertiary structure of the triad constituents, which are found in the active site of the protease. The triad seen in Fig 1.2 contains three amino acids, namely, His-57, Ser-195 and Asp-102 bonded in a network fashion. In the primary protein structure, the amino acids are far from each other but move closer to each other after the enzyme is folded (Poddar et al., 2017).

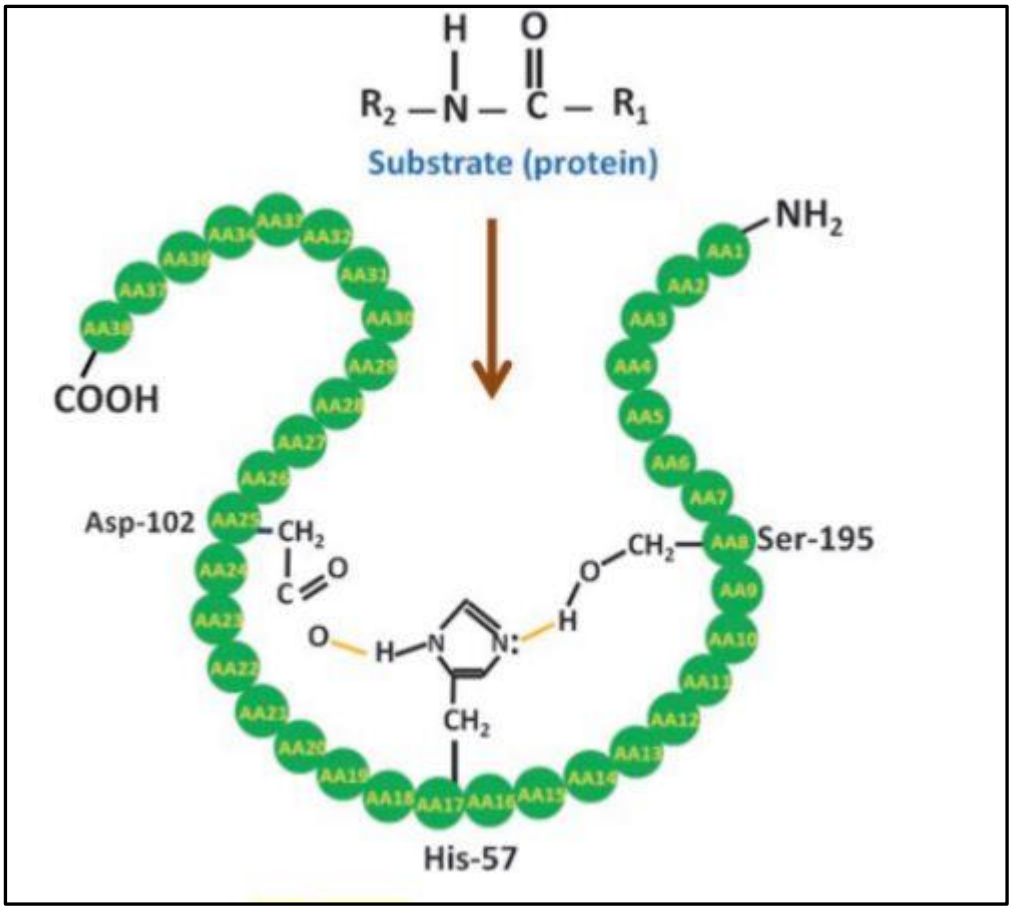

Figure 1.2 Catalytic triad in the active site of a serine protease comprising an aspartic acid residue (Asp-102), histidine (His-57) and serine residue (Ser-195). Adapted from Poddar et al. (2017).

The catalytic mechanism of serine proteases involves the formation of a covalently bonded unstable enzyme-peptide intermediate, and then the intermediate is stabilised, releasing the peptide fragment (Poddar et al., 2017). In the first step, which is acylation, the substrate binds to the protease's active site. A tetrahedral intermediate is the formed due to nucleophilic attack of serine on the carbonyl part of the peptide. The acyl-enzyme intermediate is then formed by the breakage of a peptide bond of the substrate. The next step is de-acylation, where water stabilises the cleavage peptide of carbonyl carbon giving rise to a new tetrahedral intermediate with the nitrogen of the histidine. The product is then released freeing up the active site (Poddar et al., 2017). The positive charge on the histidine residue on the active site is suggested to be stabilised by the hydrogen bond between the histidine and an aspartate that is negatively charged (Katona et al., 2002).

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), di-isopropyl fluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-l-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site (Rao et al., 1998). Serine proteases inhibited by PMSF have been identified from the fungus *Aspergillus flavus,* as well as from the nematophagous fungus, *Lecanicillium psalliotae* (Kranthi et al., 2012; Yang et al., 2005).

1.4.1.2 Aspartic proteases

Aspartic proteases are a subfamily of endopeptidases which are distinguished by their acidic pH optima (Hartley, 1960; Hsiao et al., 2014; Rao et al., 1998). They are also known as acid proteases and their catalytic activities are dependent on aspartic residues (Rao et al., 1998). Previous studies have revealed that the enzymes of the pepsin family are bilobed molecules with the active-site cleft located between the lobes with each lobe contributing one of the pair of aspartic acid residues, that is essential for the catalytic activity (Blundell et al., 1991; Sielecki et al., 1991). The lobes arise by gene duplication and are consequently homologous to one another. The molecular masses of acid proteases are normally in the range of 30 to 45 kDa and they are inhibited by pepstatin (Rao et al., 1998). Aspartic proteases are also sensitive to diazoketone compounds like diazoacetyl-dl-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions (Saravanamuthu, 2010; Rao et al., 1998.

Figure 1.1 is an example of an aspartic protease of the pepsin family, endothiapepsin, with a bi-lobal structure and active site between two lobes.

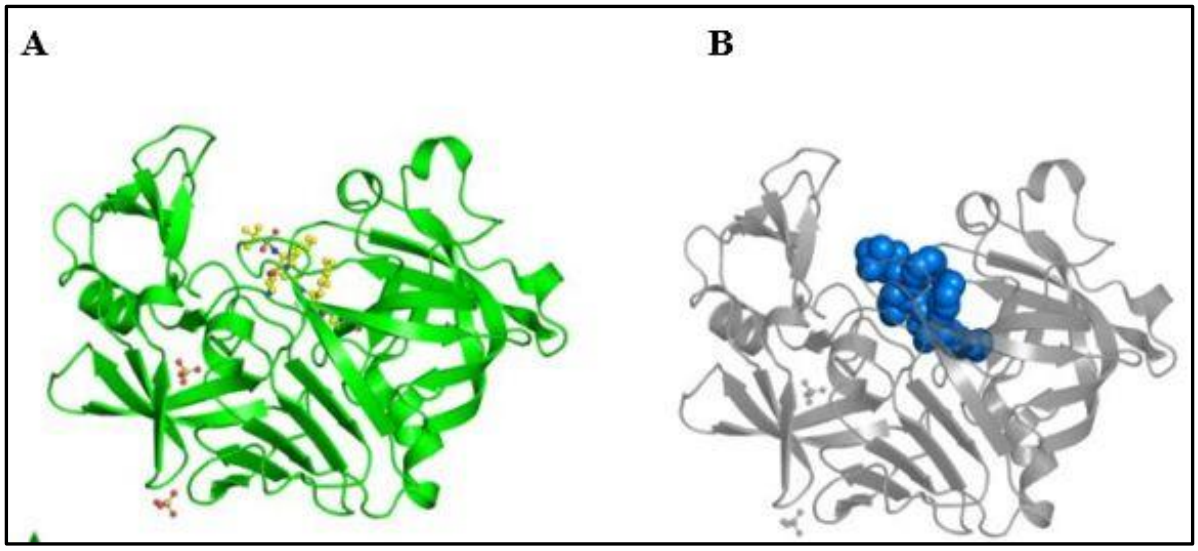

Figure 1.3 A 3-D structure of a pepsin-like protease, endothiapepsin (PDB 1od1). The protease was isolated from the fungus *Cryphonectria parasitica* (Coates et al., 2003). The non-polymetric entities are the gem-diol inhibitor PD-135,040, which is highlighted in blue in Panel B, and two sulfate ions.

Structural and kinetic studies of aspartic proteases have shown the participation of a lytic water molecule in a general acid-base catalysis mechanism (Rao et al.,1998). This mechanism is shown in Figure 1.2, with E being the bi-lobed enzyme and S as the substrate.

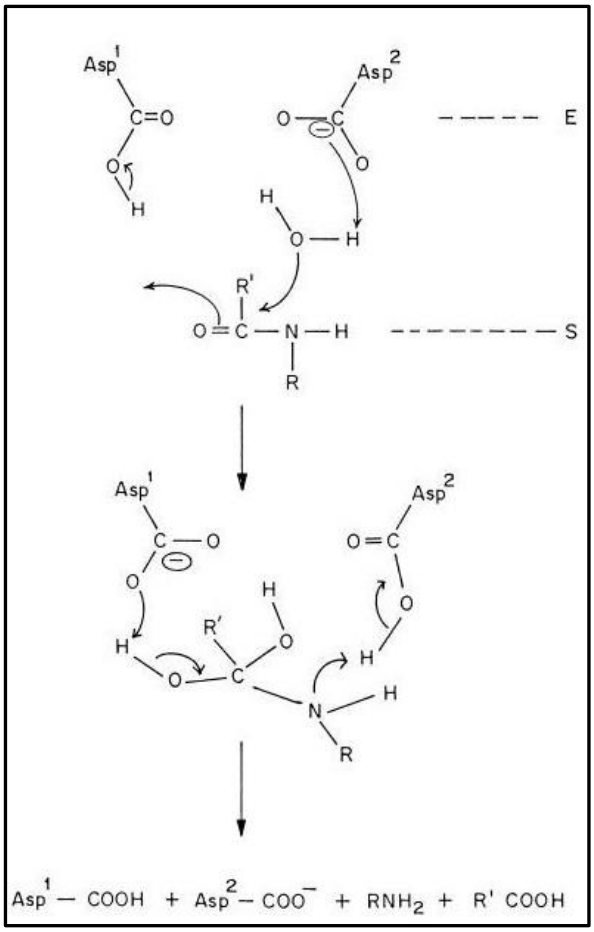

Figure 1.4 Mechanistic action of aspartic proteases. The activation of a water molecule by an aspartate residue of the protease active site is shown, facilitating a nucleophilic attack by the water molecule on the carbonyl carbon of the substrate. Adapted from Rao et al. (1998).

1.4.1.3 Cysteine proteases

This group of proteases hydrolyses carboxylic acid derivatives through a double-displacement pathway which comprises general acid-base formation and hydrolysis of an acyl-thiol intermediate (Menon and Rao, 2012; Rao et al., 1998). It contains both endo and exopeptidases (Bugg, 2012). Cysteine proteases are characterised by the presence of cysteine and histidine residues in the active centre, with papain-like cysteine proteases being complemented with asparagine at the catalytic centre (Ryzchon et al., 2004). They are produced as zymogens with a pro-domain that prohibits access to the substrate active site to prevent unnecessary protein hydrolysis (Grzonka et al., 2001).

Cysteine proteases have been identified in bacteria such as *Clostridium histolyticum*, as well as in the fungus *Aspergillus flavus* (Grzonka et al., 2001). They are typified by papain, which is characterised by a two-domain structure which forms the active site of the enzyme (Figure 1.5). Papain and cathepsins belong to the most abundant family of cysteine proteases (Verma et al., 2016).

These enzymes have broad specificity and also attack amide ester, thiol ester, and thiono ester bonds. The enzyme initially binds noncovalently to the substrate, after which acylation of the enzyme occurs together with release of the first product. Water reacts with the acyl enzyme, releasing the second product through de-acylation. (Menon and Rao 2012).

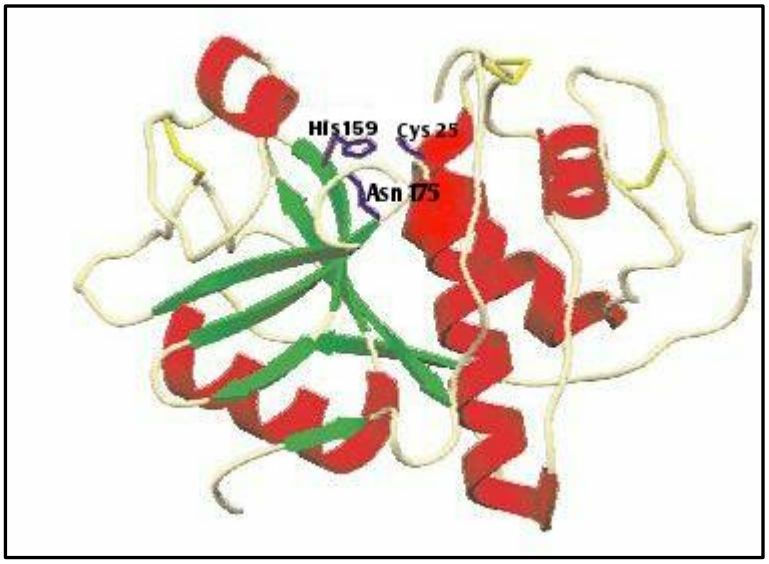

Figure 1.5 Structure of papain showing a single protein chain folded to form two domains delimiting a cleft for the purpose of substrate binding. Adapted from Grzonka et al. (2001).

A characteristic feature of the papain molecule is its bi-nuclear nature, which implies that the molecule is constructed around two hydrophobic cores. Fig 1.5 shows the folding of the main chain into two virtually independent lobes. Between the lobes, there is a large groove which can accommodate polypeptide substrates consisting of several amino acid residues. The side chain of the essential cysteine residue (Cys-25) (Fig 1.5) is found in the groove. The sulfur atom of Cys-25 is in the plane of the imizadole ring of histidine (His-159), which is situated on the opposite wall of the groove, in a β-sheet at the surface of the right domain of the enzyme (Polgar, 1989; Vernet et al., 1995). The high nucleophilicity of the sulfur atom of the cysteine residue is a vital characteristic of cysteine enzymes, including papain, because at pH values where the enzyme is active, the sulfur atom is present as a thiolate anion (Vernet et al., 1995).

The presence of a conserved aspargine residue (Asn-175) (Fig 1.5) close to the catalytic His-159 (-creating a Cys-His-Asn triad in cysteine proteases) is regarded as analogous to the Ser-His-Asp arrangement found in serine proteases (Rao et al., 1998; Saravanamuthu, 2010; Vernet et al., 1995).

Cysteine proteases catalyse the hydrolysis of peptide, amide ester, thiol ester, and thiono ester bonds (Mehta, 2010; Polgar, 1990; Rao et al., 1998). They implement a hydrolysis mechanism that involves the establishment of a general acid base followed by the hydrolysis of an acyl-thiol intermediate (Mehta, 2010; Rao, 1998). The first step of the reaction comprises the formation of the enzyme-substrate complex by the covalent binding of the substrate and the enzyme. The first product, which is the amine R'-NH_2, is then formed and released following the acylation of the enzyme. The final step is the de-acylation step, where the acyl-enzyme reacts with a water molecule resulting in the release of the second product and the enzyme is freed (Mehta, 2010).

Inhibitors of cysteine proteases include epoxysuccinates such as *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), and the effects are irreversible (Grzonka et al., 2001; Siklos et al., 2015). E-64 was first isolated from the fungus, *Aspergillus japonicus* in 1977 (Hanada et al., 1978). Epoxysuccinates are discriminatory towards cysteine proteases because of the nucleophilicity of the active site cysteine. The cysteine residue is irreversibly modified by the epoxide, resulting in the formation of a thioether bond (Siklos et al., 2015). Other inhibitors of cysteine proteases include allyl sulfones and fluoromethyl ketones (Fennell et al., 2013; Rasnick, 1985). Simple agents like iodoacetate, diisopropyl fluorophosphate as well as *N*-ethylmaleimide can also be used as cysteine protease inhibitors as they irreversibly alkylate or acylate the active site of cysteine proteases (Siklos et al., 2015).

1.4.1.4 Metalloproteases

Metalloproteases are a very diverse group made up of both endo- and exo-peptidases characterised by the presence of a divalent metal ion (usually zinc but sometimes manganese or cobalt) at their active site (Hooper, 2002; Rao et al., 1998). They are essential in biological processes such as cell proliferation, cell migration and re-modelling thus making them important in the patho-physiology of diseases such as cancer, arthritis and cardiovascular diseases (Chang and Werb, 2001; Nagase; 2006; Ramos and Selistre-de Araujo, 2001).

The metal ion, usually zinc, at the active center, is tetrahedrally coordinated by the three amino acid ligands and a water molecule. The zinc ion enhances the nucleophilicity of a water molecule and polarises the peptide bond to be cleaved prior to nucleophilic attack (Adekoya and Sylte, 2013; Grandi and Galli, 1992). Thermolysin is a representative zinc metalloprotease, which was originally isolated from the bacterium *Bacillus thermoproteolyticus* (Adekoya and Silte, 2013; Grandi and Galli, 1992; Inouye et al., 2007) (Figure 1.6). It is used in the industry for the enzymatic synthesis of *N*-carbobenzoxy L-Asp-L-Phe methyl ester (ZDFM), a precursor of an artificial sweetener, aspartame (Inouye et al., 2007; Reddy, 1991).

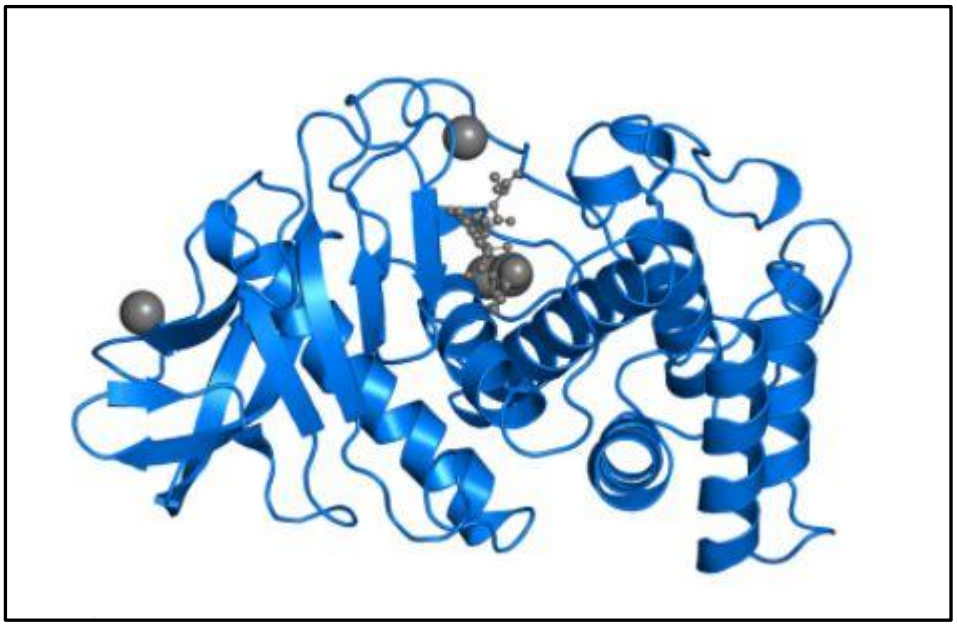

Figure 1.6 Image of thermolysin (PBD 4tmn) isolated from *Bacillus thermoproteolyticus* (Holden et al., 1987). It contains non-polymetric entities including calcium ions and a zinc ion.

Another well studied metalloprotease is the pancreatic enzyme, carboxypeptidase A. It contains one zinc ion bound to a single polypeptide chain of 307 amino acids. Its biological function involves the hydrolysis of C-terminal amino acids from polypeptide substrates, and it exhibits a preference toward those substrates possessing large hydrophobic C-terminal side chains such as phenylalanine, tryptophan or leucine (Barber and Fisher, 1972; Christianson and Lipscomb, 1989). Important residues for catalysis and binding include Glu-270, Arg-71, Arg 127, Asn-

144, Arg-145, Tyr-24S, Zn^{2+} and the zinc bound water molecule (Christianson and Lipscomb, 1989).

They can be inactivated by the addition of chelating agents such as ethylenediaminetetraacetic acid (EDTA) (Rao et al., 1998). Metalloproteases are also inhibited by 1,10-Phenanthroline, which is particularly effective against zinc metalloproteases, bestatin as well as phosphoramidon (Hooper, 2002). Metalloproteases have been identified in some fungal species, such as the thermophilic fungus *Thermoascus aurantiacus* (Merheb-Dini et al., 2009) and *Microsporum canis* (Brouta et al., 2001). Sumantha et al., (2005) reported a metalloprotease inhibited by chelating agents EDTA and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) from the fungus *Aspergillus oryzae* NRRL 2217.

1.5 RECOVERY OF SILVER FROM X-RAY FILMS USING PROTEASES

Approximately 20% of the world's silver needs are provided by the recycling of photographic waste (Parpalliwar et al., 2015). X-ray films contain about 1.5 to 2% ratio of silver in gelatin-coated film made on a polyester base layer (Fig 1.3).

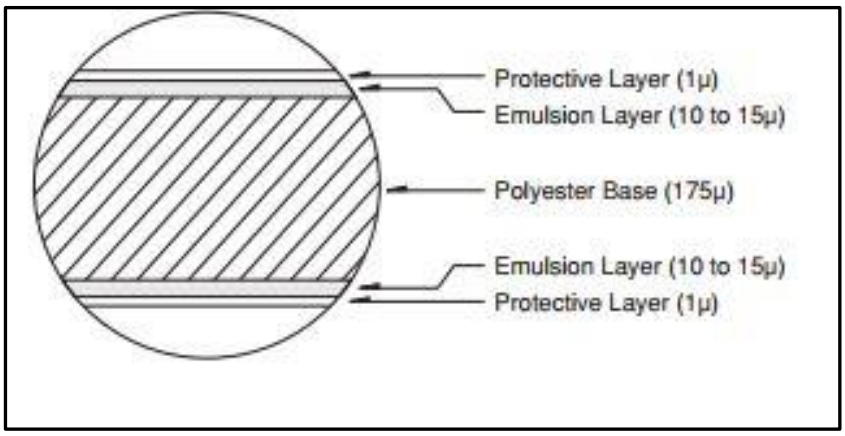

Figure 1.7 Structure of X-ray film (Adapted from FujiFilm, 2009)

The X-ray film is made of a blue-tinted polyester base that is covered by an emulsion layer on both sides. The emulsion layer contains silver halides and gelatin, and is covered by another layer containing gelatin called the protective layer (FujiFilm, 2009).

The present method of recovery of silver from photographic waste is that of directly burning the films, which causes environmental pollution and produces a foul smell (Al-Abdalall and

Al-Khaldi, 2016; Cavello et al., 2013). Another disadvantage of the incineration method is that the polyester base cannot be recovered (Cavello et al., 2013). Moreover, it is an expensive method to recover silver because of the cost of maintaining the furnace, energy requirements and treatment of the effluent soot and smoke (Cavello et al., 2013).

Since silver is linked to gelatin in the emulsion layer, it is possible to break this layer and release the silver using proteases (Nakiboglu et al., 2001). Using this method, the silver in the X-ray films is recovered and the polyester base is left intact, thus causing less pollution. For this reason, recovery of silver using proteolytic enzymes from a microbial source is a method that is currently being explored (Al-Abdalall and Al-Khaldi, 2016; Choudhary, 2013; Parpalliwar et al., 2015).

1.6 RESEARCH HYPOTHESIS AND OBJECTIVES

Proteases make up the largest percentage of industrial enzyme sales in the world and the need for more novel sources of proteases is constantly growing. Several ericoid mycorrhizal fungi have been identified as protease producing species. *Hymenoscyphus ericae*, which was isolated from acidic soils, was shown to produce an acid protease, active at an optimum pH of 2.2 (Leak and Read, 1990). Yang et al. (2004) reported protease activity from two ERM fungi from low bush blueberry roots, *Hymenoscyphus ericae* (UAMH 9264) and *Oidiodendron maius* (UAMH 9263).

Previous studies have shown protease production by *O. maius*, and there have been reports on the proteolytic activities of this fungus. Biochemical characterisation of the proteases produced by this fungus is necessary to identify possible desirable characteristics for further research and their potential use in industry, and also to further understand the habitats occupied by the fungus.

The aims of this project were as follows:

1. To culture *O. maius* in liquid media
2. To obtain crude extracts of the extracellular proteases from the cultures
3. To determine protease activity and protein concentration in crude extracts
4. To partially purify the crude extracts
5. To determine the molecular weights of the partially purified proteases
6. To characterise the proteases fractions with respect to;

a) pH
b) temperature optima and stabilities
c) metal ions
d) protease inhibitors
e) substrate specificity

7. To investigate the potential use of the crude and purified enzymes in the recovery of silver from X-ray films.

CHAPTER 2

PRODUCTION OF PROTEASES BY *OIDIODENDRON MAIUS*

2.1 INTRODUCTION

Protease production by micro-organisms is usually extracellular with the organism secreting the enzymes into the surrounding liquid medium (Souza et al., 2015). This facilitates the recovery and purification of microbial proteases in comparison to those from animal and plant sources, where they normally occur in extremely low concentrations. (Savitha et al., 2011). The importance of extracellular proteases is to degrade proteins that are present outside the cell for purposes of absorption of the products by the organism (Gupta et al., 2002).

Proteases that originate from fungi are preferable compared to those of bacterial origin because fungal mycelia can be removed by filtration without difficulty (Souza et al., 2015). Another advantage they offer is that the use of fungi as enzyme producer is regarded as safer than the use of bacteria, because they are usually recognised as GRAS (generally regarded as safe) micro-organisms (Ire et al., 2011). Fungal enzyme producers also have the advantage of utilising cheap substrates and secreting large quantities of enzymes into culture media which could possibly simplify downstream processing (Liu et al., 2013; Souza et al., 2015).

Production of the enzyme is often linked to the growth of the micro-organism, therefore the construction of a growth curve before harvesting of the enzymes is important (Bridge, 1996). A growth curve serves to illustrate the different growth phases of an organism and this can be achieved by determining the organism's biomass at various time intervals. Enzymes are produced at various stages of an organism's growth and incubation time of the organism will determine the amount of enzymes that are released into the medium (Bridge, 1996). It is of great importance to always harvest the enzyme at the same growth stage to increase precision and reduce variability amongst the experiments.

Growth of a micro-organism requires a fitting medium containing various growth requirements that provide a sufficient energy source for the organism and mimicking its natural growth environment in the process (Costa et al., 2002). The conditions in which it grows are also

important for production of the required enzymes (Souza, 2015). Several factors are responsible for the stimulation of protease production by the micro-organism, including: pH, temperature, inoculum density, as well as incubation time (Basu et al., 2015; Souza, 2015). Other requirements comprise a nitrogen source, the presence of metal ions (and their concentrations), as well as the presence of simple sugars like glucose (Bizabani and Dames, 2016; Gupta et al., 2002; Souza 2015).

The aims of this chapter were to optimise the growth of *O. maius*, develop activity assays and to confirm protease production in the liquid medium. The development of activity assays is important when studying enzymes for purposes of quantifying their presence in the medium. This is normally achieved by quantifying the amount of product formed during the reaction. Identification of proteases is made possible by their catalysed reactions with proteinaceous substrates such as casein, gelatin, haemoglobin, keratin or bovine serum albumin (BSA) where the accumulation of the product is measured (Bisswanger, 2014).

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Gelatin was obtained from Fluka Analytical. Folin's reagent and other chemicals were obtained from Merck. A pure culture of *Oidiodendron maius* (Isolate code CafRU082b/GeneBank accession number KP119480) was obtained from Professor J. Dames (Department of Biochemistry and Microbiology, Rhodes University) and UV spectroscopy was performed on a Powerwave X-1 (BioTek Instruments Inc.).

2.2.2 SUBSTRATE HYDROLYSIS ON PLATES

Pure cultures of *O. maius* (CafRU082b) were maintained on Potato Dextrose Agar (PDA) plates and grown in a 28°C incubator. The fungus was tested for hydrolysis of gelatin, casein and haemoglobin using plate assays. For gelatin plate assays, 1% (w/v) gelatin was dissolved in 100 ml of PDA and autoclaved at 121°C for 15 minutes. After cooling to approximately 50°C, the medium was poured into plates and allowed to set. The plates were inoculated with one 5 mm plug of fungal culture each and allowed to grow at 28°C in the dark. Casein and haemoglobin plates were treated similarly, except that casein plates contained 0.5 % (w/v)

casein and haemoglobin plates contained 0.2% (w/v) bovine haemoglobin in place of the gelatin. After growth for 14 days, gelatin and some haemoglobin and casein plates were flooded with 12.5 % (w/v) mercuric chloride ($HgCl_2$) dissolved in 2 M hydrochloric acid (HCl) and checked for zones of clearance after 1 h. Remaining haemoglobin and casein plates were stained with 0.1% amido-black dissolved in methanol:acetic acid (30%:10% v/v) and observed for zones of clearance (Vermelho et al., 1996). All treatments were performed in duplicate.

2.2.3 SUBMERGED CULTURING OF *O. MAIUS*

The fungus was sub-cultured in Modified Melin Norkran's media (MMN) containing 10 g/l tryptone; 3 g/l gelatin; 3 g/l yeast extract; 1 g/l malt extract; 1 g/l glucose; 0.25g/l ammonium phosphate; 0.5 g/l potassium phosphate; 0.15 g/l magnesium sulphate; 0.05 g/l calcium chloride; 0.025 g/L sodium chloride; 0.003 g/l zinc sulphate; 1.2 ml of 1% iron chloride solution and 100 µg/l of thiamine hydrochloride (Marx, 1969; Rossi and Oliveira, 2011).

The pH was adjusted to 5.5 using 1 M HCl and the medium was autoclaved at 121°C for 15 min. One plug of the fungal culture was inoculated for every 50 ml of MMN broth and the Schott bottles were covered in aluminium foil. Cultures were grown at 25°C with continuous shaking at 150 rpm.

2.2.4 ENZYME EXTRACTION

After growth of the fungus, the fungal biomass was separated from the growth medium by vacuum filtration using Whatman number 1 filter paper. This filtrate was then regarded as the crude enzyme extract.

2.2.5 PROTEASE ACTIVITY ASSAY

Crude enzyme extract (100 µl) was added to the substrate gelatin (50 µl, 0.65% (w/v) in potassium phosphate dibasic trihydrate buffer (0.05 M, pH 7.0) and the mixture was incubated at 37°C for an hour. The reaction was terminated by the addition of trichloroacetic acid (TCA) (250 µl, 10% (w/v)) and centrifuged at 8000 x g. To the supernatant (200 µl), 500 mM sodium carbonate (500 µl) was added, immediately followed by Folin Ciolcateau's reagent (100 µl) and the absorbance was read after 30 minutes at a wavelength of 660 nm using a Powerwave X-1 (BioTech Instruments Inc.) (Cupp-Enyard, 2008; Pant et al., 2015). Controls containing crude enzyme extract only and substrate only were maintained. One unit of enzyme was defined

as the amount of enzyme required to generate 1 µmol of tyrosine per ml per minute under the assay conditions (Equation 1 and 2) (Cupp-Enyard, 2008). Tyrosine concentrations of 27.5 to 275 µM were used for the construction of a tyrosine standard curve (Appendix IIB).

$$\text{Activity (U/ml)} = \frac{\text{µmol of tyrosine equivalent x total volume of assay (ml)}}{\text{volume of enzyme used (ml)x time of assay (minutes)x volume used in colorimetric determination (ml)}}$$

…**Equation 1**

Specific activity was calculated as follows:

$$\text{Specific activity (U/mg)} = \frac{\text{Activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

…**Equation 2**

Equations adapted from Cupp-Enyard, (2008).

2.2.6 PROTEIN DETERMINATION

The protein concentration of samples was measured using Bradford's method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard, and the standard curve was prepared for protein concentrations from 0 to 1 mg/ml (See Appendix IIA). The sample to Bradford's reagent volume ratio was 25:235 µl and the absorbance was read at 595 nm.

2.2.7 GROWTH KINETICS OF *O. MAIUS*

To determine the growth kinetics of the fungus, 10 flasks containing MMN medium, supplemented with 0.3% (w/v) gelatin, were inoculated with *O. maius*. One flask was removed at two-day intervals and the mycelium was harvested by vacuum filtration using pre-weighed Whatman filter paper. The mycelium was washed using Milli-Q water and dried at 60°C for 24 hours. After 24 hours, the mycelium was weighed to determine the dry biomass.

The protein concentrations of the supernatants were determined at each interval using the methods described in section 2.2.6.

2.3 RESULTS

The plates containing gelatin showed a zone of clearance around the fungus after flooding them with mercuric chloride (Figure 2.1).

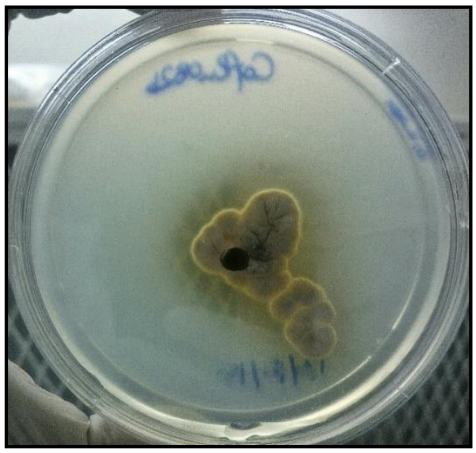

Figure 2.1 Zone of substrate clearance on a gelatin agar plate showing gelatin hydrolysis.

No zone of substrate clearance was observed on the casein plate flooded with mercuric chloride (Figure 2.2 A) but the casein plate stained with 0.1% amido-black (Figure 2.2 B) showed a slight but negligible zone of clearance.

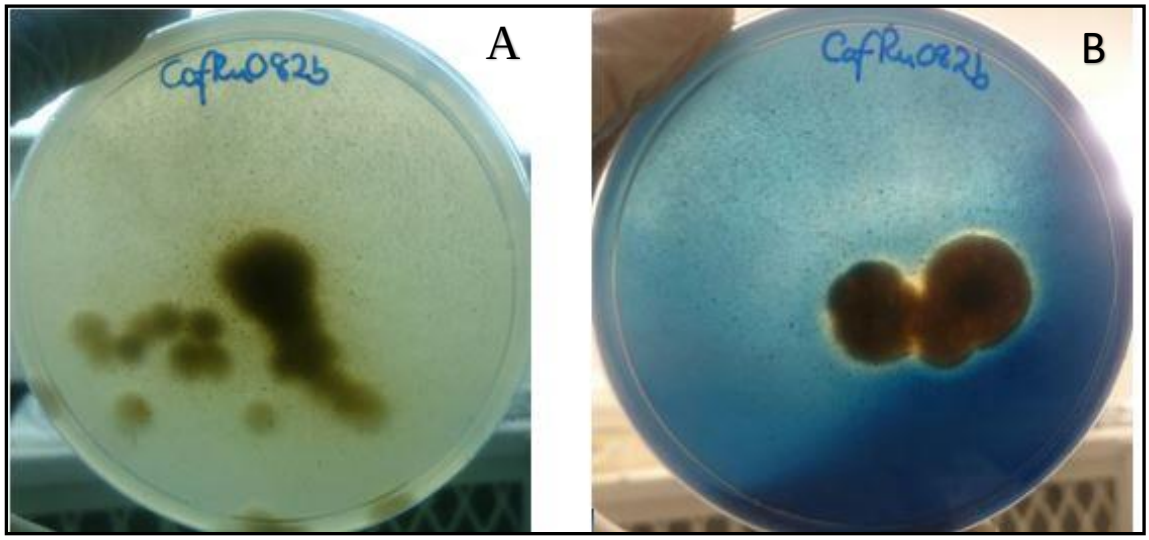

Figure 2.2 Casein agar plates inoculated with *O. maius*. Plate A was flooded with $HgCl_2$ and showed no zone of clearance. Plate B was flooded with 0.1% amido-black and showed a negligible zone of clearance.

Plates containing bovine haemoglobin showed no sign of enzymatic hydrolysis. No zone of clearance was observed on both the amido-black stained plate (Figure 2.3 A) or the plate treated with mercuric chloride (Figure 2.3 B)

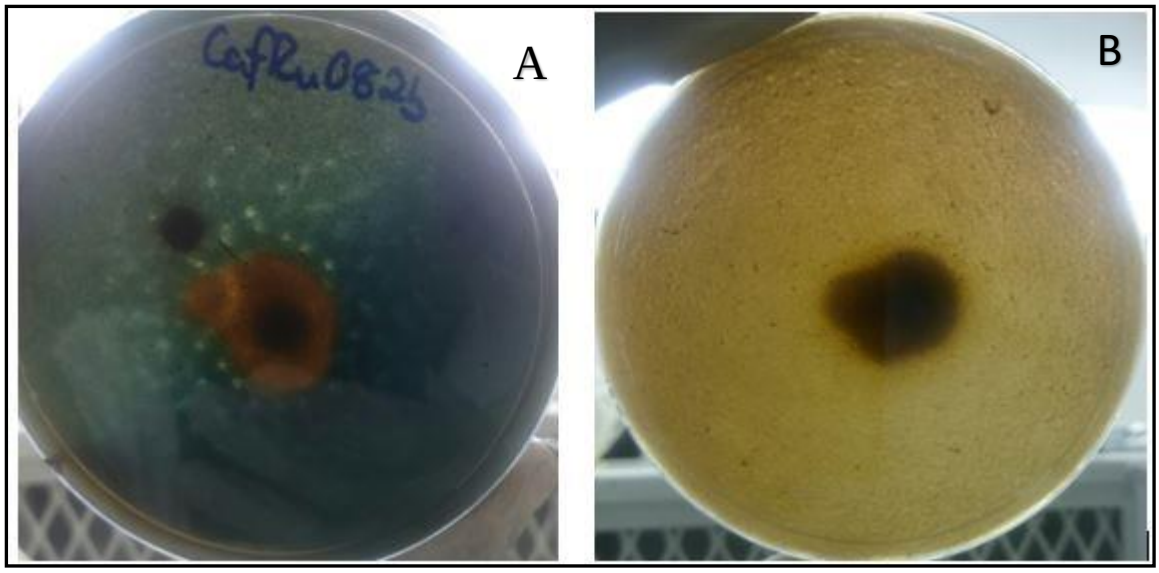

Figure 2.3 Bovine haemoglobin agar plates inoculated with *O. maius*. Plate A was stained with amido-black and showed no substrate clearance zones. Plate B was flooded with $HgCl_2$ and showed no zone of substrate clearance.

Figure 2.4 shows the formation of typical spherical mycelial clumps formed by *O. maius* in MMN liquid medium.

Figure 2.4 *O. maius* in liquid medium after growth for 2 weeks.

The growth curve of the fungus, *Oidiodendron maius*, is illustrated in Figure 2.5. The protein concentration started increasing exponentially after day 10 until day 14, where the increase slowed down. Fungal biomass, represented by the dry weight, increased from day 8 to day 14. After day 14, fungal dry weight decreased gradually.

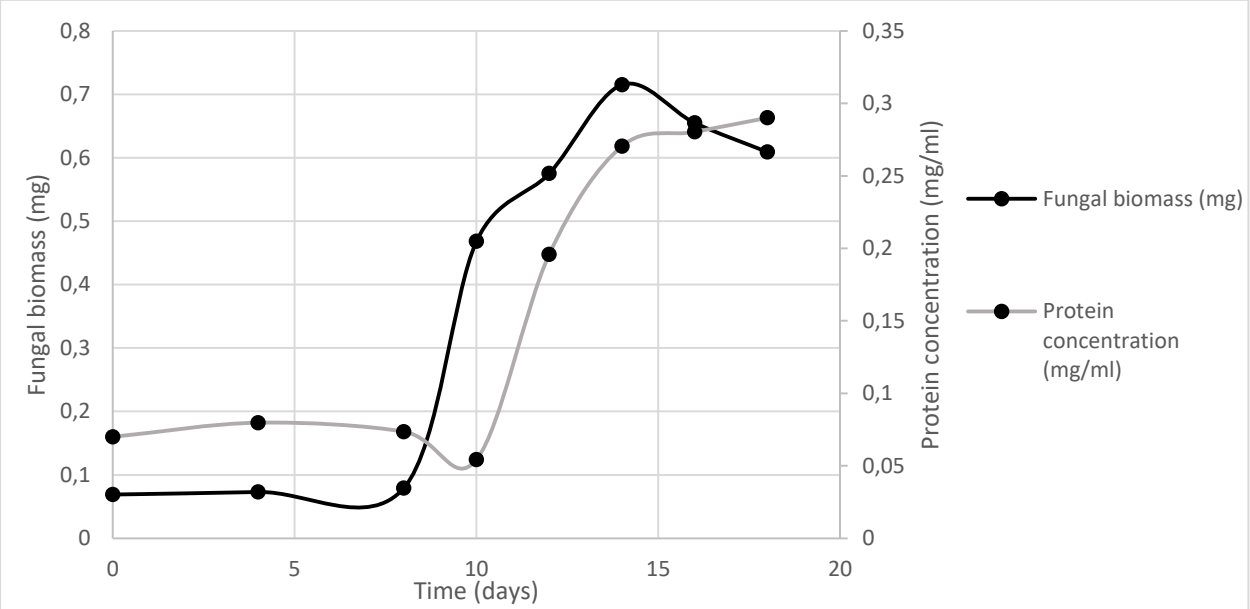

Figure 2.5 Growth curve of *O. maius* and protein production over time.

2.4 DISCUSSION

Frazier's method (1926) for detecting gelatinase activity using mercuric chloride was applied in Section 2.2.2. A clear zone around the fungus against an opaque background suggests the production of gelatinases by the fungus (Marokhazi et al., 2004). Similarly, detection of proteases by the gelatin agar plate assay has been successfully used by Alnahdi (2012), Sharma et al. (2017) and Pant et al. (2015). No zones of clearance on haemoglobin and casein plates indicated that the fungus did not produce proteolytic enzymes capable of hydrolysing these two substrates. Absence of these clearance zones also suggests that the fungus was unable to obtain sufficient N from the PDA. Vermelho et al. (1996) however, showed zones of clearance on haemoglobin agar plates stained with amido-black. Although *O. maius* did not show hydrolysis of haemoglobin and casein, it managed to grow on plates containing all substrates, as PDA contains all nutrients necessary for its growth. Fungi of the genus *Oidiodendron* have been shown to grow well on both liquid and solid synthetic culture media (Hambleton and Currah, 2000; Wei et al., 2016).

Studies on phosphorus, nitrogen, and carbon utilisation of *O. maius* (CafRu082b) have already been performed prior to this study and the nitrogen sources included arginine, BSA, ammonium phosphate as well as calcium nitrate (Bizabani and Dames, 2016). Phosphate sources included orthophosphate, phytic acid as well as DNA from salmon sperm, while carbon sources included glucose, cellobiose, carboxymethylcellulose, pectin and tannic acid. The fungus grew well in the presence of ammonium, orthophosphate and the carbon source glucose (Bizabani and Dames, 2016). In the current study, MMN media was therefore altered accordingly for the growth of the fungus. *O. maius* was successfully cultured in MMN supplemented with gelatin for the production of proteases.

Gelatin degrading enzymes were detected using the enzyme assay giving a specific activity of approximately 70 U/mg. Rice and Currah (2005) tested 33 *Oidiodendron* strains from different sources for production of gelatinases; all of them liquefied the gelatin. Gelatin has also been used as a substrate for the detection of proteases from other micro-organisms (Alnahdi 2012; Fedatto et al., 2006; Pant et al., 2015). Since gelatin was used as the inducer in the fungal growth medium, it was selected as the substrate for all subsequent proteolytic activity assays.

O. maius, like some filamentous fungi, grows in liquid media as mycelial clumps that are shaped like spheres formed by radial filamentous growth (Figure 2.4) (Moore et al., 2011). This growth "pattern" produces a culture that is non-homogenous, unlike bacterial cultures, making it difficult for the collection of representative samples from a single culture for monitoring of the growth of the fungus. Whole cultures which were cultured at the same time were used as samples for the measurement of growth rate. Mycelia were extracted at different growth intervals. For the purpose of consistency, and reproducibility, samples of a constant age were extracted. Construction of a growth curve was therefore necessary to identify the most appropriate day for enzyme extraction from the growth medium.

The production of proteins increased with time up to a protein concentration of approximately 0.68 mg/ml. The crude was extracted at day 14, where the growth of the fungus was at its peak. The decrease in fungal dry weight after day 14 could possibly have been because of cell death. Protein concentration also stopped increasing exponentially at day 14.

It was expected that proteolytic enzymes were produced by this day and this time was therefore selected as the time for harvesting in all subsequent experiments. Rice and Currah (2005) showed growth of the fungus at room temperature for 14 days before protease extraction. *O. maius* is reported to have an optimum growth temperature of 20-25°C and has been previously

grown at this temperature range (Rice and Currah, 2001; Sigler and Gibas, 2005 and Wei et al., 2016).

O. maius was grown at a pH of 5.5, which is line with reports by Rice and Currah (2001), who previously showed that *O. maius* (among other *Oidiodendron* species), was an acidophile, growing well at a pH of 5.0. Wei et al. (2016) grew *O. maius* at pH 5.2 in the dark and Bizabani and Dames (2016) used an acidic pH of 4.5 for the growth of the fungus. The optimum growth of *O. maius* under acidic conditions is affected by its normal growth in acidic soils.

Light may affect the growth of some *Oidiodendron* species, such as *O. echinulatum*, which showed stunted/ redundant growth in the light (Rice and Currah, 2005). *O. maius* was constantly grown in the dark in experiments by Sigler and Gibas (2005) as well as those by Wei et al. (2016), suggesting its sensitivity to the light. All plates in this study were therefore grown in the dark and culture bottles were covered in foil to maintain a dark environment.

2.5 CONCLUSION

Initial experiments on substrate containing agar plates (in this chapter), showed the ability of *O. maius* (CafRU082b) to produce gelatin degrading enzymes. Growth of the fungus on two other substrates suggested the organism's inability to produce caseinolytic as well as haemoglobin degrading proteases. The ascomycete fungus, *O. maius*, grew well in MMN media, producing gelatinases that showed activity on performing activity assays. Activity assays were optimised for future experiments and a growth curve of the fungus was constructed. The optimal day of enzyme extraction (for purification studies) was determined, and enzyme purification was performed in the following chapter, Chapter 3.

CHAPTER 3

PARTIAL PURIFICATION OF PROTEASES FROM *O. MAIUS*

3.1 INTRODUCTION

The fourth aim of this research was to partially purify proteases produced by *O. maius* (CafRu082b) following its growth in liquid media (Section 1.6). Purification of a protein is necessary for the purpose of studying its biochemical properties and functions as an enzyme, as well as for its identification. Several procedures such as precipitation, concentration and chromatography are followed in order to purify proteins (Berg et al., 2002).

Protein precipitation and the consequent recovery of the precipitate from the growth medium signifies one of the most important operations for the laboratory and industrial scale recovery and purification of proteins (Bell et al., 1983). Precipitation of the proteins is necessary for the removal of contaminants in the form of lipids and other undesirable cellular material (Feist and Hummon, 2015). Soluble proteins can be precipitated by interaction with a suitable precipitant that decreases the protein's attraction to the solvent and increases the protein's attraction to other protein molecules, resulting in protein accumulation and eventually precipitation (Ryan, 2011). It is used as a preliminary purification step before using more refined steps for the isolation of a specific protein. Ammonium sulphate is one of the most commonly used reagents for the precipitation of proteins (Bollineni et al., 2015). The use of organic solvents, such as ethanol or acetone, has previously been described in literature, and is a widely used technique (Panesar, 2010; Uttatree et al., 2017).

Acetone precipitation, which is a simple, quick and effective purification procedure was used in this chapter. It involves the addition of pre-chilled, ice cold acetone to the sample containing the proteins to be precipitated. The advantages of acetone precipitation include stability of the protein pellet and as well as its increased solubility (Nejadi et al., 2014)

Techniques such as gel filtration chromatography, affinity chromatography and ion exchange chromatography (IEC) have been previously used in the purification of proteases (Inouye et al., 2007; Thaz and Jayaraman, 2014; Vishwanatha et al., 2009). A combination of these

methods are often used to achieve high purification fold values (Dutta et al., 2005). In this chapter, the use of centrifugal filters for ultrafiltration of samples was used, where a semi-permeable membrane is used in the separation of proteins based on their molecular size. It is a gentle and non-denaturing method (Mohammad et al., 2012).

The second aim of this chapter was to determine the molecular weight of the protease using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography. Zymography is a common enzyme identification technique that is centred on the SDS-PAGE protocol and makes use of an enzyme's ability to degrade co-polymerised substrate (d'Avila-Levy et al., 2012). Gelatin is a cheap protease substrate that can be degraded by most proteases, thus it is the substrate of choice for zymography (Cole, 2000; d'Avila-Levy et al., 2012;). Several other substrates can also be used, e.g. haemoglobin (Ktari et al., 2014) and casein (Li et al., 2000; Quesada et al., 1996; Raser et al., 1995).

The protease is renatured after washing the gel with a detergent like Triton X-100. Staining with Coomassie Brilliant Blue reveals the zones of catalytic activity as colourless bands against a blue background (Hawkes et al., 2010; Toth et al., 2012).

SDS-PAGE gels are normally stained with Coomassie Brilliant Blue, but in cases where protein concentration is low, silver staining can be performed. Silver staining is an extremely sensitive detection technique as it has a protein detection limit of about 0.25-5 ng. This is approximately 100 times more sensitive that staining with Coomassie Brilliant Blue, which detects proteins from a concentration of 50 ng upwards (Heukeshoven and Dernick, 1985; Merril, 1986, ThermoFischer Scientific).

3.2 MATERIALS AND METHODS

3.2.1 ACETONE PRECIPITATION

Proteins were precipitated using ice cold acetone pre-chilled at -20°C overnight. Acetone was added to the extracellular crude extract in a dropwise manner with continuous stirring of the sample on ice. The volume ratio of acetone:sample was 4:1. After addition of acetone, samples were refrigerated at -20°C for 20 mins and then centrifuged at 10 000 x g for 15 mins. The supernatant was discarded, and the pellet was air dried and re-suspended in buffer.

3.2.2 SIZE EXCLUSION BY FILTRATION

Size exclusion was first performed by filtration using Amicon Ultra-15 Centrifugal Units with Ultracel-50 membranes (UFC905024 Merck), which have a nominal molecular weight limit (NMWL) of 50 kDa. The filtrates from the 50 kDa filters were collected and passed through the Amicon Ultra-15 Centrifugal Filter Units with an Ultracel-30 membrane (UFC903024 Merck) which had a NMWL of 30 kDa. Samples were run at 4000 *x g* in a swing bucket centrifuge at a temperature of 4°C for 40 min.

3.2.4 SDS-PAGE

SDS-PAGE was carried out using a 12% resolving gel and a 4% stacking gel according to the method of Laemmli (1970). Protein samples were mixed with 2X sample buffer (100 mM Tris-Cl (pH 6.8); 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 200 mM 2-mercaptoethanol) using a volume ratio of 1:1. These were heated at 100°C for 5 mins on the AccuBlock Digital Dry bath (Labnet) and then left to cool to room temperature. Samples of 15 µl were loaded into the wells of the gel parallel to an unstained marker (BioRad Precision Plus Protein Unstained Standard, Cat No. 1610363)). Electrophoresis was conducted at a constant voltage of 125 V in a Mini Protean Trans Block Cell tank containing running buffer (25 mM Tris base; 192 mM glycine; 1% (w/v) SDS) until the dye had reached the bottom of the gel.

Gels were removed from the glass plates and washed twice with distilled water. They were then stained for 30 mins with Coomassie staining solution (0.1% (w/v) Coomassie Brilliant Blue G250; 20% (v/v) methanol and 15% (v/v) glacial acetic acid) and thereafter, de-stained with de-staining solution (30 % (v/v) methanol and 10% (v/v) glacial acetic acid). De-staining was performed until dark protein bands were seen against a light background. Silver staining was performed using a Silver Stain kit (Pierce^{TM} Silver Stain Kit CN 24612) according to the manufacturer's protocol. All gels were visualised and captured using the BioRad Molecular Imager Chemidoc XRS.

3.2.5 ZYMOGRAMS

Gelatin zymography was performed using a 10% resolving gel protocol containing gelatin of 2.65 mg/ml concentration, as well as a 4% stacking gel. The samples were mixed, using a 1:1

ratio, with a 2X sample buffer containing: 2 ml 1 M Tris, pH 6.8; 4.6 ml 50% (v/v) glycerol; 1.6 ml 10% SDS (w/v) and 0.4 ml 0.5% (w/v) bromophenol blue. Samples were loaded into the wells of the gel alongside the Precision Plus Protein Unstained Standard from BioRad (Cat No. 1610363). The gel was run in running buffer containing 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS in a Mini Protean Trans Block Cell tank at a constant voltage of 120 V for 75 min.

After the run, the gel was incubated in a renaturing buffer containing: 2.5% (v/v) Triton X-100; 50 mM Tris HCl, pH 7.4; and 5 mM $CaCl_2$. After 30 min, the buffer was discarded, and the gel was incubated in fresh renaturing buffer for another 30 min. The gel was then incubated at 37°C overnight in a developing buffer containing: 1% (v/v) Triton X-100; 50 mM Tris-HCl and 5 mM $CaCl_2$.

The gel was stained with Coomassie staining solution (0.5% w/v Coomassie Brilliant Blue G-250, 40% (v/v) methanol and 10% (v/v) acetic acid) for 30 min and de-stained with a de-staining solution (40% (v/v) methanol and 10% (v/v) acetic acid) until clear bands were observed against a dark background. Gels were visualised and captured using the BioRad Molecular Imager Chemidoc XRS.

The sequence of the steps used for purification of the protease is shown in Figure 3.1.

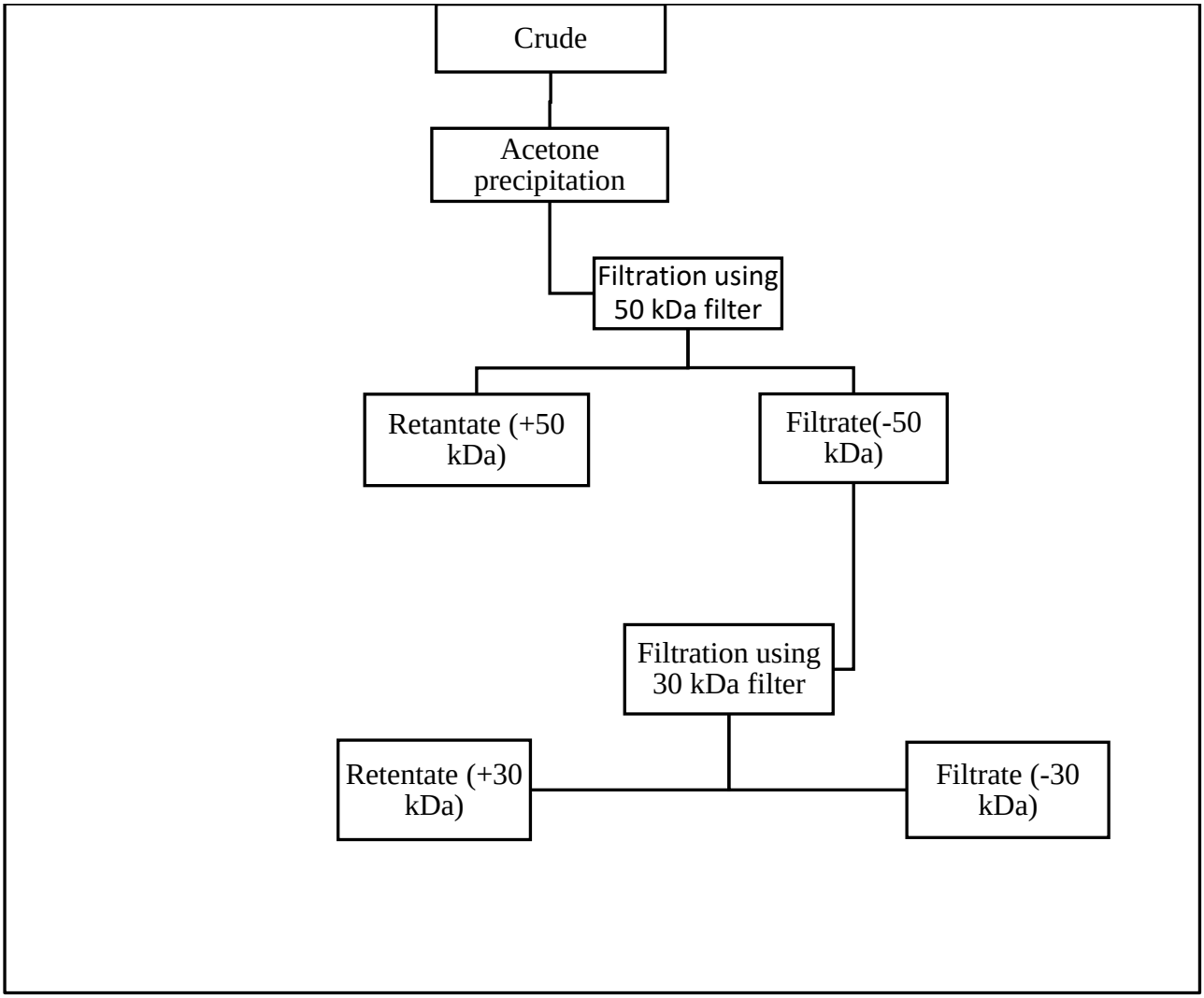

Figure 3.1 Purification steps for the partial purification of the proteases from the crude extract of *O. maius.*

3.3 RESULTS

Table 3.1 shows a purification table which was constructed using results obtained from protein and activity assays of samples at each purification step. All activity assays were performed under standard assay conditions (Section 2.2.5) using gelatin as the substrate. Protein determination was performed according to the Bradford method in Section 2.2.6.

Table 3.1 Purification table for partially purified fractions of the protease.

Purification Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific Activity (U/mg)	Purification fold	Yield/ %
Crude extract	85	0.2517	21.396	18.128	1 541	72	1	100
Acetone Precipitated Fraction	70	0.2895	20.265	22.456	1 572	78	1.1	102.01
Filtrate from 50 kDa filter	60	0.0288	1.732	35.965	2 158	1 246	17.3	140.04
Filtrate from 30 kDa filter	58	0.0192	1.116	52.105	3 022	2 707	37.6	196.12

Proteases from *O. maius* were partially purified with the acetone precipitation giving a 1.1-fold increase in specific activity of the protease and a yield of 102%. Further purification by the 50 kDa filter showed a 17.3 purification fold and a 38% increase in yield from the acetone precipitated fraction. The 30 kDa filter filtration step showed a 37.6-fold increase in purification compared to the crude extract and a yield of 196%. Yields of above 100% were obtained in all purification steps, which was highly unusual.

The samples that were collected after filtration with the 30 kDa filter were subjected to SDS-PAGE to visualise presence and purity of the protein. Figure 3.2 shows the SDS-PAGE gel with the lanes 1, 2 and 3 containing the partially purified protein from the 30 kDa flow through. A band was observed at approximately 35 kDa and it was considered to be the protease as it coincided with the cleared bands on the zymogram.

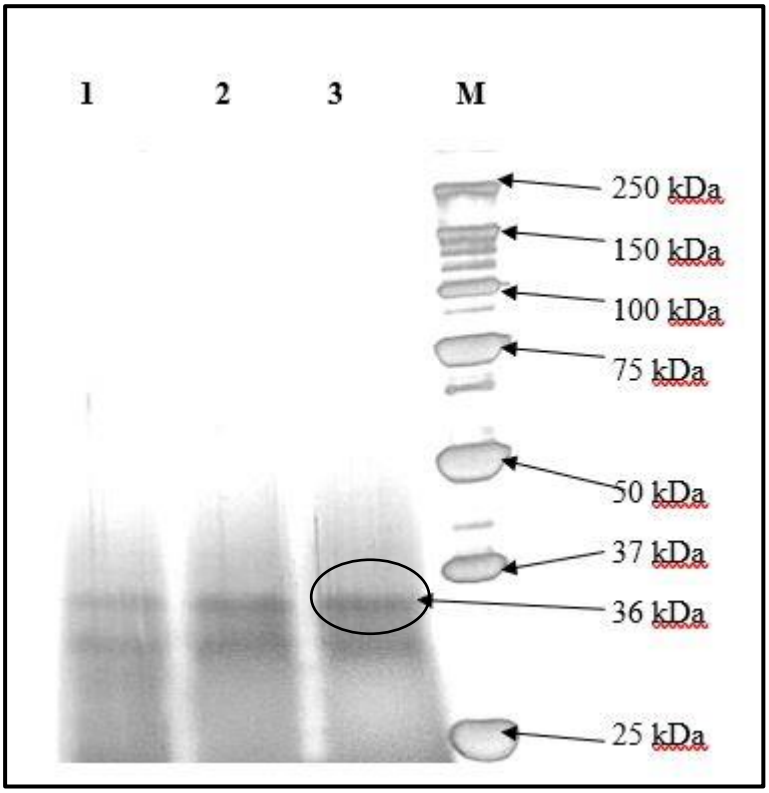

Figure 3.2 SDS-PAGE gel of partially purified enzymes with silver staining. Lane M: Molecular weight marker (Precision Plus Protein™ Unstained Protein Standards from Biorad, UK); Lane 1, 2 and 3: Partially purified filtrate from 30 kDa filter.

Figure 3.3 shows a gel co-polymerised with the substrate, gelatin. Samples from each stage of purification were loaded onto the gel. The samples in lane 1, 2, 3 and 4 showed clear bands on a blue background slightly below the 37 kDa marker protein.

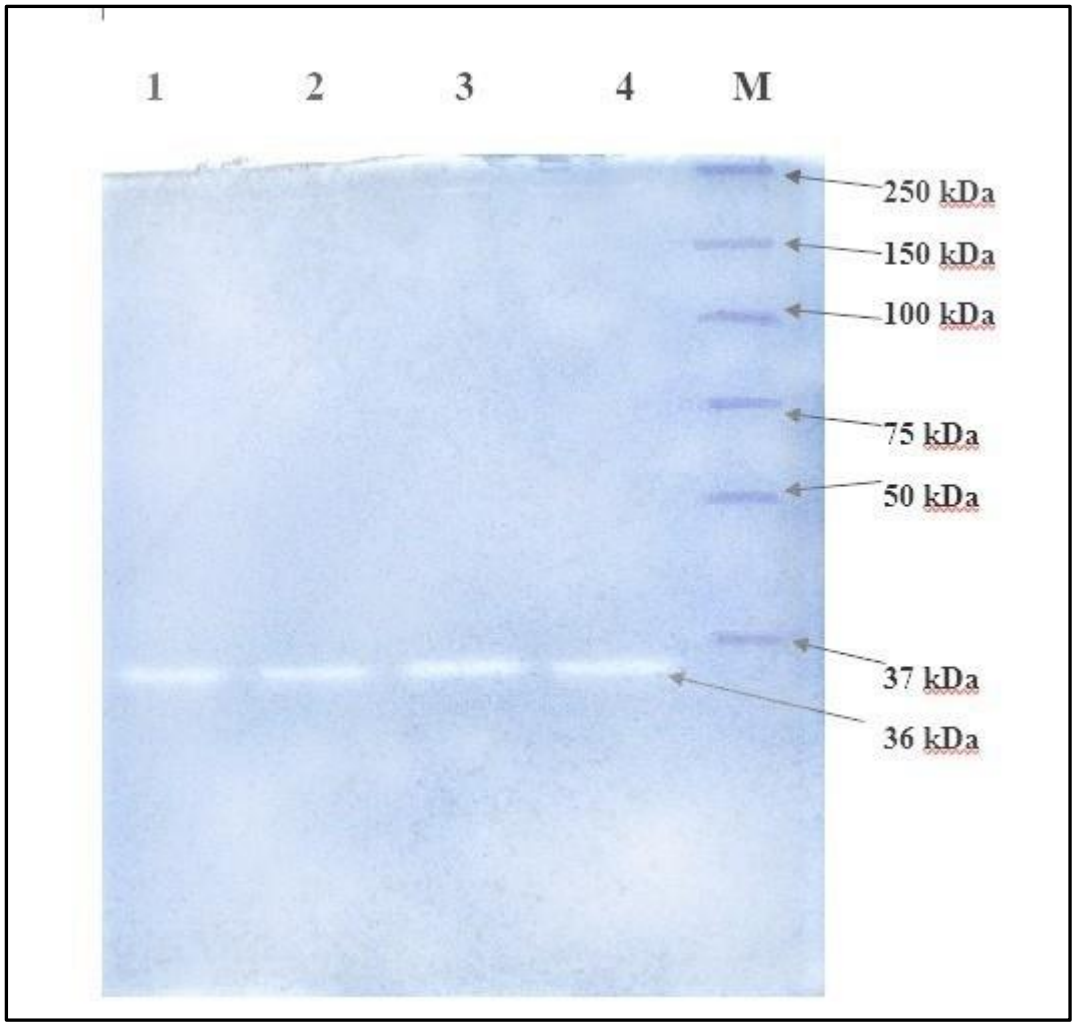

Figure 3.3 Gelatin zymogram of protease fractions from *O. maius*. Lane 1: 30 kDa fraction; Lane 2: 50 kDa fraction; Lane 3: Acetone precipitated fraction; Lane 4: Crude fraction and Lane M: Molecular weight marker (Precision Plus Protein™ Unstained Protein Standards from Biorad, UK).

3.4 DISCUSSION

The gelatin zymogram (Figure 3.3) confirmed protease activity which was shown by the hydrolysis of the substrate co-polymerised in the gel. The protease band was consistent with all purified fractions and was found to be approximately 36 kDa in size.

Partial purification of the protease was successful, with a purification fold of 37.6 after filtration using the 30 kDa filters. This fraction exhibited high specific proteolytic activity of 2 707 U/mg. Previously purified proteases from the fungus *Aspergillus niger* I1 showed specific activity as high as 13 429 U/mg with a purification fold of 3.55 (Siala et al., 2009).

The retentate from the 30 kDa filter, though much lower than that of the filtrate, also showed some protease activity (data not shown). This suggests that some proteases did not pass through the membrane. Manufacturers of the ultra-centrifugal filters (Sartorius Stedim) suggest the use of a molecular weight cut-off (MWCO) membrane that is half the molecular weight of the protein for maximum protein recovery. This explains how a protein of molecular weight 36 kDa passes through a 30 kDa cut-off filter. A MWCO membrane of approximately 15 kDa would, therefore, have been the best for maximum recovery of the proteases.

Enzyme purity was confirmed by SDS-PAGE. The protease was observed to be ~35 kDa in size, with the band on the gelatin zymogram corresponding with the band observed on the SDS-PAGE (Figure 3.2). This is similar to a gelatin degrading protease of a molecular weight of 36 kDa reported by Yang et al. (2004), which was produced by a different strain of *O. maius.* Proteases of 34 kDa and 39 kDa from the fungus, *Rhizopus oryzae* have also been reported (Hsiao, 2014; Kumar et al., 2005).

There were several other bands observed on SDS-PAGE gel, indicating only partial purity of the protease. Slight smears below suggest protein degradation by the protease. The presence of trace amounts of these proteases will result in significant amounts of proteolytic degradation. (Walsh, 2013).

An increase in protease activity (U/ml) resulted in yields over 100%. Similar results have been reported for a protease obtained from *Vibrio vulnificus* (Miyoshi, 1987), which showed yields of 111% and 118% after the first and second purification steps, respectively. The yields over a 100% suggests that there was removal of an inhibitory substance during purification. Another possibility would be that the proteases were produced as zymogens. These are inactive enzymes which require activation for them to function. Most microorganisms are known to produce their proteolytic enzymes as zymogens for purposes of prevention of the breakdown of crucial proteins within the cell (Khan and James, 1998). The activation of zymogens is either autocatalytic, where a simple change in environmental conditions such as pH induces zymogen activation, or the zymogen requires peptide hydrolysis of an inhibitory fragment (Khan and James, 1998; Neurath, 1999). Examples of previously isolated proteases that are initially secreted as zymogens include porcine pepsin A, chymosin, as well a protease from *Candida tropicalis* (Barrett et al., 2012; Hiramatsu, 1989). McDonald and Kunitz (1941) reported the conversion of trypsinogen into trypsin in presence of calcium ions.

The organism, *O. maius*, grows at a pH of 5, thus the proteases are produced at this pH. After acetone precipitation, however, the pellet is re-dissolved in a potassium phosphate buffer of pH 7. This shift in pH could possibly be responsible for activating the proteins, as the yield increased to over 196% after the final purification step (Table 3.1).

3.5 CONCLUSION

This chapter showed both qualitative (Figure 3.2 and Figure 3.3) and quantitative (Table 3.1) data on the protease produced by *O. maius*. Silver staining was used in place of staining with Coomassie Brilliant Blue G 250 for the SDS-PAGE due to the low protein concentrations of the partially purified proteases. Higher concentrations and a purer protein fraction could have been obtained by more purification methods with a higher discriminatory ability, such as ion exchange chromatography (IEC). The proteases were however, successfully partially purified using acetone and centrifugal filters. Therefore, a partially pure sample of the protease was produced for all subsequent characterisation studies in the following chapter (Chapter 4) as well as for silver recovery studies (Chapter 5).

CHAPTER 4

CHARACTERISATION OF PARTIALLY PURIFIED PROTEASES FROM *O. MAIUS*

4.1 INTRODUCTION

The increasing demand for proteolytic enzymes in industry, with suitable specificity and stability to pH, temperature, metal ions and surfactants continues to drive the isolation of new capable strains that can produce large amounts of enzyme with desirable traits at a low cost (Saxena and Singh, 2010)

Enzymes display their highest activity at their respective optima, and their characterisation allows for application of these optimum parameters in industry. For purposes of industrial use, enzymes that are able to carry out a reaction at the fastest reaction rate per unit of enzyme are required, as this indicates the maximum effect for minimum amount of added catalyst. Enzyme kinetic studies describe the rates at which reactions occur. The kinetic parameters regulating the enzyme's rates of reaction include initial substrate concentration, initial enzyme concentration as well as temperature (Dutta et al., 2005; Ghobadi Nejad et al., 2014). Initial substrate concentration is an important kinetic parameter that affects enzyme activity (Dutta et al., 2005).

Enzymes exhibit structural and functional diversity with respect to their catalytic activities, thereby determining their application in industry. For example, enzymes that do not require Ca^{2+} to function are required in the laundry detergent industry because they'll be interacting with chelating agents (Lund et al., 2012). Thermo-stability is also necessary for these enzymes because they are used at different temperatures for a prolonged time (Banerjee et al., 1999)

There has been extensive research performed on fungal protease isolation and characterisation for their potential use in industrial processes. Table 4.1 shows some crucial characteristics of some of the fungal proteases that have been discovered.

Table 4.1 Biochemical characteristics of some fungal proteases

Name of fungus	Molecular weight	pH optimum	Temperature optimum (°C)	Inhibitor	Reference
Rhizopus oryzae	34 kDa	5.5	60	Pepstatin	Kumar et al. (2005)
Aspergillus niger	38 kDa	10	50	EDTA[1]	Devi et al. (2008)
Aspergillus foetidus	50.6 kDa	5	55	Pepstatin	Souza et al. (2017)
Aspergillus oryzae MTCC 5432	47 kDa	3-4	55	Pepstatin	Vishwanatha et al. (2009)
Alternaria solani	42 kDa	9-10	50	PMSF[2]	Chandrasekaran and Sathiyabama (2014)
Lecanicillium psalliotae	32 kDa	10	70	PMSF	Yang et al. (2005)

The regulatory role of proteases is intimately coupled to their inhibitors. These molecules are present in numerous tissues of all living organisms to prevent unwanted proteolysis (Polgar, 1989). Different types of inhibitors act on different classes of proteases. Serine and cysteine proteases are often inhibited by PMSF, acid proteases by Pepstatin, and metalloproteases by EDTA and other chelating agents. The importance of metal ions in the functionality of an enzyme include their roles as structural regulators (Riordarn, 1977). Inhibition of enzyme activity can be a result of removal or replacement of a metal ion by a different one, either with the same charge or size (Shaw et al., 2004).

This chapter reports on the biochemical characterisation and substrate kinetic studies of the protease partially purified in Chapter 3.

[1] EDTA- Ethylenediaminetetraacetic acid
[2] PMSF- Phenylmethylsulfonyl fluoride

4.2 MATERIALS AND METHODS

4.2.1 PHYSICO-CHEMICAL CHARACTERISATION

4.2.1.1 Relative and residual activity

For the biochemical characterisation studies performed, the enzyme activity was expressed as either relative or residual activity. Relative activity is the percentage enzyme activity of the sample relative to the activity of the sample with the highest activity and is calculated as per equation 3:

$$\text{Relative activity (\%)} = \frac{\text{Activity of sample (U/mg)}}{\text{Activity of sample with the highest activity (U/mg)}} \text{ x } 100 \quad \ldots\text{Equation 3}$$

Residual activity is the percentage enzyme activity of the sample relating to the activity of the control sample and is calculated as per equation 4:

$$\text{Residual activity (\%)} = \frac{\text{Activity of sample (U/mg)}}{\text{Activity of control (U/mg)}} \text{ x } 100 \quad \ldots\text{Equation 4}$$

Adapted from Helvin (2013).

4.2.1.2 Effect of temperature on enzyme activity

The effect of temperature was determined by performing activity assays at 5°C intervals from 21°C to 86°C. Protease activity assays were carried out as previously described (Section 2.2.5). Activity was expressed as a percentage of the maximum, i.e. relative activity (Section 4.2.1.1).

4.2.1.3 Effect of pH on enzyme activity

The effect of pH was examined using a wide range of pH, i.e. from pH 1-11. The universal buffer (50 mM Tris, 50 mM boric acid, 33 mM citric acid, and 50 mM Na_2PO_4 was adjusted using either HCl or NaOH to the required pH) was used for pH optima assays. Substrate controls as well as enzyme controls were maintained throughout. Activity was expressed as a percentage of the maximum, i.e. relative activity (Section 4.2.1.1).

4.2.1.4 Thermostability of enzyme

Temperature stability of the enzyme was determined at 37°C, 68°C and 100°C. The enzyme was incubated at the above-mentioned temperatures for 1 h, 2 h and 4 h and residual activity was measured using activity assays (Section 2.2.5). Residual activity was calculated according to Equation 3 (Section 4.2.1.1). Substrate controls and enzyme controls were maintained for each assay.

4.2.1.5 Effect of metal ions

The compounds $FeSO_4$, $MnSO_4$, $CaCl_2$, $MgSO_4$, $CuSO_4$, $AgNO_3$, $CoSO_4$ and $ZnSO_4$ were dissolved in Milli-Q water thus providing the metal ions in solution. The divalent cations were added to the assays to make final concentrations of 1 mM, 5 mM and 10 mM, respectively. Controls containing enzyme and metal only were included for each assay and residual activity was calculated (Section 4.2.1.1).

4.2.1.6 Effect of known protease inhibitors

The effect of some known protease inhibitors on enzyme activity was investigated. The partially purified protease was assayed in the presence of 1 mM, 5 mM and 10 mM concentrations of phenylmethylsulfonyl fluoride (PMSF), *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), ethylenediaminetetraacetic acid (EDTA) as well as pepstatin A. The effect of 2-mercaptoethanol was also investigated. Residual activities were calculated for each assay (Section 4.2.1.1).

4.2.2 SUBSTRATE SPECIFICITY

Protease assays were carried out with possible protease substrates which included azocasein, bovine haemoglobin, casein, bovine serum albumin (BSA) as well as gelatin. Assays for bovine haemoglobin, casein, BSA and gelatin were performed under standard assay conditions (Section 2.2.5) and proteolytic activity was calculated.

The azocasein protease assay was performed according to a method by Charney and Tomarelli (1947).

4.2.2.1 Azocasein Protease assay

The partially purified enzyme extract (100 µl) was added to the substrate azocasein (100 µl, 3% (w/v)) in potassium phosphate dibasic trihydrate buffer (0.05 M, pH 7.0) and the mixture was incubated at 37°C for an hour. Termination of the reaction was achieved by the addition of trichloroacetic acid (TCA) (800 µl, 5% (w/v)) and it was centrifuged at 8000 *x g* for 5 min. Sodium hydroxide (0.5 N, 500 µl), was added to the supernatant (500 µl) and the absorbance was read after 30 mins at a wavelength of 440 nm using a Powerwave X-1 (BioTech Instruments Inc.). Controls containing partially purified enzyme extract only and substrate only were included. One unit of enzyme was defined as the amount of enzyme required to produce an increase of 0.01 optical density (OD) per millilitre of partially purified enzyme extract, under the assay conditions specified (Sandhya et al., 2005).

Zymograms were also performed according to the method in Section 3.2.5 except gelatin was replaced with azocasein, haemoglobin and casein. The gels were stained with Coomassie Brilliant Blue to determine substrate hydrolysis.

4.2.3 SUBSTRATE KINETICS

Enzyme was incubated with different concentrations of gelatin from 0,05% (w/v) to 1% (w/v) and assays were conducted for an hour. K_m and V_{max} values were calculated graphically using the Lineweaver-Burk plot as well as the Michaelis-Menten graph using GraphPad Prism™.

4.2.5 STATISTICAL ANALYSIS

All experiments were performed in triplicate. The standard deviations and means were calculated using Microsoft™ Excel (Microsoft Corporation, Redmond, USA). All graphs were plotted using Microsoft Excel except the Michaelis-Menten graph.

4.3 RESULTS

The temperature optimum for the enzymatic activity of proteases from *O. maius* was observed to be 68°C (Figure 4.1). The enzyme was approximately 90% active at temperatures as high as 81°C.

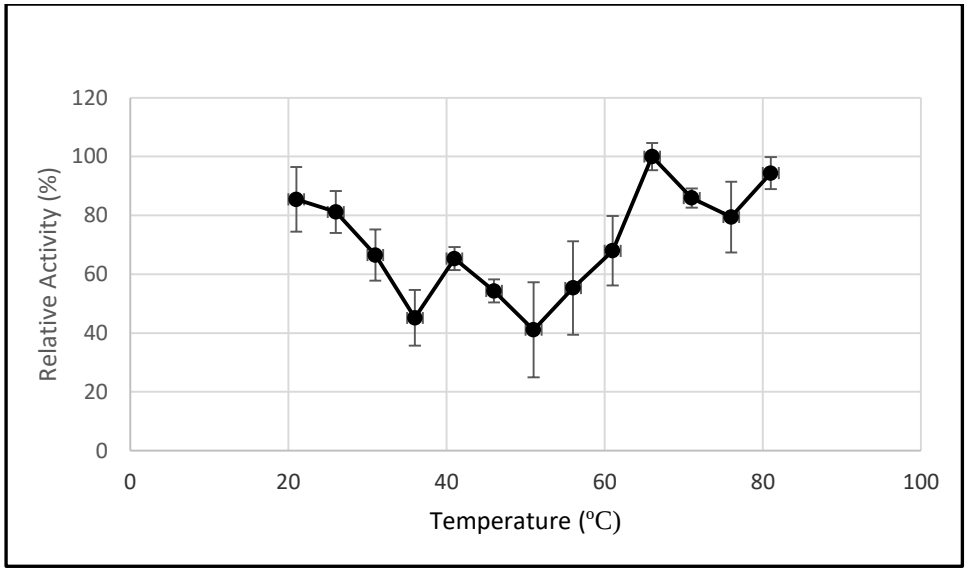

Figure 4.1 Effect of temperature on *O. maius* protease activity. Data points are shown as mean values ± SD (n=3).

The enzyme showed maximal activity at pH 3.0 and 7.0 (Figure 4.2) No activity was detected at pH values higher than 9.0. The two peaks in Figure 4.2 suggests the presence of two species of proteases, one species with a pH optimum of 3.0 and another with a pH optimum of 7.0.

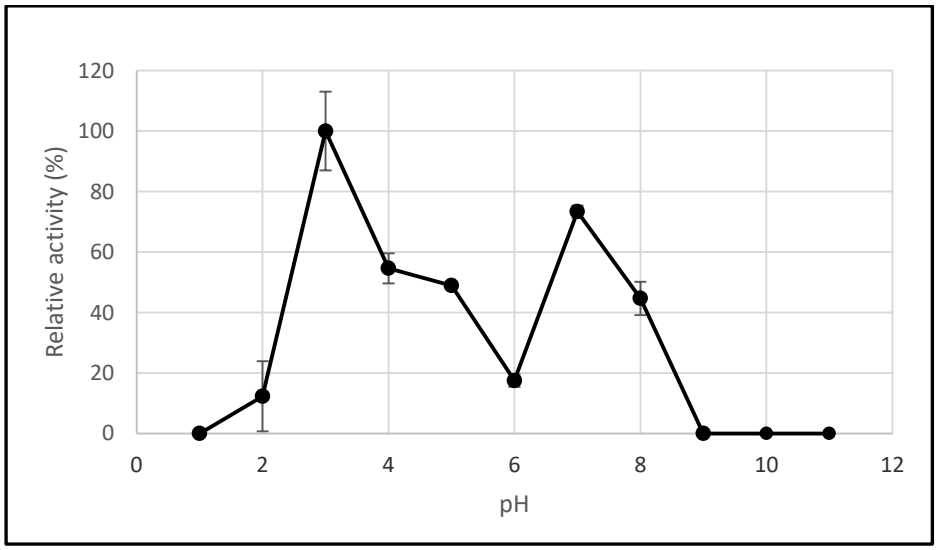

Figure 4.2 Effect of pH on protease activity. Data points are shown as mean values ± SD (n=3).

Figure 4.3 shows the thermostability of the proteases at 37°C. At this temperature, the protein concentration decreased slightly over a period of 4 hours, with the enzyme sample retaining more than 90% of the protein after 4 hours. Enzyme activity decreased over time and the proteases displayed 45% activity after 4 hours.

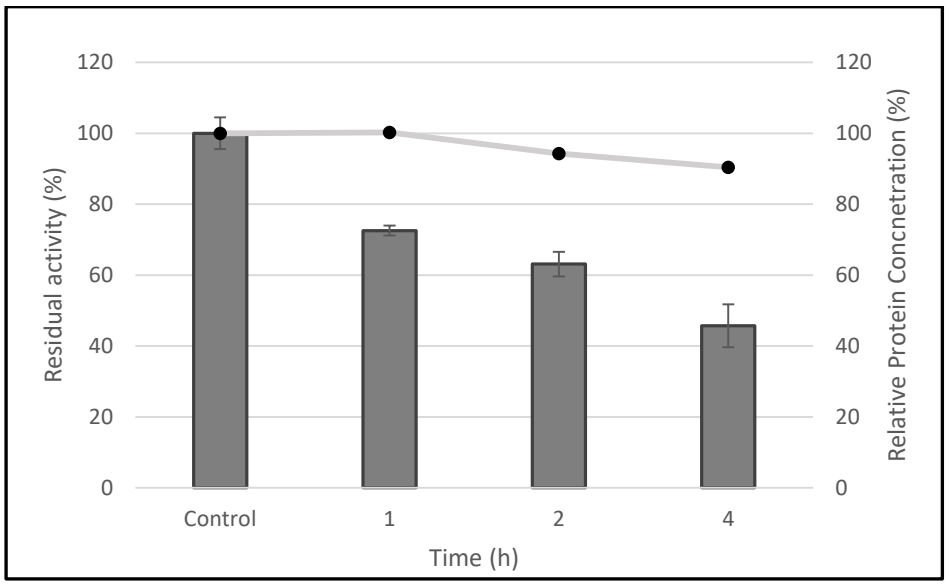

Figure 4.3 Thermostability of the proteases at 37°C. Data points are shown as mean values ± SD (n=3).

Figure 4.4 shows the thermostability of the proteases at 80°C. Protein concentrations of the enzymes and activity both decreased to 40% after 4 hours.

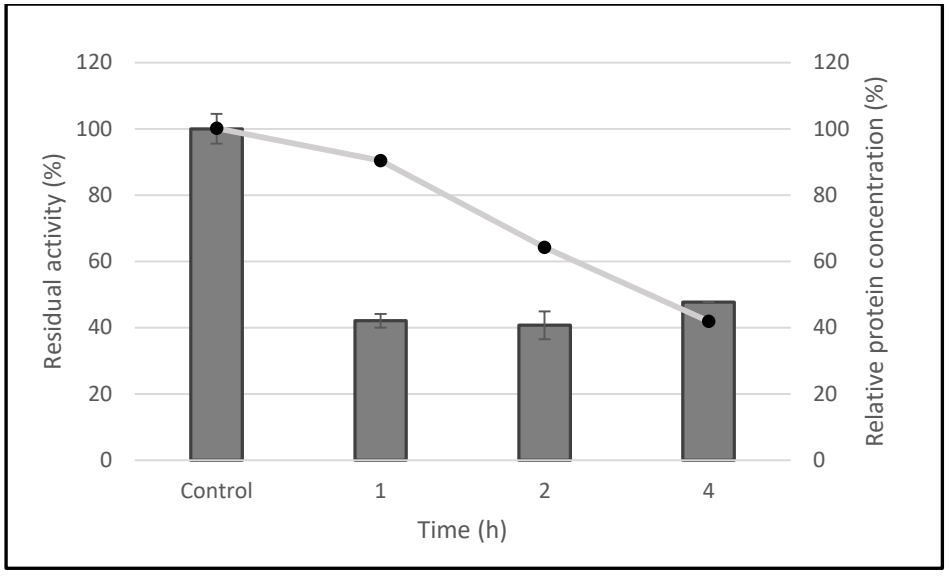

Figure 4.4 Thermostability of the proteases at 80°C. Data points are shown as mean values ± SD (n=3).

The thermal stability of the enzyme at 100°C is illustrated in Figure 4.5, where protein concentration decreased to less than 10% of the initial concentration after 4 hours. Enzyme activity remained at 100 % after 1 hour but decreased thereafter to 40% after 4 hours of incubation.

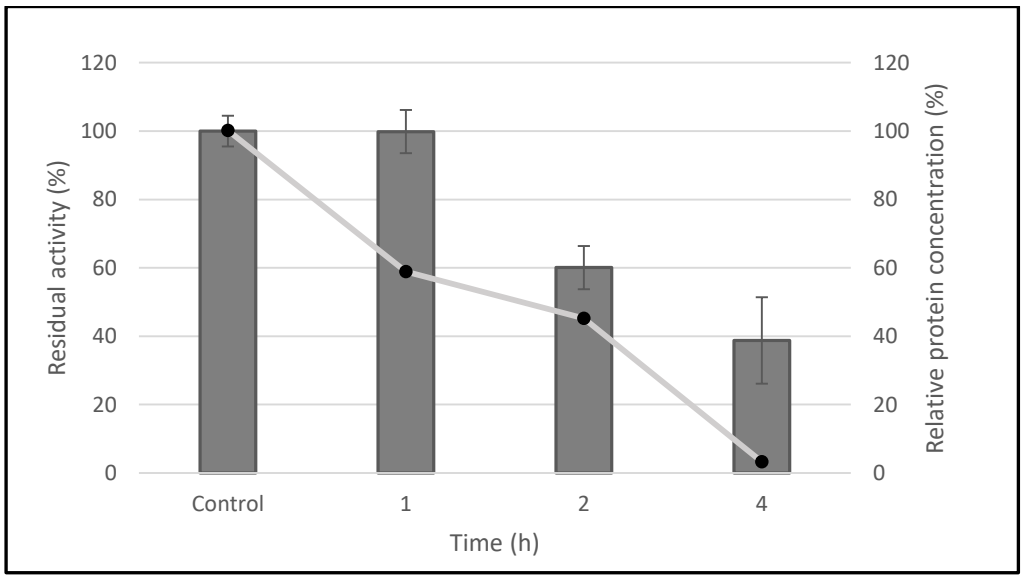

Figure 4.5 Thermostability of the proteases at 100°C. Data points are shown as mean values ± SD (n=3).

Figure 4.6 shows the effect of divalent metal ions on protease activity at different metal ion concentrations. Enzyme activity increased at a metal ion concentration of 1 mM for all metal ions except for Ca^{2+}. An increase in metal ion concentration resulted in an increase in enzyme activity in the cases of Fe^{2+}, Mg^{2+} as well as Mn^{2+}. Ca^{2+} did not have a significant effect on protease activity, with an increase in the metal ion concentration showing no change on enzyme activity. Zn^{2+} exhibited the highest enzyme activation at 1 mM, with an enzyme activity of approximately 3 times as much as the control. An increase in its concentration to 10 mM decreased enzyme activity to 215%, relative to the control.

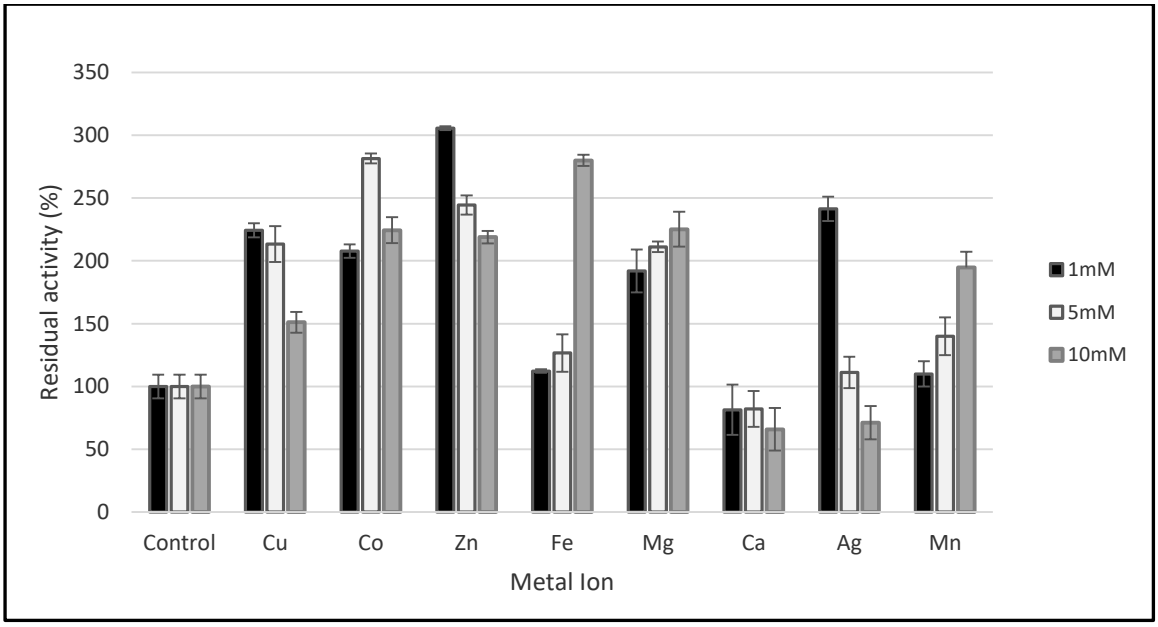

Figure 4.6 Effect of metal ions on protease activity. Data points are shown as mean values ± SD (n=3).

Results in Figure 4.7 show the effect of PMSF and EDTA, some known protease inhibitors, as well as 2-mercaptoethanol on protease activity. PMSF slightly inhibited the enzyme by 20% at 10 mM. A 1 mM concentration of EDTA inhibited protease activity by approximately 40%, and an increase in the concentration of the inhibitor to 5 mM and 10 mM did not show any further inhibition. The enzyme was activated by 2-mercaptoethanol, with a concentration of 1 mM increasing enzyme activity by 40%.

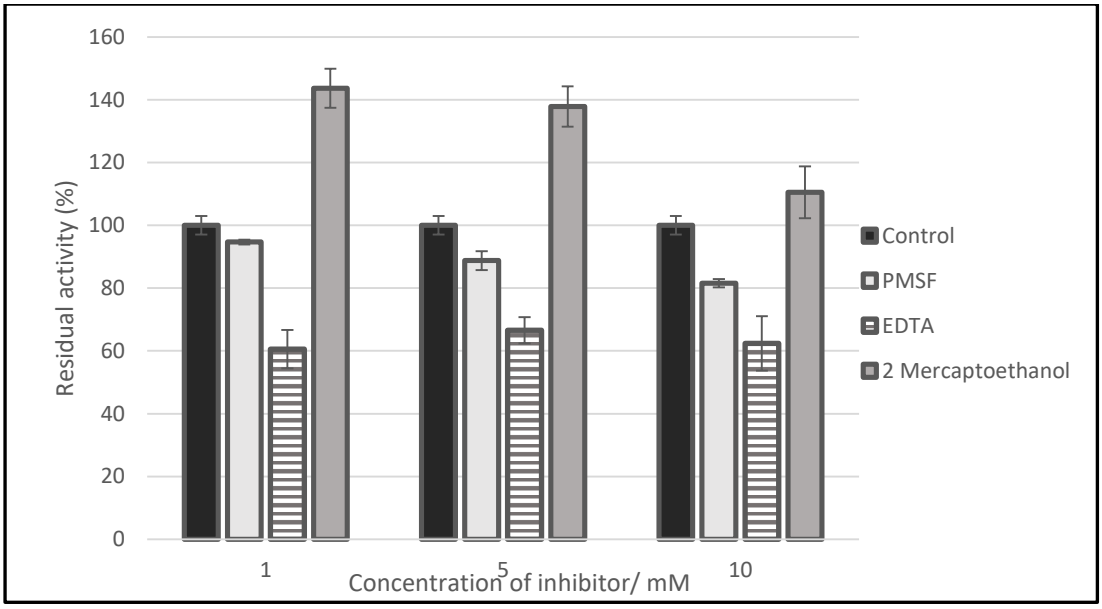

Figure 4.7 Effect of EDTA, PMSF and 2-Mercaptoethanol on protease activity. Data points are shown as mean values ± SD (n=3).

The effects of pepstatin A and E-64 are shown in Figure 4.8. The enzyme was inhibited by pepstatin A at a concentration of 5 µM and an increase in its concentration decreased enzyme activity. Complete inhibition was observed at 25 µM of pepstatin A. E-64 showed no effect on enzyme activity at 5 and 10 µM. An increase in activity was observed at 25 µM of E-64.

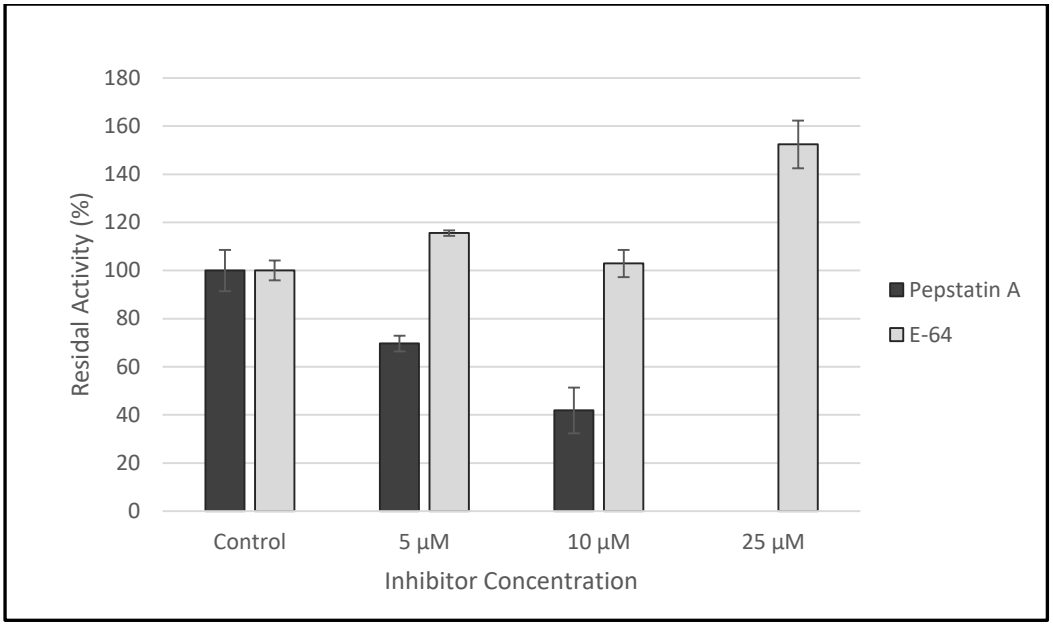

Figure 4.8 Effect of pepstatin A and E-64 on protease activity. Data points are shown as mean values ± SD (n=3).

The enzyme showed no activity on all the different protease substrates except for gelatin. Casein, azocasein and bovine haemoglobin zymograms did not show any cleared bands, indicating the inability of the protease to hydrolyse these substrates.

Determination of the maximum velocity of the reaction (V_{max}) and the Michaelis constant (K_m) of the acid protease was performed at various substrate concentrations. These were estimated by plotting data on a Michaelis-Menten graph (Figure 4.9) which showed a V_{max} of 55.25 U/ml and a K_m of 0.27% (w/v) gelatin (2.7 mg/ml).

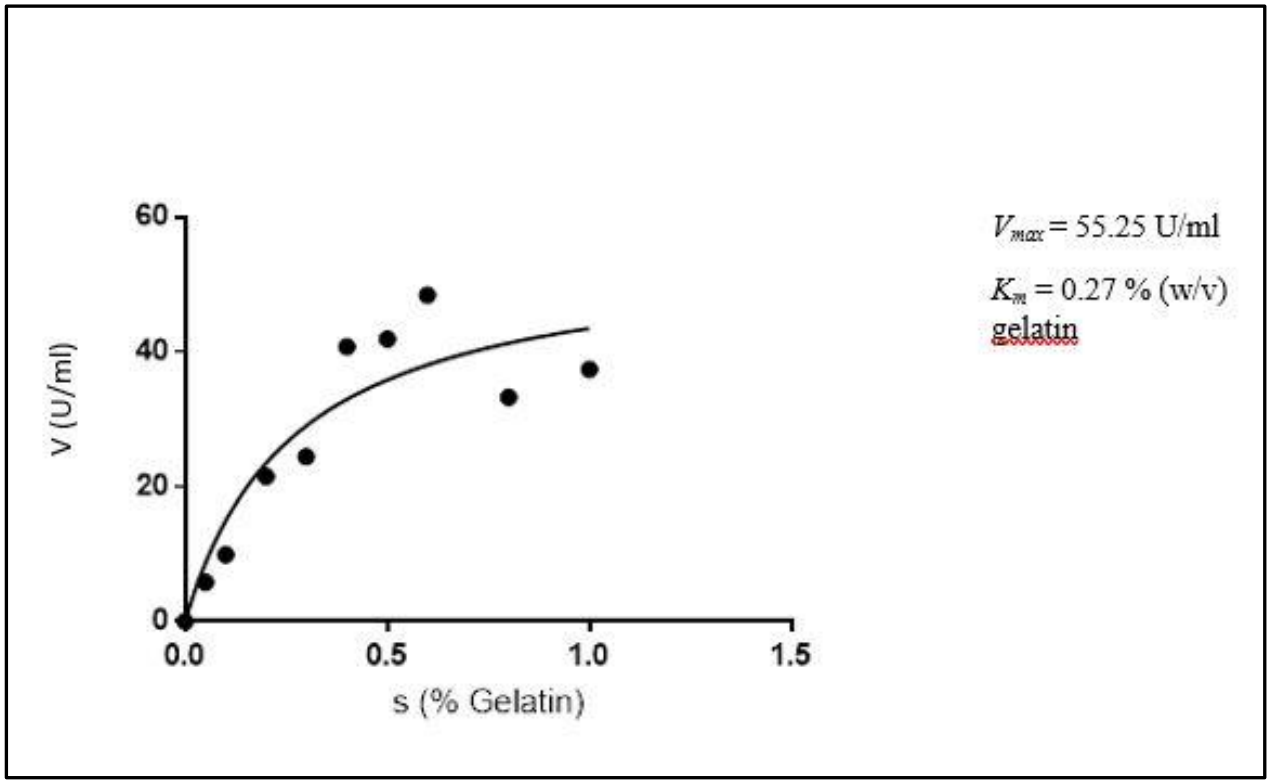

Figure 4.9 Michaelis-Menten Graph showing effect of substrate concentration on protease activity. Data points are shown as mean values ± SD (n=3).

The Lineweaver-Burk plot was constructed using Excel and is shown in Figure 4.10. It shows the enzyme had a maximum velocity, V_{max}, of 82.645 U/ml and a Michaelis constant, K_m, of 0.66% (w/v) gelatin, which is 6.6 mg/ml of substrate.

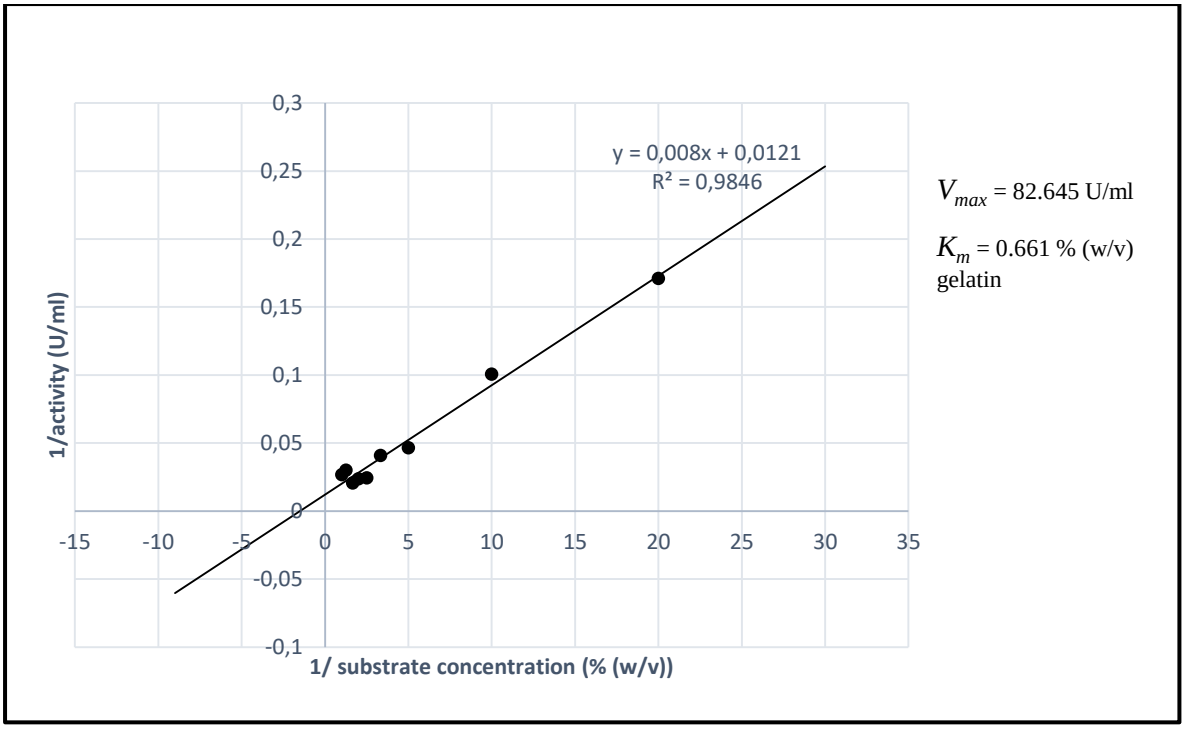

Figure 4.10 Lineweaver-Burk plot. Data points are shown as mean values ± SD (n=3).

4.4 DISCUSSION

The pH optima of 3.0 suggests that the protease is an aspartic protease also known as an acid protease. Rao et al., (1998) suggested that most aspartic proteases exhibit optimal activity at low pH. Similar pH optimum values have been recorded for proteases from other fungal species such as *Rhizopus oryzae* (Hsiao et al., 2014), which had a pH optimum of 3.4, and *Aspergillus oryzae* BCRC 30118 with an optimum pH of 3 (Yin et al., 2013). Fungi are recognised as one of the top producers of aspartic proteases (Szecsi, 1992).

Relatively high activity, approximately 75% of the pH optimum (Figure 4.2), was also exhibited at pH 7.0 after a decrease in activity at pH 6.0 and this may indicate the presence of a second protease species with its own pH optimum of pH 7.0. Proteases produced by *Pleutorus ostreatus* and *Gleophyllum sepiarium* showed an optimum pH of 7.0 (Shaba and Baba, 2012). The optimum temperature and pH conditions of protease production are likely to reflect the climatic conditions found in environments inhabited by the original host plant (Zaferanloo et al., 2013).

Most metals were observed to boost enzyme actvity of the protease (Figure 4.6). Similar results on protease activity activation by Mn^{2+} ions have been reported by Nascimento and Martins

(2004), Rahman et al. (1994), as well as Shivanand and Jayaraman (2011). Protease activation by Cu^{2+} has also been observed by Shivanand and Jayaraman (2011). Saxena and Singh (2010) isolated a protease from a *Bacillus* strain which was activated by Mn^{2+} ions at 5 mM and activity increased after an increase in concentration to 10 mM. Also, Cu^{2+} ions increased enzyme activity at 5 mM but an increase to 10 mM decreased enzyme activity, though it was still higher than the control (Saxena and Singh, 2010). A similar trend was observed in this study with respect to Mn^{2+} and Cu^{2+} ions (Figure 4.6). Zinc is ubiquitous in most environments and acts as a co-factor for a number of enzymes. It also holds together structural components of protein sequences (Martino et al., 2000). This explains the enzyme's activation by Zn^{2+} as seen in Figure 4.6.

Based on the evidence that the protease activity is increased by the addition of metal ions (Figure 4.6), it is possible that the protease is metallo-dependent. The metal ions may play an important functional role in the enzyme's structure, possibly as co-factors and may also be involved in stabilising as well as activating the enzymes (Li et al. 2013; Saxena and Singh 2010; Zeng et al. 2014). The ability of the proteases to tolerate metal ions suggests that the fungus may originate from a polluted environment with high metal ion concentrations. Mycorrhizal fungi are known to protect their host plants from metal toxicity which would explain the enzyme's tolerance levels (Khouja et al., 2013). Comparison of an *O. maius* strain from a metal ion polluted site and one from a non-polluted site, however, showed that both fungal isolates were tolerant to zinc ions (Martino et al., 2000).

The proteases cannot, however, be classified as metalloproteases due to the fact the EDTA did not significantly inhibit enzyme activity (Figure 4.7). EDTA is a strong chelating agent capable of binding to metal ions forming structures called chelates, thus making the metal ions unavailable to the enzyme (Bowen and Kerwin, 1954; Flora and Pachauri, 2010). The proteases still managed to retain approximately 60% of their activity in the presence of this chelating agent, thus they were not completely dependent on metal ions for their function. Silver nitrate did not inhibit proteolytic activity, supporting its potential role in silver recovery. In contrast, previous studies by Yang et al. (2004) showed that the proteases produced by *O. maius* were inhibited by EDTA as well as silver nitrate. The activity of proteases produced by *O. maius* (CafRu2038b), however, was increased by almost 2.5-fold by the addition of 1 mM of silver nitrate. Only a concentration of 10 mM was shown to have a negative effect on the protease.

Complete inhibition of the protease by pepstatin A, confirmed the enzyme is an aspartic protease. Inhibition of a protease from *Aspergillus fumigatus* by pepstatin A confirmed its classification as an aspartic protease (Vickers et al., 2007). Proteases from *Aspergillus oryzae* MTCC 5431 (Vishwanatha et al., 2009) and *Rhizopus oryzae* (Hsiao et al., 2014) were inactivated by pepstatin A and were established as aspartic proteases.

Optimum gelatinase activity was observed at 68°C and the protease still exhibited high activity at 80°C, which suggests that the proteases are stable at high temperatures. In this respect, these proteases resemble those produced by *Pleutorus ostreatus* and *Gleophyllum sepiarium*, which showed a temperature optimum of 70°C; and those produced by *A. niger* which had a temperature optimum of 60°C (Shaba and Baba, 2012; Siala et al., 2009). *Rhizopus oryzae* aspartic proteases displayed a temperature optimum of 60°C (Kumar et al., 2005).

Thermostability of the proteases was assayed at 37°C (which is the temperature at which the assay reactions were run), 80°C and 100°C. High temperatures are known to denature proteins, thus the protein concentration decreased drastically at 100°C. However, even at a protein concentration of 3.3% of the initial concentration, the protease still managed to retain 40% of activity. This is a desirable trait of enzymes for their use in industry. Activity assays require a temperature where the enzyme is stable for the duration of the assay. (Daniel and Danson, 2013). Thermostability studies confirmed that the proteases could retain their catalytic activity for an hour at 37°C - the standard assay conditions at which reactions were conducted.

The industrial application of a protease is dictated by type of substrate it is able to degrade (Shankar et al., 2011). The protease displayed high specifity towards gelatin. No activity was observed for casein, bovine haemoglobin, azocasein and BSA. Casein, azocasein and hemoglobin zymograms confirmed the inability of the protease to degrade these substrates as no digestion bands were observed. This restricts its potential application of this protease in the industry of silver recovery.

On the Lineweaver-Burk plot, the K_m value was calculated to be 6.6 mg/ml, and on the Michaelis-Menten graph, the K_m value was 2.7 mg/ml. This shows that the protease has a high affinity for its substrate and does not require high substrate concentrations to reach V_{max}. Amid et al. (2014) reported a thermoalkaline protease from *Hylocereus polyrhizus* with a K_m of 2.8 mg/ml and Shankar et al. (2011) reported a protease with K_m of 5.1 mg/ml with casein as the substrate from *Beauveria* sp. An aspartic protease from *Rhizopus oryzae* has been shown to

have a K_m value of 5 mg/ml (Kumar et al., 2005). Values as low as 0.13 mg/ml have also been observed for proteases obtained from a goat skin surface metagenome. (Pushpam et al., 2011).

The maximum velocity, V_{max}, was 55.25 U/ml and 82.625 U/ml as calculated from the Michaelis-Menten graph and Lineweaver-Burk plots respectively. *Aspergillus niger* was shown to produce an acid protease with a V_{max} of 56 U/ml and an alkaline protease with a much higher V_{max} of 166 U/ml (El-Shora and Metwally, 2008). A different *Aspergillus* species, *Aspergillus terreus*, produced alkaline proteases with a low V_{max} of 29 U/ml (El-Shora and Metwally, 2008).

4.5 CONCLUSION

The proteases from *O. maius* were successfully characterised as aspartic proteases because of their low pH optimum as well as complete inactivation by the aspartic protease inhibitor, pepstatin A. Bovine haemoglobin, azocasein and casein were not hydrolysed by the proteases, which showed high substrate specificity for gelatin. The enzymes proved to be quite thermostable, displaying activity even after incubation at temperatures as high as 100°C for 4 hours. EDTA did not completely inactivate the proteases, despite the high activities observed when the enzymes were exposed to most divalent metal ions.

Silver did not inhibit the activity of the proteases at low concentrations, thus supporting their use in the application of silver recovery in the following chapter, Chapter 5.

CHAPTER 5

RECOVERY OF SILVER FROM X-RAY FILM USING PARTIALLY PURIFIED PROTEASES FROM *O. MAIUS*

5.1 INTRODUCTION

Enzymes are biodegradable and environmentally friendly, and their use has been preferred to conventional methods in many industrial processes. This use has proved to be effective in reducing global warming, acidification as well as eutrophication and in the process, saves a lot of energy (Abdelmoez and Mustafa, 2014; Jegannathan and Nielsen, 2013).

Silver is one of the least expensive metals in the world, characterised by it ductile and malleable properties (Drake and Hazelwood, 2005). It also possesses high electrical and thermal conductivity thus its use in the manufacturing of electrical appliances. Silver has antimicrobial properties and is used in the medical field for prevention of infections (Politano et al., 2013). It is also used for making coins, jewellery as well as silverware. Recycling of silver from these uses is vital for its continuous supply.

Due to its photosensitivity, silver has been used in the photographic industry (Belloni, 2002). Waste photographic film, negatives, paper, and old x-ray film may contain silver at concentrations of five ppm or more, causing them to be toxic hazardous wastes. X-rays films are made of a polyester film which is covered with a thin coating of gelatin that is impregnated with silver grain. A large amount of silver is released into the environment during the manufacture and disposal of certain photographic and X-ray films (Lakshmi and Hemalatha, 2016).

Chemical recovery of silver involves the use of chemicals like nitric acid, oxalic acid, sodium hydroxide, trioxonitrate (V) acid, cyanide solution as well as ammonia (Aktas et al., 2010; Ajiwe and Anyadiegwu, 2000; Arslan et al., 2011). X-ray films that are discarded by incineration produce hazardous smoke with a foul smell and the film cannot be recycled afterwards. Approximately 30% of silver is lost during incineration, reducing the efficiency of the method (Radha and Arun, 2010). The most effective way to reduce hazardous waste

responsibly and cost effectively is to reduce the amount or toxicity of the waste generated, thus enzymes present a better option for the recovery of silver from X-ray film.

The objective of this chapter was to recover silver from X-ray films by treating them with the crude enzyme as well as the partially purified protease obtained from Chapter 3. The collected film can be reused for the manufacturing of X-ray film or other things such as packaging films and soft-drink bottles (Radha and Arun, 2010). The silver can be re-used for jewellery, silver plating of utensils, electrical manufacture and in photography.

5.2 MATERIALS AND METHODS

5.2.1 RECOVERY OF SILVER

X- ray film was obtained from Professor Dames (Rhodes University, Department of Biochemistry and Microbiology). Potassium phosphate buffer (50 mM, pH 7.0) was used in all experiments.

The film was cut into 0.5 cm x 1 cm pieces, and incubated at 37°C in various treatments as follows;

Tube 1: 10 ml buffer + 1 g of film

Tube 2: 5 ml buffer + 5 ml enzyme sample

Tube 3: 5 ml buffer + 5 ml enzyme sample + 1 g film

Samples were incubated for 16 h and protein concentrations of the supernatants were measured at 0 h, 1 h, 2 h, 4 h and 16 h for experiments with the crude samples. Protein concentrations were measured at 0 h and 16 h for experiments with the partially purified sample. The Bradford assay described in Section 2.2.6 was used for protein determination. Samples were checked at each interval for film clearance. All experiments were performed in triplicate.

5.2.2 SCAN ELECTION MICROSCOPY

The pieces of X-ray film were subjected to analysis by scanning electron microscopy (SEM) to determine the presence or absence of silver before and after the recovery process. All samples were air dried for 2 hours before being mounted onto aluminium stubs. To identify and quantify the elements on the pieces of film, the energy dispersive X-ray spectrometry (EDS) method was applied using a INCAPenta FET X 3 Oxford Instrument at an accelerating

voltage of 10 keV. Thereafter, the samples were coated with gold using a Quorum Q150 S machine and examined using the Tescan Vega LMU at a magnification of 2.00 kx.

5.3 RESULTS

Protein concentrations in all the tubes (with supernatants from crude enzyme samples) remained fairly constant from 0 h to 4 h. After 16 h, the protein concentrations had increased in all tubes, with the tubes containing buffer, enzyme and film showing the highest increase from 0.17 mg/ml to 0.6 mg/ml. An increase of approximately 0.1 mg/ml was observed after 16 h in the control tubes containing buffer and enzyme.

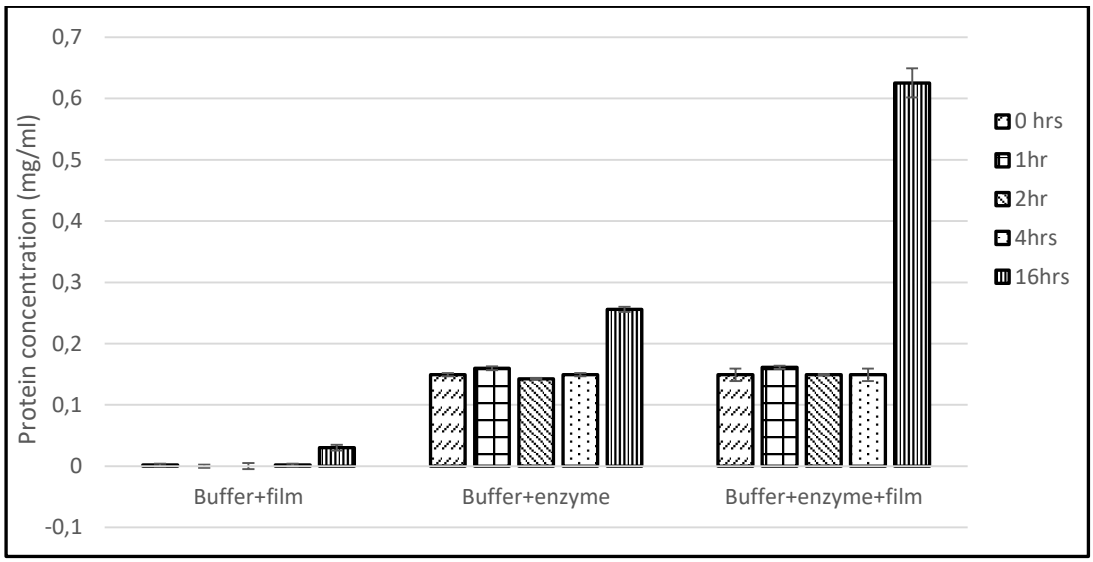

Figure 5.1 Protein concentration of silver recovery in the supernatants (crude samples) Data points are shown as mean values ± SD (n=3).

Figure 5.2 shows uncleared film in panel B after being incubated with buffer only. Panel C shows cleared film after being incubated with the crude fraction containing enzyme, suggesting removal of the silver containing coating.

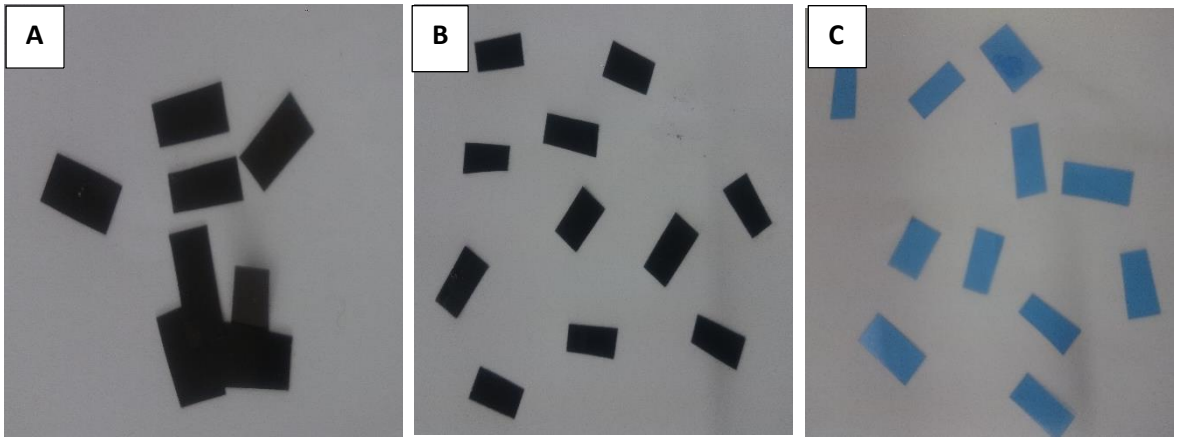

Figure 5.2 Images of the X-ray film; A - before treatment, B – after incubation in buffer for 16 h and C –after incubation in crude enzyme sample for 16 h.

For supernatants containing partially purified enzyme (PPE), there were no significant increases in protein concentration in the tubes containing buffer and film only. The tubes containing the enzyme sample and buffer only showed an increase in protein concentration of approximately 0.05 mg/ml after 16 h. The tubes containing the X-ray film, enzyme sample and buffer showed a protein concentration of 0.089 mg/ml higher than the control tubes with buffer and enzyme sample after 16 h (Fig 5.3).

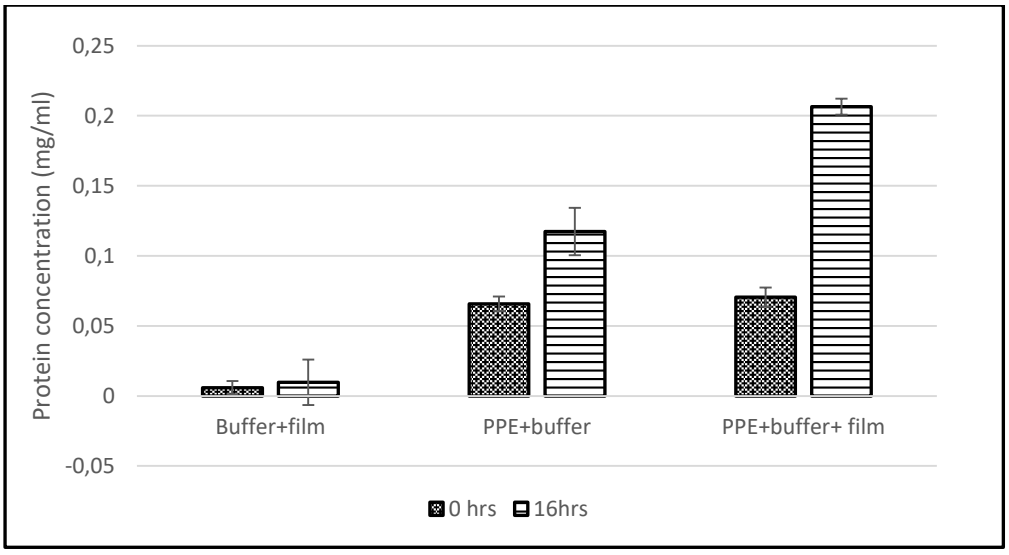

Figure 5.3 Protein concentration of silver recovery supernatant (Partially Purified Enzyme – PPE). Data points are shown as mean values ± SD (n=3).

All sections of X-ray film remained unchanged after incubation with buffer only (Figure 5.4 A). Some sections were partially cleared and others remained unchanged after incubation with

the partially purified enzyme (Figure 5.4 B). Some sections were completely cleared in Figure 5.4 B.

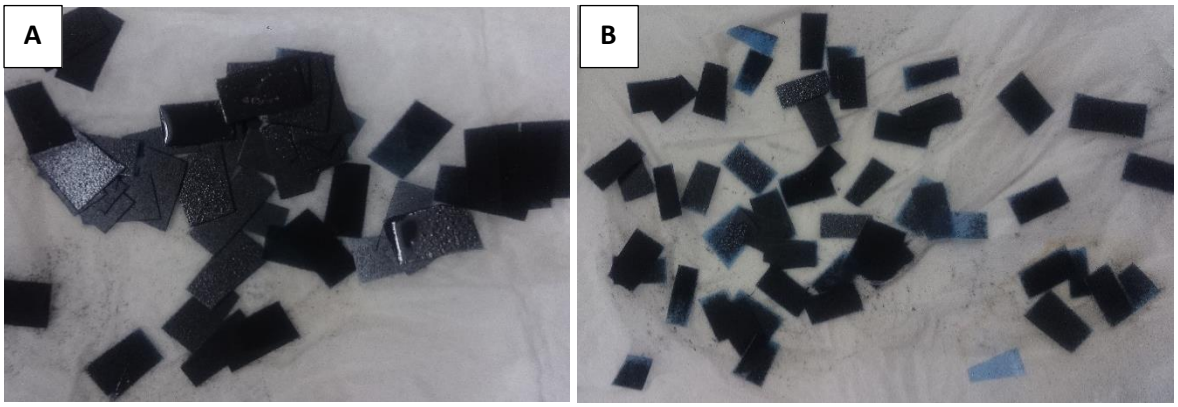

Figure 5.4 Images of the X-rayfilm; A –after incubation with buffer only for 16 h and B – after incubation with partially purified enzyme for 16 h

A distinct characteristic peak of silver (Ag) is shown in the EDS image in Figure 5.5 showing the presence of silver in the film before incubation with the crude containing the protease.

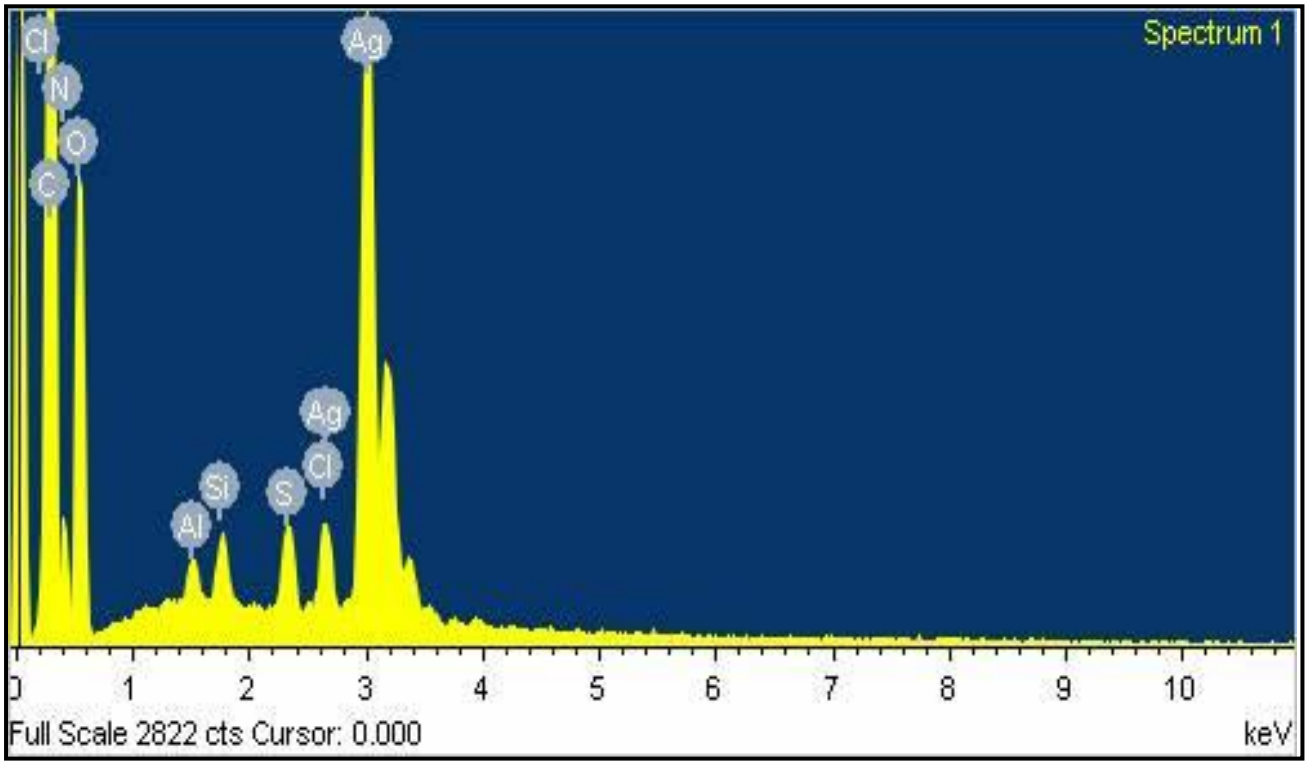

Figure 5.5 EDS profile of the X-ray film before incubation with the protease.

The absence of a peak of silver (Ag) is observed in the EDS profile in Figure 5.6. This is the clear film from Figure 5.2 C that was incubated with the crude protease sample for 16 h.

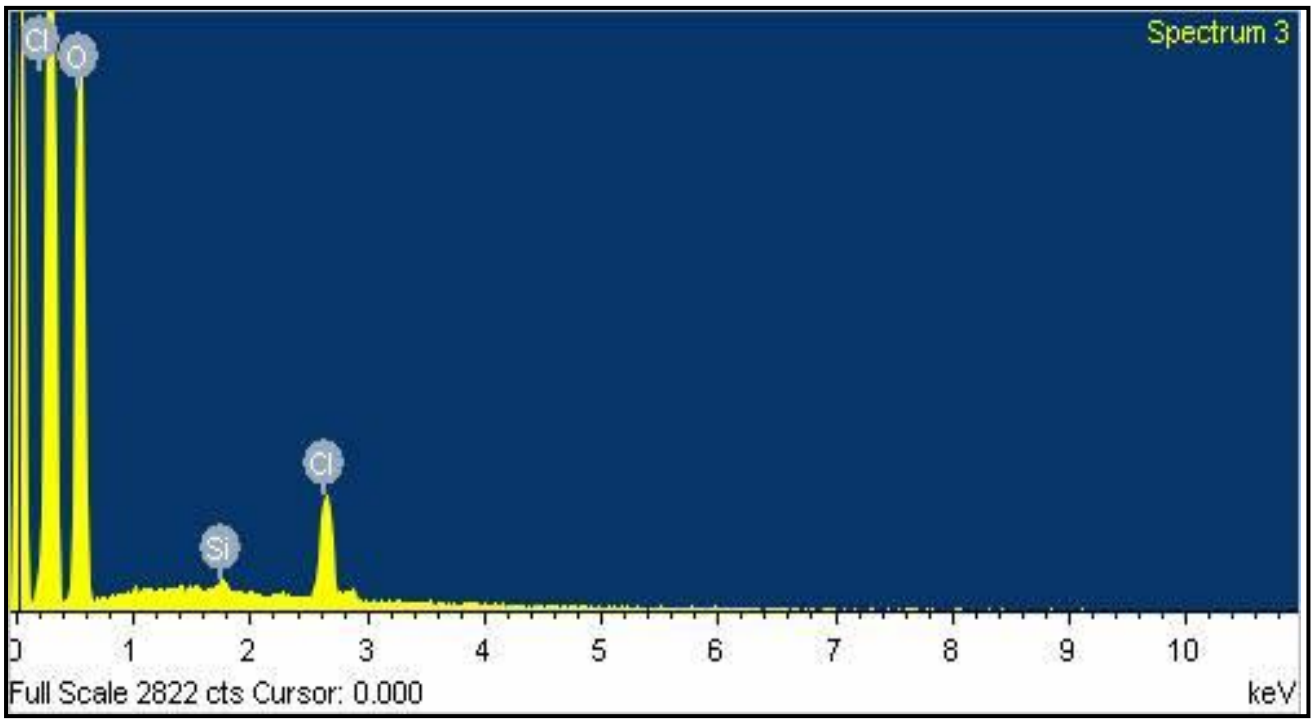

Figure 5.6 EDS profile of the X-ray film after incubation with the protease.

Figure 5.7 shows clearance of the film (panel B) on SEM after exposing it to the crude enzyme sample. The emulsion layer consisting of gelatin and silver halides seen in panel A was completely removed in panel B.

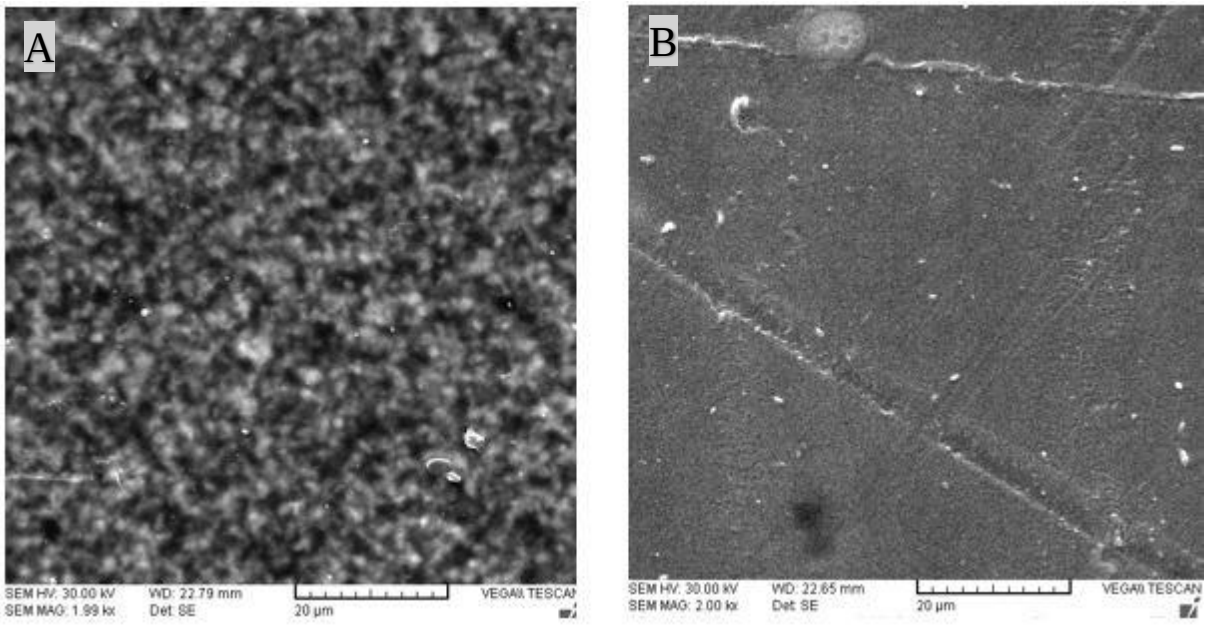

Figure 5.7 SEM images of the X-ray film, A: before incubation with the enzyme and B: after incubation with the enzyme.

5.4 DISCUSSION

The overall increase in protein concentration in tubes containing enzyme and film compared to the other two controls indicates removal of protein from the X-ray film into the solution. This is suspected to be the gelatin that is embedded in the film. The silver that is impregnated in the gelatin layer is removed with hydrolysis of the layer. This is confirmed by the EDS images showing a peak of silver before (Figure 5.5) and the absence of the peak after (Figure 5.6) clearing of the film by the enzyme. Similar SEM images confirming film clearance (Figure 5.7) have also been reported by Radha and Arun (2010). Clearing of X-ray films after incubation with a protease has also been observed by Radha and Arun (2010) using bromelain from pineapples, Choudhary (2013), using a protease from *Aspergillus versicolor,* as well as Parpalliwar et al. (2015), using an alkaline protease from *Bacillus subtilis* (NCIM 2724).

The crude extract containing protease from *O. maius* showed complete silver extraction from the film after 16 h. Pathak and Deshmukh (2012) showed silver extraction after 24 h of incubation. Nakiboglu et al. (2001) and Choudhary (2013) isolated proteases, from *Bacillus subtilis* and *Aspergillus versicolor,* respectively, that extracted silver in 15 min.

The partially purified fraction was not as effective in recovery of the silver from the film as the crude fraction. This is possibly because of its low protein concentration in the partially purified

sample. Another explanation would be the absence of stabilisers that are normally found in the crude sample. Many industrial processes, however, do not necessarily require a high degree of enzyme purity. Crude protease solutions are acceptable provided there is no interference by other enzymes and no reduction in quality of the product (Rao et al, 1998).

5.5 CONCLUSION

The proteases were able to successfully recover silver from X-ray film with the crude extract showing a higher efficiency compared to the partially purified sample. Silver was completely removed from the film, leaving it clear. The protease from *O. maius* (CafRU082b) is therefore a potential contender for use in the recovery of silver from photographic film.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSION, AND FUTURE RECOMMENDATIONS

Proteases are extensively applied in a variety of industrial applications. They make up the largest single family of enzymes and constitute a large percentage of the enzyme needs in industry. There is an increased necessity for proteases that are better adapted to industrial applications.

This study investigated the production, purification as well as characterisation of proteolytic enzymes from the fungus, *Oidiodendron maius* (CafRU082b). Also investigated, was the potential use of this protease in the recovery of silver from X-ray films

Like most fungi, the proteases from *O. maius* were expressed extracellularly in the liquid medium. Preliminary studies in Chapter 1 revealed the production of proteases with the ability to degrade gelatin on PDA plates containing the substrate gelatin. This phenomenon was observed as a zone of clearance around the growing fungus after incubating the plate cultures with mercuric chloride dissolved in HCl. Comparable qualitative studies using agar plates incorporated with gelatin have been reported and showed similar results (Pant et al., 2015). Plates containing bovine haemoglobin and casein as substrates for the detection of proteases did not contain any clearance zones. It was therefore concluded that the fungus, *O. maius* (CafRU082b), was not capable of producing haemoglobin and casein hydrolysing proteases but had the ability to produce gelatinases.

Growth of the fungus in Modified Melin Norkran's medium was subsequently supplemented by gelatin for the induction of gelatinase production. The fungus grew well in the medium into spherical fungal masses at room temperature in the dark, since it was suspected to be light sensitive (Rice and Currah, 2005; Sigler and Gibas, 2005; Wei et al., 2016). Extracellular proteases produced showed activity using activity assays with gelatin as a substrate and they were extracted on day 14 in all subsequent experiments following the construction of the fungal growth curve. Asker et al. (2013) as well as Pant et al. (2015) also used gelatin as a substrate for their protease activity assays.

Chapter 3 described the partial purification of the protease that was produced and extracted as per experiments detailed in Chapter 2. The first step of purification involved precipitating proteins from the extracellular fraction, thus removing all other non-protein cellular contents. Precipitation was achieved using pre-chilled acetone and the protein precipitate was re-dissolved in buffer. Proteases have been previously partially purified by acetone precipitation in combination with other methods (Dutta and Banerjee, 2006; Laver and Trikojus, 1955; Mukhtar, 2013; Vishwanatha et al., 2009). Confirmation of the presence of gelatin-hydrolysing proteases, as well as an estimation of their size, was obtained by performing zymograms. Triton X-100 was used as the detergent in renaturation of the enzyme. Zymography was successfully carried out with the zymogram images showing gelatin hydrolysis after staining with Coomassie Brilliant Blue G 250. Wei et al. (2016) used 0.1% amido black to stain the zymogram gels in place of Coomassie Brilliant Blue. The molecular weight of the bands was observed to be 36 kDa. This justified the use of 50 kDa and 30 kDa molecular weight cut off filters during purification. Larger proteins remained in the retained fractions and the enzyme of interest passed through.

SDS-PAGE was performed to confirm protein purity and silver staining was used because of the low protein concentration in the partially purified samples. The enzyme showed partial purity, with one other band and some protein smears on the gel. These were possibly due to proteolytic degradation during purification. The partially purified enzyme, however, showed a high purification fold of 37 after the final purification step, as well as a final yield of 196%.

For a purer protein, ion exchange chromatography could be used, where proteins are separated according to their charge (Panesar, 2010). Another possibility would be to isolate and clone the gene for the protease from the fungus, insert it into a vector, probably yeast cells, for expression of the protease. Yamashita et al. (1987) made use of the yeast *Saccharomyces cerevisiae* to express protease genes from the fungus *Mucor pusillus.* The gene of a serine protease from the fungus *Chaetomium thermophilum* was also successfully expressed in the yeast *Pichia pastoris* (Li and Li, 2009). This method results in the production of pure protein samples in large quantities. However, it was not pursued in this study as it is not always successful, and optimisation is a tedious process which was not possible in the given time-frame.

Physico-chemical characterisation of the partially purified enzyme was performed. The enzyme had an acidic optimum pH of 3.0, characteristic of an acid/aspartic protease. Ellaiah et

al. (2002) suggests that most fungal aspartic proteases are not stable at pH values above pH 7.0, which would explain why the enzyme did not show any activity at all from pH 9.0 onwards.

A high optimum temperature of 68°C was observed for the protease. The protease proved to be quite thermostable with samples retaining 90% of protein concentration after incubation at 37°C for 4 hours. It also showed over 50% residual activity after incubation at 100°C for 2 hours, a temperature at which most enzymes are completely inactivated and denatured. Further experiments to increase the protease's thermostability could be performed by the addition of stabilisers that prevent thermal inactivation (Bhosale et al., 1995). The effect of stabilisers such as mannitol, sorbitol and xylitol could be explored. These additives, including glycerol and trelahose, were found to be very effective in increasing the stability of a fungal alkaline protease produced by *Conidiobolus brefeldianus* MTCC 5184 (Nirmal and Laxman, 2014). Shivanand and Jayaramann (2011) also found detergents such as CTAB, Triton X-100 and SDS to contribute significantly to the stability of the protease produced by *Bacillus aquimaris* VITP4. The addition of sorbitol, PEG-8000, as well as glycine, increased thermostability of the protease isolated from *Conidiobolus coronatus* (NCL 86.8.20) (Bhosale et al., 1995).

The protease showed a high tolerance to divalent metal ions. All metal ions except Ca^{2+} enhanced protease activity at a concentration of 1 mM. At a concentration of 10 mM, all metal ions (except Ca^{2+} and Ag^{2+}) still displayed activities higher than the control. The metal chelating agent and metalloprotease inhibitor, EDTA, did not completely inhibit the enzyme, confirming that the enzyme is not a metalloprotease. Similar results were observed by Thaz and Jayaraman (2014), where metal ions enhanced protease activity, but activity was only slightly inhibited by EDTA. The protease was found to be a serine protease, inhibited by PMSF (Thaz and Jayaraman, 2014). Chittoor et al. (2016) suggested that these metal ions are not necessarily directly involved in protease activity but improve stability of the enzyme, which was seen with the protease from *Conidiobolus coronatus* (NCL 86.8.20), where the half-life of the enzyme was tripled by the addition of Ca^{2+} (Bhosale et al., 1995).

Further experiments on the effects of stabilisers could be coupled with experiments on optimum storage conditions of the protease. Long shelf life is a desirable characteristic for an enzyme in industry. The addition of stabilisers could result in the enzyme being stable for longer at optimum storage conditions. The alkaline protease from *Aspergillus terreus* gr. was found to be stable at 4°C, 28°C and -20°C (Niyonzima and More, 2015).

Inhibition by PMSF as well as E-64 was negligible, with high concentrations of E-64 enhancing protease activity. Complete inhibition by pepstatin A confirmed its classification as an aspartic protease suggested by its low pH optimum. Pepstatin is a known aspartic protease inhibitor (Ellaiah et al., 2002). Proteases from the ripe fruits of *Salpichroa origanifolia* were also classified as aspartic proteases due to inhibition by pepstatin A and activity at acidic pH (Rocha et al., 2015).

Substrate specificity experiments revealed that the proteases did not exhibit activity on the proteolytic substrates casein, azocasein, haemoglobin and BSA. However, they exhibited high specificity towards gelatin. With gelatin as the substrate, the protease displayed a V_{max} of 82.625 U/ml and a K_m of 6.6 mg/ml.

The efficiency of the protease to recover silver from X-ray films was explored. Silver recovery was successful with SEM images of the film and EDS profiles showing complete disappearance of the silver (Ag) peak after the recovery experiments. This was also confirmed by visual inspection of the film after incubation, with the crude extract completely clearing all sections of film and the partially purified protease only clearing a few sections after the same reaction times. Protein concentration increase was also more pronounced with the crude protease samples. Further experiments involving recovering of the actual metal from the solution after removal from film are recommended. Smelting the slurry attained from treatment of the film in the presence of borax is a method suggested by Nakiboglu et al. (2001). Parpalliwar et al. (2015) smelted the slurry obtained in the presence of borax at 900°C in a furnace. Another method, used by Radha and Arun (2010), involved smelting the dried slurry in the presence of sodium carbonate at 750°C in a furnace. Alternatively, the silver in the slurry can be recovered as silver chloride, which is used as pottery glazes as well as in wound healing products (Shankar et al., 2010).

Experiments on the reusability of the protease could also be performed. Shankar et al. (2010) showed reusability of protease for four cycles of gelatin hydrolysis and Masui et al. (1999) used the alkaline protease from *Bacillus* sp for five cycles of silver recovery. However, an increase in treatment time after every re-use of the proteases was observed (Shankar et al., 2010).

A study of the effect of temperature on hydrolysis of the gelatin layer is also necessary. The proteolytic decomposition of the gelatin layer occurred more rapidly at higher temperatures. Masui et al. (1999) suggests swelling of gelatin on the film before hydrolysis by the protease,

as there is a lag phase prior to the protein being released rapidly from the film. Pre-incubation of the X-ray film at the reaction temperature may be necessary before adding enzyme to allow the gelatin to swell. This would minimise exposure of the enzyme to the high temperatures, thus possibly decreasing incubation time and improving reusability. Lag periods before hydrolysis of the gelatin layer could also be due to slow adsorption of the protease onto the surface (Shankar et al., 2010).

The temperatures used in previous studies ranged from 40°C -50°C (Al-Abdalall and Al-Khaldi 2016; Nakiboglu et al., 2001; Shankar et al., 2010). The protease from *O. maius* showed activity at high temperatures as well as satisfactory thermostability in characterisation experiments, thus delaying inactivation of the enzyme due to high temperature.

The protease, although it required a longer time compared to other proteases studied in literature, to recover silver from X-ray film, proved to be a potential contender for use in the industry. This silver recovery feature, coupled to its impressive thermostability, metal ion tolerance and kinetic characteristics make it desirable for commercialisation.

For purposes of upscaling of the recovery of silver from X-ray film to industrial scale using this protease, additional studies would need to be performed on the actual amount of X-ray film being produced in the current digital age. A survey of the amounts of film that have already been produced and have not yet been properly disposed of, would also be necessary to determine the economic viability of this approach.

Recovery and purification of the enzyme add to the cost of industrial production, with industries such as the detergent industries requiring crude enzymes, whereas medical industries require enzyme samples that have gone through extensive purification processes (Saggu and Mishra, 2017; Sawant and Nagendran, 2014). The silver recovery process does not necessarily require a purified enzyme, and the crude protease fraction from *O. maius* proved to work better than the partially purified enzyme. Previous silver recovery studies using microbial proteases only made use of crude enzyme preparations and did not explore using purified proteases (Choudhary, 2013; Nakiboglu et al., 2001; Parpalliwar et al., 2015; Shankar et al., 2010). The use of a crude protease preparation would reduce costs in the event of commercialisation. Crude enzyme preparations also have the advantage of being produced in bulk and this, coupled with the inexpensive fermentation media requirements for *O. maius* would contribute to the economical production of the proteases for silver recovery (Sawant and Nagendran, 2014).

Further studies, however, are recommended for optimising the growth of the fungus for maximal protease production. The effect of different metal ions on enzyme activity were explored, but not their effect on the growth of the fungus itself. Future studies on the identification of the protease by molecular methods coupled to in-silico structure predictions would also be interesting.

The main significance on this study and contribution of this study to the field was the discovery of efficient proteases from a novel fungal source (*O. maius* CafRu082b) and their application in recycling silver as well as reducing environmental pollution simultaneously.

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APPENDICES

APPENDIX I: LIST OF REAGENTS

2-mercaptoethanol	Sigma (CAT No. M6250)
Acetone	Merck (Cat No. 822251)
Acrylamide	Sigma (CAT No. A8887)
Ammonium persulfate	Sigma (CAT No. A3678)
Azocasein	Sigma (CAT No. A2765)
Bovine haemoglobin	Sigma (CAT No. H2625)
Bovine Serum Albumin	Sigma (CAT No. A7906)
Bradford's Reagent	Sigma (CAT No. B6916)
Bromophenol blue	Sigma (CAT No. 114391)
Calcium chloride dihydrate	Merck (CAT No. 208290)
Calcium chloride	Sigma (CAT No. C1016)
Casein	Sigma (CAT No. C7078)
Chloramphenicol	Sigma (CAT No. C0378)
Cobalt (II) sulphate heptahydrate	Sigma (CAT No. C6768)
Coomassie Brilliant Blue G 250	Merck (CAT No. 115444)
Copper (II) sulphate pentahydrate	Merck (CAT No. 102790)
di-Ammonium hydrogen phosphate	Merck (CAT No. 1.01217)
di-Potassium hydrogen phosphate	Merck (CAT No. 137010)
Ethylenediaminetetraacetic acid (EDTA)	Sigma (CAT No. E9884)
Folin Ciolcateau's reagent	Sigma (CAT No. F9252)
Gelatin	Fluka Analytical (CAT No 48723)

Glacial acetic acid	Merck (CAT No. 1.00063)
Glucose	Merck (CAT No. 346351)
Glycerol	Merck (CAT No. 818709)
Glycine	Merck (CAT No. 104169)
Hydrochloric acid	Merck (CAT No. 100319)
Iron (III) chloride	Sigma (CAT No. 1557740)
Iron (II) sulfate heptahydrate	Sigma (CAT No. F7002)
L-tyrosine	Sigma (CAT No. T3754)
Magnesium sulphate anhydrous	Merck (CAT No. 106067)
Malt Extract	Merck (CAT No. 105391)
Manganese (II) sulphate monohydrate	Sigma (CAT No. M7364)
Methanol	Merck (CAT No. 822283)
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma (CAT No. T9281)
N,N-methylenebisacrylamide	Sigma (CAT No. M7279)
Pepstatin A	Sigma (CAT No. P5318)
Phenylmethylsulfonyl fluoride (PMSF)	Sigma (CAT No. P7626)
Potato Dextrose Agar	Merck (CAT No. 110130)
Protein Unstained Marker	BioRad (CAT No. 1610363)
Silver nitrate	Sigma (CAT No. 209139 M)
Sodium azide	Merck (CAT No. 8.22335)
Sodium carbonate	Merck (CAT No. 106392)
Sodium chloride	Merck (CAT No. 137017)
Sodium dodecyl sulphate	Merck (CAT No. 817034)
Sodium nitrite	Sigma (CAT No. 237213)

Thiamine HCl	Calbiochem (CAT No. 5871)
Trans-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64)	Sigma (CAT No. E3132)
Trichloroacetic acid	Sigma (CAT No. T6399)
Tris (hydroxymethyl) aminomethane	Merck (CAT No. 108382)
Triton X-100	Merck (CAT No. 108643)
Tryptone	Merck (CAT No. 107213)
Yeast Extract	Merck (CAT No. 111962)
Zinc sulphate heptahydrate	Sigma (CAT No. Z0251)

APPENDIX II: STANDARD CURVES

II A: Bradford Standard Curve

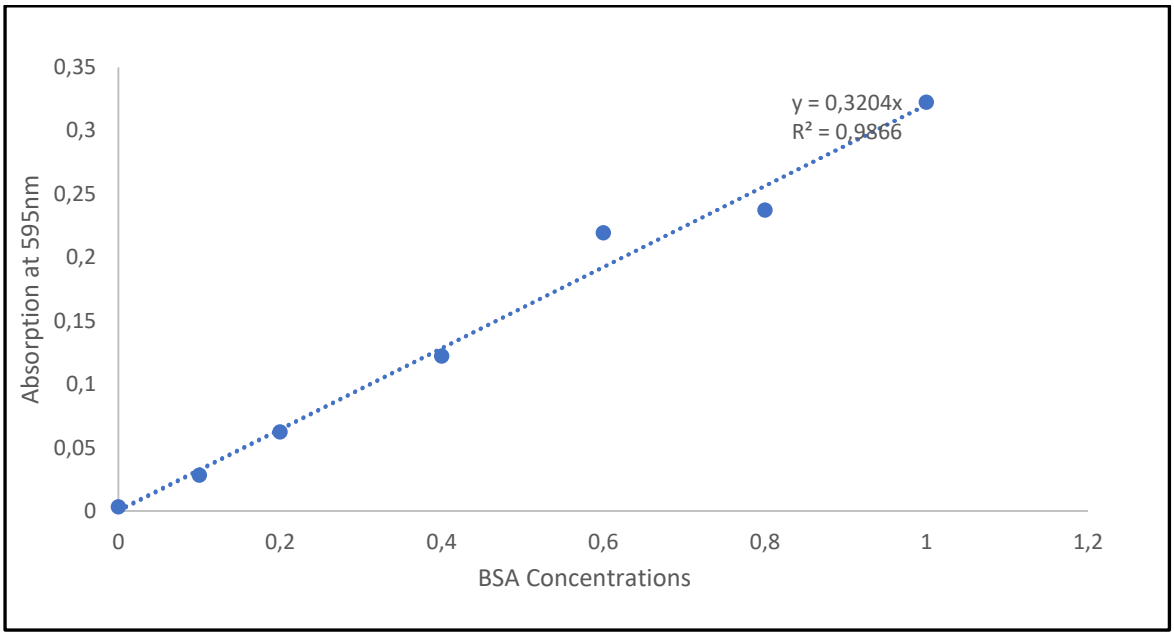

Figure II A. Bradford standard curve for protein concentration determination using BSA as a standard. Data points shown represent means ± SD (n=3).

II B Tyrosine standard curve

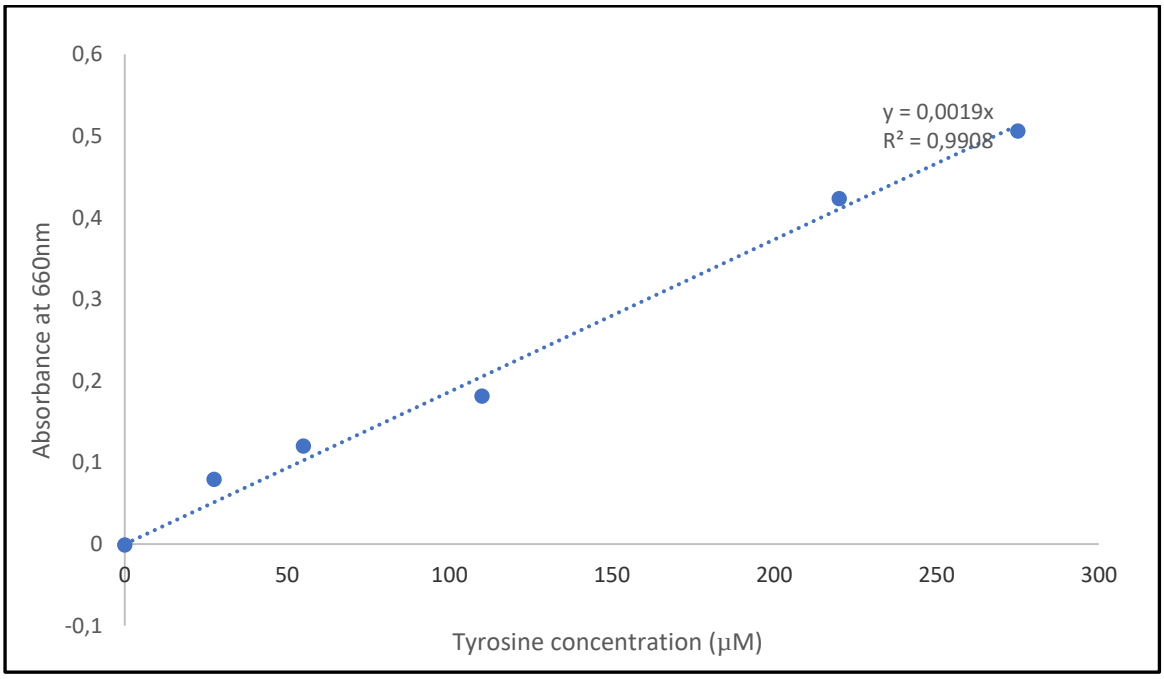

Figure II B. Tyrosine standard curve for calculation of protease activity with tyrosine as a standard. Data points shown represent means ± SD (n=3).