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IDENTIFICATION, PURIFICATION AND CHARACTERISATION OF A KERATINOLYTIC ENZYME OF *Chryseobacterium carnipullorum*

By

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Dissertation submitted in fulfilment of the requirements in respect of the Master of Science Degree

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DECLARATION

I Elebert Pauline Mwanza declare that the Master's degree research dissertation that I herewith submit for the Master of Science Degree at the University of the Free State in the Faculty of Natural and Agricultural Science is my own independent work and has not previously been submitted by me at another University/Faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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E. P. Mwanza

2018

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
BLAST	Basic Local Alignment Search Tool
CFB	*Cytophaga-Flavobacterium-Bacteriodes*
cfu	Colony forming units
^{o}C	Degrees Celcius
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii*
FPLC	Fast protein liquid chromatography
g	Gram
gDNA	Genomic deoxyribonucleic acid
h	Hour
HCl	Hydrochloric acid
H_2S	Hydrogen sulphide
$HgCl_2$	Mercuric chloride
IMAC	Immobilised metal ion affinity chromatography
IPTG	Isopropyl-b-D-thiogalactopyranoside
LB	Luria-Bertani / Lysogeny Broth
LB-Kan	Luria-Bertani / Lysogeny Broth containing 50 mg/ml kanamycin
Log	Log_{10}
LC-MS/MS	Liquid Chromatography-tandem mass spectrometry
mg	Milligram
min	Minute
ml	Millilitre
NA	Nutrient Agar
NB	Nutrient Broth
NH_4HCO_3	Ammonium bicarbonate
PMSF	Phenylmethyl sulfonyl fluoride
PRP	Proteasome resistant protein
PRP^{SC}	Proteasome resistant protein scrapie form
PRP^{C}	Proteasome resistant protein cellular form
rDNA	Ribosomal Deoxyribonucleic acid

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TTS	Tris-Tricine-Sodium dodecyl sulphate
WFM	Whole feather medium

ABSTRACT / SUMMARY

Microbial enzymes are essential for sustainable technology and green chemistry coupled with a wide scope of genetic manipulation. *Chryseobacterium carnipullorum* 9_R23581^{T}, isolated from raw chicken is a potential keratin degrader. Feather degradation is a challenge for most conventional proteases due to the structure of keratin material which makes up more than 90% of the feathers. Keratin is composed of tightly packed α–helix and β–sheets that are further assembled into supercoiled polypeptide chains. Furthermore, the presence of hydrophobic interactions, disulphide bridges and hydrogen bonds in keratin, contribute to its recalcitrant property, resulting in an extremely stable structural protein. Keratinases have huge potential applications in various industries that include the poultry processing industry, production of rare amino acids and semi-slow nitrogen release fertilizers in organic farming among others. This study focused on identifying, purifying, and characterising a proteolytic enzyme produced by *C. carnipullorum*. Growth studies were conducted to determine the stage of enzyme production and it was observed that secretory enzymes are produced during the exponential growth phase of *C. carnipullorum*. The secretory proteins were visualised using SDS-PAGE and identified using LC-MS/MS. Primers were designed on selected genes of interest, which were amplified from the genome of *C. carnipullorum* (accession number NZ-FRCD01000002.1). Peptidase M64 was identified as the most likely main component of the keratinolytic enzymes produced by the strain used in this study. The keratinolytic enzyme (peptidase M64) was expressed in *E. coli* BL21[DE3] cells and purified using Immobilised Metal Affinity Chromatography (IMAC). The molecular mass of the keratinase was determined to be about 50 kDa while its optimum temperature and pH were 50 ^{o}C and 8.5, respectively. Different enzyme assays were conducted to test activity. The enzyme activity was inhibited by PMSF and it was enhanced by the presence of divalent metal ions such as $MgSO_4$ and $CaCl_2$.

Key words: *Chryseobacterium carnipullorum,* degradation, keratinase, keratin, feather waste, proteins

CHAPTER 1: INTRODUCTION

Chryseobacterium is one of the genera that evolved from the family Flavobacteriaceae after reclassification of some poorly described species in the genus *Flavobacterium* (Holmes, 1992; Hugo and Jooste, 2003). It encompasses some spoilage and psychrotolerant species, which are widely distributed in nature (Hugo *et al.,* 2003; Hugo and Jooste, 2012, Bernardet *et al.,* 2011). The significance of these bacteria in food and feed has been the topic of investigation for a number of years since these organisms may play different roles in the food and feed industry especially by their ability to exhert strong proteolytic activity (Bernardet *et al*., 2011). These proteases may therefore be involved in spoilage of food, which have a negative impact on the food industry. On the other hand, these enzymes may have positive applications if they have the ability to break down certain pollutants in the food industry.

Proteases are associated with the largest sector of the worldwide market for industrial enzymes (Sarrouh *et al*., 2012) and they are extensively applied in several industrial sectors, research as well as biotechnology (Mótyán *et al*., 2013). Some proteolytic enzymes are keratinolytic in nature due to their ability to degrade keratin, a highly rigid sulphur containing fibrous protein made from either α-helices or β-pleated sheets held together by disulphide crosslinks and hydrogen bonds (Suzuki *et al*., 2006; Wang *et al*., 2016).

Projections by Alexandratos and Bruinsma (2012) indicated an increase in global poultry production among other variables, this implied a growing threat of pollution from the poultry industry since huge amounts of feathers are produced as by-products during poultry processing. Feathers can not be easily digested by proteolytic enzymes such as pepsin, trypsin and chymotrypsin, while keratinolytic enzymes are able to do so (Sivakumar *et al*., 2013). Keratinolytic enzymes or keratinases are a group of proteases that have a unique ability to specifically degrade keratin (Ramnani and Gupta, 2004). These enzymes have other potential biotechnological applications which include solid waste management (Sahni *et al.,* 2015), dehairing of hides during leather processing (Vigneshwaran *et al.*, 2010), production of feed, glues, films, fertilizers, and rare amino acids, such as cysteine, proline and serine from feathers (Gupta and Ramnani, 2006; Cai *et al*., 2009), prion decontamination (Gupta *et al*., 2013), detergent formulations (Paul *et al*., 2014) as well as in cosmetics and pharmaceuticals that target conditions such as acne, corn and callus (Gupta *et al.*, 2013).

Considering the extensive potential applications of keratinases in the different industries, it is essential to probe their biological activity and optimise their production where necessary. The investigation of the biological activity of candidate genes can be done by cloning and

recombinant protein expression studies. These studies involve amplifying the gene of interest from the host genome, ligating the gene into a vector plasmid, transforming the vector plasmid into a suitable expression host which can then be inoculated on selective media containing a specific marker to enable the selection of positive clones (Zhang, 2016).

Chryseobacterium carnipullorum, a species isolated from raw chicken was observed to possess keratinolytic activity in a PhD study by Charimba (2012). This added to the number of the few Gram-negative keratinolytic micro-organisms known to date. The keratinase encoding genes from *C. carnipullorum* have not yet been cloned, expressed or characterised, hence, the motivation for this study.

Therefore, the aims of this study are to:

1. Identify keratinolytic enzymes produced by *C. carnipullorum*
2. Express the enzyme(s) in a suitable expression system
3. Purify and characterise the expressed enzyme(s)

The objectives are to:

1. To determine the stage of enzyme production
2. To identify secretory enzymes with proteolytic activity
3. To clone the gene(s) coding for the proteolytic enzyme(s) in an expression vector and express the enzyme(s) in a bacterial expression system
4. To purify the enzyme(s) and conduct enzymatic assays

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

The genus *Chryseobacterium* encompasses some spoilage and psychrotolerant species (Hugo *et al.*, 2003; Hugo and Jooste, 2012). The original members of this genus were *Chryseobacterium gleum, C. indologenes, C. indotheticum, [C. meningosepticum], C. balustinum* and *C. scopthalmum*, which were all from the genus *Flavobacterium* (Holmes, 1992; Hugo and Jooste, 2003). Most of the food spoilers are still referred to as flavobacteria or CDC Group IIb organisms in literature (de Beer, 2005). Both the *Chryseobacterium* and *Flavobacterium* genera form part of the 141 genera in the family Flavobacteriaceae (Euzéby, 2017).

Species of *Chryseobacterium* occur in various ecological niches; which include food products and the production environment, the hospital environment, freshwater and marine environments, soil, diseased plants, mammals, molluscs, amphibians, fish, crustaceans, reptiles, sea urchins, digestive tract of insects and vacuoles or cytoplasm of amoebae (Jooste and Hugo, 1999; Hugo and Jooste, 2003; 2012; Bernardet and Nakagawa, 2006).

Chryseobacterium species are significant in food spoilage due to the production of proteolytic and lipolytic enzymes which contribute some off-odours and off-flavours to the food (Sørhaug and Stepaniak, 1997). The first description of *Chryseobacterium* given by Vandamme *et al.* (1994) noted that the members of the genus present strong proteolytic activity. Proteolytic and lipolytic activities were also reported in two *Chryseobacterium* strains from raw milk in a study done by Hantsis-Zacharov *et al.* (2008a). Recent studies by Bekker *et al.* (2015) also reviewed strong proteolytic activity for both *C. joostei* and *C. bovis,* making them likely candidates in spoilage of food products.

Chryseobacterium has also been associated with keratinolytic activity (Riffel and Brandelli, 2006; Charimba, 2012; Park *et al*., 2014; Gurav *et al*., 2016). Keratinolytic enzymes belong to a group of proteases that have the ability to specifically degrade keratin (Ramnani and Gupta, 2004). The presence of hydrophobic interactions, disulphide bridges and hydrogen bonds in keratin makes it an extremely stable structural protein with a low degradation rate (Sahni *et al.*, 2015). Hence, keratinases play an important role in applications involving keratin degradation such as in degrading chicken feathers, as they are made up of at least 90% keratin which is not easily digestible by other enzymes. Feathers are usually produced in enormous quantities in poultry processing plants leading to disposal problems (Santos *et al.*, 1996; Parry and North, 1998; Charimba *et al.*, 2013). Keratinases may also be used during the de-hairing process in leather production (Vigneshwaran *et al.*, 2010) as well as in

prion degradation as an eco-friendly alternative to chemicals and other harsh methods (Langeveld *et al.*, 2003; Gupta *et al.*, 2013).

This literature review is aimed at discussing a brief background of *Chryseobacterium*, the significance of *Chryseobacterium* species in food and feed as well as the keratinolytic characteristics of *Chryseobacterium* in terms of keratin degradation, potential applications, factors affecting keratinase production and production/harvesting of microbial keratinases.

2.2 THE GENUS *Chryseobacterium*

2.2.1 Ecology

Species of *Chryseobacterium* occur in a wide range of habitats, which include food, natural environments such as water and soil, industrial environments and clinical environments.

A variety of food sources have been reported to exhibit spoilage defects due to *Chryseobacterium* species. The products include meat and meat products, fish, poultry and dairy products (Bernardet *et al.*, 2006). The CDC Group IIb strains are widely distributed in nature and may be found in foods such as dairy products, raw meats, vegetables and poultry (Jooste *et al.*, 1985; Hugo, 1997).

Other foods in which *Chryseobacterium* species have been found include fish (*C. arothri*, Campbell *et al.*, 2008; *C. balustinum*, Holmes *et al.*, 1984; *C. piscium*, de Beer *et al.*, 2005; and *C. scophthalmum*, Mudarris *et al.*, 1994), chicken (*C. vrystaatense*, de Beer *et al.*, 2005, *C. carnipullorum*, Charimba *et al.*, 2013; *C. gallinarum*, Kämpfer *et al.*, 2014), milk (*C. joostei*, Hugo *et al.*, 2003; *C. haifense*, Hantsis-Zacharov and Halpern, 2007; *C. bovis*, Hantsis-Zacharov *et al.*, 2008a; *C. oranimense*, Hantsis-Zacharov *et al.*, 2008b;) and in a lactic acid beverage (*C. shigense*, Shimomura *et al.*, 2005). *Chryseobacterium indologenes* and *C. gleum* were among the dairy isolates in a study conducted by Hugo *et al.* (1999).

On the other hand, some species that have been isolated from water sources include *C. daecheongense* (Kim *et al.*, 2005), *C. aquaticum* (Kim *et al.*, 2008), *C. aquifrigidense* (Park *et al.*, 2008), *C. yonginense* (Joung and Joh, 2011), *C. defluvii* (Kämpfer *et al.*, 2003) and *C. angstadtii* (Kirk *et al.,* 2013).

Furthermore, *Chryseobacterium* species isolated from soil samples are not uncommon. Some members of the CDC group IIb, *C. indologenes* and *C. gleum,* are widely found in soil and water. Strains of *C. indologenes* isolated in Spain from soil samples had the capacity to degrade various toxic compounds that included 5-hydroxymethylfurfural, furfural and ferulic acid (López *et al.*, 2003). Similar results were reported by Radianingtyas *et al.* (2003) from a

study in Indonesia on soil samples where *C. indologenes* could degrade toxic compounds such as 4-chloroaniline and aniline.

Kook *et al.* (2014) reported the isolation of *C. camelliae* from plants. *Chryseobacterium* species were reported by Rosado and Govind (2003) to be among bacteria that degrade complex carbohydrates, and these species were isolated from biotopes of sub-tropical regions in the Caribbean belt and from the normal microbiotain marigold flowers (Luis *et al.*, 2004).

Some industrial sources of *Chryseobacterium* species include activated sewage sludge (Kämpfer *et al.,* 2003), paper mill slimes (Oppong *et al*., 2003) and drain outlets attached to the waste disposal unit of a sink (McBain *et al.*, 2003). Some species isolated from industrial waste include *Chryseobacterium defluvii* from a wastewater treatment plant (Kämpfer *et al.*, 2003), *C. daeguense* from wastewater of textile dye works (Yoon *et al.*, 2007) and *C. caeni* from a bioreactor sludge (Quan *et al.*, 2007). In 2010, Pires *et al.* reported the isolation of *C. palustre* from sediments that were industrially contaminated.

Chryseobacterium spp. are also frequently isolated from the hospital environment since water is their natural habitat. The bacteria come in contact with patients through indwelling devices like breathing tubes and catheters, and they are the most frequently isolated flavobacteria in clinical laboratories (Holmes and Owen, 1981). They are, however, not part of the normal microbiota of humans.

Various *Chryseobacterium* strains have been isolated from diseased animals. *Chryseobacterium indologenes* has been found in diseased frogs (Olson *et al.*, 1992) while *C. balustinum* and *C. scophthalmum* have been reported as pathogens of fish (Bernardet and Nakagawa, 2006).

2.2.2 Taxonomy

Chryseobacterium is a genus originating from the then genus *Flavobacterium* in the family Flavobacteriaceae under the phylum Bacteroidetes (Bernardet *et al.*, 2011), previously referred to as the "*Cytophaga-Flavobacterium-Bacteroides* group (CFB)". The family Flavobacteriaceae falls in the class *Flavobacteriia*, order *Flavobacteriales* and domain Bacteria (Bernardet, 2011).

In 1985, Jooste proposed the family Flavobacteriaceae which was accepted by Reichenbach (1989). Jooste (1985) in addition suggested the inclusion of the genus *Flavobacterium* in the family Flavobacteriaceae (Holmes, 1992). The family Flavobacteriaceae was then validated by Reichenbach (1992) followed by an emended description, published by Bernardet *et al.*

(1996). The genera that made up the family Flavobacteriaceae then included *Flavobacterium, Chryseobacterium, Bergeyella, Ornithobacterium, Capnocytophaga, Riemerella, Empedobacter, Weeksella* and organisms that were later classified as *Myroides* and *Tenacibaculum* (Bernardet *et al.*, 1996). According to Brady and Marcon (2012) the family Flavobacteriaceae has undergone extensive revision and now contains genera that include *Chryseobacterium, Elizabethkingia, Flavobacterium, Weeksella, Bergeyella, Empedobacter* and *Myroides*. At the date of writing, there were 141 genera included in the family Flavobacteriaceae (Euzéby, 2017).

Bernardet *et al.* (2002) described the family as strictly aerobic organisms which may either be non-pigmented or have yellow-orange pigmentation, are non-gliding and are retrieved from a variety of environmental and clinical sources.The features of the genus *Flavobacterium* were the basis of the family description. Flavobacteria were first described as Gram-negative, rod shaped, chemoorganotrophic species, non-endospore forming and comprised of 46 species that are yellow pigmented (Bergey *et al.*, 1923). As the years progressed, researchers kept on removing some features and adding others to the description of *Flavobacterium*. Most species from the genus *Flavobacterium,* which were initially associated with pathogenicity and food spoilage, were regrouped into other genera within the family Flavobacteriaceae such as *Chryseobacterium, Weeksella, Bergeyella, Myroides* and *Empedobacter* (Holmes, 1992; Hugo and Jooste, 2003).

The genus *Chryseobacterium* was comprised of six species soon after re-classification from the genus *Flavobacterium* by Vandamme *et al.* (1994). Some of the food spoilage bacteria in literature are, however, still being referred to as flavobacteria or organisms belonging to CDC Group IIb (de Beer, 2005). The number of species started to increase when *C. joostei* (Hugo *et al.*, 2003) and *C. defluvii* (Kämpfer *et al.*, 2003) were described. Addition of more species to the list of approved bacterial names with standing in nomenclature was done in the year 2005, while further description of six more species was done in the year 2006 (Herzog *et al.*, 2008). Over the years, the number of species in the genus *Chryseobacterium* has continued to increase. Currently, this genus consists of 97 validly published species (Euzéby, 2017).

2.3 SIGNIFICANCE OF *Chryseobacterium* SPECIES IN FOOD AND FEED

Several species of *Chryseobacterium* have been isolated from food sources and some were associated with spoilage of food. In studies done by Hugo and Jooste (1997) and Hugo *et al.* (1999), *C. indologenes, C. gleum, C. joostei* and CDC group IIb organisms were isolated from milk. Other *Chryseobacterium* species that have been isolated from raw milk include *C.*

haifense (Hantsis-Zacharov and Halpern 2007), *C. oranimense* (Hantsis-Zacharov *et al.*, 2008b) and *C. bovis* (Hantsis-Zacharov *et al.*, 2008a).

Chryseobacterium species have also been encountered in meat, fish and poultry products on several different occasions (Engelbrecht *et al.,* 1996; García-López *et al*., 1998; Bernardet *et al.,* 2005; de Beer *et al*., 2005; Olofsson *et al.,* 2007; Charimba *et al*., 2013). The microorganisms have been mainly found to contaminate feathers, intestines, skins and the flesh of poultry products (Mead, 1989; Charimba *et al*., 2013). The high occurrence of *Chryseobacterium* throughout the chicken processing unit and in raw broiler carcasses was suspected to have been due to environmental sources such as poor hygiene. These organisms exhibited diverse proteolytic activity and H_2S production which also contributed to off-odours that included fruity, stale as well as pungent odours (Engelbrecht *et al.,* 1996).

Some species of *Chryseobacterium* have been found to produce enzymes that play crucial roles in the degradation of hard to degrade substrates such as keratin (Riffel and Brandelli, 2006; Charimba, 2012; Park *et al*., 2014; Gurav *et al*., 2016) hence, they may be utilised in the processing of feathers for different applications. Feathers are a huge by product of the poultry industry and typically, they are made up of 91% keratin protein, 1% lipids and 8% water. This makes them a rich source of protein that is of high nutritional value and can be used as a supplement livestock feed as well as fish feed in aquaculture (Chiturri *et al*., 2015).

2.3.1 Enzyme activities

Valuable information regarding the spoilage potential can be obtained from the organism's enzyme production ability. Reichenbach (1989) reported that various types of organic macromolecules can be degraded by the different enzymes produced by bacteria. The type of enzymes produced is influenced by the biopolymers available in different habitats (Kirchman, 2002). Several new taxa were described after some enzymatic screenings of environmental isolates such as *C. proteolyticum* (Yamaguchi and Yokoe, 2000), *Zobellia galactanovorans* (Barbeyron *et al.,* 2001), *Flavobacterium frigidarium* (Humphry *et al.,* 2001) and *Fucobacter marina* (Sakai *et al.,* 2002). The model hosts for production of bacterial enzymes have always been *Bacillus* species and these also contribute about 50% to the total worldwide enzyme production (Quax, 2006). Table 2.1 shows the enzymatic possibilities of different genera and it can be seen that *Bacillus* species have been recorded to produce enzymes from almost all the different classes listed.

Table 2.1: Enzymatic possibilities of different genera (Quax, 2006)

ENZYME	GENERA
Proteases	*Bacillus*
Lipases	*Pseudomonas*
Amylases	*Bacillus*
	Aspergillus
Isomerases	*Actinomyces*
Esterases	*Bacillus*
Cellulases	Alkalophilic
	Bacillus
	Actinomyces
Xylanases	Thermophilic
	Bacilli
Phytases	*Bacillus*

2.3.1.1. Lipolytic activity

Lipolytic enzymes may contribute either desirable or undesirable flavours to products. These enzymes may also generate off-odours in the contaminated food products due to production of certain metabolites or gas (Banwart, 1989). The off-flavours produced are due to oxidation of unsaturated fatty acids produced as well as aldehyde and ketone formation during lipolysis (Chen *et al.*, 2003). Many lipases can cause lipolysis by attacking fat globules that are intact without prior activation. They are relatively heat stable and hence, may cause lipolysis even in some products that are heat treated (Mottar, 1989).

Several species in the genus *Chryseobacterium* have been reported to have significant lipolytic activity. A study conducted by Hantsis-Zacharov and Halpern (2007), reviewed lipase activity in *C. haifense*. Other species with lipolytic activity include *C. oranimense* (Hantsis-Zacharov and Halpern 2008b), *C. joostei* (Hugo *et al.,* 2003), *C. bovis, C. shigense* and *C. indologenes* (Tsôeu *et al.*, 2016).

The GC-MS (Wang and Xu, 2009), fluorimetric (Stead, 1983), spectrophotometric (Versaw *et al.*, 1989; Humbert *et al.*, 1997) and reflectance colorimetric (Blake *et al.*, 1996) techniques can be used to measure lipolytic activity. Changes in levels of free fatty acids (FFA) can also be used as an indirect measurement of lipolytic activity using solvent extraction and then titrating with an alkaline solution (Deeth *et al.*, 1975; Bekker *et al.*, 2016).

2.3.1.2. Proteolytic activity

Vandamme *et al.* (1994) initially reported that strong proteolytic activity is exhibited by members of the genus *Chryseobacterium*. Several species in the genus *Chryseobacterium* have been associated with proteolytic activity and food spoilage (Forsythe, 2000). These include *C. indologenes, C. gleum, C. joostei* and CDC group IIb organisms (Hugo and Jooste, 1997; Hugo *et al.,* 1999, 2003), *C. balustinum* (Engelbrecht *et al.,* 1996), *C. haifense* (Hantsis-Zacharov and Halpern, 2007), *C. oranimense* (Hantsis-Zacharov and Halpern, 2008b) and recently, Bekker *et al.* (2015) reported strong proteolytic activity in *C. bovis* and *C. joostei*.

Off-odours and flavours are often produced from the proteolytic enzymes produced by *Chryseobacterium* species that contaminate some food products. The off-flavours are due to formation of short peptides by the proteases (Venter *et al.*, 1999). The production of specific metabolites containing indole and alcoholic compounds as well as production of gas contribute to some flavour compounds (Banwart, 1989). Putrid off-flavours are associated with continued proteolysis and degradation products such as ammonia, sulphides and amines (Frank, 1997). Proteolytic enzymes also cause astringent off-flavours and are the main cause of bitter peptides that form in milk leading to bitterness in milk (Springett, 1996). Gelation of milk as well as coagulation of milk proteins may also occur due to the activity of proteolytic enzymes (Harwalker *et al.,* 1993; Tondo *et al.,* 2004). The problem of milk 'flocculation' may also be due to proteolytic enzymes in milk (Tsôeu *et al.*, 2016). Proteolytic enzymes play a significant role in the virulence of pathogenic strains from different environments (Bernardet and Nakagawa, 2006). Venter *et al.* (1999) revealed that proteolytic enzymes produced by species of *Chryseobacterium* were resistant to pasteurisation. Some proteolytic enzymes also have keratinolytic activity and these are predominantly active in the presence of keratin substrates, in such cases, they are referred to as keratinases (Gopinath *et al.*, 2015).

Spectrophotometry using the azocasein method (Christen and Marshall, 1984), fluorimetric as well as radiometric techniques (Christen, 1987) can be used to determine the proteolytic activity in products such as milk.

2.4 KERATINOLYTIC CHARACTERISTICS

Keratinases form a class of proteolytic enzymes, which have the ability to degrade insoluble keratin substrates (Chitturi and Lakshmi, 2016). Primarily, keratinases can be obtained from bacteria, fungi and actinomycetes (Korniłłowicz-Kowalska and Bohacz, 2011) as depicted in Table 2.2. They are largely serine or metalloproteases, possess a broad range of substrate specificity and are robust enzymes that remain active over a wide range of temperatures and

pH. Keratinases are stable in the presence of solvents, detergents and metals. Their molecular weights range from 18 to 200 kDa although it can be as high as 440 kDa for pathogenic fungi (Gupta and Ramnani, 2006).

Table 2.2: Keratinolytic microorganisms (Jin *et al*., 2017)

MICROORGANISM	REFERENCE
Bacillus licheniformis	Lin *et al*., 1992
Fervidobacterium pennavorans	Friedrick and Atranikian, 1996
Aspergillus fumigatus	Santos *et al*., 1996
Streptomyces sp. S.K$_{1-02}$	Letourneau *et al*., 1998
Bacillus subtilis KS-1	Kim *et al*., 2001
Thermoanaerobacter keratinophilus	Riessen and Atranikian, 2001
Stenotrophomonas sp. D1	Yamamura *et al*., 2002
Chryseobacterium sp. kr6	Riffel *et al*., 2003
Microbacterium arborescens Kr 10	Thys *et al*., 2004
Bacillus subtilis S 14	Macedo *et al*., 2005
Bacillus subtilis NRC 3	Tork *et al*., 2013
Actinomadura keratinilytica Cpt29	Habbeche *et al*., 2014
Bacillus safensis LAU 13	Lateef *et al*., 2015
Actinomadura viridilutea DZ50	Ben *et al*., 2016
Caldicoprobacter algeriensis	Bouacem *et al*., 2016
Bacillus pumilus AT16	Herzog *et al*., 2016
Bacillus subtilis DP1	Sanghvi *et al*., 2016
Thermoactinomyces sp. RM4	Verma *et al*., 2016

2.4.1. Keratin structure

Keratin is a structural protein that cannot be degraded by conventional proteases (Gupta *et al.*, 2013). The presence of hydrophobic interactions, disulphide bridges and hydrogen bonds in keratin contribute to its recalcitrant property and makes it an extremely stable structural protein with a low degradation rate (Sahni *et al.*, 2015). McKittrick *et al.* (2012) reported keratin as the third most abundant polymer after cellulose and chitin. Keratin helps a wide spectrum of animals to withstand biotic attacks and abiotic stress because it forms part of their outer protection (Lange *et al.*, 2016).

Keratin is a biopolymer with a hierarchical character and a three-dimensional fibrous structure consisting of small polymerized nano-amino acids. It also has about 60% hydrophobic and 40% hydrophilic chemical groups in its structure which are dependant on

the amino acid sequence (Brebu and Spiridon, 2011; Staroń *et al*., 2011; Staroń *et al*., 2014).

Keratins are categorised into α- / β-keratin based on their secondary structure. α-keratin is made up of α-helical-coils self-assembled into intermediate filaments with a diameter of 7 nm (Meyers *et al.*, 2008; McKittrick *et al.*, 2012; Wang *et al*., 2016) whereas β-keratin is made from supramolecular fibril bundles (Bodde *et al.*, 2011) and is rich in β-pleated sheets that have intermediate flaments with a diameter of 3 nm (Meyers *et al.*, 2008; Wang *et al*., 2016). Intermolecular hydrogen bonds as depicted in Figure 2.1 stablise the helical structure of α-keratins and hold the strands of the β-pleated sheet which are made from polypeptide chains (Wang *et al*., 2016). In α-keratins, the intramolecular hydrogen bonds are located between an amino group of one amino acid and a carbonyl group of another amino acid while β-pleated sheets are characterized by the interchain hydrogen bonds located between carbonyl and amino groups (Brebu and Spiridon, 2011; Staroń *et al*., 2011). A mixture of the keratins is present in almost all keratinaceous materials. Different degrees of bio-accessibility are obtained in different keratinous materials due to the differences in keratin characteristics (Lange *et al.*, 2016). Feather keratin fibers consist of 41% α-helix, 38% β-sheet, and 21% random structure bringing the total molecular weight of feather keratin to about 10,500 Da (Brebu and Spiridon, 2011; Staroń *et al*., 2011; Staroń *et al*., 2014). Some keratinases more easily access β-keratin for degradation than α-keratin because of its lower number of disulphide bridges besides its fibril and porosity structure (Gupta and Ramnani, 2006).

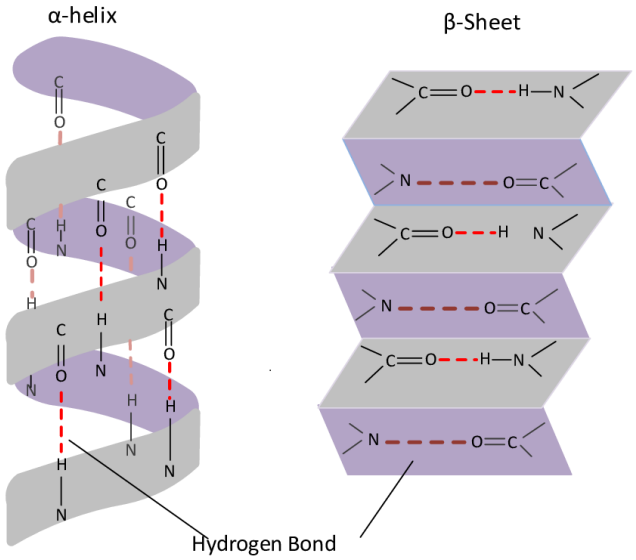

Figure 2.1: An illustration of the α-helix of α-keratin and the beta-pleated sheet of β-keratin showing the location of hydrogen bonds (Kabir, 2015).

2.4.2. Keratin decomposition

Keratins may be decomposed through either chemical (acid, base, catalyst) or enzymatic hydrolysis with the former having disadvantages that include aggressive reaction conditions, risk of environmental pollution and a product of low nutritional value which is not the case with enzymatic hydrolysis. Enzymatic hydrolysis is however costly and lengthy. The amino acid composition determines the nutritional value of the final product of hydrolysis (Grazziotin *et al*., 2006; Grazziotin *et al*., 2007).

The recalcitrant keratin structure cannot be fully decomposed by a single purified enzyme (Ramnani and Gupta, 2007; Inada and Watanabe, 2013; Lange *et al.*, 2016). Keratinolytic activity may be exhibited by several proteases but full keratin decomposition can only be achieved through a concerted effort by different enzymes with keratinolytic activity (Lange *et al.*, 2014; Huang *et al.*, 2015; Lange *et al.*, 2016). Lange *et al.* (2016) revealed that a minimum of three keratinases are required to break down keratin, these include an exo-acting, endo-acting and an oligopeptide-acting keratinase. Furthermore, some co-factors such as sulphite may be required to boost the enzyme activity during keratin breakdown (Grumbt *et al.*, 2013). Yamamura *et al.* (2002) reported that sulphite gives improved access of the enzymes to the keratinaceous substrate by breaking the sulphur bridges hence contributing to decomposition. Keratin decomposition may also be achieved through the synergistic action between enzymatic and biochemical mechanisms (Yamamura *et al.*, 2002; Lange *et al.*, 2014; Huang *et al.*, 2015).

Lange *et al.* (2016) reported that observation of breakdown of keratinolytic materials such as feathers, skin, hair and bristles can be used in an assay for keratinase activity. The author further stated that information on the expressed or induced proteins after growing a strain on keratinaceous materials can be obtained by testing the composition of the secretome of that strain.

Brandelli and Riffel (2005) recorded a strain of *Chryseobacterium* (strain kr6) that was found to produce keratinases while Charimba (2012) in a study found *Chryseobacterium carnipullorum* to be potentially keratinolytic. Riffel *et al.* (2007) purified and characterised a keratinase from *Chryseobacterium* sp. kr6 as belonging to the M14 metalloprotease family. Keratin was degraded by metalloproteases from different microorganisms of which a *Chryseobacterium* sp. (Wang *et al.*, 2008a; Silveira *et al.*, 2012) was one of them and these metalloproteases were sensitive to inhibition by EDTA.

Gupta and Ramnani (2006) noted that there is no overlap between the kinetics of keratin degradation and keratinase production hence, one cannot serve as a marker for the other.

2.4.3. Applications of keratinases

Keratinases can be applied in a variety of areas that require the degradation of recalcitrant, highly cross-linked structural proteins such as keratin found in hair, feathers, prions and nails. A blend of keratinases and perhaps other enzymes which target post translational modifications of the keratin such as glycosylation and disulphide bridges may be required for efficient degradation of keratin in industrial processes (Lange *et al.*, 2016).

2.4.3.1. Waste management

Keratinases play a significant role in degrading chicken feathers that are insoluble and cause disposal problems as they mostly contain 90% keratin protein that cannot be degraded by most proteolytic enzymes. Keratinolytic enzymes have potential in non-polluting processes involving keratin hydrolysis of keratin-containing waste from the leather and poultry industries as well as in biotechnology (Shih, 1993; Onifade *et al.,* 1998; Riffel *et al.*, 2003; Casarin *et al.,* 2008). In feather processing, structurally modified feather keratin is produced using keratinase and this feather keratin has reduced resistance to attack by other digestive enzymes (Burrows *et al.*, 2002).

2.4.3.2. Feed industry

The biological conversion of feather into feather meal produces high value dietary proteins that can be used as food and feed supplements (Burrows *et al.*, 2002; Riffel *et al.*, 2003; Grazziotin *et al.*, 2006; Chitturi *et al.*, 2015). Odetallah *et al.* (2003) reported an improvement in broiler performance when crude keratinase was used as a nutraceutical product in poultry feed. According to Yoshioka *et al.* (2007) bio-processing of keratin rich waste from poultry, using keratinases, produces enzymatic hydrolysates with high amounts of sulphur containing polypeptides and specific amino acids such as leucine, serine, glutamate and arginine.

2.4.3.3. Organic farming

Gupta and Ramnani (2006) reported that hydrolysis of a keratinous waste leads to production of a keratin hydrolysate that is rich in hydrophobic amino acids and high in nitrogen content (15%). Therefore, the concentrated, protein rich feather meal can be used as a semi-slow release, nitrogen fertilizer in organic farming (Hadas and Kautsky, 1994; Choi and Nelson, 1996; Kainoor and Naik, 2010). The microbially hydrolysed feather meal may serve as a readily available and cheaper alternative to other nitrogen-rich organic amendments such as guano in organic farming. In addition to its high nutritive value, it upsurges the soil's water retention, promotes microbial activity and structures the soil in order to improve plant growth (Gupta and Ramnani, 2006).

2.4.3.4. Leather industry

Vigneshwaran *et al.* (2010) reported that keratinase is a potential enzyme for the eco-friendly de-hairing process during leather processing as opposed to the use of sulphide, which is toxic. Keratinolytic enzymes can also be used to improve the leather quality in beam house operations of the leather and tanning industry (Chitturi *et al.*, 2015). The use of keratinase would help in pulling out intact hairs without altering the tensile strength of the leather by selectively breaking down keratin tissue in the follicle (Macedo *et al.*, 2005; Gupta and Ramnani, 2006). A significant amount of organic waste originating from keratin is produced during leather production. Use of keratinases is one of the greatest innovative achievements in the context of total solid reduction and solid waste management (Sahni *et al.,* 2015).

2.4.3.5. Prion decontamination

Keratinases can be used to efficiently degrade or inactivate pathogenic forms of prions, e.g. the scrapie protein (Yoshioka *et al.*, 2007). According to Saunders *et al.* (2008), prions are infectious agents that causes fatal and communicable brain diseases. A prion is composed of protein in misfolded form and PrP (proteasome resistant protein) is the major prion protein. It is predominantly expressed in the nervous system. PrP^{sc} is the disease-causing, scrapie form while PrP^{c} is the cellular form (Sahni *et al.*, 2015). The infectious PrP^{sc} facilitates conversion of harmless PrP^{c} to PrP^{sc} in order to cause an infection (Gupta and Ramnani, 2006). Keratinases cleave the β-keratin of prion proteins (PrP^{sc}) with an improved rate in the presence of detergents and heat treatments than conventional proteases hence, are promising candidates for prion decontamination (Langeveld *et al.*, 2003; Gupta *et al.*, 2013). Taylor (2000) stated that the use of detergent-based prion decontamination formulations are not only eco-unfriendly, energy intensive and harsh but also does not guarantee complete loss of infectivity. Furthermore, medical devices can be damaged by continuous use of detergent-based formulations to decontaminate prions (Rutala and Weber, 2010). Microbial keratinases may serve as a better alternative in decontaminating lab equipment, medical instruments as well as interchangeable items like dentistry tools and contact lenses (Langeveld *et al.*, 2003; Gupta and Ramnani, 2006).

2.4.3.6. Pharmaceutical and biomedical industries

Keratinases can be used as additives to increase the efficacy of topical drugs since they are able to attack skin and nail keratin. Most nail diseases especially those that occur beneath the nail plate are difficult to treat. Keratinolytic enzymes may help to improve the permeability of the drugs across the nail plate. Additionally, keratinases find application in the treatment of several skin conditions such as acne, corn and callus (Gupta *et al.*, 2013). Selvam and Vishnupriya (2012) describes acne as a skin condition that occurs due to

excessive keratin blocking the sebaceous glands. Keratinases can be used in acne treatment as they can dissolve the keratin that blocks the sebaceous glands as well as the dead cells. Areas involving removal of scars and regeneration of the epithelium also makes use of keratinolytic enzymes (Gupta *et al.,* 2013). Keratinases can also be used as additives in skin-lightening agents due to their property of stimulating keratin degradation (Vignardet *et al.*, 2001; Gupta and Ramnani, 2006).

Corn and calluses usually form on the dorsal surface of toes and fingers as painful thickenings of dead skin. Keratinases can be used to dissolve the keratin on the corn and thick layer of dead skin as a greener alternative to the use of salicylic acid, which is mostly used as a keratinolytic agent in the removal of the horny layer of the skin (Gupta and Ramnani, 2006; Encarna and Elena, 2011; Gupta *et al.*, 2013).

2.4.3.7. Detergent industry

Keratinases are additionally postulated for application in the detergent industry as additives to the detergent formulation (Brandelli *et al.,* 2010). Paul *et al.* (2014) observed that supplementation of detergent formulation with a keratinolytic protease significantly improved the stain removal as compared to the removal of stains by detergent or enzymes alone. Furthermore, these workers reported that the fibre strength and structure of the fabrics was not damaged after the use of detergents supplemented with keratinases. The important property of keratinases is their ability to hydrolyse and bind solid keratinous soils often encountered during laundry on which most proteases fail to act (Gassesse *et al.*, 2003; Gupta and Ramnani, 2006). They can thus be used as additives in hard-surface cleaners and for cleaning up of drains congested with keratinous wastes (Farag and Hasan, 2004; Gupta and Ramnani, 2006).

2.4.4. Factors affecting keratinase production and activity

Some carbon and nitrogen sources affect the production of keratinases by bacteria. The presence of dextrose, citric acid and glucose were reported to inhibit keratinase production in a study by Kainoor and Naik (2010). Previous studies by Ramnani and Gupta (2004) also showed that carbon sources such as sucrose, glucose and glycerol suppress the secretion of keratinase. A similar observation on the inhibitory effect of carbohydrates on keratinase production was made in 2005 by Brandelli and Riffel.

In the presence of a rigid protein such as feather keratin, the presence of simpler carbon sources such as glucose results in catabolite repression. The bacterium first utilizes the simpler nutrient source such as glucose, before breaking down the difficult nutrient source such as feathers, hence, resulting in delayed production of the enzyme (Mehta *et al.*, 2014).

On the other hand, nitrogen sources that include ammonium nitrate, peptone, urea and sodium nitrate were reported to suppress keratinase production in a study by Sivakumar *et al.* (2013). These researchers also reported that starch and yeast extract, which are extra carbon and extra nitrogen sources respectively, had positive effects on the production of keratinase by *Bacillus cereus* TS1.

Keratinases have mostly been grouped as inducible enzymes basing their nature on keratinolytic activity although a few constitutive keratinases whose nature is based on caseinolytic activity have also been reported (Gassesse *et al.*, 2003; Manczinger *et al.*, 2003; Gupta and Ramnani, 2006). A high concentration of inducer such as the concentration of feather or keratin in the medium was found to repress the production of keratinase by *Chryseobacterium* sp. kr6 (Brandelli and Riffel, 2005). Kainoor and Naik (2010) also reported a suppression of keratinase production with increased concentration of inducer (feather) in the medium.

Microorganisms require an optimum temperature to achieve the highest enzyme production and activity. Temperature controls the metabolism of energy as well as enzyme synthesis (Frankena *et al.*, 1986; Mehta *et al.*, 2014). The range of temperature for keratinase producing bacteria, actinomycetes and fungi, is 28 ^{o}C to 50 ^{o}C while for *Thermoanaerobacter* and *Fervidobacterium* spp. it may be as high as 70 ^{o}C (Friedrich and Antranikian, 1996; Rissen and Antranikian, 2001; Nam *et al.*, 2002). The activity optimum for most keratinases lies in the range of 30 °C – 80 °C (Cai *et al.*, 2008). Most species of *Chryseobacterium* are mesophilic in nature (Sreenivasa and Vidyasagar 2013) although some psychotolerant food spoilers also exist (Overmann, 2006), hence, keratinase can be produced during their normal growth. Keratinase activity is retained up to a certain temperature after which the enzyme stability may be lost (Kainoor and Naik, 2010). Friedrich and Kern (2003) records some keratinases active at temperatures as high as 90 ^{o}C.

pH also affects the production and activity of keratinases, since microorganisms require an optimum pH to achieve the highest enzyme activity (Kainoor and Naik, 2010). Cai *et al.* (2008) reported that most keratinases are active in neutral to alkaline pH, ranging from pH 7.0 to pH 9.5. Friedrich and Antranikian (1996) stated that an alkaline pH from 6 to 9 favours keratinase production and the degradation of feathers by most microorganisms as cysteine residues are modified to lathionine at high pH creating easy access for keratinase action.

Furthermore, the incubation time affects keratinase production by microorganisms. Some microorganisms have maximum production of keratinases within a short incubation period after which the enzyme production decreases whereas others require a lengthy period of incubation to achieve maximum enzyme production. Different strains of the same species

may also differ in the incubation period required for maximum production the of enzyme (Kainoor and Naik, 2010).

Chemicals such as solvents, metal ions, inhibitors and reductants have a diverse effect on keratinase activity. The activity and production of keratinases may be enhanced, inhibited or not affected by the presence of chemicals (Kainoor and Naik 2010). Some bacterial keratinases are stimulated by divalent metal ions such as Ca^{2+}, Mn^{2+} and Mg^{2+} (Nam *et al.*, 2002; Riffel and Brandelli, 2002; Sivakumar *et al.*, 2013). Mg^{2+} a divalent ion, is present in all cell membranes and cell walls acting as a co-factor for several enzymes and plays an important role in enzyme activity, stability as well as cell mass build-up (Mehta *et al.*, 2014). Some heavy metal ions inhibit keratinase production by bacteria. $HgCl_2$ was found to completely inhibit the enzyme activity of a keratinase isolated from *Streptomyces albus* in a study by Sreenivasa and Vidyasagar (2013). In the same study, the most potent inhibitor was phenylmethyl sulphonyl fluoride (PMSF). Its presence indicated serine residues and a protease as it is known to inhibit serine proteases.

2.4.5. Microbial keratinase production and harvesting

Gopinath *et al.* (2015) highlighted some important considerations for the efficient production of keratinases from microorganisms. Firstly, microbes with proteolytic as well as keratinolytic activity can be isolated from non-keratinolytic microbes by conducting screening tests. Methods of screening include; plate screening, spectrophotometric methods, sequence-based amplification and keratin baiting (Gopinath *et al*., 2015). Plate screening and keratin baiting are the two popular methods used because they enable the observation of keratinophilic species visually on the substrate such as on media, feathers or hair. Plate screening involves the plate-clearing assay for keratinolytic microorganisms hence, is easier to use in addition to being less expensive. The pour plate method can also be used as an alternative. *Chryseobacterium* was recorded as one of the four species (*Chryseobacterium, Pseudomonas, Burkholderia* and *Microbacterium*) that were recovered on milk agar plates after isolation from feather waste by Riffel and Brandelli (2006).

To obtain excess keratinase production from the isolated microbes, they can be cultivated on an appropriate artificial growth medium under optimum conditions. Most keratinophilic microbes grow well under neutral and alkaline pH, the range being 6.0 to 9.0 (Jain and Sharma, 2012). In terms of temperature, Cai *et al.* (2008) reported that the activity optimum for most keratinases lies in the range of 30 – 80 ^{o}C.

The keratinase obtained can then be purified. Purification is essential to hasten the effectiveness of the keratinase action for further industrial applications (Gopinath *et al*., 2015). Higher stability under diverse conditions has been exhibited by purified enzymes from

various species. Enzyme purification through precipitation followed by column chromatography is the most common approach (Gopinath *et al.*, 2015). The aim of precipitation is to concentrate the proteins as well as to eliminate interfering compounds. There are various precipitation methods that may be used and these rely on different chemical principles (Zellner *et al.*, 2005). These methods include acetone, ethanol, trichloro-acetic acid (TCA), chloroform/methanol and ammonium sulphate precipitation. Jiang *et al.* (2004) compared the different protein precipitation methods and concluded that an efficient desalting and sample concentration for a proteomic analysis can be obtained using precipitation with acetone and TCA. All proteins are soluble at very low TCA concentrations. However, at TCA concentrations of between 15 and 40%, protein precipitation of even the highly TCA soluble proteins occurs. Therefore, a suitable concentration of TCA should be used to precipitate almost all proteins with minimal protein modifications. These modifications may occur when the concentration of the acid is elevated above 50% as the proteins re-dissolve back into the solution, possibly due to acid-induced structural changes in the protein (Zellner *et al.*, 2005).

Ethanol precipitation involves diluting the protein sample in a surplus of ethanol, which is a 9-fold volume of ethanol and this aids the precipitation of all proteins. Although ethanol is readily miscible with water, it tends to denature proteins particularly at temperatures higher than 0 ^{o}C and yields a substantial heat of solution. Hence, in protein fractionation experiments, a cold ethanol precipitation is used in which the solvent temperature is always kept below 0 ^{o}C and this enables the preparation of non-denatured proteins (Zellner *et al.*, 2005).

Protein solubility usually decreases at higher salt concentrations leading to precipitation and ammonium sulphate is the reagent of choice for salting out, due to its higher solubility than any of the phosphate salts (Green and Hughes, 1955; Wingfield, 2001). Suntornsuk *et al.* (2005) reported the purification of a keratinase from a feather degrading bacterium via ammonium sulphate precipitation followed by ion-exchange chromatography and then gel filtration. The purified keratinase exhibited high specific activity, a molecular mass of 35 kDa and was thermotolerant. When suitable anti-keratinase antibodies are available, immune-precipitation can be used to achieve keratinase purification with greater efficiency (Gopinath *et al.*, 2015).

Acceleration of the overproduction of keratinase is also necessary after enzyme purification. This can be achieved using recombinant DNA technology as well as statistical optimization. Some keratinases already have corresponding amino acid sequences in the data bank.

Statistical modelling studies using certain formulae can also be used to improve the production levels of an enzyme (Gopinath *et al.*, 2015).

2.5 CONCLUSIONS

The genus *Chryseobacterium* contains species with the ability to proliferate in different environments which include food, industrial and clinical sources. Some *Chryseobacterium* species are able to produce different metabolic products such as proteolytic, lipolytic and keratinolytic enzymes which contributes to their spoilage capabilities in a variety of food products and may have potential biotechnological applications. The ability of proteolytic enzymes of these species to survive pasteurisation, the organisms to grow over a wide range of pH, temperature and in the presence of NaCl (0-5%) increases the chances that they may cause spoilage of different food products.

Microbial keratinolytic proteases is a subject that requires further exploration since keratinases have significant application potential in industry involving keratin hydrolysis as a cheaper and eco-friendly alternative to the use of chemicals, heat-treatments and other eco-unfriendly methods of keratin breakdown. Value added products can be obtained from keratinous wastes due to the diverse substrate specificity of keratinases coupled with the ability to remain active over a wide range of pH, temperature as well as in the presence of detergents, solvents and metals. Species of *Chryseobacterium* such as *C. carnipullorum* and *Chryseobacterium* strain kr6 can contribute to bridging the gap that currently exists between demand for microbial keratinases and supply if they can be harvested on a large scale.

CHAPTER 3: PRODUCTION AND IDENTIFICATION OF PROTEOLYTIC ENZYMES

3.1 INTRODUCTION

Owing to the increase in enzymes required by industries, it is imperative that methods that lead to large-scale enzyme production within a short period of time are adopted. These include the use of microbial enzymes that have shorter generation times, are more stable, dynamic with an extensive scope of genetic manipulation than their animal and plant counterparts (Anbu *et al*., 2015).

The *Chryseobacterium* genus comprises some species which exhibit strong proteolytic activity (Bernardet *et al.*, 2011). The most important category of enzymes from an industrial point of view are proteases as they account for more than 65% of the total market for industrial enzymes (Banik and Prakash, 2004; Wang *et al.*, 2008b). The authors Venter *et al.* (1999) showed that a specific metalloprotease from *C. indologenes* was heat resitant. Some species of *Chryseobacterium* have been found to have proteases that are keratinolytic in nature (Riffel and Brandelli 2006; Wang *et al.*, 2008a, 2008b; Charimba, 2012; Park *et al.*, 2014; Gurav *et al.*, 2016). Keratinolytic activity is the ability to degrade highly recalcitrant and cross-linked structural proteins such as keratin. This protein widely occurs in feathers, hair, nails, wool and horns. Keratin is generally designed to be unreactive and resistant to most forms of stress encountered by animals as it is a class of proteins that is mechanically strong due to the presence of disulphide bridges, hydrophobic interactions and hydrogen bonds (Sahni *et al.*, 2015).

Various applications of proteases have been reported in literature and these include, food processing, production of protein hydrolysates, leather processing (Banik and Prakash, 2004) as well as in the detergent industry (Paul *et al.*, 2014). Some keratinolytic proteases have been used in accelerating the cost effectiveness and efficiency of a wide range of industrial systems and processes as an alternative to chemicals (Gupta *et al.*, 2002; Paul *et al*., 2014). Keratinases stand out among proteases in developing cost-effective by-products from feathers for feed as well as fertilizers (Gupta and Ramnani, 2006).

The first aim of this chapter was to evaluate at which growth stage *C. carnipullorum* actively secretes proteins into the growth medium. The second aim was to identify whether the proteins secreted are enzymes and to identify the types of enzymes produced in order to find potential proteolytic enzymes.

3.2 MATERIALS AND METHODS

3.2.1. Sample collection, resuscitation and identification

The type strain of *C. carnipullorum* 9_R23581^T was obtained from the culture collection housed at the Food Science division at the University of the Free State, Bloemfontein, South Africa, in freeze dried form. The strain was resuscitated in 10 ml nutrient broth (NB; Oxoid CM0067) and incubated at 25 ^oC for 48 hours. A loopful of the broth culture was streaked on a nutrient agar (NA; Oxoid CM0003) plate to check for purity and the plate was incubated at 25 ^oC for 48 hours. Pure single colonies were obtained from the NA plates and streaked on NA slants, which were incubated at 25 ^oC for 48 hours after which they were maintained at 4 ^oC and used as working cultures. Sub-culturing the working cultures was done after every 7-8 weeks to maintain their viability. The odour produced was noted and a Gram-staining reaction was performed on the strain.

3.2.2. Extraction of genomic DNA

Genomic DNA (gDNA) was extracted from cells of *C. carnipillorum* using the method described by Labuschagne and Albertyn (2007) with slight modifications: 1.5 ml of a previously grown culture in NB was pipetted into a 1.5 ml Eppendorf tube and the cells were spun at 3000 x *g* for 5 minutes. The supernatant was decanted then 500 µl of lysis solution (50 mM EDTA; Merck, pH 8.0, 1 M Tris-HCl; Roche Diagnostics and Merck respectively, pH 8.0, 1% w/v SDS; BDH Laboratory Supplies, England) was added. The mixture was vortexed for 1 minute followed by cooling on ice for another minute. Vortexing and cooling was repeated for one minute each. Then, 275 µl of 7 M ammonium acetate (Merck) at pH 7.0 was added. Incubation was at 65 ^oC for 5 minutes followed by incubation on ice for 5 minutes. Addition of 500 µl chloroform (Merck) was done and the mixture was gently inverted for about 5 times. Centrifugation was performed at 20 000 x *g* for 2 minutes at a temperature of 4 ^oC. The supernatant (top layer) was transferred into a clean tube. 750 µl of isopropanol (Merck) was added to the supernatant. The mixture was centrifuged at 20 000 x *g* at 4 ^oC for 5 minutes. The supernatant was discarded and the pellet was washed with 750 µl of ice-cold 70% (v/v) ethanol (Merck). Centrifugation was repeated under the same conditions for 2 minutes. The pellet was air dried under a laminar flow hood. It was then re-dissolved in 50 µl TE (1 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0) mixed with 50 mg/ml RNase (Qiagen) and incubated at 37 ^oC for 1 hour to digest the RNA.

The extracted gDNA quantity and quality was assessed using the Nanodrop ND-1000 (v3.3.0) spectrophotometer. The extracted DNA was stored at -20 ^oC.

3.2.3. 16S rDNA Polymerase Chain Reaction (PCR)

Chryseobacterium carnipullorum was subjected to a 16S rDNA polymerase chain reaction (PCR) according to the manufacturer's instructions; dNTPs (2 µl of 10 mM) were added to the 0.2 ml PCR tubes, followed by 1 µl of 10 µM primers (forward and reverse). The forward primer used was 27F (5'-AGAGTTTGATCCTGGCTCAG-3', Intergrated DNA Technologies) while the reverse primer was 1492R (5'-GGTTACCTTGTTACGACTT-3', Intergrated DNA Technologies). Buffer (ThermoPol®; 5 µl of 10x concentration) and 5 µl template DNA (sample) were also added. A control sample was prepared where the template DNA was replaced by addition of 5 µl nuclease-free water. In addition, 0.3 µl of 5000 U/ml *Taq* DNA Polymerase (New England BioLabs Inc.) was added. The reaction volume was adjusted to 50 µl by addition of nuclease-free water and a quick spin of 1 second was done to collect all the liquid at the bottom.

Thermal cycling was conducted using a 2720 Thermocycler (Applied Biosystems) that was programmed as follows: initial denaturation at 94 ^{o}C for 5 minutes, 32 amplification cycles of; denaturing at 94 ^{o}C for 30 seconds, annealing at 50 ^{o}C for 30 seconds and elongation at 68 ^{o}C for 1.45 minutes. Final elongation was allowed at 68 ^{o}C for 7 more minutes and the reaction was kept at 4 ^{o}C until further processing.

3.2.4. Media preparation for bacterial cultivation

Chicken whole feathers were collected from a poultry processing plant in Bloemfontein. They were thoroughly washed using a detergent (Lemon bright; SA Polichem) and rinsed using tap water. Air drying of the feathers was done for 36 hours followed by oven drying for 72 hours at 65 ^{o}C (Santos *et al.*, 1996; Charimba, 2012).

Whole Feather Medium (WFM) was prepared by suspending 3.5 g of the dried chicken feathers in 350 ml basal medium consisting of (g/l): K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 0.175; NaCl, 0.175 and $FeCl_3$, 0.035; pH 7.5 in 1000 ml Erlenmeyer flasks. A total of 16 flasks containing WFM were prepared. Four (4) of the flasks were supplemented with 1% (w/v) glucose (Merck), another set of four flasks were supplemented with 1% (w/v) starch (Merck) and another set of four flasks were supplemented with 0.014 M phenylmethylsulfonyl fluoride (PMSF; Roche Diagnostics) dissolved in isopropanol (Merck).

3.2.5. Secretory proteins, with focus on enzyme production

Enzyme production during growth of *C. carnipullorum* was studied according to Nagal *et al.* (2010) with some modifications. A loopful of growth of the strain on NA slant was inoculated in 10 ml NB and incubated at 25 ^{o}C for 48 hours. Then, 1 ml from the 10 ml broth was inoculated into three of each of the four 350 ml flasks containing different media

compositions. One of the flasks for each type of medium was left as a control. All the flasks were incubated at 25 ^{o}C aerobically on a shaking incubator (150 rpm) for 60 hours. Keratinolysis was visually assayed by observation of feather degradation in the flasks.

Growth of the culture was monitored by measuring the colony counts at various stages of growth. This was achieved by obtaining 1 ml from the 350 ml culture flask followed by preparing 10^{-1} serial dilutions using 0.1 M phosphate buffer. For the first 24 hours of growth, the 10^{-1} dilutions were plated on NA from the zero dilution to 10^{-5} but after 24 hours, only dilutions 10^{-4} to 10^{-8} were plated. The plates were incubated at 25 ^{o}C for 48 hours. Colonies were enumerated using a colony counter and values were expressed as colony forming units per ml (cfu/ml). Analyses were conducted in triplicate.

At each stage when plating was done, 15 ml of the medium in the flask was obtained and centrifuged at 7197 x *g* for 10 minutes at 4 ^{o}C. The supernatant was collected and stored at -20 ^{o}C as the crude enzyme extract until further use.

3.2.6. Protein extraction

The supernatant preparations stored at -20 ^{o}C were thawed on ice. The samples were gently mixed by shaking for a few seconds.

3.2.6.1. Centrifugal Ultrafiltration

Concentration of the sample was done using centrifugal filter units (Amicon® Ultra-15) of sizes ultracel® 50 kDa and 10 kDa. Of the supernatant, 12 ml was pipetted into the 50 kDa centrifugal filter unit and centrifuged at 7197 x *g* for 30 minutes at 4 ^{o}C. The sample that filtered through the 50 kDa column was introduced to the 10 kDa centrifugal filter unit and centrifuged at 7197 x *g* for 30 minutes at 4 ^{o}C. The concentrate was washed using 6 ml 1x phosphate buffer and centrifuged using the same conditions for one hour. The concentrated sample was collected from the sample reservoir of the filter device using a pipette and stored in 2 ml Eppendorf tubes at 4 ^{o}C.

3.2.7. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed according to the method of Laemmli (1970) in a discontinuous SDS-PAGE consisting of resolving gel (12% w/v, pH 8.8) and stacking gel (4.2% w/v, pH 6.8). The concentrated sample (14 µl) was mixed with 5 µl of 4x SDS-sample buffer (1 M Tris-HCl, 1% w/v SDS, 0.1% w/v Bromophenol blue, 100% v/v glycerol) and 1 µl β-mercaptoethanol (14.3 M; Sigma) in 0.2 ml PCR tubes. The tubes were boiled at 94 ^{o}C and cooled to 25 ^{o}C using a PCR thermal cycler (Vacutec), followed by micro-centrifugation (Hermle Z100M) for 1 second. 15 µl of each of the sample-SDS mixtures and 5 µl of

Precision Plus Protein™ dual color standards marker were run on the acrylamide gel at a constant voltage of 7 $V.cm^{-1}$ until the dye-front reached the bottom of the gel. The electrophoresis buffer used in the chamber was Tris-Tricine-SDS (TTS) buffer (0.1 M Tris; Roche Diagnostics, 0.1 M Tricine; BioRad, pH 8.3, 0.1% w/v SDS; BDH Laboratory Supplies).

3.2.8. Gel staining and viewing

The gel was rinsed with distilled water for a few seconds before starting with the Fairbanks staining procedure according to Fairbanks *et al*. (1971). It was then soaked in Coomassie stain where Coomassie brilliant blue R-250 (0.05% w/v) in a mixture of isopropanol (Merck), glacial acetic acid (Merck) and distilled water (25%, 10% and 65% v/v respectively) was used. The gel was boiled for about 45 seconds in a microwave then staining was continued on an orbital shaker for 30 minutes. Destaining of the gel was done using 10% (v/v) acetic acid for 2 hours or until some active bands appeared (usually overnight).

Bands on the gel were viewed on a Gel Doc™ EZ Imager (Bio-Rad) and photographed using ImageLab™ software (version 5.0, Bio-Rad).The most prominent bands were cut and kept at -20 ^{o}C in 1.5 ml microcentrifuge tubes until further investigation by LC-MS/MS.

3.2.9. In-gel digestion of proteins

In-gel digestion of proteins was conducted using standard laboratory procedures. The excised gel pieces were cut into smaller cubes of about 1 mm^3 and washed in 100 µl water twice for 15 minutes each. The cubes were also washed in 50% (v/v) acetonitrile (HPLC grade; LAB-SCAN) for 15 minutes. Washing in water for 15 minutes followed by washing in 50% (v/v) acetonitrile for another 15 minutes was repeated. The gels were shrunk using 50 µl of 100% (v/v) acetonitrile, and the residual liquid removed.

The washing step was followed by reduction and alkylation. During this stage, the gel particles were swollen in 10 µl dithiotreitol (DTT, Apollo Scientific LTD, BIMB1015) in 1 ml of 0.1 M ammonium bicarbonate (NH_4HCO_3, Sigma, 99%, A6141) and incubated at 56 ^{o}C for 45 minutes. The tubes were chilled to room temperature after which excess liquid was removed and immediately replaced with 50 µl of 10 mg iodoacetamide (Sigma, 99%) in 1 ml of 0.1 M NH_4HCO_3. The soaked gel pieces were incubated in the dark at room temperature for 30 minutes. The iodoacetamide solution was then removed with a pipette.

The washing step with water, followed by acetonitrile, was repeated and so was the shrinking of gels with 100% (v/v) acetonitrile. The acetonitrile was removed using a pipette after which the gels were air dried in a fume hood.

The gel particles were digested using freshly prepared trypsin (Promega, seq grade modified). Of the supplied buffer, 40 µl was added to the trypsin vial. The digestion solution consisted of 8 µl dissolved trypsin in 400 µl of 0.1 M NH_4HCO_3. Digestion solution (30 µl) was used to rehydrate the gel particles. More digestion solution was added in cases where the initially added volume was absorbed by the gel pieces. The gel pieces in the digestion solution were incubated overnight at 37 ^oC.

Extraction of peptides from the gel was done after the overnight incubation. During this stage, the digestion solution was transferred into a clean 1.5 ml tube. 20 µl of 5% (v/v) formic acid (Merck) was added to the gel pieces that remained in the tube. A quick vortex was done followed by 15 minutes of incubation on the bench. The 5% formic acid was transferred from the tube containing the gel pieces to a 1.5 ml tube and immediately replaced with 20 µl of 100% acetonitrile. Quick vortexing was done followed by 15 minutes of incubation on the bench. This was followed by 5 minutes of room temperature centrifugation at maximum speed and transferral of the 100% acetonitrile to the 1.5 ml tube. Extraction of the peptides in 20 µl of 5% formic acid followed by 20 µl of 100% acetonitrile was repeated and the supernatants combined in the same 1.5 ml tube. The solution containing the extracted peptides was concentrated under vacuum (SpeedVac, Eppendorf concentrator plus) drying at 45 ^oC for 1 hour and 30 minutes.

The peptides were re-dissolved in 20 µl of 5% formic acid and identified using a Liquid chromatography-tandem mass spectrometry (LC-MS/MS; AB SCIEX). Mascot software version 2.2.04 was used to identify the proteins (Perkins *et al*., 1999). The peptide sequences were further analysed using the protein Basic Local Alignment Search Tool (BLASTp; https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.,* 1990).

3.2.10. Amplification of genes, coding for proteins of interest

Chryseobacterium carnipullorum was subjected to PCR with Q5 Hot Start High Fidelity DNA polymerase (New England Biolabs Inc.) as per manufacturer's protocol using primers (Integrated DNA Technologies) that were designed on the genes (Genbank Accession number NZ-FRCDO1000002.1) that code for the proteins of interest. Primer design was based on the results obtained after BLAST analysis of the peptide sequences. Primers were designed on the nucleotide sequence coding for the corresponding peptide, which indicated peptidase M64 and glutaminase as the proteolytic enzymes produced by the *C. carnipullorum* strain. The restriction sites *Nde*I (CATATG) and *Xho*I (CTCGAG) were introduced via forward primer 5'-GCGCCCATATGAAAAAAACTTTATTAGCCTTATTAATC-3' and reverse primer 5'-GGCGCCTCGAGTTCTTTAATGATTTTCTGGGAAATG-3' for peptidase M64 as well as 5'-

CCGCGCATATGATGAAAAATCTTTTTTTATCAATGATGGCCTTTGTG-3' for the glutaminase forward primer with 5'-CCGCCCTCGAGAAATCCACAGCTGGATACATCCG-3' being its reverse.

The PCR mixture consisted of 10 mM dNTPs, 5X Q5 Reaction buffer (Biolabs Inc. New England), 5X Q5 High GC Enhancer (New England Biolabs Inc.), template DNA (sample), 10 µM of each primer (forward and reverse) and Q5 Hot start High Fidelity DNA polymerase (2000 U/ml; New England Biolabs Inc.) with a total reaction volume of 50 µl, which was adjusted using nuclease-free water. A negative control sample was similarly prepared except that the template DNA was replaced by addition of nuclease-free water. A quick spin of 1 second was done to collect all the liquid at the bottom.

PCR was performed using a 2720 Thermocycler (Applied Biosystems) with cycling conditions which included an initial denaturation at 98 ^{o}C for 30 seconds, 35 amplication cycles of; denaturation at 98 ^{o}C for 10 seconds, annealing at 46 ^{o}C for the peptidase gene while the glutaminase gene was annealed at 53 ^{o}C both for 30 seconds and elongation was performed at 72 ^{o}C for 45 seconds. Final elongation after cycling was performed at 72 ^{o}C for 2 minutes after which the reaction was cooled and kept at 4 ^{o}C.

Several optimisation steps were done for the gene coding for glutaminase. A gradient PCR was performed using the same cycling conditions described above and allowing the annealing temperatures to range from 45 to 55 ^{o}C. In addition, whole cell PCR was performed as opposed to using the genomic DNA of the culture, in case of the gene being plasmid encoded. Furthermore, different magnesium ion (Mg^{2+}) concentrations were added to the PCR mixture containing genomic DNA in order to have a final Mg^{2+} ion concentration of 2, 3, 4 and 5 mM in the final reaction.

3.2.11. Visualisation of PCR Amplicons

The PCR products were visualized on a 1% w/v agarose (Seakem® LE Agarose, Lonza) gel to which ethidium bromide stain (~20 mg/ml) was added. The agarose gel was prepared using 1x TAE buffer (containing 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0)

The gel wells were loaded with 1 µl of 6x loading buffer mixed with 5 µl of the PCR product. The DNA marker Thermo-Scientific O'gene Ruler™ (3 µl) was also loaded alongside the samples. Electrophoresis was performed for 34 minutes at 9 $V.cm^{-1}$

The PCR amplicons were viewed using a Gel Doc™ EZ Imager (Bio-Rad) and photographed using ImageLab™ software (version 5.0, Bio-Rad). Upon amplicon confirmation, the remainder of PCR products were loaded on a 2% w/v Low Melt Agarose gel (Pronadisa,

Conda) and electrophoresis was performed at 7 V/cm for 45 minutes. The gels were viewed by a UV Transilluminator (Spectroline). The amplicons of interest were excised and purified.

3.2.12. Amplicon clean-up and sequencing reactions

Excised gel slices were purified using the Wizard® SV Gel and PCR Clean-Up system (Promega) per manufacturer's instructions except that the gel was incubated for 5 minutes on the minicolumn instead of 1 minute during binding of DNA. In brief, the gel slice (10 mg) was dissolved in 10 µl of membrane binding solution at 50-65 ^{o}C. Binding of DNA was allowed by first transferring the dissolved gel mixture to an SV minicolumn that was inserted in a collection tube and incubation was done for 5 minutes at room temperature. Centrifugation was conducted at 16 000 x *g* for 1 minute. The bound DNA on the minicolumn was washed using 700 µl membrane wash solution and centrifugation was performed at 16 000 x *g* for 1 minute. The washing step was repeated using 500 µl of the membrane wash solution and centrifuged (16 000 x *g,* 5 minutes). The collection tube was emptied and evaporation of any residual ethanol was allowed by opening the lid and recentrifuging the minicolumn for 1 minute. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and elution of the DNA was done using 50 µl Nuclease free water (16 000 x *g*, 1 minute). The concentration of the purified DNA was determined using the Nanodrop ND-1000, v3.3.0 spectrophotometer.

Sequencing reactions were performed using the BigDye terminator v.3.1 kit (Applied Biosystems) according to manufacturer's instruction by the ddNTP chain termination method (Sanger sequencing). Sequencing clean-up reactions were performed using the EDTA/ethanol precipitation protocol according to the BigDye manual. The samples were then stored in the dark at -20 ^{o}C until sequencing was performed at the University of the Free State.

Sequence data were analysed using Geneious version R9 software (http://www.geneious.com, Kearse *et al*., 2012), and compared with sequences obtained on Genbank using the nucleotide Basic Local Alignment Search Tool (BLASTn)(http://www.ncbi.nlm.nih.gov; Altschul *et al*., 1990).

3.3 RESULTS AND DISCUSSION

3.3.1. Identification of the culture

The preliminary tests on the culture confirmed that *C. carnipullorum* was a Gram-negative rod-shaped organism with bright yellow colonies that were smooth and shiny with smooth edges as described by Bernardet *et al.* (2011).The PCR amplicon for the 16S rDNA gene gave a band of approximately 1500 bp as depicted in Figure 3.1, and the sequencing results

showed *Chryseobacterium carnipullorum* 9_R23581^T as one of the strains with significant alignment scores of the 16S rDNA gene (Figure 3.2).

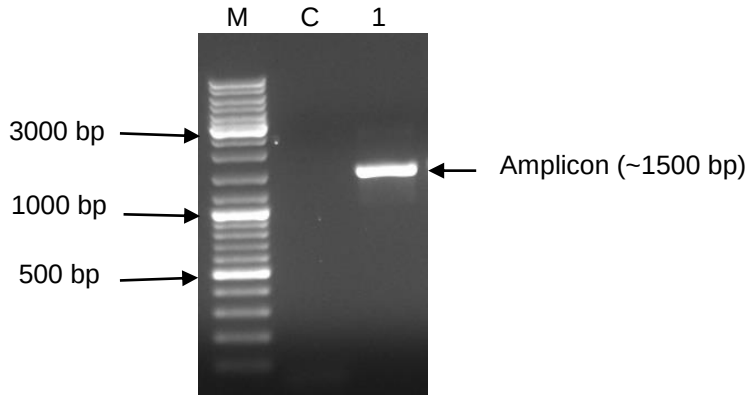

Figure 3.1: 16S rDNA amplicon obtained from the Polymerase Chain Reaction (PCR). Lane M: 10 kb O'geneRuler (marker); Lane C: negative control; Lane 1: 1500 bp amplicon indicative of 16S rDNA gene from *C. carnipullorum*.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chryseobacterium RF-5 16S ribosomal RNA gene, partial sequence	1349	1349	100%	0.0	100%	KX230031.1
Chryseobacterium shigense strain G188 16S ribosomal RNA gene, partial sequence	1349	1349	100%	0.0	100%	JX100823.1
Chryseobacterium carnipullorum strain 9_R23581 16S ribosomal RNA gene, partial sequence	1349	1349	100%	0.0	100%	NR_118332.1
Chryseobacterium carnipullorum strain b68 16S ribosomal RNA gene, partial sequence	1345	1345	100%	0.0	99%	KR778801.1

Figure 3.2: Sequences showing significant alignment of the 16S rDNA gene from the culture used in the study. *Chryseobacterium carnipullorum* strain 9_R23581^T was among the first few hits with a 100% identity.

3.3.2 Assay for keratinolytic activity

Keratinolysis was visually assayed during the growth of *C. carnipullorum* by observation of feather degradation in the flasks as shown in Figure 3.3. No feather degradation was observed in the control flasks throughout the study as these were devoid of the bacterial inoculum. This was an indication that there was no keratinolytic effects present without the *Chryseobacterium* culture. However, all the flasks that were inoculated with *C. carnipullorum* showed substantial degradation of feathers between 48 to 60 hours of growth except for the flasks that were supplemented with PMSF. This indicated that there are potentially keratinolytic enzymes being produced during growth of the culture. Keratinases are

predominantly extracellular enzymes and would thus be secreted into the growth medium (Smith *et al*., 1998; Selvam and Vishnupriya, 2012) of *C. carnipullorum*.

Figure 3.3: Assay for keratinolytic activity. Left: Control flask without *C. carnipullorum* with no feather degradation. Right: Feather degradation observed in flask cultured with *C. carnipullorum,* after 48 hours of incubation at 25 ^{o}C.

Enzymes which selectively break down the β-keratin present in feathers may be produced by some strains of bacteria to assist them in obtaining nutrients needed for their growth and maintenance such as carbon, sulphur and energy (Govarthanan *et al.,* 2011). The results in this study showed that the keratinase produced by *C. carnipullorum* was an efficient enzyme for β-keratin (chicken feathers), hence, demonstrated potential in disposal of feather waste. The colony counts revealed maximum growth of the culture during the exponential phase from about 20 hours to about 50 hours (Figure 3.4), hence, enzyme production could be a survival mechanism for this micro-organism as nutrients get depleted in the media to utilize the feathers as a nutrient source.

There are a few other *Chryseobacterium* species that have been recently reported to possess keratinolytic activity. These include *C. bovis* DSM 19482^{T} (Laviad-Shitrit *et al*., 2017), *C. camelliae* Dolsoni-HT1 (Kim *et al*., 2018) and *C. gallinarium* strain DSM 27622^{T} (Park *et al*., 2015). Previously, only *Chryseobacterium* sp. strain kr6 (Riffel *et al*., 2003) was reported among the Gram-negative bacteria described to have feather degrading activity.

Figure 3.4 illustrates the growth of *C. carnipullorum* in different media compositions. These include whole feather medium (WFM), WFM supplemented with 1% (w/v) glucose, WFM supplemented with 1% (w/v) starch and WFM supplemented with 0.014 M phenylmethyl

sulfonyl fluoride (PMSF). It was observed that whole feather medium promotes the growth of the culture as cells continued to multiply throughout the period of study. The whole feathers were possibly being utilised as a complete source of carbon and nitrogen for the bacterium to survive. When the whole feather medium was supplemented with glucose, a rapid increase in the growth of bacteria was noted within the first 24 hours but eventually there was a gradual decrease in the total bacterial count. This could be due to catabolite repression as glucose is a simpler carbon source, hence, it is easily utilised by the bacteria as an energy source (Mehta *et al*., 2014), however, when it gets exhausted, the bacteria starts to enter into the stationary phase, followed by a diauxic growth pattern. Starch supplemented feather medium on the other hand, showed a gradual increase in bacterial growth up to about 50 hours, followed by a decrease in the total bacterial count. Starch is an extra carbon source (Sivakumar *et al*., 2013) in addition to the feathers, hence it had a positive effect on the growth of the culture. The presence of starch in the media may also force the microorganism to produce keratinase in order to utilise the whole feathers as a source of nutrients, as starch is a polymer of glucose so the cells need to liberate the free glucose units for utilization. The growth pattern of the microorganism in the WFM was very similar to that of the WFM supplemented with either glucose or starch as depicted in Figure 3.4, hence, this was likely an indication that the keratinolytic activity of enzymes produced by *C. carnipullorum* was very high to allow growth on such a minimal media with only chicken feathers as complete nutrient source. On the otherhand, phenylmethyl sulfonyl fluoride (PMSF), a serine protease inhibitor, when added to the whole feather medium prolonged the lag phase of the microorganism in the flasks and supressed the growth of the culture to some extent compared to growth in the flasks without PMSF. This indicated that the enzyme could possibly be a serine protease (Böckle *et al*., 1995; Sreenivasa and Vidyasagar, 2013). The PMSF will inhibit the released enzymes (Figure 3.5), until all the PMSF is utilized, although the cells continue to divide. The new cells release the enzyme and utilization of feathers as a nutrient source can occur, hence, the observed growth of microorganisms in the flasks supplemented with PMSF.

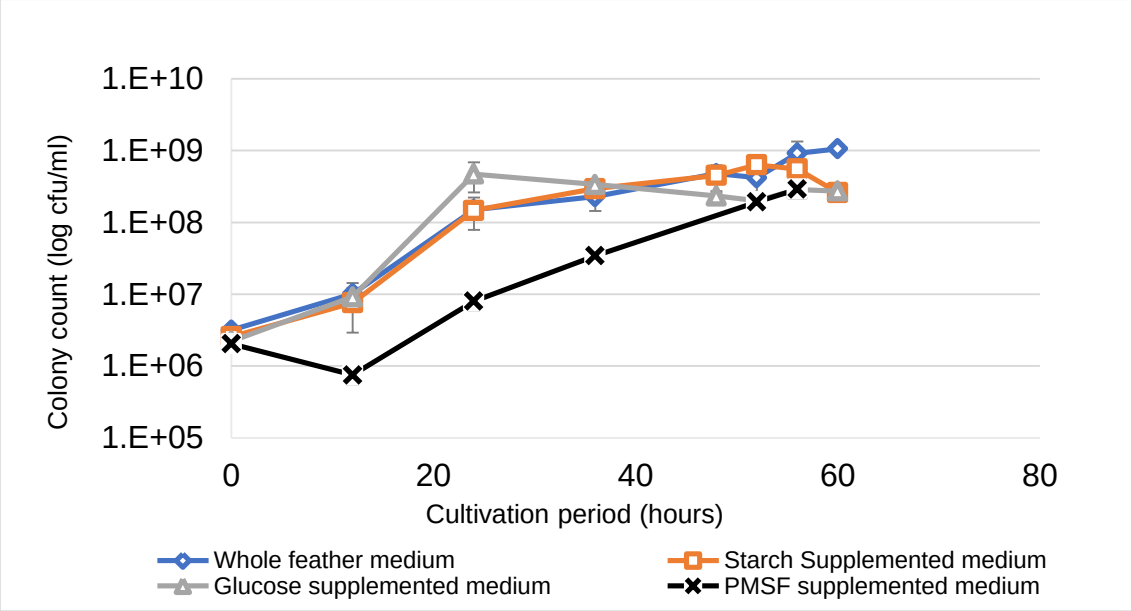

Figure 3.4: Growth (log cfu/ml) at 25 ^{o}C for 60 hours of *Chryseobacterium carnipullorum* in whole feather medium supplemented with either 1% (w/v) glucose, 1% (w/v) starch or 0.014 M phenylmethyl sulfonyl fluoride.

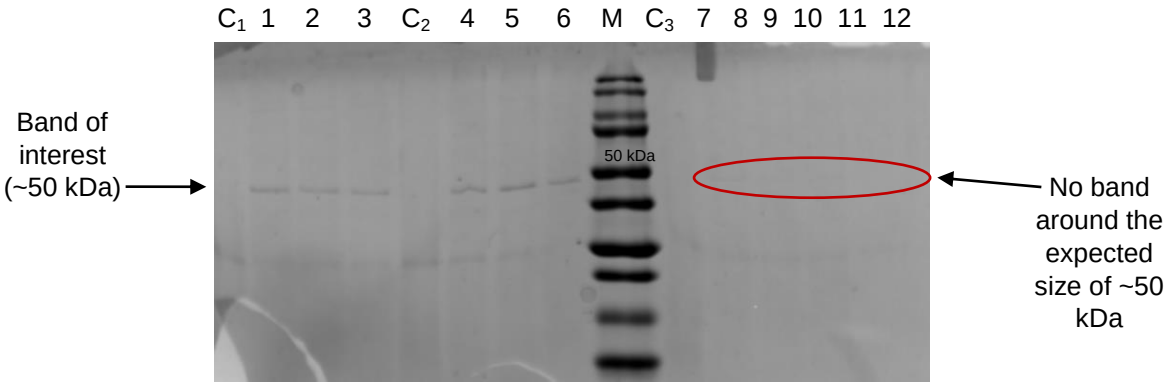

Figure 3.5 SDS-PAGE showing proteins produced by *C. carnipullorum* in glucose supplemented medium and in PMSF supplemented medium after 24 and 48 hours respectively. Lanes C_1 and C_2; negative controls for the glucose supplemented medium after 24 hours and 48 hours respectively, Lanes 1 to 6 triplicates after 24 and 48 hours respectively in glucose supplemented medium, Lane M; precision plus protein standards marker, Lane C_3 Control for the PMSF medium after 24 hours, Lanes 7 to 12; triplicates after 24 hours and 48 hours respectively in PMSF medium, no band observed around the expected size.

3.3.3. Secretory protein production and enzyme identification

Secreted proteins were obtained and observed within 24 hours of the organism's growth in all the types of medium used. This was seen from the faint bands obtained on the SDS-

PAGE as shown in Figure 3.5 for the glucose supplemented medium. PMSF supplemented medium, however, did not give bands on the SDS-PAGE (Figure 3.5) during this period and this result could be an indication of serine protease inhibition. In all other types of medium, the production was more pronounced after 48 hours of growth of the culture (Figure 3.6). The same bands were obtained for all the types of medium, only the intensity varied with the starch supplemented medium giving the most prominent bands. SDS-PAGE gels showed that there were different proteins produced by the culture ranging from 20 kDa to about 162 kDa (Figure 3.6).

Identification of proteins using LC-MS/MS revealed the presence of different proteins expressed extracellularly during the growth of *C. carnipullorum*. The proteins identified were peptidase M64 (~50 kDa), glutaminase (~20 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ~37 kDa), superoxide dismutase (~25 kDa), citrate synthase (~<50 kDa) as well as some outer membrane proteins (OMPs; ~100 kDa). A large majority of the proteins were therefore enzymes (Figure 3.6). The 50 kDa filter unit was used to concentrate proteins below 50 kDa, as with LC-MS/MS identification of crude extracts, it was seen that the peptidase proteins were smaller than 50 kDa.

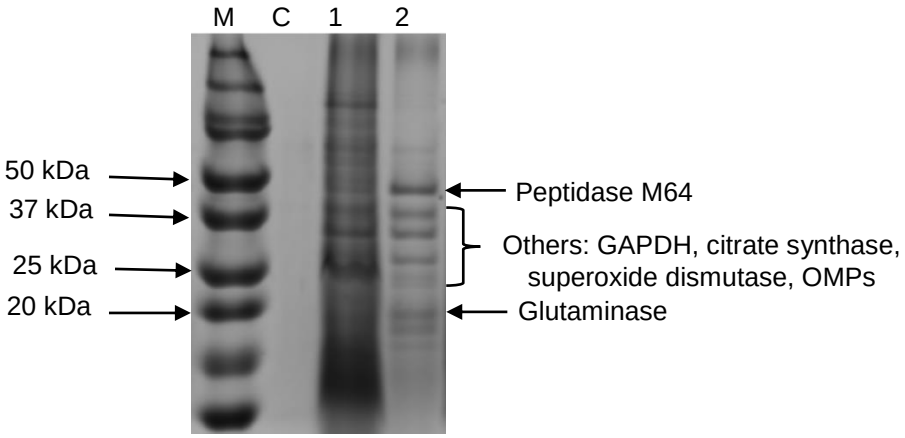

Figure 3.6: SDS-PAGE protein bands obtained after centrifugal ultrafiltration of the culture supernatant using 50 kDa and 10 kDa ultracel filter units. Lane M: Bio-Rad Precision Plus Protein™ Standard marker; Lane C: Negative control sample – medium with no bacterial culture after 48 hours; Lane 1: Sample after 48 hours, filtered through 50 kDa filter unit; Lane 2: Sample after 48 hours filtered through 10 kDa filter unit.

Although some of the enzymes identified may not have a significant role to play in keratin degradation, their activities complement each other towards the growth and survival of the culture. These enzymes play a role in amino acid breakdown and subsequent energy production for survival and growth of the bacteria. Secreted proteins are more easily purified

than cytoplasmic proteins due to less contaminating proteins in the culture medium than in the cytoplasm (Kikuchi *et al*., 2008).

Peptidase M64 is a member of highly selective metallo-endopeptidases, which belong to the MEROPS peptidase family M64 (http://www.ebi.ac.uk/interpro/entry/IPR019026). The peptidases contained in the family M64 are attached to the bacterial cell wall (Barreti *et al*., 2012), hence, the reason why they were found in the supernatant of the bacterial culture in this study. Peptidases, as the name suggests, are enzymes that generally target peptide bonds of peptides and/or proteins. Some are involved in catalysing extensive protein-processing events such as the breakdown of intake proteins in addition to remodelling, maintenance and development of tissues (Cerda-Costa and Gomis-Ruth, 2014).

Glutaminase is an enzyme that deaminates L-glutamine to L-glutamic acid and ammonia (Nandakumar *et al*., 2003; Altman *et al*., 2016). Glutamine from glutamate is further converted to alpha-ketoglutarate, an essential component of the citric acid cycle. Alpha (α)-ketoglutarate provides energy for the cell when it enters the citric acid cycle by production of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) or it can produce citrate through reductive carboxylation which supports the synthesis of acetyl CoA and lipids (Altman *et al*., 2016). In the food industry, glutaminase is used as a flavour-enhancing enzyme (Nandakumar *et al*., 2003).

GAPDH is an enzyme that catalyses the irreversible conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate for energy generation and carbon molecules through glycolysis. It contains four identical subunits with a molecular mass of 37 kDa (Zhang *et al*., 2015) and this corresponds to the size at which it was identified in this study. GAPDH also has a moonlighting property of initiating apoptosis in response to cell stress (Hara *et al*., 2005). Apoptosis is the principal mechanism through which cells undergo physiological death (Ucker *et al*., 2012). During apoptosis, there is an increase in the gene expression for GAPDH (Barbini *et al*., 2007). Ucker *et al*. (2012) reported that GAPDH is among the enzymes that are externalized during cell death, hence, it serves as an early biomarker of apoptosis. GAPDH is upregulated in stress conditions, hence, increasing the rate of glycolysis serving as a survival mechanism for the bacteria (Velasco *et al*., 1994).

Superoxide dismutases belongs to a class of metallic proteins that catalyse the detoxification of superoxide radicals (O_2^-) to oxygen and hydrogen peroxide (H_2O_2) (Bojadzhieva and Emanuilova, 2005). The H_2O_2 can be broken down into water (H_2O) by the action of peroxidases and catalases (Boyadzhieva *et al*., 2010). Superoxide dismutase therefore, provides a protective effect to the cell against the superoxide radicals, which are by products

of oxygen metabolism and cause damage of proteins, lipids as well as DNA (Perry *et al*., 2010).

Citrate synthase (CS) is an enzyme with a crucial role in carbohydrate metabolism for energy generation. It belongs to a set of transferases which transport an acetyl from acetyl-CoA to a 2-oxo-acid with the release of CoA (Marco-Urrea *et al*., 2011). Citrate synthase is responsible for catalysing the first reaction of the citric acid cycle that is the production of citrate from acetyl CoA and oxaloacetic acid (Chen *et al*., 2014). The initiation of both anabolic and catabolic reactions in aerobic organisms is achieved by the action of citrate synthase leading to the biosynthesis of products such as glutamate and glutamine via 2-oxoglutarate (Marco-Urrea *et al*., 2011). Citrate synthase is assumed to be the rate-limiting enzyme for the citric acid cycle (Mukherjee *et al*., 1976; Chen *et al*., 2014). In this study, citrate synthase was identified as part of the secretome of *C. carnipullorum* perhaps due to cytosolic leakage as cells were dying out in the media.

Some outer membrane proteins were also identified in the culture supernatant of *C. carnipullorum*. These proteins play a key role in signal transduction as well as protein and solute translocation across the bacterial cell mostly in Gram-negative bacteria (Koebnik *et al*., 2000).

3.3.4. Amplification of genes, coding for proteins of interest

From the LC-MS/MS data, only proteolytic enzymes were selected for further investigations, these were peptidase M64 and glutaminase. The gene coding for the peptidase M64 was successfully amplified from the genome of *Chryseobacterium* (Figure 3.7) whereas the amplification of the glutaminase gene was not successful even after several optimisation attempts. In one of the reactions for glutaminase, Mg^{2+} ions were added at different concentrations since they play an important role during the incorporation of nucleotides to the primer by DNA polymerase (Yang *et al*., 2004), however, no amplification was observed for all the concentrations. Hence, the study could not be continued for glutaminase but only for peptidase.

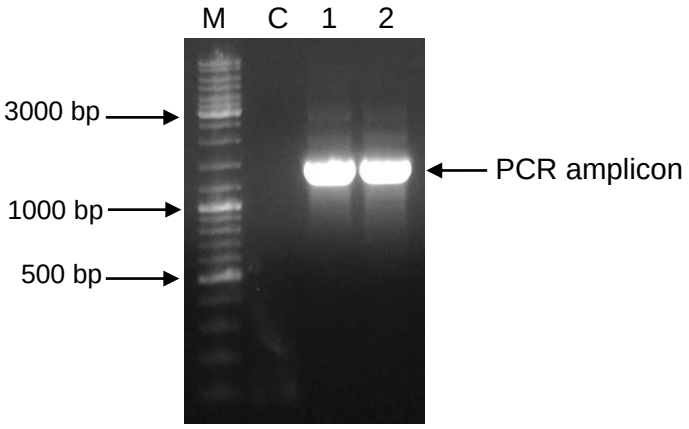

Figure 3.7: PCR amplification of the peptidase M64 gene. Lane M: 10 kb O'geneRuler (marker); Lane C: Control without genomic DNA from *C. carnipullorum*; Lane 1 and Lane 2: PCR amplification of the gene from the genome of *C. carnipullorum*

3.4 CONCLUSIONS

Chryseobacterium carnipullorum 9_R23581^T is a Gram-negative bacterium that grows at room temperature (25 ^oC) and does not require special conditions to produce proteolytic enzymes including keratinases. It therefore, has great potential in being used as an eco-friendly alternative of chemicals and heat treatments when handling keratinous materials.

The protein production by *C. carnipullorum* was observed to begin in the exponential phase of growth around 24 hours. Nearly complete degradation of feathers was observed within 48 hours in this study whether the minimal chicken feather medium was used alone or supplemented with either glucose or starch. This was a positive indication of the high keratinolytic activity of the enzyme produced by *C. carnipullorum* with or without supplements when using whole feather medium. The keratinolytic enzymes produced by *C. carnipullorum* may help to alleviate the feather waste disposal problem being experienced by the poultry processing industry. Other industries that deal with keratinous substrates such as the leather industry, feed industry, waste management sector and production of semi-slow release nitrogen fertilizer in organic farming may also exploit the potential of keratinases from *C. carnipullorum*.

Most of the secreted proteins were identified to be enzymes although the proteolytic enzymes were only peptidase M64 and glutaminase. Their relative molecular masses were approximately 50 kDa and 20 kDa respectively. Peptidase M64 was successfully amplified from the genome of *C. carnipullorum,* hence, it was cleaned-up for use in gene cloning and expression (Chapter 4) for further study.

CHAPTER 4: EXPRESSION, PURIFICATION AND CHARACTERISATION OF KERATINASE

4.1 INTRODUCTION

Keratins are structural proteins that are known to be resistant to proteolytic hydrolysis due to disulphide bonds, which results in high mechanical stability (Kornikowicz-Kowalska and Bohacz, 2011). The huge amount of feathers produced by the poultry industry can be converted to feather meal using microbial enzymes which are not only simple to work with but also economically viable (Tiwary and Gupta, 2012) as opposed to chemical treatments and steam pressure, which require substantial energy input and destroy amino acids (Papadopoulos *et al*., 1986; Thys and Brandelli, 2006). Proteases produced by certain micro-organisms can be used to convert keratin to useful biomass, protein concentrates as well as amino acids with improved digestibility of feather keratin (Thys and Brandelli, 2006).

Improving enzyme production levels and mining of keratinase resources are the focus of most studies due to the increasing industrial demand for keratinases (Rao *et al*., 1998). Expression of keratinases using heterologous systems would lay the foundation for its genetic manipulation, large scale enzyme preparation and protein engineering although the self-degradation mechanism limits the expression levels of the keratinase (Hu *et al*., 2013).

This study is aimed at expressing the keratinolytic enzymes of *C. carnipullorum* in *Escherichia coli*, purifying the expressed enzyme and characterising it. The objectives are to clone the gene that codes for peptidase M64 into pET28b(+), propagate the cells and express the enzyme in *E. coli* BL21 (DE3) for further purification and characterisation.

4.2 MATERIALS AND METHODS

The type strain of *C. carnipullorum* (strain 9_R23581^T) was obtained from the freeze-dried culture collection of the Food Science division, University of the Free State, South Africa. The competent cells of *Escherichia coli* 10-β for gene cloning and *Escherichia coli* BL21 (DE3) for expression were maintained in the Veterinary Molecular Laboratory, University of the Free State, South Africa, and stored at -80 ^oC in 50 µl Luria Bertani (LB) medium. pGEM-T Easy^{TM} vector, plasmid extraction and gel elution kits were obtained from Promega, Madison, WI, USA. The pET28b(+) vector was obtained from Novagen. Restriction enzymes were obtained from Thermo-Scientific, South Africa. Azocasein was obtained from Sigma-Aldrich, USA. Taq DNA polymerase, T4 DNA ligase, T4 DNA ligase buffer and bovine serum albumin (BSA) were obtained from BioLabs Inc, New England.

All the techniques for cloning and transformation were performed according to standard molecular biology techniques (Sambrook and Russell, 2001).

4.2.1. Subcloning into pGEM-T Easy™ vector

Subcloning was performed according to the manufacturer's instruction. A ligation reaction of the PCR product into pGEM-T Easy™ was done. The reaction contained 1 µl of T4 DNA ligase, 1 µl of 10x T4 DNA ligase buffer, 0.25 µl pGEM-T Easy™, 0.5 µl BSA (10 mg/µl), 6.25 µl nuclease-free water and DNA insert ranging between 1-3 µl, depending on the amount needed by the formula:

DNA insert = [(50 ng vector X insert size)/3 kb vector] X (3/1)

A negative control sample was also prepared in the same manner except that it had no DNA insert. The total volume of the mixture was 10 µl. Ligation was allowed to take place overnight at 4 ^{o}C.

4.2.1.1. Transformations

Competent cells (50 µl) of *E. coli* 10-β were obtained from -80 ^{o}C and thawed on ice for 5 minutes. All the DNA (10 µl) from the ligation step was added. The mixture was incubated on ice for 20 minutes then heat shocked at 42 ^{o}C in a water bath for 1 minute. It was then incubated on an ice slurry for 2 minutes. The cells were supplemented with 1 ml LB, 20 µl of 1 M glucose and 10 µl of 2 M magnesium chloride. The cells were grown at 37 ^{o}C in a waterbath for 60 minutes. Centrifugation of the cells was done at 9000 x *g* for 30 seconds. Approximately, 800 µl of the supernatant was removed and the cells were re-suspended in the remaining media. Spread plating was done on LB Ampicillin-IPTG-X-Gal (AIX) plates which were prepared by weighing 1 g Tryptone, 1 g NaCl, 0.5 g yeast extract and 1.5 g Agar bacteriological in 100 ml distilled water (pH 7.0). After autoclaving, 0.2 ml ampicillin (100 mg/ml), 0.2 ml IPTG (4.8 mg/ml) and 0.2 ml X-gal (20 mg/ml) was added after cooling the media to 55 ^{o}C. The plates were sealed with parafilm and incubated at 37 ^{o}C for 20 hours. Colonies were observed. Blue / White selection was done.

Blue and white selection is a method used to identify colonies that are carrying the recombinant plasmid. It involves picking white colonies as positive transformants since the insertion of a gene in the multiple cloning site of a plasmid interrupts the activity of the β-galactosidase gene (Sherwood, 2003) that catalyses the hydrolysis of X-gal causing nonenzymatic oxidation and dimerization forming a blue coloured pigment (Julin, 2014). Hence, colonies that appear pale or deep blue are those containing plasmids without insert.

4.2.1.2. Plasmid Extraction – Small scale plasmid isolation.

A single white colony from the transformation stage was inoculated in 5 ml LB broth supplemented with 5 µl of 100 mg/ml ampicillin and incubated overnight at 37 ^{o}C. Further incubation was done on a shaking incubator at 37 ^{o}C for 3 hours.

One millilitre of cell culture was pelleted at 5000 x *g* for 5 minutes.The pellet was re-suspended in 350 µl STET buffer (8 g sucrose, 5 ml Triton X-100, 10 ml 0.5 M EDTA, 5 ml 1 M Tris-HCl, pH 8, in 100 ml distilled water). Addition of 4 µl lysozyme (50 mg/ml) was done followed by mixing. The mixture in the tube was placed in a boiling water bath for 44 seconds. It was then immediately placed on ice for 10 minutes and centrifuged for 15 minutes at full speed (20000 x *g*).

The supernatant was transferred to a new 1.5 ml microcentrifuge tube. The supernatant was supplemented with 420 µl isopropanol and 40 µl sodium acetate (2.5 M, pH 5.2). The mixture was vortexed and then incubated on ice for 30 minutes. The tube was centrifuged at 4 ^{o}C for 5 minutes at maximum speed (20000 x *g*). The supernatant was removed and 1 ml of 70% v/v ice-cold ethanol was added. Gentle mixing was done followed by centrifugation at 20 000 x *g* at 4 ^{o}C for 5 minutes. The supernatant was removed and the pellet was air dried for about an hour in a laminar flow cabinet. 40 µl Tris-EDTA buffer (TE; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with RNase (50 mg/ml) was added, followed by incubation at 37 ^{o}C for 30 minutes. The quality and quantity of the plasmid DNA was determined using 1% agarose gel eletrophoresis and spectrophotometrically by means of a Nanodrop ND-1000. The extracted plasmid DNA was stored at -20 ^{o}C until further use.

The insert in the pGEM-T Easy^{TM} vector was confirmed through Sanger sequencing according to Big Dye manual using SP6 promoter and T7 terminator primers (Integrated DNA Technologies).

4.2.1.3. Digestion of insert from pGEM-T Easy^{TM} vector

Digestion of the plasmid DNA was conducted by mixing 1 µl of each of the restriction digest enzymes (*Xho*I and *Nde*I) with 2x Tango buffer and 22 µl of plasmid DNA and the final volume was adjusted to 30 µl using nuclease-free water. The mixture was incubated in a water bath at 37 ^{o}C for 2 hours. Electrophoresis was performed at 9 V cm^{-1} on a 1% agarose gel.

After observation of the gel using the Gel Doc^{TM} EZ Imager (Bio-Rad), the remainder of the reaction mixture was run on a low melt 2% agarose gel. The band of interest was excised and gel clean-up was performed using the Wizard® SV Gel and PCR Clean-Up system (Promega) according to the manufacturer's instructions. The concentration of the plasmid DNA was determined using a Nanodrop ND-1000 spectrophotometer.

4.2.2. Subcloning into pET28b(+) vector

4.2.2.1. Transformations

Escherichia coli 10-β was transformed with the pET28b(+) vector by adding 1 µl of the vector to 50 µl of the competent cells. In a different tube, no pET28b(+) was added to the competent cells and this served as the negative control. The mixture was incubated on ice for 20 minutes then heat shocked at 42 ^{o}C for 1 minute. The rest of the steps were as described in sub-section 4.2.1.1. The only difference was that LB-kanamycin (50 mg/ml) media was used for plating while LB-kanamycin broth was used to grow the cells, as kanamycin is the antibiotic selection marker for pET28b(+).

4.2.2.2. Plasmid extraction and Digestion of the pET28b(+) vector

Plasmid DNA from *E. coli* 10-β cells transformed with pET28b(+) vector was extracted using the small scale plasmid extraction method. Digestion was then performed using the same restriction enzymes (*Xho*I and *Nde*I) used to digest the insert from pGEM-T Easy^{TM} vector. The digest was run on a 1% agarose gel followed by electrophoresing the remainder of the reaction on a 2% agarose gel and gel clean-up was performed as outlined in section 3.2.12.

4.2.2.3. Ligation reactions and transformations into *E. coli* 10-β for propagation

Ligation of the purified insert digested from the pGEM-T Easy^{TM} vector into the digested and purified pET28b(+) vector was conducted. The ligation mixture was transformed into *E. coli* 10-β cells and positive transformants were inoculated in LB-kanamycin (50 mg/ml) broth. Incubation was at 37 ^{o}C for 20 hours. Plasmid isolations were performed.

4.2.2.4. Digestion and sequencing of the recombinant plasmid vector

The recombinant plasmid was digested by first using single digestion with 1 µl *Xho*I, 1x Red buffer (Thermofischer Scientific), 5 µl plasmid DNA and adjusting the final volume to 25 µl with nanopure water. Digestion was allowed to take place at 37 ^{o}C for 1 hour then 1 µl of the mixture was run on a 1% agarose gel to see whether linearization had occurred. To the remaining reaction mixture, a double digestion was performed by adding 1 µl *Nde*I and 0.5 µl Red buffer followed by re-adjusting the final volume to 30 µl using nanopure water. The restriction digest was further incubated at 37 ^{o}C for 1 hour. In another tube, a single digestion was performed on 5 µl of plasmid with 1 µl *Nde*I in 1x Orange buffer and the final volume was adjusted to 30 µl with nanopore water.

Confirmation of the insert was done by running 1 µl of the double digested product on a 1% agarose gel alongside the products of the single digestions and a 1 kb O'gene ruler. In all cases, electrophoresis was conducted at 9 V/cm for 34 minutes.

After viewing the digests using the Gel Doc^{TM} EZ Imager, further confirmation of insert in the pET28b(+) vector as well as its orientation were achieved by Sanger sequencing using T7 promoter and T7 terminator primers (Integrated DNA Technologies).

Analysis of sequence data was done using Geneious version R9 software (http://www.geneious.com, Kearse *et al*., 2012), and consensus sequences were compared with sequences obtained on Genbank using the nucleotide Basic Local Alignment Search Tool (BLASTn; http://www.ncbi.nlm.nih.gov; Altschul *et al*., 1990).

4.2.3. Expression of the recombinant keratinase

The recombinant plasmids with a concentration of up to 100 ng/µl were transformed into *E. coli* BL21[DE3] cells. Some positive transformants were picked and colony PCR was performed using gene specific primers (peptidase M64) to determine whether the insert was present. Each of the same colonies was inoculated in 5 ml LB-kanamycin (50 mg/ml) broth and glycerol stocks were prepared upto a concentration of between 10 – 20% glycerol followed by freezing at -20 ^{o}C until further use in order to keep the recombinant cultures viable.

a) Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction

IPTG was used to express the proteolytic enzyme by inoculating cells from glycerol stocks in 5 ml LB-kanamycin broth up to an OD_{600} of between 0.5 and 0.8 followed by addition of IPTG to final concentrations of 0.5, 1 and 2 mM to the cell cultures. Induction was allowed to take place at 25 and at 37 ^{o}C for 4, overnight and 24 hours. Centrifugation was conducted at 7197 x *g* for 10 minutes.

b) Auto Induction

Expression of the recombinant keratinase was also exploited using ZYP-5052 auto induction medium (Studier, 2005). The ZYP-5052 medium was prepared by combining ZY medium, 20x NPS and 50x 5052 medium as outlined below:

- ZY medium contained 10 g Tryptone, 10 g NaCl and 5 g yeast extract in 1 litre of distilled water. The medium was autoclaved.
- 20x NPS made up of 0.5 M ammonium sulphate ($(NH_4)_2SO_4$; Merck), 1 M potassium dihydrogen orthophosphate (KH_2PO_4; Merck) and 1 M di-sodium hydrogen phosphate (Na_2HPO_4; Merck). The mixture was filter-sterilised (0.20 µm).
- 50x 5052 medium was made up of 5 g glycerol (Merck), 0.5 g glucose (Merck) and 2 g α-lactose (Merck) in 50 ml distilled water. The mixture was filter-sterilised (0.20 µm).

- Magnesium sulphate ($MgSO_4$, 1 M; Merck) was also prepared and filter-sterilised (0.20 µm).

The ZY medium (950 ml) was mixed with 50 ml of 20x NPS, 20 ml of the 50x 5052 and 2 ml of 1 M $MgSO_4$ to have the ZYP-5052 auto induction medium. Cells (25 ml) previously grown in LB-kanamycin broth were inoculated in 500 ml ZYP-5052 medium and the mixture was incubated at 37 ^oC with shaking (150 rpm) for 24 hours. Analyses were conducted in triplicate. The cell culture was centrifuged at 7197 x *g* for 10 minutes and the supernatant was discarded. The pellet was resuspended in binding buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM Immidazole, pH 7.4) to a final concentration of 0.1 g/ml and immediately placed on ice. Cell disruption was conducted using a constant cell disruption system (Constant Systems, UK) at a pressure of 30 kPsi (2.068 x 10^5 kPa). The disrupted cells were centrifuged (7197 x *g*, 10 minutes) and the supernatant was collected as the expressed enzyme extract.

SDS-PAGE was performed on the supernatant to visualise the expression of the recombinant keratinase using 12% resolving gel and 4.2% stacking gel at 7 V/cm for 1 hour 45 minutes in TTS buffer (0.1 M Tris, 0.1 M Tricine, pH 8.3, 0.1% w/v SDS). The bands of interest were excised and trypsinised as described in section 3.2.9 for identification using LC-MS/MS.

4.2.4. Purification of the recombinant keratinase

The purification of the recombinant keratinase was performed according to Jin *et al*. (2017) with some modifications. The enzyme extract was centrifuged at 7197 x *g* to get rid of excess cells and the pH was measured and maintained at pH 7.4. Ultracentrifugation was performed using an Optima^{TM} L-100 XP ultracentrifuge (Beckman Coulter) at 150 000 x *g* for 1 hour at 4 ^oC. The rotor used for ultracentrigation was a Beckman Coulter SW32Ti swing-bucket rotor.

The supernatant was filtered through a 0.20 µm sterile filter membrane and loaded on an immobilised metal ion affinity chromatography (IMAC) gravity flow column which had cobalt as the ligand. The filtered supernatant was also loaded on an IMAC HisTrap_HP_1_ml column which had nickel as the ligand. The column was equilibrated using 10 ml binding buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 7.4) before loading the sample.

An ÄKTA FPLC (Fast Protein Liquid Chromatography; GE Healthcare) was used to run the sample on the HisTrap column using UNICORN^{TM} 5.11 software. The pumps were washed with binding and elution buffers (50 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, pH 7.4) before equilibrating the loop with binding buffer. The column was washed with 10 ml binding buffer before eluting the bound protein in 1 ml fractions using a linear gradient of imidazole

ranging from 20 mM to 500 mM. The eluents were concentrated by centrifugal ultrafiltration using MWCO membranes with a 50 kDa cutoff and dialysed against 5 ml of 50 mM Tris-HCl buffer (pH 7.4) on the ultrafiltration column. The washed sample was then concentrated up to about 100 µl then diluted in 1 ml Tris-HCl buffer (50 mM).

The purified and dialysed enzyme fractions were visualised on SDS-PAGE (12% resolving gel and 4.2% stacking gel) which was stained using Coomassie brilliant blue R-250 and destained using 10% (v/v) acetic acid according to Wong *et al*. (2000).

4.2.5. Determination of protein content of the purified enzyme

Protein determination was performed according to the Pierce™ BCA protein assay kit (Thermo-Scientific) using bovine serum albumin (Thermo-Scientific) as the protein standard.

4.2.6. Characterisation of the recombinant protein

The recombinant protein was analysed for its optimum temperature, optimum pH, effect of metals, effect of inhibitors and effect of detergents using azocasein as the substrate for further characterisation. The absorbance range for azocasein is between 440-460 nm, thus 450 nm was selected due to the instrumentation used, but it is within the absorbance range of azocasein.

4.2.6.1. Determination of optimum temperature

The optimum temperature was determined according to Cai *et al*. (2008) and Vigneshwaran *et al*. (2010) with some modifications by incubating the enzyme substrate mixture at 37 and at 50 ^{o}C after which the reaction was terminated using 10% (v/v) TCA. Analyses were conducted in triplicate. The enzyme activity was determined by the standard enzyme assay.

4.2.6.2. Determination of optimum pH

The optimum pH was studied according to Cai *et al*. (2008) with slight modifications. Azocasein (Sigma-Aldrich) was used as the substrate using a buffer system of phosphate (pH 5.0 – 8.0) and Tris buffer (pH 7.5 – 9.0). The substrate mixture comprised of 1% azocasein in each of the buffers at different pH. The mixture was incubated at the optimum temperature and the reaction was terminated by the addition of TCA upto a concentration of 10%. Analyses were conducted in triplicate.

4.2.6.3. Enzyme assay

The procedure for enzyme assay were done using azocasein as substrate (Sarath *et al*., 1989; Govinden and Puchooa, 2012). The substrate mixture (250 µl) containing 1% azocasein was mixed with 250 µl of the buffer at the same pH. The control reaction consisted of the buffer with the substrate mixture only. Since 50 ^{o}C was selected as the

optimum temperature based on the preliminary assays, the mixture was equilibrated at 50 ^{o}C, before adding the enzyme solution (20 µl). Incubation was done at the optimum temperature for 1 hour. The reaction was stopped by the addition of TCA up to 10% concentration. The mixture was gently mixed and immediately centrifuged at 10 000 x *g* for 15 minutes at 4 ^{o}C. The absorbance was measured in triplicate using Gen 5 program version 1.11 at a wavelength of 450 nm. One unit of enzyme was described as one that caused a 0.01 increase in absorbance for a reaction time of 1 hour (Ramnani and Gupta, 2004).

The proteolytic activity was determined by mixing 1% azocasein in Tris buffer (480 µl of 50 mM, pH 8.5) with the enzyme solution (20 µl) and incubating the reaction mixture at 50 ^{o}C for 1 hour. The reaction was terminated using 500 µl of 10% TCA. Centrifugation was done (10 000 x *g*, 15 minutes) and the absorbance was determined at 450 nm. The mixture of azocasein, Tris buffer, and TCA without enzyme was used as the control.

4.2.6.4. Effect of Inhibitors, Metals and Detergent on enzyme activity

The effect of inhibitors, metals and detergent on the enzyme activity was determined according to Gupta *et al*. (2015) and Su *et al*. (2016) with modifications. All analyses were conducted in triplicate. Inhibitors (10 µl, PMSF and EDTA) at 1 mM and 5 mM concentrations were added to 10 µl of the enzyme solution and incubated for 30 minutes at room temperature. A blank solution was also prepared by replacing the enzyme solution with distilled water.

The effect of metals on enzyme activity was studied using $MgSO_4$ and $CaCl_2$ at 2.5 and 5 mM concentrations. After pre-incubating the enzyme solution (10 µl) with the metals (10 µl), the residual activity was determined. A blank sample was prepared with water instead of the enzyme solution.

To study the effect of detergents on enzyme activity, sodium dodecyl sulphate (SDS) was used at 0.1% and 5% concentration after pre-incubating the detergent-enzyme mixture for 30 minutes at room temperature. The enzyme solution (10 µl) was mixed with 10 µl of the detergent, incubated at 50 ^{o}C for 1 hour and residual activity determined. A control sample was similarly prepared except that the enzyme solution was replaced with distilled water.

4.3 RESULTS AND DISCUSSION

4.3.1. Subcloning into pGEM-T Easy™

Ligation of the insert into the pGEM-T Easy™ vector and transformation into competent cells revealed the presence of clones as some white colonies (positive clones) were seen on the plate apart from the blue colonies which were indicative of negative transformation. Turbidity was observed in the LB-ampicillin broth after an incubation period of 20 hours with

inoculation of a single white colony. The growth of cells in broth indicated the presence of an ampicillin resistance gene which is found in the PGEM-T Easy™ vector.

The restriction digest of the pGEM-T Easy™ plasmid vector after transformation revealed two different bands (Figure 4.1) indicating that the insert was successfully cloned and digestion took place. The size of the plasmid backbone was about 3015 bp, whereas the band of interest (cloned fragment) was approximately 1362 bp. Hence, the cloned fragment was cut and purified for use in subcloning into the pET28b(+) vector.

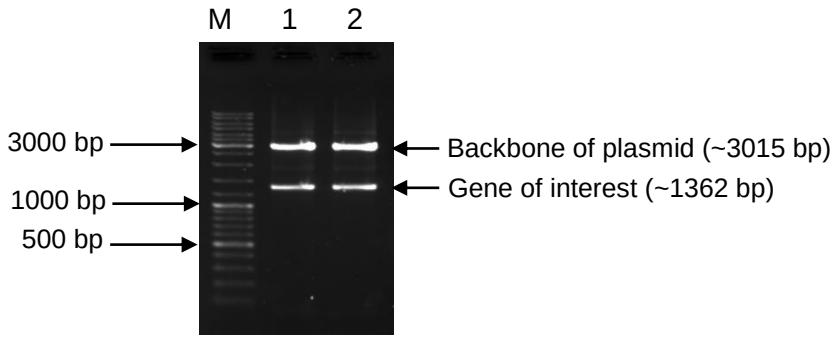

Figure 4.1: Digestion of pGEM-T Easy™ vector containing insert using *Xho*I and *Nde*I. From left to Right: Lane M: 10 kb O'geneRuler (marker); Lane 1 and Lane 2: pGEM-T Easy™ plasmid backbone and cloned fragment.

4.3.2. Subcloning into pET28b(+) vector

Transformation of the pET28b(+) vector into competent cells revealed white colonies on the LB-kanamycin plates. Turbidity was seen in the LB-kanamycin broth indicative of cells resistant to kanamycin due to the presence of the pET28b(+) vector. The restriction digest gave only one band of approximately 5400 bp on the gel (Figure 4.2). Hence, the band was cut and purified for further analysis.

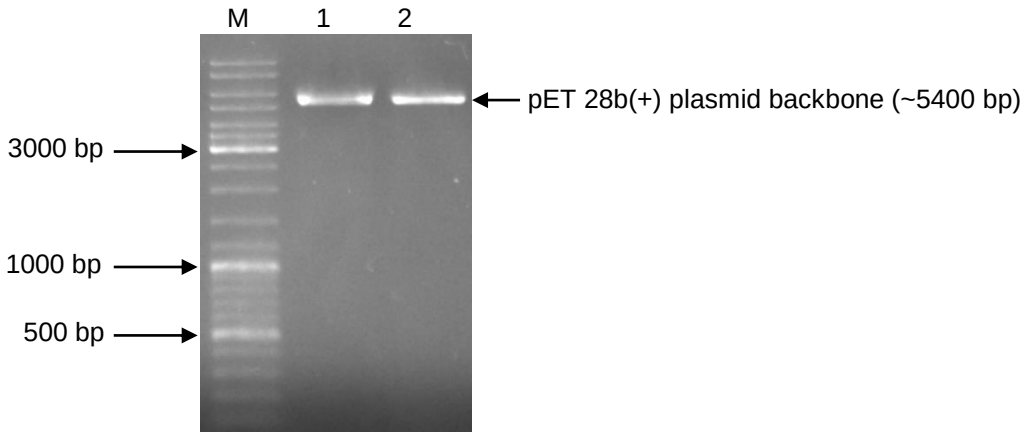

Figure 4.2: Digestion of the pET28b(+) vector using *Xho*I and *Nde*I. From left to right: Lane M: 10 kb O'geneRuler (marker); Lane 1 and Lane 2: pET28b(+) vector backbone after restriction digest.

Upon ligation of the insert (cloned fragment) into the pET28b(+) vector and digestion using *Xho*I first followed by *Nde*I, two bands were obtained on the gel after electrophoresis at 9 V/cm for 34 minutes. The band with a larger size was indicative of the plasmid backbone while the smaller size represented the gene of interest (Figure 4.3, lanes A5 and B5).

The sequences from the recombinant plasmid were compared with sequences of peptidase M64 on the NCBI database (Accession number NZ-FRCDO1000002.1). The analysis results confirmed that the insert was in-frame and consensus sequences revealed 100% similarity to those from the NCBI database.

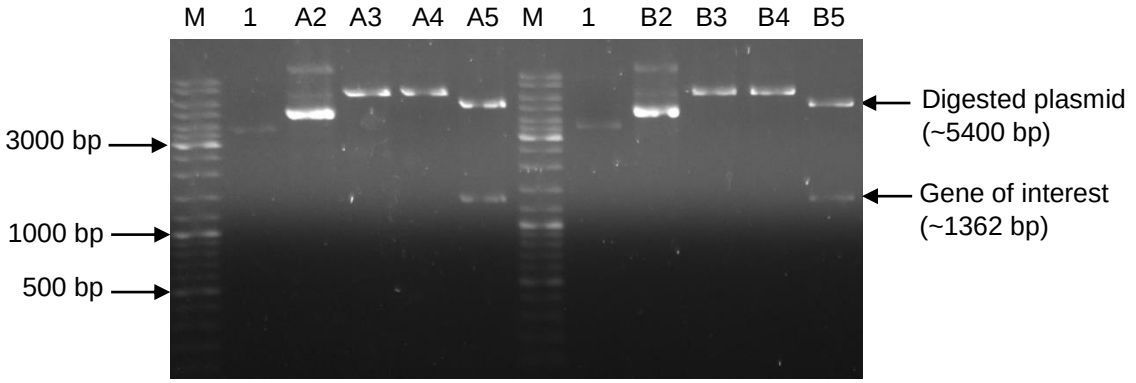

Figure 4.3: Digestion of the recombinant plasmids A and B. Lane M: 10 kb O'geneRuler (marker); Lane 1: pET28b(+) plasmid only; Lanes A2 and B2: recombinant plasmid before digestion; Lanes A3 and B3: recombinant plasmid after digestion with *Xho*I only; Lanes A4 and B4: recombinant plasmid after digestion with *Nde*I only; Lanes A5 and B5: recombinant plasmid after digestion with both *Xho*I and *Nde*I.

4.3.3. Expression of the recombinant keratinase

After growing the cells containing the insert (peptidase M64 gene) and inducing protein expression, some proteins were observed in the cell lysate and supernatant using SDS-PAGE as shown in Figure 4.4 and there was a dominant band around the expected size of about 50 kDa. From the SDS-PAGE, the expression was better using the auto induction medium than IPTG induction, which gave faint bands (data not shown). LC-MS/MS results revealed the band of interest (~50 kDa) as peptidase M64 and the alignment of the obtained peptide sequences to that on the NCBI database was homologous.

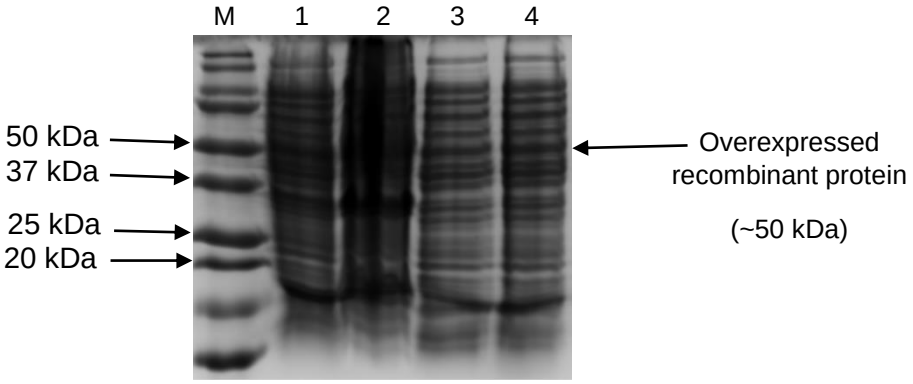

Figure 4.4: Expression of the recombinant keratinase using ZYP-5052 auto induction medium. Lane M: Bio-Rad Precision Plus Protein™ Standard marker; Lane 1: cell lysate; Lane 2: pellet resuspended in phosphate buffer; Lanes 3 and 4: Supernatant.

4.3.4. Purification of the recombinant keratinase

The purification of the recombinant keratinase revealed a band around the expected size of about 50 kDa (Figure 4.5) after IMAC purification using cobalt as ligand. However, after purifying with an IMAC HisTrap column that had nickel as the ligand, the dominant band size was slightly more than the expected size of about 50 kDa (Figure 4.6) and this could be due to the added hexahistidine tags at both the C and N terminal ends of the primer sets which added more base pairs to the initial size of the gene. Although the band migrated above 50 kDa, it was confirmed by LC-MS/MS to be peptidase M64. Purification was repeated on a HisTrap column in order to increase the enzyme yield for downstream processing.

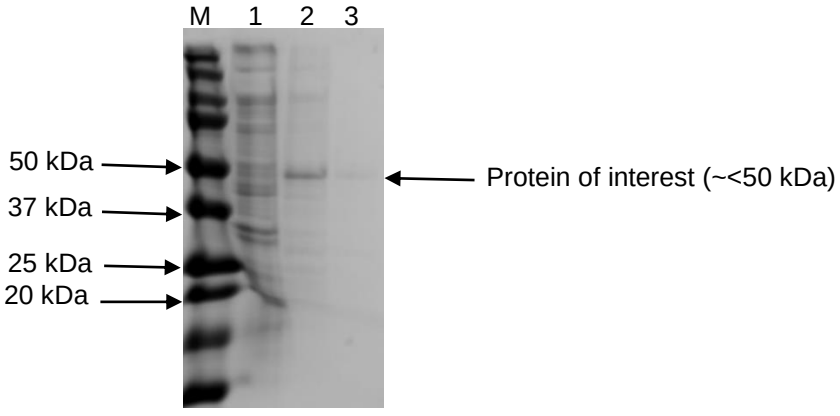

Figure 4.5: Purification using gravity flow IMAC – Cobalt column. Lane M: Bio-Rad Precision Plus Protein™ Standard marker; Lane 1: Flowthrough; Lanes 2 and 3: purified and concentrated protein using a 50 kDa & 10 kDa MWCO membranes respectively.

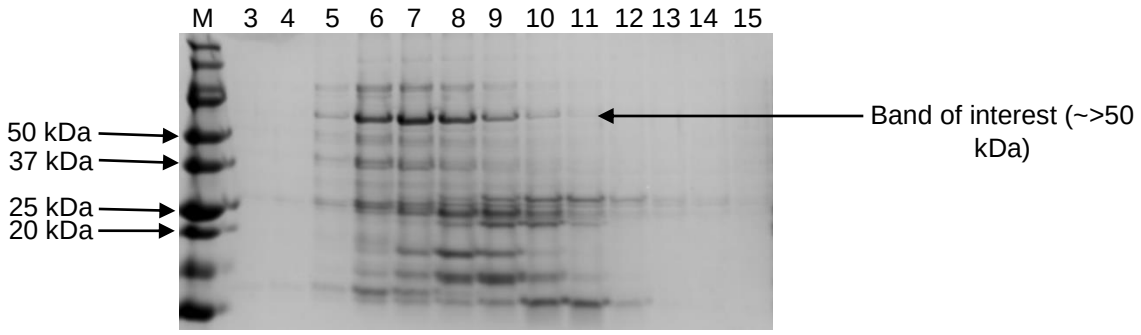

Figure 4.6: Purification using FPLC IMAC – Nickel HisTrap column. Lane M: Precision plus protein standards marker; Lanes 3 to 15: Eluent (1 ml) fractions.

Using the LC-MS/MS, the 50 kDa band in Figure 4.5 as well as the band slightly above 50 kDa in Figure 4.6 were identified as peptidase M64 and this confirmed the success of cloning and expression studies.

4.3.5. Determination of the purified enzyme concentration

The average concentration of the purified recombinant keratinase analysed in triplicate was 0.24 mg/ml.

4.3.6. Characterisation of the recombinant keratinase

The optimum temperature for enzyme activity was determined to be 50 ^{o}C, whereas the optimum pH was 8.5 in Tris buffer (Figure 4.7).

The molecular weight determination conducted by SDS-PAGE using 12% polyacrylamide gel was about 50 kDa.

Strong inhibition by PMSF, a serine protease inhibitor was observed both for the crude keratinase in which the band of interest was absent as discussed in Chapter 3 and illustrated in Figure 3.5 and for the purified keratinase in which the enzyme activity was substantially inhibited compared to the control sample.

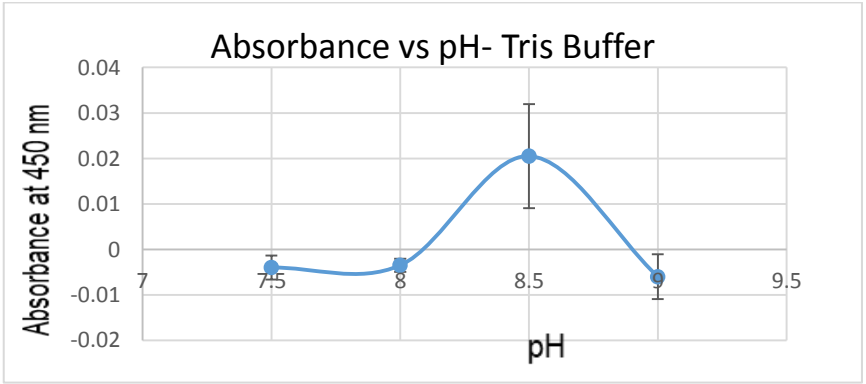

Figure 4.7 Graph of the relationship between Absorbance at 450 nm and pH using Tris buffer

$MgSO_4$ and $CaCl_2$ enhanced keratinase activity better at 5 mM than at 2.5 mM concentration. This is in agreement with previous findings in which divalent metal ions where found to stimulate keratinase activity (Nam *et al.*, 2002; Riffel and Brandelli, 2002; Sivakumar *et al.*, 2013). The enzyme activity was not affected by the SDS (data not shown) meaning it may be used by the detergent industry.

4.4 CONCLUSIONS

Chryseobacterium carnipullorum exerts an extraordinary activity against insoluble substrates such as keratins. Although the strain is mostly known for food spoiling capabilities, it also has great potential in biotechnological applications involving keratin hydrolysis.

This study reviewed that the most likely main component of the keratinolytic enzymes produced during the growth of *C. carnipullorum* is peptidase M64, a serine-metalloprotease endopeptidase as it was inhibited by PMSF which is a well known serine protease inhibitor. The gene coding for peptidase M64 was successfully cloned, propagated and expressed in *E. coli* BL 21[DE3] cells in which the activity after expression was confirmed.

The molecular weight of this keratinolytic enzyme was about 50 kDa and its optimum temperature and pH were determined to be 50 ^oC and pH 8.5, respectively. This was in accordance with literature as most keratinases have been reported to have optimum activity at temperatures ranging from 37-85 ^oC and optimum pH from 7.5 to 9 (Böckle *et al*., 1995; Riffel and Brandelli, 2002; Govinden and Puchooa, 2012; Selvam and Vishnupriya, 2012). This is desirable for industry as these are the conditions that are mostly employed during processing in biotechnological applications. Microbial keratinases exhibit various properties depending on the microorganism producing it. The activity of the proteolytic enzyme in this study was enhanced especially by media that contained divalent metal ions such as $MgSO_4$ and $CaCl_2$. Metal ions help to maintain enzyme conformation by acting as an ion bridge or as a salt (Grappel, 1976).

The keratinase produced by *C. carnipullorum* may be easily cultivated due to the strain's ability to grow at room temperature (25 ^oC) aerobically without the need for co-factors or supplements, hence, it may be recommended as one of the best candidates for enzyme production intended for further use in industry and feather processing.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

Keratinolytic microorganisms have continued to be identified although the majority fall under the Gram-positive bacteria (Jim *et al*., 2017). *Chryseobacterium carnipullorum* is among the few Gram-negative microorganisms that have been discovered to have keratinolytic activity by its ability to degrade keratinolytic materials such as feathers. The cloning and propagation of most of the keratinolytic species have not been totally successful due to hindrances that include loss of activity after expression in a different host.

Species of the genus *Chryseobacterium* have been mostly known for spoilage of foods due to production of proteolytic and lipolytic enzymes which lead to off-odours and off flavours (Bernadet *et al*., 1996; Hugo *et al.,* 2003; Venter *et al.,* 1999). Originally isolated from poultry, *C. carnipullorum* has been found to produce fruity, sulphide-like, evaporated and fishy off-odours in poultry (Banwart, 1989).

The type strain of *C. carnipullorum* was resuscitated from a freeze dried culture and preliminary identification conducted. Using SDS-PAGE and LC-MS/MS, proteins annotated to be proteolytic enzymes were selected and the genes coding for them were amplified from the host genome. Subcloning of the gene of interest into pGEM-T Easy^{TM} allowed for propagation after which the cloned fragment was digested and subcloned into the expression vector pET28b(+). The enzyme was expressed in *E. coli* BL21[DE3] cells and it was extracted using a constant cell disruption system (French press, 30 kPsi) followed by centrifugation in order to collect it in the supernatant. Purification was achieved using immobilised metal ion affinity chromatography (IMAC). A better quantity of the enzyme solution was obtained after purification using the HisTrap 1_ml column on the ÄKTA- FPLC compared to when the cobalt column was used. This is because the binding capacity of FPLC columns are a lot higher than the resin used in gravity flow IMAC and more than one fraction of the eluent can be collected and pooled as well as concentrated to improve the enzyme concentration. However, when this was done the band of interest was seen to have slightly shifted above the expected size of 50 kDa. This could have been due to the added hexahistidine tags at both the N and C terminus during primer design, hence, it was more pronounced after purification. Enzyme assays were performed on the purified enzyme to enable its further characterisation, as this has not been previously done.

Secretory enzymes are produced during the exponential growth phase of this strain. The observed feather degradation (Figure 3.3) was an indication of the keratinolytic potential of the strain as the uncultured flasks did not show any feather degradation. Furthermore, using SDS-PAGE analysis of proteins, the negative control samples (from the uncultured flasks) did not show any bands indicating that the bands observed for the supernatants of the

cultured flasks were solely produced by the culture (*C. carnipullorum*). Of the proteolytic enzymes identified, peptidase M64 was successfully amplified from the host genome and its activity was determined by exposing it to inhibitors, metals and a detergent (SDS). Since the keratinase was inhibited by the serine protease inhibitor PMSF, it can be concluded that peptidase M64 is a serine metalloprotease. The presence of divalent metal ions and SDS did not affect the enzyme activity negatively, hence this enzyme may be used in biotechnological processes and industries such as detergent industries without losing its activity. Peptidase M64 has a molecular weight of about 50 kDa, an optimum temperature of 50 ^{o}C and an optimum pH of 8.5 which is in agreement with the documented literature regarding the ranges of molecular weight (20 – 70 kDa, Selvam and Vishnupriya, 2012; 18 – 200 kDa, Gupta and Ramnani, 2006), temperature (40 – 80 ^{o}C) and pH (7.5 – 9) (Selvam and Vishnupriya, 2012).

This study helped to add to the knowledge base on the limited information regarding keratinolytic *Chryseobacterium* species. Species of *Chryseobacterium* have only been recently reported to have keratinolytic activity and include a *Chryseobacterium* sp. strain kr6 (Riffel *et al*., 2003), *C. bovis* DSM 19482^{T} (Laviad-Shitrit *et al*., 2017), *C. camelliae* Dolsoni-HT1 (Kim *et al*., 2018) and *C. gallinarium* strain DSM 27622^{T} (Park *et al*., 2015). The findings in this study also helped to obtain a better understanding of the keratinase produced by *C. carnipullorum,* which has been known to be a food spoilage microorganism while it can also be exploited for its keratinolytic potential. The use of *C. carnipullorum* as a keratin degrader may help to alleviate feather waste disposal problems experienced by the poultry processing industry, as it may be an eco-friendly alternative to the use of heat and chemical treatments. Industries such as feed, leather processing, waste management, detergent, biomedical and pharmaceutical industries are among the potential beneficiaries of this enzyme if it may be harvested on a large scale.

5.1. Recommendations for future research:

1. The future research should include determination of amino acid composition of the breakdown products such as feathers in order to appreciate its nutritional value when added as a supplement to animal feed.
2. To determine the stability of the enzyme at different temperatures and pH.

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