SCREENING, PURIFICATION AND CHARACTERISATION OF AN ACTIVE HYDROXYNITRILE LYASE (NITRILASE) FROM INDIGENOUS SOUTH AFRICAN PLANTS

by

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DISSERTATION

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CO-SUPERVISOR: Dr KC Lucas

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DEDICATION

I give thanks to the Lord my creator who gave me the opportunity to be part of this project, without you this project would have not been a success.

I would like to dedicate this to the following special people:

- My parents: Mr Mopai M.D and Mrs Mopai D.D. for loving, caring and supporting in most of the good choices I made. I love you.
- My daughter: Ditebogo Mopai for being my loving little girl and motivator, I love you.
- My siblings: Agnes Gafane, Mahlodi Tlhatlha, Edmond Mopai, Phillip Mopai and Khutso Mopai for giving me words of encouragement, support, care and love. I love you guys.
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who have tirelessly made provision for me emotionally through tough times and given me support during study period. They all continue to be a constant source of inspiration and role model in defining the true meaning of success and achievement.

DECLARATION

I declare that **SCREENING, PURIFICATION AND CHARACTERIZATION OF AN ACTIVE HYDROXYNITRILE LYASE (NITRILASES) FROM INDIGENOUS SOUTH AFRICAN PLANTS** is my own work. This report is being submitted for the degree of Master in biochemistry at the University of Limpopo. This report has not been submitted to any other University and I further declare that all sources quoted are indicated and acknowledged by means of a comprehensive list of references.

Kgaugelo Lydia Mopai

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Full names	**Signature**
[redacted]	
……	**……**
Student number	**Date**

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TABLE OF CONTENTS **PAGE**

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACE:	Angiotensin-converting enzyme
*At*HNL:	*Arabidopsis thaliana* hydroxynitrile lyase
*Bm*HNL:	*Baliospermum montanum* hydroxynitrile lyase
BSA:	Bovine serum albumin
Con A:	Concanavalin A Sepharose 4B
DEP:	Diethylpyrocarbonate
DMSO:	Dimethyl sulfoxide
DTT:	Dithiothreitol
*Ej*HNL:	*Eriobotrya japonica* hydroxynitrile lyase
FAD:	Flavine adenine dinucleotide
GMC:	Glucose-methanol-choline
HCN:	Hydrocyanic acid
HNL:	Hydroxynitrile lyase
KCN:	Potassium cyanide
*Ks*HNL:	*Kalanchoe species* hydroxynitrile lyase
*Pe*HNL:	*Passiflora edulis* hydroxynitrile lyase
*Pm*HNL:	*Prunus mume* hydroxynitrile lyase
*Sa*HNL:	*Sorbus aucuparia* hydroxynitrile lyase
*Sb*HNL:	*Senecio species* hydroxynitrile lyase
*Sb*HNL:	*Sorghum bicolor* hydroxynitrile lyase
SDS:	Sodium dodecyl sulphate

ABSTRACT

Hydroxynitrile lyases (HNLs) are enzymes that catalyse enantioselective cleavage of the substrate in a reaction and are also used as important industrial biocatalysts for the synthesis of chiral cyanohydrins. The aim of the study was to screen indigenous South African plants for potential hydroxynitrile lyase activity, purify and biochemically characterise the active hydroxynitrile lyase(s) from the selected plants. Several indigenous plants were randomly collected, identified and screened for HNL activity. The plant parts (leaves, seeds or fruits) were processed using established experimental protocols in order to obtain the crude enzyme extracts. The enzymatic conversion of benzaldehyde and potassium cyanide to mandelonitrile was optimised and consequently used for the screening of HNL activity. Enzyme activity was detected in the crude enzyme extracts of *Kalanchoe spp* and *Senecio spp* and these were then designated as *Ks* and *Sb*, respectively. Ammonium sulphate fractionation, DEAE Toyopearl 650M and Concanavalin A chromatography techniques were then used in the purification process of the active crude enzyme extracts. Subsequently, two purified active fractions were isolated from each plant species with molecular masses estimated at 64.64 kDa and 64.06 kDa for the *Ks*HNL enzymes and 70.60 kDa and 74.04 kDa for *Sb*HNL enzymes. The optimum temperature and pH of all the isolated enzymes were determined as 50°C and pH 5, respectively. The experimental K_m and V_{max} values of the enzymes were respectively determined to be 0.33 and 0.73 mM and 1.238 and 1.948 µM/min for *Ks*HNL; while that for *Sb*HNL enzymes were 5.86 and 0.22 mM and 9.741 and 1.905 µM/min. The effect of additives and metal ions (*viz*., DTT, DEP, mercury chloride, magnesium chloride and zinc chloride) was determined. The experimental data obtained alluded to the notion that both *Ks*HNL and *Sb*HNL enzymes may contain the cysteine and serine residues next to their active sites and that a histidine residue may be involved in the catalytic activities of both the isolated *Ks*HNL enzymes and one of the *Sb*HNL enzymes. All the isolated enzymes from the two plant species did not seem to contain an FAD group. These findings compared favourably to the theoretical type II HNLs, although with a slight difference in that they displayed high molecular weights. *Kalanchoe spp* and *Senecio spp* are the two indigenous South African plants that were found to contain active HNLs. The isolated HNLs from the two plants have a potential to be

purified to homogeneity, cloned and overexpressed into robust recombinant enzymes that can be used for large scale industrial applications.

CHAPTER 1

1.1 LITERATURE REVIEW

1.1.1 Introduction

Cyanohydrins are compounds composed of a chiral carbon bound to an alcohol group, a cyano- and hydroxyl-group [$R_2C(OH)CN$]. Due to their stereochemistry, biochemical industries (e.g., agro- and pharm-industries) use cyanohydrins for the synthesis of value-added chiral substances as the versatile polyfunctional building blocks (Ueatrongchit *et al.,* 2010). These compounds are used as intermediates in industries producing veterinary products, fine chemicals, pharmaceuticals, crop protecting agents, food additives and vitamins (Fechter and Griengl, 2004).

These industrially important enantiomers are either synthesized by chemical catalysts or by stereoselective enzymes from various sources. Although there are several enzymes utilized to achieve stereo- and region-selective chemical transformations (Liu *et al*., 2011), nature has evolved *(R)*- and *(S)*-selective hydroxynitrile lyase (HNL) enzymes (Blaser, 2004) that catalyse the direct enantioselective enzymatic addition of hydrocyanic acid (HCN) to the carbonyl group and produce pure enantiomeric products (Purkarthofer *et al*., 2007). This process thus takes advantage of the naturally occurring reversible reaction that normally transpires in plants where HNL enzymes cleave cyanohydrins into hydrocyanic acid (HCN) and aldehydes or ketones (Figure 1) (Johnson *et al*., 2000; Sharma *et al*., 2005). Thus, HCN is released during *in vivo* catalysis of plants, its functions being to defend different plants against microbial attack and herbivores (Conn, 1981; Hickel *et al*., 1996) and to serve as a nitrogen source in the biosynthesis of asparagine.

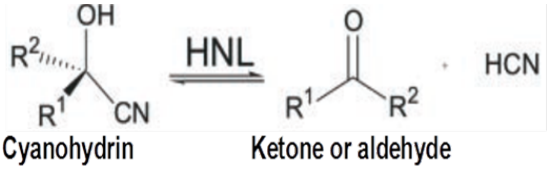

Figure 1: The reversible enzymatic reaction of cyanohydrin by HNL to its corresponding aldehyde/ketone and hydrocyanic acid (Sharma *et al*., 2005)

Hydroxynitrile lyases are naturally found in plants and HNL activity was detected for the first time in almond by Friedrich Wöhler in 1837 (Sharma *et al*., 2005). Many plants have since been screened and found to contain HNL activity which shows stereoselectivity. For example, the extract from *Baliospermum montanum* leaves showed *(S)*-HNL activity, whilst *(R)*-HNL activity was detected in the extract of *Passiflora edulis* leaves and in *Eriobotrya japonica* seeds (Ueatrongchit *et al*., 2008).

Due to the scanty background information regarding the available knowledge of HNL enzymes in indigenous South African plants, it was important to embark on this study and screen South African indigenous plants for HNL activity, in order to generate and increase new knowledge and enhance the prospect of discovering novel and robust hydroxynitrile lyase enzymes that can be applied industrially.

1.1.2 Cyanogenesis

The phenomenon of cyanogenesis (hydroxynitrile lyase-mediated release of a cyanide group in the form of HCN) is observed in higher plants and is also reported in taxonomically diverse groups of organisms such as bacteria, lichen, fungi, arthropods, millipedes and insects (Sharma *et al*., 2005). The reaction in plants includes the enantioselective cleavage of cyanohydrin from cyanogenic glycosides catalysed into aldehydes or ketones and HCN by HNL (Caruso *et al.*, 2009).

Cyanogenic glycosides are compounds found in most plants that produce highly toxic prussic acid or hydrogen cyanide (HCN). These substances belong to the plant antibiotic class of phytoanticipins (classified as secondary metabolites present in the plant that protect it against microbial attack) (Zagrobelny *et al*., 2008). For example, sorghum contains the glucoside dhurrin that is hydrolysed by the enzyme,-glucosidase yielding p-hydroxymandelonitrile. The p-hydroxymandelonitrile will be dissociates by hydroxynitrile lyase to produce HCN and p-hydroxybenzaldehyde (Stout *et al.*, 1980). The cyanogenic glycosides have a high structural diversity which is achieved by galloylation or glycosylation of the cyanohydrin to modify the sugar moiety (Neilson *et al*., 2011). These cyanogen glycosides play a role in the primary metabolism of plants in nitrogen storage and transport used to form asparagine (Møller, 2010).

There is convincing evidence that cyanogenesis is a mechanism that can serve as protection for the plant against predators such as the herbivores. Specific plant enzymes are released when a plant cell is damaged by crushing, wilting, chewing, or freezing, which results in the hydrolysis of the glycosides to cyanide (Knight and Walter, 2002). Cyanogenic glycoside biosynthesis occurs primarily in young and developing tissues, and the levels of which decrease in older plant parts due to the *de novo* biosynthesis that proceeds at a lower rate than catabolic turnover, or does not keep up with the net gain in total biomass (Zagrobelny *et al*., 2008).

The level of cyanogenic glycosides produced is thus dependent upon the variety and age of the plant, but has also been found to be affected by environmental factors (Francisco and Pinotti, 2000). Cyanogenesis has been reported in more than 3000 species of vascular plant taxa encompassing 105 families of pteridophytes (ferns), gymnosperms and flowering plants. Prominent among all these plants, are the plants belonging to families Linaceae, Clusiaceae, Euphorbiaceae, Gramineae (monocotyledons), Olacaceae, Rosacea and Filitaceae (ferns) (Stumpf and Conn, 1981). Cyanogenesis is mostly a two-step process. For the first step, a sugar moiety is cleaved by one or more glycosidase resulting in cyanohydrins and secondly, the α-hydroxynitrile (cyanohydrin) is degraded either spontaneously or catalysed by HNL in order to create prussic acid and the carbonyl compound (Nahrstedt, 1985).

There are many plant species that are known to contain cyanogenic glycosides that are likely to produce HCN poisoning. For example *Cassava* roots may contain up to one gram of HNC (http://www.chm.bris.ac.uk/motm/HCN/HCN.htm). However, relatively few of these plants regularly cause cyanide poisoning in humans or animals because they are infrequent food sources for animals or humans. However, some of the plants that are frequently associated with cyanide poisoning in animals are grown as food sources for humans and animals. Clover (*Trifolium spp*.), sorghum (*Sorghum spp*.) and cassava (*Manihot esculenta*) are some of the plants that can be used safely provided the circumstances under which these plants accumulate cyanogenic glycosides are monitored (Knight and Walter, 2002).

1.1.3 Nitrilases

Nitriles are predominantly synthetic compounds containing C≡N functionality. There are few naturally occurring nitriles produced from cyanide by cyanogenic plants (Vetter, 2000), whilst others are produced during amino acid anaerobic degradation (Sorokin *et al*., 2009). These nitriles are widely used for transformation into amines, esters, amides and carboxylic acids. They are also used for the synthesis of fine chemicals such as agricultural chemicals, medicines and dyes as intermediates (Chmura *et al.,* 2008).

Non-enzymatic hydrolysis of a nitrile in the form R-C≡N produces the corresponding acid amide, R-C=O(NH_2) with one water addition, and the corresponding acid, R-CO_2^- with the second water addition (Pace and Brenner, 2001). Chemical nitrile hydrolysis has several disadvantages especially for highly functionalized chiral molecules. This kind of catalyst may cause racemization, side reactions, or decomposition due to the fact that they take a long time and require high temperatures (Osprian *et al.,* 2003).

Nitrilases form a group of homologous enzymes that are widely distributed in (but not restricted to) plants, bacteria and fungi (Piotrowski, 2008). These nitrilases are one of the lyase class of enzymes that catalyse the substance addition to a double bond or the elimination of a group, which results in an unsaturated bond producing a chiral compound (Schulze and Wubbolts, 1999). The enzyme is able to readily facilitate the chemical hydrolysis processes in organic chemical processes and to detoxify cyanide waste (Liu e*t al*., 2011). Nitrilases (EC 3.5.5.1) are important in synthesis and degradation pathways for naturally occurring, as well as xenobiotically derived, nitriles. They also catalyse the hydrolysis of nitriles directly to the corresponding carboxylic acids with the concomitant release of the nitrile nitrogen as ammonia (Janowitz *et al*., 2009). Several nitrilases have been explored for the preparation of single enantiomer carboxylic acids, and thus the development of nitrilases that could be used as viable synthetic tools is on-going (DeSantis *et al*., 2002; Reetz, 2010).The enzymes belong to a subfamily of the carbon-nitrogen hydrolase or nitrilase superfamily. Members of the superfamily share similar topologies, characteristic sequence motifs and an α-β-β-α sandwich fold (Robertson *et al.,* 2004).

The benefit that nitrilases have in comparison to nitrile hydrolases is their higher stability and enantioselectivity (Martı´nkova´ and Kr˘en, 2010). They also produce the acid without releasing an acid amide by virtue of covalent, thiol-mediated catalysis. The enzyme binds to a nitrile substrate covalently, and produces ammonia with the first water addition, with the concomitant release of an acid and a regenerated enzyme with the second water addition (Pace and Brenner, 2001). In plants, hydroxynitrile lyase enzymes that catalyse the cleavage of α-hydroxynitriles to cyanide and an oxo compound [aldehyde or ketone (compound containing an oxygen atom)] (Wajant and Effenberger, 1996) have a common name ‘‘oxynitrilase” which represents one of the earliest usages of the term ‘‘nitrilase” in the scientific literature (Piotrowski, 2008).

1.1.4 Classification of HNL

HNL or oxynitrilase refers to four groups of completely unrelated classes of proteins, with HNL activity belonging to different fold families, (Ueatrongchit *et al.,* 2008), which are mandelonitrile lyase (E.C. 4.1.2.10), *p*-hydroxymandelonitrile lyase (E.C. 4.1.2.11), acetone-cyanohydrin lyase (E.C. 4.1.2.37) and hydroxynitrilase (E.C. 4.1.2.39) (Effenberger, 1999). The enzymes probably arise from various protein families. These enzymes were originally classified based on electroselectivity into two major groups (HNL-I and HNL-II) based on the presence (HNL-I) or absence (HNL-II) of flavine adenine dinucleotide (FAD^+) (Hickel *et al.,* 1996*;* Hu and Poulton, 1997). HNL-I (*R*-selective HNL) enzymes are N-glycosylated, whereas HNL-II (*S*-selective HNL) enzymes are unglycosylated (Sharma *et al.,* 2005).

HNLs with completely different selectivities, substrate specificities, and other biochemical features like optimum pH, or cofactor requirement are known to enable the enzymatic production of a large variety of different *(R)*- and *(S)*-cyanohydrins despite catalysing the same reaction (Dreveny *et al*., 2009). Heterogeneity of their properties suggests that most of these enzymes have independently evolved (convergent evolution). The lack of homologies among the amino acid sequences of the recently cloned HNLs from *Prunus serotina* (*Ps*HNL), *Manihot esculenta* (*Me*-

HNL), and *Sorghum bicolor* (*Sb*HNL) emphasises this concept (Wajant and Pfizenmaier, 1996).

Successful applications of the *S*-HNLs in industrial processes were introduced recently. Characterization studies of *S*-HNLs are significantly scarce compared to those for *R*-HNLs although there are few reports describing the cloning of genes encoding HNLs from both classes (Dadashipour *et al*., 2011).

The FAD-dependent enzyme is an enzyme with a characteristic molecular mass between 50 and 80 kDa and contains up to 30% carbohydrate content. These enzymes are characterised to a certain extent by single chain proteins with *(R)*-mandelonitrile as their natural substrate (Gruber *et al*., 2004). FAD-independent HNLs differ in molecular mass (28–42 kDa), primary structure, and subunit polymerization structure and are more heterogeneous (Hickel *et al*., 1996). FAD-independent HNLs enzymes contain up to 9% carbohydrate, adopt homo- or heterodimeric conformations *in vivo*, and accept acetone cyanohydrin, *(S)*-mandelonitrile, 4-OH-*(S)*-mandelonitrile, or *(R)*-2-butanone cyanohydrin as substrates (Caruso *et al*., 2009).

The detection of *R*-selective HNLs (*R*-HNLs) representing the first class of HNL enzymes was found in the seeds of *Eriobotrya japonica* and the leaves and seeds of *Passiflora edulis*, *Sorbus aucuparia, Prunus persica*, *Prunus mume* and *Chaenomeles sinensis* (Asano *et al*., 2005). The HNLs from *E. japonica* (*Ej*HNL) (Ueatrongchit *et al*., 2009), *P. edulis* (*Pe*HNL) (Ueatrongchit *et al*., 2010), and *B. montanum* (*Bm*HNL) (Dadashipour *et al*., 2011) were purified to homogeneity and their enzymatic properties were characterised. There is no evidence found that the cofactor participates directly in the catalysis by this enzyme (Gruber and Kratky, 2003). The FAD prosthetic group provides structural stability to the enzyme structure by covalently binding to a hydrophobic region near the catalytic site of the enzyme, even though it's not involved in the net redox reaction (Jorns, 1980). FAD-dependent HNLs catalyse a condensation reaction and closely resembles activity of glucose-methanol-choline (GMC) oxidoreductases despite the fact that it does not catalyse a redox reaction (Dreveny *et al*., 2009). Inactivation of this enzyme is caused by the removal of the FAD cofactor (Jorns, 1979).

The second class of HNLs was isolated from *Manihot esculenta and Hevea brasiliensis.* This class is closely related to the α/β-hydrolases class characterized by α/β/α–sandwich formed from a β-sheet core of five to eight strands connected by α-helices (Dreveny *et al*., 2001). Most of the family members' β-strands are parallel, but an inversion in some, results in an antiparallel orientation (Hotelier *et al.,* 2004). Enzymes with the α/β-hydrolase fold, for example esterases, are also found in the α/β-hydrolase superfamily. The catalytic amino acid residues are presented on loops in enzymes, where the nucleophile elbow is the most conserved feature of the fold. The catalytic mechanism of HNL is different from that of esterases. Esterase reactions have two transitions states for the formation and for the release of the acyl enzyme, whilst the HNL reaction has a single transition state that does not involve an additional covalent complex with an enzyme or an acyl enzyme intermediate. The role of the serine residue also differs between the two types of enzymes as it becomes a hydrogen bond donor instead of being a nucleophile. The serine residue does not interact with its carbonyl carbon as in the esterase reaction, but with its carbonyl oxygen because a threonine residue in the HNL blocks the oxyanion hole compelling an aldehyde carbonyl to position differently in the active site of the enzyme. Thus, HNL does not activate the carbonyl group of the substrate by the use hydrogen bonds from the oxyanion hole (Santosh *et al*., 2010).

The third class of HNLs is represented by the oxynitrilase isolated from *Sorghum bicolor*. This oxynitrilase has 60% amino acid sequence homology with a carboxypeptidase (Wajant *et al*., 1994). When synthesised, a 510-amino acid long polypeptide is produced which is post-translationally cleaved into two smaller peptides, designated as α- and β-subunits. The active enzyme is a *N*-glycosylated α_2, β_2 heterotetramer consisting of two α, β heterodimers (Gruber and Kratky, 2003).

The zinc-dependent HNL from flax (*Linum usitatissimum*) represents the fourth class with homologies to alcohol dehydrogenases that are important in catalysing oxidation of primary and secondary alcohols to ketones and aldehydes (Trummler and Wajant, 1997). The biological substrates for this HNL are acetone and butanone and the enzyme shows *(R)*-enantioselectivity. It occurs as a homodimer of 87 kDa and is unglycosylated with an optimum pH of 5.5. Different HNLs have structural

dissimilarity, and architectures of their active site are vastly different, in spite of their affinity for identical or similar biological substrates. Furthermore, there are structural similarities with the family of highest sequence homology [e.g., carboxypeptidases, glucose-methanol-choline (GMC)-oxidoreductases and α/β hydrolases] even though their similarities in the mechanism and type of the catalysed reaction are not paralleled (Gartler *et al*., 2007).

1.1.5 HNL application

Enantiomeric aldehydes are mainly used as perfumes, solvents and flavouring agents or intermediates in the manufacturing of pharmaceuticals, dyes and plastics. The nitroaldol reaction or Henry reaction is a classically named reaction found in organic synthesis. The generated nucleophile from a nitroalkane couples with a carbonyl compound as an electrophile in this reaction (Fuhshuku and Asano, 2011). The first report of an asymmetric Henry reaction was in 1992 (Sasai *et al*., 1992). This reaction involved the release of β-nitro alcohols as the products that were used as valuable building blocks in synthetic organic reactions. There are various reports on the development of non-metal and metal-based catalysts that have been reported in the literature (Boruwa *et al*., 2006; Palomo *et al*., 2007). However, the new chiral biocatalyst developments have stimulated the interest of many groups, because of the wide applicability of the reaction (Uraguchi *et al*., 2010).

HNL is a biocatalyst that enables the transformation of unnatural substrates with high degrees of chemo-, regio- and enantioselectivity. They provide cyanohydrins that are important building blocks for the production of valuable chiral pharmaceuticals and veterinary products such as the angiotensin-converting enzyme (ACE), inhibitors which may be used to cure congestive heart failure and hypertension, and Clopidogrel, an oral thienopyridine class antiplatelet agent (Zhao *et al*., 2011). The enzymes function under mild conditions (temperature range between 20–60°C, normal pressure, pH range between 5–8), are environmentally friendly and compatible to each other to enable sequential reactions (Turcu, 2010).

Several enzymes have been increasingly exploited for organic synthesis as practical catalysts (Gruber-Khadjawi *et al*., 2007) hence there are reports of enzyme-

catalysed Henry reactions (Purkarthofer *et al*., 2006; Tang *et al*., 2010; Wang *et al*., 2010). Among them, reactions that are catalysed by *S*-HNL from *Hevea brasiliensis* (*Hb*HNL) indicate enantioselectivity (Fuhshuku and Asano, 2011). A study on *R*-HNL containing an α/β-hydrolase fold isolated from the non-cyanogenic plant *Arabidopsis thaliana* (*At*HNL) (Andexer *et al*., 2007), indicated that the *R*-HNL accepted nitromethane ($MeNO_2$) as a donor in a reaction yielding aromatic aldehydes and optically active β-nitro alcohols. The reaction conditions were optimised, including the reaction medium, pH profile and the amount of enzyme used. A newly developed *At*HNL-catalysed asymmetric Henry reaction for the synthesis of *(R)*-β-nitro alcohols was proposed (Fuhshuku and Asano, 2011). There was development and application of these enzymes in industry that produced a large scale of efficient manufacturing of enantiomerically pure products. Indeed, this property makes these enzymes a valuable resource in the fine chemical manufacturing industries.

Lack of operational stability and the high price of enzymes are the main impediments in the commercial production sector. For an industrial process to be economically viable it is important to produce large quantities of HNLs as recombinant enzymes to accommodate large-scale industrial application. This can be achieved by using microbial sources and recombinant techniques that will produce and secrete the target enzyme into the fermentation supernatant (Bauer *et al.,* 1999). Although *R*-HNLs have been purified from many kinds of plants, HNL gene sequences have been reported from only almond, black cherry and Japanese apricot. There is thus a limit on the variation of HNLs that have been expressed using biotechnology. However, HNLs from different plants in different countries have been isolated and cloned. In Japan for example, *Pm*HNL extracted from Japanese Apricot *Prunus mume* was cloned from the genomic DNA and cDNA from *P. mume* seedlings and the enzyme was produced in microbial host *Pichia pastoris* (Fukuta *et al*., 2011). An inner partial HNL gene fragment from *E. japonica* with a length of 1450 base pairs has also been amplified (Zhao *et al*., 2011).

The enzyme from the rubber tree *Hevea brasiliensis* was cloned and overexpressed in several organisms such as *Saccharomyces cerevisiae, Escherichia coli*, and *Pichia pastoris* (Schulze and Wubbolts, 1999). Recombinant *Hevea brasiliensis*

HNLs has been expressed and used in the formation of enantiopure *(S)*-meta-phenoxybenzaldehyde cyanohydrin. This cyanohydrin is used as a building block in the synthesis of pyrethroids applied as insecticides. It is also suitable in the conversion of a number of alicyclic, aliphatic, unsaturated heteroaromatic and aromatic aldehydes as well as ketones, to the corresponding *(S)*-cyanohydrins (Zagrobelny *et al*., 2004).

During industrial application the enzymes are used under a variety of extremely unfavorable conditions such as heavy metal contamination, high temperature and low pH. It is noteworthy to mention that these recombinant enzymes should display extreme stability under these conditions. Increased stability under low pH is of great significance for the enantioselective synthesis of cyanohydrins because of the spontaneous unselective addition of HCN to the carbonyl group occurring as a background reaction at elevated pH (Bauer *et al.,* 1999). The enantiomeric product yield as well as enzyme stability are enhanced at low temperature from −5 to 4°C (Bauer *et al.,* 1999). However, the water in the reaction mixture freezes with further decrease in temperature below −5°C, leading to a complete loss of enzyme activity (Sharma *et al*., 2005). It is therefore necessary, to study and optimize all the parameters for a reaction to obtain a high initial velocity and enantiomeric purity of the enzyme because the reaction may be influenced by other parameters in a complex manner (Ueatrongchit *et al.,* 2010). Additionally, protein engineering techniques such as directed evolution or *de novo* design should be used to improve enzyme function and stability under the adverse conditions used industrially to make the process economically viable, as mentioned above. Immobilised enzymes provide several advantages for biotechnological and industrial applications, including repeated use. Immobilisation of enzymes alters the properties of the enzyme, and allows for continuous operation in a packed-bed reactor, improvement of enzyme stability and ease of separation of reaction products from the biocatalyst (Tükel *et al*., 2010).

In order to obtain a good candidate enzyme for industrial application that has characteristics similar to those mentioned above, or that can be altered by protein engineering to accommodate various conditions, new sources of the enzyme need to be found. Very little information is available on HNL activity in South African

indigenous plants and these may provide a new source of enzyme for industrial application. Screening of South African indigenous plants as HNL sources needs to be carried out and potential enzymes isolated and characterised to find possible industrially useful enzymes.

1.2 PURPOSE OF THE STUDY

1.2.1 Aim

The main aim of this study was to screen indigenous South African plants for potential hydroxynitrile lyase activity, and to further purify and biochemically characterise the active hydroxynitrile lyase(s) from the selected plants.

1.2.2 Objectives

The objectives of the study were to:

I. select South African indigenous plants, prepare crude enzyme extracts and screen for presence of HNL activity;
II. purify the active HNLs using various protein isolation methods;
III. estimate the molecular mass of the purified HNLs;
IV. determine the kinetic parameters (K_m, V_{max} and k_{cat}) and prosthetic group requirement of the purified HNLs;
V. determine the optimum temperature and pH of the purified HNLs;
VI. establish the effects of metal ions and other additives on the kinetic efficiency of the purified HNLs.

1.3 HYPOTHESIS

Indigenous South African plants are a source of active HNL enzymes.

CHAPTER 2

2.1 MATERIALS

The following materials were used and purchased from different manufacturers:

Potassium phosphate diabasic trihydrate, potassium cyanide, benzaldehyde, bovine serum albumin (BSA), diethylpyrocarbonate (DEP), dimethyl sulfoxide (DMSO), (*R*)-(+)-mandelonitrile, citric acid, dithiothreitol (DTT), dialysis tubing cellulose membrane, DEAE-Toyopearl 650M column matrix, Concanavalin A Sepharose 4B gel, sodium dodecyl sulphate and methyl-α-D-mannopyranoside were all purchased from Sigma-Aldrich. Sodium chloride was purchased from SMM Instruments. Ammonium sulphate, acetic acid, sodium hydroxide, zinc chloride, sodium acetate and polyvinylpyrolidone were all sourced from Rochelle Chemicals. Coomassie brilliant blue R-250 was purchased from ICN Biomedicals. Tris (hydroxymethyl) aminomethane was purchased from Melford Laboratories. Acrylamide and calcium chloride were obtained from Fluka Chemika. Mercury chloride, magnesium chloride, manganese chloride and hydrochloric acid were sourced from Saarchem. Tetramethylethylenediamine (TEMED) was manufactured from Invitrogen Life Technologies. Purchased from Merck Millipore were Amicon ultra centrifugal filter tubes.

The study plants *Senecio spp* (leaves) (*Sb*), *Kalanchoe spp* (leaves) (*Ks*), *Bulbine spp* (leaves), *Sclerocarya spp* (unripe fruit), *Euphorbia spp* (leaves), *Ficus spp* (unripe fruits), *Colophospermum spp* (leaves), *Englerophytum spp* (leaves), *Sclerocarya spp* (leaves), *Croton spp* (leaves), *Erythrina spp* (seeds), *Aloe spp* (raceme) and *Adansonia spp* (pericarp) were collected from the Limpopo Province, South Africa.

The following equipment were used: Variable SpeedLaboratory Blender (Waring Laboratories); ChemiDoc, Microplate Reader, Power pack Biologic LP and Fraction Collector (Bio-Rad Laboratories); Beckman Coulter® DU® 730 Life Science UV/VIS Spectrophotometer, Avanti® J-E centrifuge and DTX 880 Multiple Detector (Beckman

Coulter), Glomax Multi^{+} Detection System (Promega) and pH meter f(Mettler Toledo).

2.2 METHODOLOGY

2.2.1 Collection and screening of indigenous plants

Several indigenous South Africa plants were collected around the Limpopo Province and then screened for HNL activity. Plant parts such as leaves, seeds and fruits were used. The screening process preferably targeted the leaves, fruits and seeds of the selected plant species as a source of HNL enzymes since this approach resulted in a low negative impact on the plant population, as opposed to the use of roots.

2.2.2 The preparation of the crude extract of HNL from indigenous plants

Plant specimens were collected, frozen at -22°C, and processed using an established experimental procedure (Asano *et al.,* 2005). Briefly, 5 g of the seeds, fruits or leaves were homogenised with a variable speed laboratory blender in 50 ml of 10 mM potassium phosphate diabasic trihydrate buffer (pH 6.0) with 0.02% polyvinylpyrolidone and stirred overnight at 8°C. The enzyme crude extract from all plants was extracted with a buffer containing polyvinylpyrrolidone as it protects the enzyme against protease degradation by inactivating the proteases (Ueatrongchit *et al*., 2008). The suspension was filtered through 3 layers of mutton cloth to remove the insoluble debris. The resultant suspension was then centrifuged at 6,100 x *g* for 90 min at 8°C on an Avanti® J-E centrifuge. The resultant crude enzyme extract was screened for HNL activity and the subsequent active extracts were further purified and characterised.

2.2.3 HNL activity assays

The HNL activity of the crude enzyme extracts was assayed by measuring the conversion of benzaldehyde to the production of the optically active (*R*, *S*)–mandelonitrile using the modified methods of Asano *et al.* (2005) and Willeman *et al.* (2000). The synthesis of (*R*, *S*)-mandelonitrile (cyanohydrin) from benzaldehyde and potassium cyanide (as shown on figure 1 where benzaldehyde is represented by an aldehyde and potassium cyanide by HCN) was followed by continuously measuring

the decrease in absorbance at 280 nm. The assay procedure was designed in this manner since the absorbance of benzaldehyde is maximal at 280 nm (Figure 2).

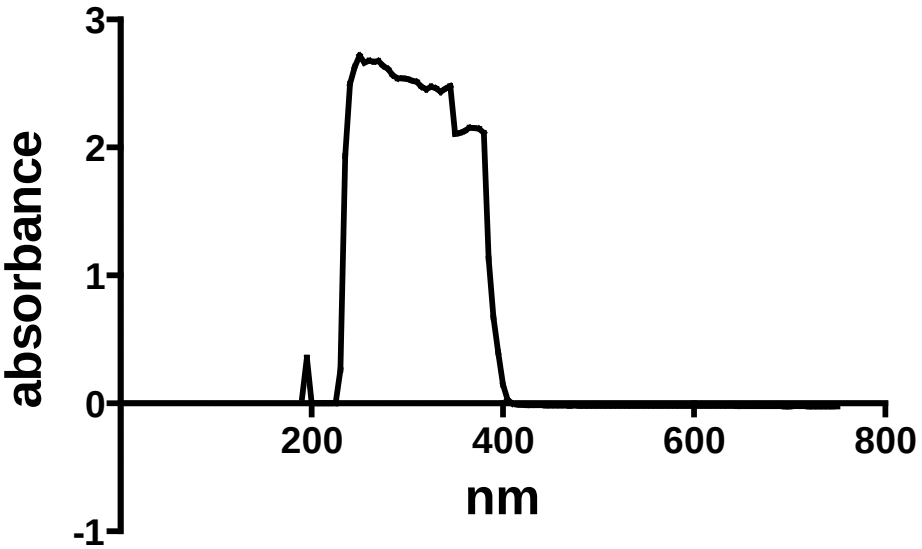

Figure 2: Absorption spectrum of benzaldehyde.

The HNL reverse reaction was performed in a quartz cuvette using a Beckman-Coulter® DU® 730 Life Science UV/VIS spectrophotometer software and / or alternatively, using a DTX 880 multiple detector. The standard assay solution contained 760 µl of 400 mM citrate buffer (pH 5.0), 40 µl of 750 mM benzaldehyde, 100 µl of 1 M potassium cyanide and 100 µl of the enzyme extract in a final volume of 1 ml. The final concentrations of the reactants in the assay were thus 304 mM citrate buffer, 30 mM benzaldehyde and 0.1 M potassium cyanide.

The activity of HNL forward reaction using the crude extracts was also assayed by measuring the release of aldehyde/ketone from the decomposition of *(R)*-(+)-mandelonitrile cyanohydrin. The release of aldehyde/ketone from *(R)*-(+)-mandelonitrile cyanohydrin was followed by continuously measuring the increase in absorbance at 280 nm, also using a Beckman-Coulter® DU® 730 Life Science UV/VIS spectrophotometer. The standard assay solution contained 1.6 ml of 400 mM citrate buffer (pH 5.5), 200 µl of 2 mM *(R)*-(+)-mandelonitrile, and 200 µl of the enzyme extract in a final volume of 2 ml. The final concentrations of the reactants in the assay were thus 320 mM citrate buffer and 0.4 mM *(R)*-(+)-mandelonitrile.

Both the forward and reverse reactions were initiated by the addition of the enzyme extract and the absorbance read in 10 sec interval for 5 min using a Beckman-Coulter® DU® 730 Life Science UV/VIS spectrophotometer or 20 sec interval for 10 min using DTX 880 multiple detector. The linear change in absorbance at initial activity was used in the calculation of the enzyme activity. The slope of absorbance was determined and subtracted from the rates obtained from the blank to remove the effect of the spontaneous blank reaction (an example is shown in Figure 3. The enzyme activity was then calculated using ε_{280}=1.4 $mM^{-1}.cm^{-1}$, where a unit of HNL activity (1 decomposition unit) was defined as the amount of enzyme that produced 1 µmol of mandelonitrile from benzaldehyde and potassium cyanide or 1 µmol of benzaldehyde from mandelonitrile per min under the standard assay conditions of enzyme activity. The increase in benzaldehyde concentration increased the noise of the slope, and thus 1 µmol or lower concentrations of substrate were used in the studies.

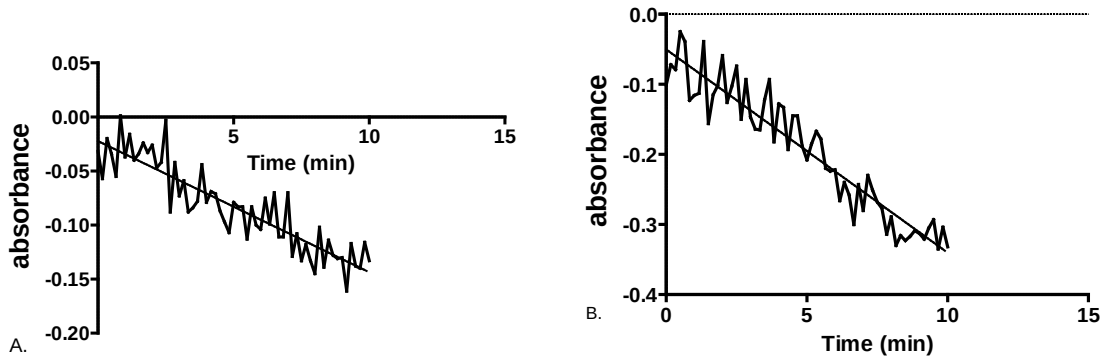

Figure 3: Determination of benzaldehyde reduction slope, (A) blank slope (-0.012) and (B) enzymatic slope using *Sb* crude extract (-0.029).

2.2.4 Enzyme purification

It is important to study enzymes in a simple system so as to understand their structure, kinetics, reaction mechanisms, regulations, and their role in a complex system. The crude plant extracts from two plant species, *Ks* and *Sb,* displayed substantial HNL activity and the active HNL was purified from each extract. During the purification the protein extracts were either kept at 4°C or on ice, unless otherwise specified.

2.2.4.1 Ammonium sulphate precipitation

Different proteins precipitate in different concentrations of ammonium sulphate. Generally, precipitation in lower concentrations of ammonium sulphate occurs for proteins of higher molecular weight. Proteins are soluble in aqueous media due to their hydrophilic amino acid side-chains interacting with water. These are provided by the basic, acidic and neutral hydrophilic amino acids. Proteins with relatively few hydrophilic regions will precipitate and aggregate at a relatively low concentration of ammonium sulphate saturation. In contrast, the protein with considerably more hydrophilic regions will remain in solution until the concentration of ammonium sulphate is at a considerably higher saturation (david-bender.co.uk/simulations/enzpur/amso4.htm). Ammonium sulphate precipitation does not purify protein highly. It only assists in the elimination of some unwanted proteins in a mixture and with sample concentration. Further, the salts in the solution are removed by dialysis through porous cellulose tubing.

In this study, extracted proteins from the enzyme crude extract were precipitated at different percentages (10% - 90%, in 10% increments) of ammonium sulphate saturation. Ammonium sulphate was added and stirred slowly at 8°C until the ammonium sulphate was dissolved completely. The precipitate was collected by centrifugation at 6,100 x *g* for 30 min using an Avanti® J-E centrifuge. The precipitate was dissolved in 10 mM potassium phosphate dibasic trihydrate buffer (pH 6.0) before use. As a result of the high salt content in the supernatant fractions, the dissolved pellets, as well as the supernatant fractions, were dialysed in 2 L of the same buffer overnight. The resultant fractions were assayed for HNL activity. The active fractions were further purified with ion-exchange and affinity column chromatography using a Biologic Low Pressure System (Ueatrongchit *et al.,* 2008).

2.2.4.2 DEAE-Toyopearl 650M column chromatography

The DEAE-Toyopearl 650M is an ion-exchange chromatography that separates proteins according to their molecular charge. The protein mixtures are applied onto the chromatographic matrix and the various proteins are then bound by reversible, electrostatic interactions. The proteins which are adsorbed are then eluted in the order of least-to-most-strongly bound molecules. On an anion exchange

chromatography, the negatively charged molecules are attracted to a positively charged solid support. Elution of the attracted proteins is achieved by applying a gradient of linearly increasing salt concentration, retaining the pH of the mobile phase buffer, that causes the molecule to become protonated and hence more positive, thus altering the attraction with the solid support causing the molecule to elute from the column. The proteins are then collected as individual chromatographic fractions, and analysed separately (www.biotech.kth.se/courses/gru/courselist/BB2040_ENG/ChromMethods.pdf).

In the study, DEAE-Toyopearl 650M column matrix (50 ml) was equilibrated with 3 column volumes of 10 mM potassium phosphate dibasic trihydrate buffer pH 6.0 at room temperature. The active dialysed protein fractions from ammonium sulphate precipitation were concentrated to a smaller volume (approximately 2X concentration) with concentrating centrifugal filter tubes. The protein sample was loaded onto the column and then washed with the same buffer to elute the unbound proteins. The bound proteins were eluted with 0 to 0.5 M NaCl gradient in 10 mM potassium phosphate buffer pH 6.0 at a flow rate of 1 ml/min with approximately 350 ml phosphate buffer. The eluent was collected in 5 ml fractions. The fractions were then assayed for HNL activity, and the fractions with higher activity from the same peak were pooled together and further purified (Ueatrongchit *et al.,* 2008).

2.2.4.3 Concanavalin A sepharose 4B (conA) column chromatography

Concanavalin A is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). The column binds molecules containing α-D-glucopyranosyl, α-D-mannopyranosyl and sterically related residues. The requirement for the binding sugar is the presence of C-3, C-4 and C-5 hydroxyl groups that react with conA. This lectin, coupled to Sepharose 4B, is generally used for separation and purification of glycolipids, glycoproteins and polysaccharides. For a substance to bind to a conA column the presence of Mn^{2+} and Ca^{2+} are required. The protein-metal ion complex remains active and is stable at neutral pH even in the absence of free metals. However, to preserve the binding activity of the conA molecules below pH 5.0 the free metal ions mentioned above should be present. There are other application areas where conA has been used for IgM purification, enzyme-antibody conjugates

purification, membrane vesicles separation, and isolation of cell surface glycoproteins from detergent solubilised membranes. It is also used in studies involving the composition of carbohydrate-containing substances (www.med.unc.edu /pharm/sondeklab/Lab%20Resources/protein_purification_handbooks/Affinity%20chromatography.pdf).

ConA column matrix (5 ml) was firstly washed with 3 column volumes of salt buffer containing 1 M NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ at pH 6.0, to activate the column recommended by the manufacturer. Following this, the column was equilibrated with 0.5 M NaCl in 10 mM potassium phosphate dibasic trihydrate buffer pH 6.0 at room temperature for 2 hours. The active enzyme fraction from the DEAE column was concentrated 10 times with Amicon ultra centrifugal filter tubes and loaded on to the column. The 0.5 M NaCl in 10 mM potassium phosphate dibasic trihydrate buffer was used to wash the column to elute the unbound proteins. The bound proteins were eluted using 1 M methyl-α-D-manno-pyranoside prepared in 0.5 M NaCl in 10 mM potassium phosphate buffer pH 6.0 at a flow rate of 0.5 ml/min approximately 25 ml of the buffer. The active protein fractions were pooled together which resulted in a bound active fraction as well as an unbound active fraction. The two fractions were dialysed in 2 L of 10 mM potassium phosphate buffer pH 6.0 at 8°C for 12 hours, concentrated and subjected to different analyses (Ueatrongchit *et al.,* 2008).

2.2.5 Protein assay

Protein concentrations of the crude enzyme extracts and purified enzymes were determined by the Bradford method (1976). The Bradford assay is a colorimetric protein assay which is based on the shift of coomassie brilliant blue G-250 absorbance, in which under acidic conditions the red form of the dye is converted into a blue form when bound to the proteins. Two types of interaction take place during the binding: firstly the red form of coomassie dye donates its free electron to the ionized groups on the protein that disrupt the native state of the protein, causing the protein to expose its hydrophobic pockets. The pockets bind to the non-polar region of the dye non-covalently via van der Waals forces, positioning the positive amine groups in proximity with the negative charge of the dye (www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf). The blue form of the coomassie dye is stabilised by the binding of the protein.

The current study used a Bio-Rad protein assay kit, with BSA concentrations of 0 – 0.5 mg/ml as a standard. Ten microlitres of the sample was added to a 96 well plate with 200 µl of the dye, mixed and incubated for 30 min. The absorbance was read at 595 nm on a micro-plate reader; this was done in duplicates. The standard curve was drawn from the averages and the unknown concentrations of the protein samples were extrapolated.

2.2.6 Gel electrophoresis of purified HNLs

Protein molecular mass determination is of fundamental importance to protein biochemical characterisation. Protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) can be used in relative molecular mass estimation and to assess the purity of protein samples. Different staining methods are used in the detection of rare proteins and to learn about their biochemical properties. SDS-PAGE uses an anionic detergent (SDS) to denature proteins. SDS molecule binds to amino acids and the charge to mass ratio of all the denatured proteins becomes constant in the mixture allowing for a more accurate molecular mass determination. The SDS-PAGE gel matrix is formed of polyacrylamide. The polyacrylamide chains are crosslinked by N,N-methylene bisacrylamide co-monomers (www.ruf.rice.edu/~bioslabs/studies/sdspage/gellab2.html).

Ammonium persulphate (radical source) initiates polymerisation and this polymerisation is catalysed by tetramethylethylenediamine (TEMED) (a free radical donor and acceptor). Polyacrylamide gels restrict large molecules from migrating as fast as small molecules and SDS disrupts secondary, tertiary, and quaternary structure of the protein to produce a linear polypeptide chain coated with a negative charged SDS molecule. These protein molecules move in the gel (towards the anode) on the basis of their molecular mass. The charge to mass ratio varies for each protein resulting in protein separation and the molecular mass determination. The final separation of proteins depends on the differences in relative molecular mass of polypeptides (Sameh, 2012).

A 12% (w/v) SDS-PAGE gel was used to estimate the molecular mass and the purity of the HNLs. The protein samples were concentrated using acetone in the ratio of 1:3 (sample:acetone, v/v). Gel electrophoresis was performed under standard electrophoresis conditions using precision Plus proteins (Bio-Rad Laboratories) as the standard molecular weight markers indicator (i.e., 250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). The gels were run at 180 V for 60 min to separate the proteins and stained with coommasie blue stain for an hour, and further destained with a destaining solution until clear. The molecular mass of the purified protein was estimated using the known standard molecular weight markers. The visible protein bands on the gels were sliced into 1 mm pieces, placed in 2 ml Eppendorf tubes and desiccated in a rotor vapour. A 100 µl of acetonitrile was then added and allowed to incubate for 10 min. The acetonitrile was later discarded, and the gels were speed-vacuumed for 5 min to dryness before being sent for peptide mass fingerprinting, which was done at Centre for Proteomic and Genomic Research (University of Cape Town).

2.2.7 Determination of the prosthetic group on the isolated proteins.

An absorbance spectrum is often used to determine the wavelength of light at which absorbance is greatest. Different molecules have unique absorbance properties and the absorbance spectrum is thus used to determine structural characteristics of unknown substances (www.marietta.edu/~spilatrs/biol309/labexercises/Spectrophotometry.pdf). In the current study the absorption spectra of the purified enzymes were measured with a Beckman spectrophotometer at an absorbance wavelengths range of 190 to 600 nm.

2.2.8 Determination of the kinetic parameters of the isolated proteins.

Biochemists usually determine the enzyme kinetics parameters using an expression for the velocity of product formation known as the Michaelis-Menten equation. In order to use Michaelis-Menten kinetics, the enzyme reaction should be assayed as a first-order or as a pseudo-first order reaction. In this assay, the concentration of the KCN was kept high, whilst the benzaldehyde concentration was varied, leading to pseudo first-order conditions. A Michaelis-Menten plot (v vs [S]) was used to establish the first order kinetics. The initial velocity (v_o) of the enzymatic conversion

of benzaldehyde was determined under the standard reaction assay, as described above (section 2.2.3). The reaction assay was repeated twice using a DTX 880 multiple detector. The $[E]_{total}$ was calculated from the concentration and the molecular weight (daltons) of the isolated enzymes. The enzyme activity was determined using citrate buffer pH 5.0 at various substrate (0–3 mM, benzaldehyde and *(R)*-(+)-Mandelonitrile) concentrations at room temperature (25–27 °C) (Ueatrongchit *et al.,* 2008). The V_{max} and the K_m values were determined using a Hanes-Woolf plot ([benzaldehyde]/v versus [benzaldehyde]. The enzyme turnover (k_{cat}) and enzyme efficiency were calculated.

The equations used were as follows:

$$k_{cat} = \frac{K_m}{E_{total}}$$

$$\text{Enzyme catalytic efficiency} = \frac{k_{cat}}{K_m}$$

2.2.9 Effect of pH and temperature on HNL

Enzymes are active within a limited pH range, the limits of which may be wide or narrow. Within the limited pH range of activity there is an optimum pH at which the maximum activity is attained (Knut, 1997). Graphs reflecting the effect of pH on enzyme activity (v vs pH) are usually bell-shaped curves indicating the pH limits as well as the optimal pH. Temperature affects enzyme activity the same way as it affects other chemical reactions. Reaction rates increase exponentially by between 4–8% per degree Celsius, although at high temperatures the enzyme protein denatures resulting in a decrease of the product formation (Knut, 1997). The optimum pH and temperature for the purified HNL activity were assayed at a pH range of 3.0–6.5 and a temperature range of 25–70°C repeated twice, each assay in duplicate, using standard assay procedures. The assays were performed on a DTX 880 multiple detector.

2.2.10 Effects of metal ions and other additives

Metal ions affect enzyme catalysis in different ways, either by binding to the enzyme directly, using substrate as a bridge; binding as a bridge between substrate and

enzyme and/or binding to a different binding site of the enzyme (Medyantseva *et al*., 1998). The effects of 10 mM DTT and 1 mM of DEP, mercury chloride, magnesium chloride and zinc chloride on the activity of the purified HNL were determined using the Glomax Multi^{+}detection system. The enzyme solutions were incubated with the above mentioned additives in 10 mM potassium phosphate buffer, at optimum pH and room temperature for 30 min. The remaining activity of the enzyme was assayed according to standard procedures and repeated twice.

2.2.11 *Statistical analysis*

Statistical analyses were done using GraphPad InStat Software. The results of each series of experiments, where appropriate, are expressed as the mean values ± standard error of the mean (SEM).

CHAPTER 3

3.1 RESULTS

Hydroxynitrile lyases or oxynitrilases have been isolated from a wide variety of plant sources (Fechter and Griengl, 2004). In this study various plants were selected and the leaves and seeds were collected from within the Limpopo Province, South Africa. Leaves, seeds or fruit crude extract of the plants were screened for HNL activity. The activity was detected in the crude extracts of 6 plants (Table 1). Two easily accessible plants that showed highest activity as compared to the other plants were selected and thus formed the core of this study (viz., *Kalanchoe species (Ks)* and *Senecio species (Sb)*). Both *Kalanchoe spp and Senecio spp* were collected from the North-West side of a granite rock outcrop on the University of Limpopo, Turfloop campus, during the summer and winter seasons.

3.1.1 Kalanchoe species

Kalanchoe spp. is a member of family of *Crassulaceae*. It is a robust, erect succulent plant with squared or ridged stems and attractive, broad green leaves of 5.8 to 12.7 cm long (www.smgrowers.com/products/plants/plantdisplay.asp?strSearchText= kalanchoe%20spp&plant_id=3121&page=).

Figure 4: The leaves of *Kalanchoe spp* (Picture captured by Mopai KL)

The leaves of this plant are thick and heavy with irregularly lobed margins. They are green with red edging in colour when the plant is grown in a slightly shaded position (Figure 4). The plant is about 60 to 91 cm high and its leaves turn an unusual dark wine-red in full sun or cold weather or turn ruby- red in winter. The inflorescence rises 30 cm or more above the plant, bearing bright yellow flowers in flat topped thyrsi. This plant is found in the North of Durban, KwaZulu-Natal, Mpumalanga, and

Limpopo provinces in South Africa. It is also found in Zimbabwe, Swaziland, and Mozambique. It grows on rocky slopes or larger shrubs (kumbulanursery.co.za/ plants/kalanchoe-spp).

3.1.2 Senecio species

The name *Senecio* is derived from the Latin *senex* (old man) referring to the fluffy white seed head borne by this species.

Figure 5: The leaves of *Senecio spp* (Picture captured by Mopai KL)

This plant species belongs to the family Asteraceae. It grows on rocky hills throughout most of the Limpopo Province and scattered in the eastern, central south-western Swaziland. The characteristics of this habitat are hot and dry climate, long periods of drought, with some summer rains, and winter temperatures near freezing. This plant is reproduced by seeds or by cuttings of stems in spring or summer and leaving the cut to heal. It grows in full sun to light shades, well drained to sandy soil and needs watering occasionally (www.smgrowers.com/products/plants/plantdisplay .asp?strSearchText=senecio%20spp&plant_id=3121&page=).

This plant is an ever green subshrub that grows between 91 and 152 cm high, with brittle fleshy stems bearing 5 cm long rounded succulent bright green leaves pointing upwards along the branches and crowded toward the tips of its stem (Figure 5). It is one of the largest finger-leaved *Senecio spp* in description. Its green colouration distinguishes it easily from the other finger-leaved plants with grey leaves. The flowers are borne typically in the late autumn, and in winter they become bright orange yellow in compact terminal clusters. Inflorescence terminal branched corymb is short and barely exceeding the erect leaves, it bears many flower heads on stalks up to 1 cm along. Its capitol is 0.4 to 1 cm in diameter to 8 green bracts, flowers 16

tubulées to 1.4 cm long and 1.2 cm long pappus (Klatt, 1896). Painted Lady butterfly are mainly attracted to this plant and help facilitate its pollination.

Table 1: Selected plants screened for hydroxynitrile lyase activity

Plant Parts	Activity
Senecio spp (leaves)	Active
Kalanchoe spp (leaves)	Active
Bulbine spp (leaves)	Active
Sclerocarya spp (unripe fruit)	Active
Euphorbia spp (leaves)	Active
Ficus spp (unripe fruits)	Active
Colophospermum spp (leaves)	No activity
Englerophytum spp (leaves)	No activity
Sclerocarya spp (leaves)	No activity
Croton spp (leaves)	No activity
Erythrina spp (seeds)	No activity
Aloe spp (raceme)	No activity
Adansonia spp (pericarp)	No activity

The activity of the enzyme from the crude extract of the selected plants was found to vary seasonally. The extracts from the leaves of *Senecio spp and Kalanchoe spp* collected in winter showed activity, whilst the extracts from the leaves of the same plants collected in summer displayed no detectable activity (Table 2). The enzyme assays were performed using both the forward and the reverse reactions with the substrates benzaldehyde and KCN, and *(R)*-(+) mandelonitrile respectively. Benzaldehyde and KCN demonstrated activity whilst there was no activity seen with *(R)*-(+) mandelonitrile as a chosen substrate (Table 3). Further studies were thus continued using benzaldehyde and KCN as substrates as opposed to *(R)*-(+)-mandelonitrile.

Table 2: Senecio spp and *Kalanchoe spp* crude extract enzyme activity assay data for winter and summer samples with benzaldehyde and KCN as a chosen substrate.

Season	***Ks* crude (µM/min)**	***Sb* crude (µM/min)**
Winter	247.86	194.29
Summer	-	-

Table 3: Senecio spp and *Kalanchoe spp* conA enzyme fractions activity assay data for both mandelonitrile and, benzaldehyde and KCN as substrates.

Substrates	***Ks* bound (µM/min)**	***Ks* unbound (µM/min)**	***Sb* bound (µM/min)**	***Sb* unbound (µM/min)**
Benzaldehyde and KCN	177.14	114.29	50.71	320.71
Mandelonitrile	-	-	-	-

3.1.3 Enzyme isolation from Senecio spp and Kalanchoe spp extracts

HNL from *Ks* and *Sb* was purified using the procedure described in section 2. Ammonium sulphate precipitation preliminary studies indicated that the best fractionation of HNL occurred between 30-80% ammonium sulphate saturation. Thus, crude enzyme extracts were precipitated between 30 and 80% ammonium sulphate saturated solution. A large amount of enzyme activity, and hence protein of interest, was lost, during this step. This step was meant to reduce all the superfluous contaminating proteins and components that may interfere in the reaction, and to assist with downstream purification techniques. All dialysed resulting fractions from the ammonium sulphate precipitation step were concentrated to minimize peak spreading, and hence loss of active fraction, for the remaining isolation techniques. DEAE anion exchange chromatography was used to further isolate the proteins as shown in Figure 6 (*Ks*) and Figure 7 (*Sb*). In both extractions the active enzyme fraction was located in the predominant eluted peak during the elution on bound proteins using NaCl as an eluent as indicated in the figures.

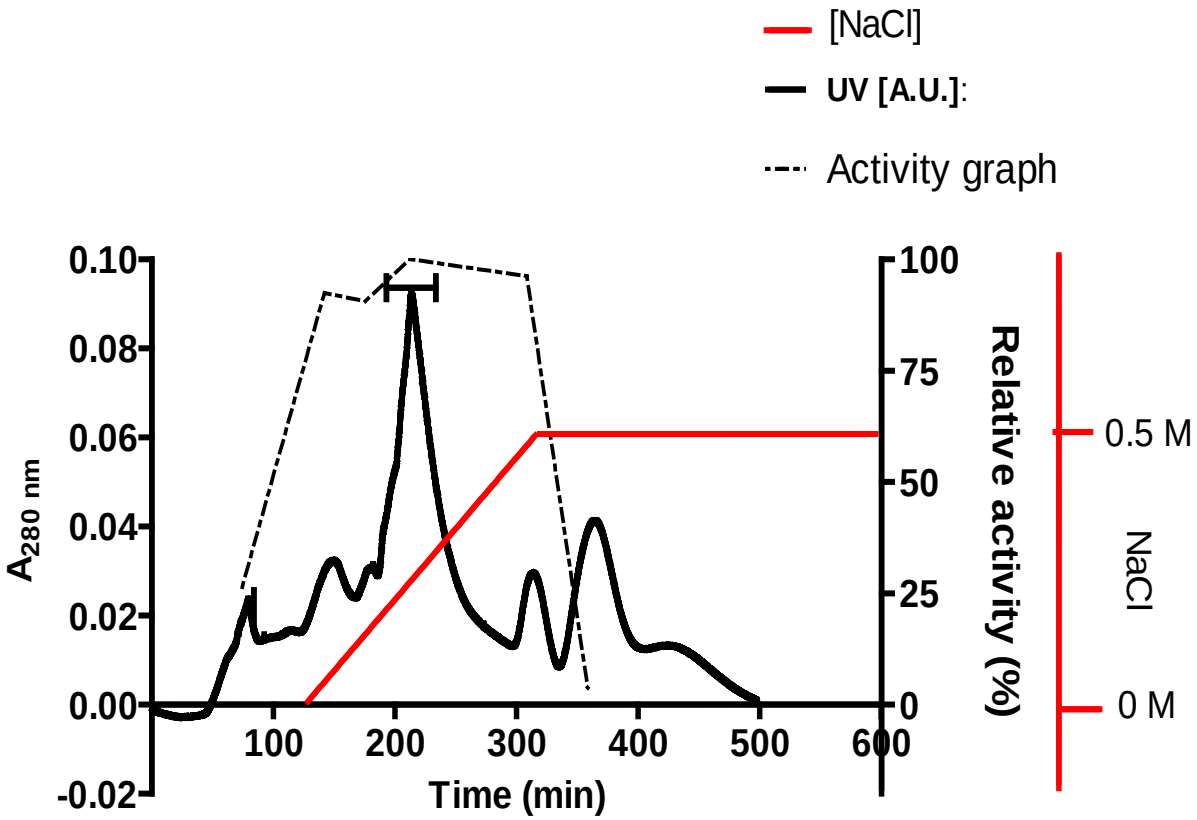

Figure 6: DEAE chromatogram of *Ks* crude enzyme extract at 280 nm. This was done on a 50 ml DEAE column matrix at 1 ml/min flow rate. The solid line indicates the different fractions of protein eluted at different ionic strengths of sodium chloride as a gradient (started at the 138th minute). The peak that indicated the highest activity is indicated by capped line, whereas the dotted line indicates the % activity of different peaks relative to the one with the highest activity.

Although activity was highest in the predominant peak, sample was collected and pooled from active fractions either side of the peak. This led to the fraction still containing contaminating proteins, but a higher activity was retrieved for further purification. Protein fractions were collected and concentrated (*Sb* 10 ml and *Ks* 4 ml) and used for further isolation by affinity chromatography.

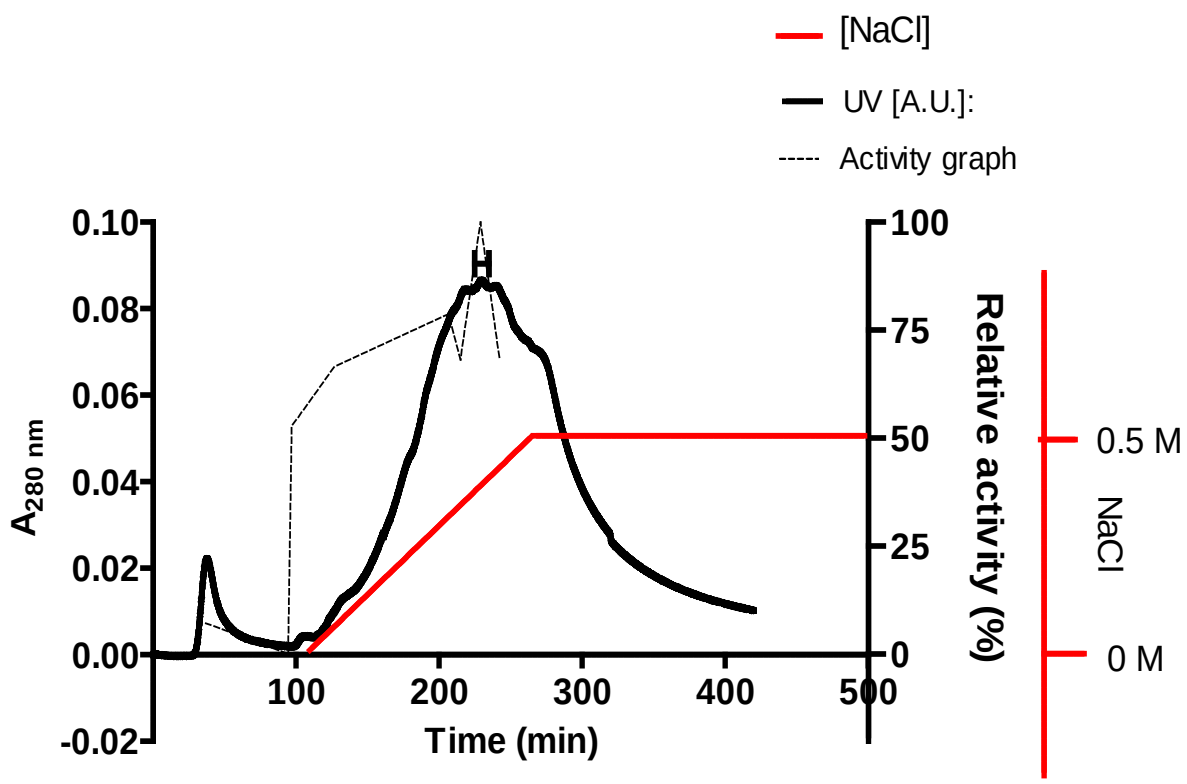

Figure 7: DEAE chromatogram of *Sb* crude enzyme extract at 280 nm. The solid line indicates different fractions of protein eluted at different sodium chloride ionic strength (started at the 110th minute). The peak that showed the highest activity is indicated by the capped line, whereas the dotted line indicates the % activity of different peaks, relative to the one with the highest activity.

The active fraction obtained from the DEAE column was then fractionated on ConA column. Two prominent protein peaks were observed from the ConA column, with the larger peak emanating from the unbound proteins. The second peak, bound proteins, eluting off the column with the elution buffer (Figures 8 and 9). Both protein peaks had very low protein concentrations as can be seen from the absorbance values which are below 0.1. Proteins that did not bind to the column (first peak) are possibly unglycosylated, or perhaps do not possess an α-D-mannopyranosyl and/or an α-D-glucopyranosyl as mentioned above, while the bound proteins (second peak) could possibly be glycosylated.

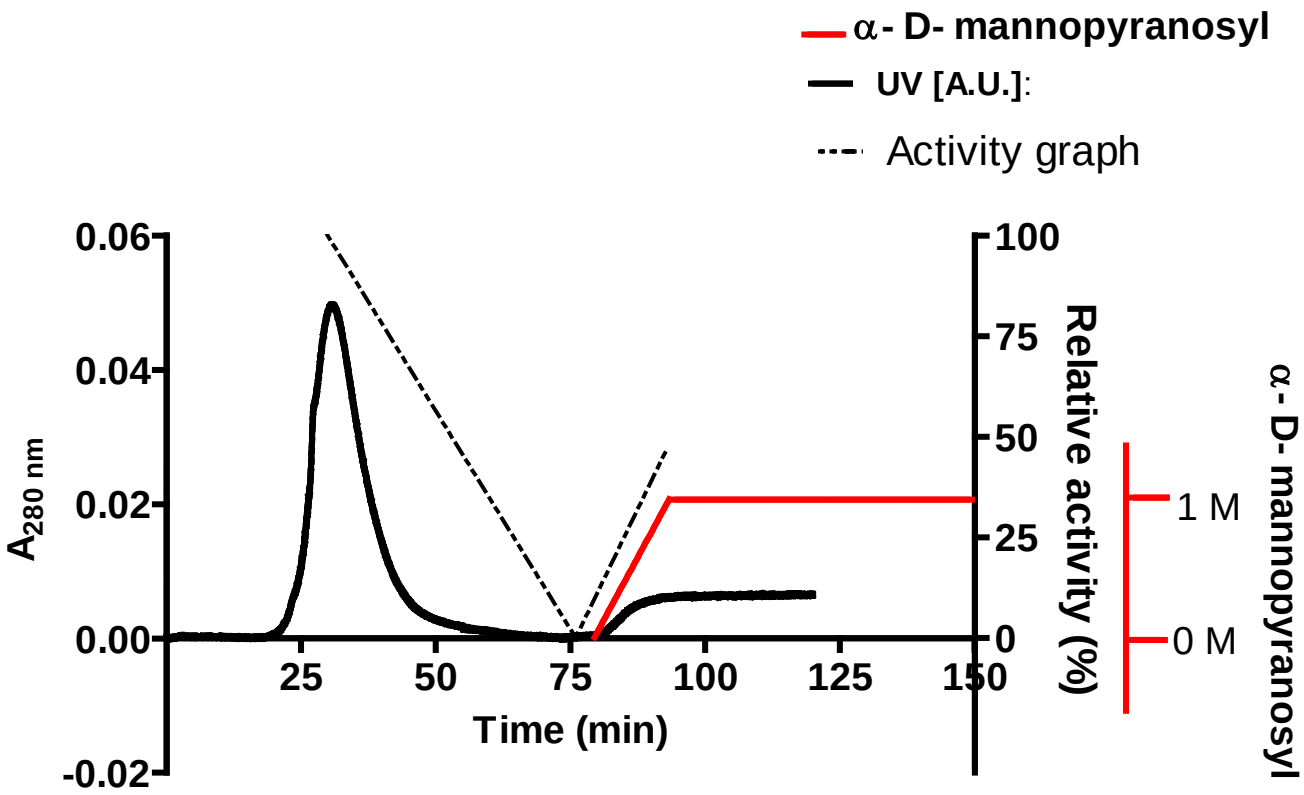

Figure 8: ConA chromatogram of *Ks* HNL enzyme at 280 nm. The first peak indicates the proteins which were not adsorbed to the column and the second peak indicates the proteins which were adsorbed to the column and eluted by α- D- mannopyranosyl gradient (started at the 80th min). The dotted line indicates the % activity of different peaks, relative to the one with the highest activity.

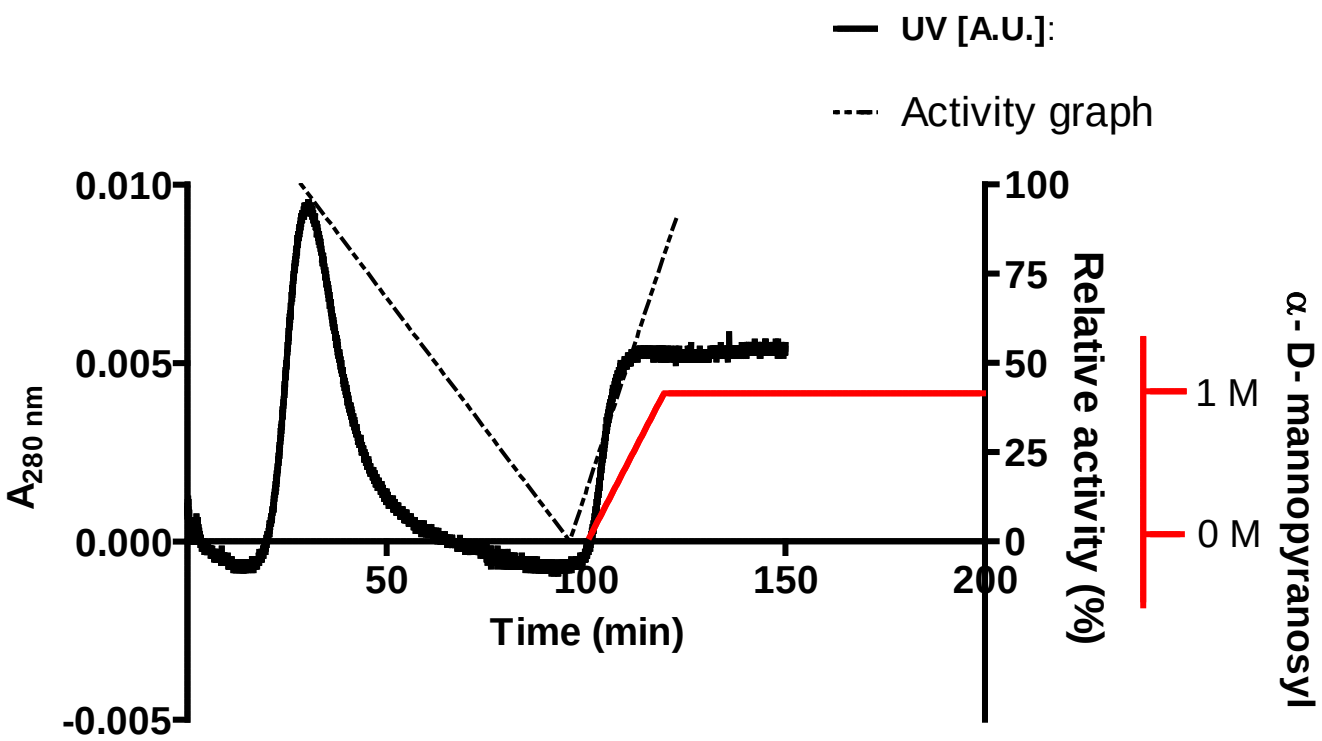

Figure 9: ConA chromatogram of *Sb* HNL enzyme at 280 nm. The first peak indicates the proteins which were not adsorbed to the column and the second peak indicates the proteins which were adsorbed to the column and eluted by α- D- mannopyranosyl gradient (started at the 100th min). The dotted line indicates the % activity of the different peaks, relative to the one with the highest activity.

3.1.4 Purification of hydroxynitrile lyase from Kalanchoe spp and Senecio spp

Below are the purification tables, Table 4 and 5, of the HNL enzyme separation steps for *Ks* and *Sb* respectively. Hydroxynitrile lyases from the leaves of *Ks* and *Sb* were isolated and purified. Crude enzyme extracts with respective activities of 32.48 and 138375 units were obtained from 50 g of the *Ks* and *Sb* leaves extracted with potassium phosphate buffer. Both *Sb*- and *Ks*HNL enzymes were precipitated between 30 and 80% ammonium sulphate saturation which resulted in approximately 25.6% of the activity remaining with a 0.5-fold purification drop for *Ks*HNL enzyme, while a purification fold of 1.1-fold for *Sb*HNL enzyme was achieved with 27% activity remaining. As mentioned previously, this is a large reduction in activity for *Ks*HNL, however, due to the removal of contaminating proteins it was decided that the step proved to be valuable in the overall purification process of the HNLs.

Table 4: Purification table of hydroxynitrile lyase from *Kalanchoe spp*

Sample fractions	Volume (ml)	[Protein] (mg/ml)	Enzyme activity (Units/ml)	Total [protein] (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Fold purified	% yield
Crude	43	0.44	14.29	18.92	614.47	32.48	1	100
30 – 80% ammonium sulphate precipitation	11	0.83	14.29	9.13	157.19	17.22	0.5	25.6
DEAE Toyopearl 650M	4	0.18	35.71	0.72	142.84	198.39	6.1	23.2
Concavalin A Sepharose 4B bound	2	0.01	16.43	0.02	32.86	1643	50.6	5.3
Concavalin A Sepharose 4B unbound	3	0.14	35	0.42	105	250	7.7	17.1

After further fractionation of the extract on DEAE Toyopearl 650M matrix, the specific activity for *Ks*HNL and *Sb*HNL enzyme increased approximately 6.1- and 6.2-fold respectively, with a slight loss in enzyme activity. The specific activity, purification fold and percentage yield of the enzyme fractions were calculated as follows:

$$\text{Specific activity} = \frac{\text{Total enzyme activity (units)}}{\text{Total protein (mg)}}$$

$$\text{Fold purified} = \frac{\text{Specific activity of each fraction (units/mg)}}{\text{Specific activity of the crude (units/mg)}}$$

$$\%\text{yield} = \frac{\text{Total enzyme activity of each fraction (units)}}{\text{Total enzyme activity of the crude (units)}} \times 100$$

Concanavalin A Sepharose 4B fractionation of the proteins resulted in a higher HNL specific activity for bound proteins as compared to that of the unbound proteins. The final purification fold for unbound protein was 50.6-fold with an enzyme activity yield of 5.3% conA fraction of the *Ks*HNL enzyme and a 7-fold purification with an enzyme yield of 3.8% for unbound conA fraction of the *Sb*HNL enzyme. For bound-*Ks* and *Sb* proteins, a 7.7- and 3.3-folds and the yields of 17.1% and 3.6% were obtained, respectively. The separation of the proteins on conA column suggest that there might

be two proteins with HNL activity, one being unglycosylated, and the other a glycoprotein from both the *Sb* and *Ks* plants.

Table 5: Purification table of hydroxynitrile lyase from *Senecio spp*

Sample fractions	Volume (ml)	[Protein] (mg/ml)	Enzyme activity (Units/ml)	Total [protein] (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Fold purified	% yield
Crude	45	0.412	3075	18.5	138375	7480	1	100
80% ammonium sulphate precipitation	9	0.485	4197	4.4	37773	8584	1.1	27
DEAE Toyopearl 650M	10	0.057	2794	0.6	27940	46567	6.2	20
Concavalin A Sepharose 4B bound	6.5	0.009	811	0.1	5272	52720	7	3.8
Concavalin A Sepharose 4B unbound	5.5	0.029	900	0.2	4950	24750	3.3	3.6

3.1.5 Gel electrophoresis of hydroxynitrile lyase from Kalanchoe spp and Senecio spp

SDS-PAGE was used to estimate the molecular mass of the conA column-proteins against the standard molecular weight markers. The protein fractions were estimated to have molecular weights of 64.64 kDa for the bound and 64.06 kDa for unbound-*Ks*HNL enzymes fractions; 70.60 kDa for bound and 74.04 kDa for unbound-*Sb*HNL enzymes (Figures 10 and 11). SDS-PAGE results indicated the presence of a single band for each fraction. The bands were faint possibly due to low concentration of the proteins loaded on the gel. The molecular weights of the proteins ranged between 64-74 kDa. This is very similar to the molecular weight of the glycosylated HNL isolated from *Eriobotrya japonica* (Loquat), 72 kDa (Ueatrongchit *et al*., 2008) and that from a *Prunus sp*. of 61 kDa as characterised by Dreveny *et al.* (2001). HNL enzymes have been found to be either monomeric such as those mentioned above, or have found to be dimeric such as that isolated from *Arabidopsis thaliana* by Andexter *et al.* (2009) or multimeric such as the HNL isolated from *Phlebodium aureum* by Wajant *et al.* (1995). The enzymes isolated in this study all seem to be monomeric.

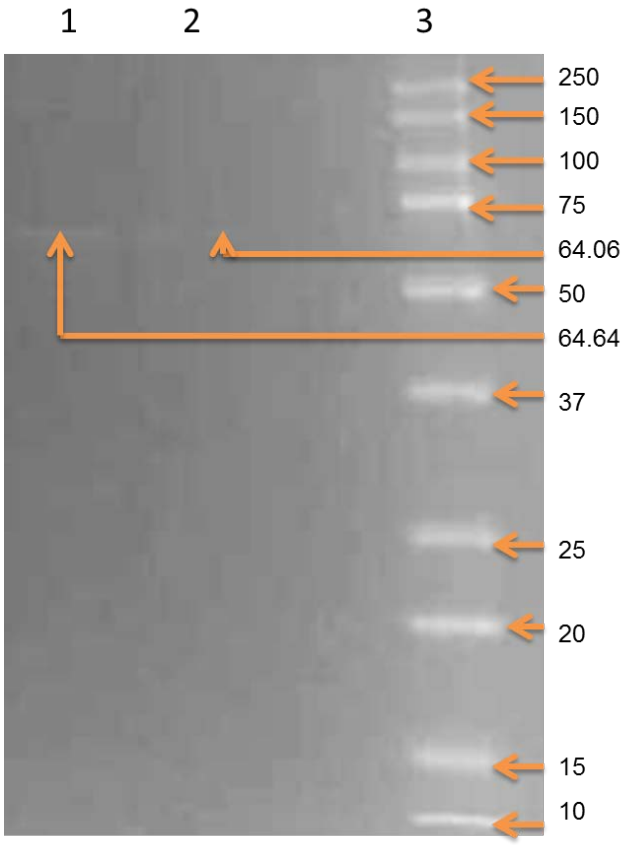

Figure 10: A 12% SDS-PAGE profile of the purified HNL from *Ks*. Lane 1, proteins eluted from conA chromatogram with NaCl; whereas lane 2 indicates the unbound proteins eluted from conA column. Lane 3 shows the molecular weight standards in kDa.

The bands were sliced and submitted to the Centre for Proteomic and Genomic Research at University of Cape Town, for peptide mass fingerprinting. Gel resolved bovine serum albumin (BSA), was included as standard process control. Unbound-*Ks*HNL protein was identified to be rabbit Glycogen phosphorylase B (EC 2.4.1.1) (t-state with AMP) (Table 6). The other isolated proteins from this study were inconclusive due to the low protein concentration supplied and therefore could not be conclusively identified.

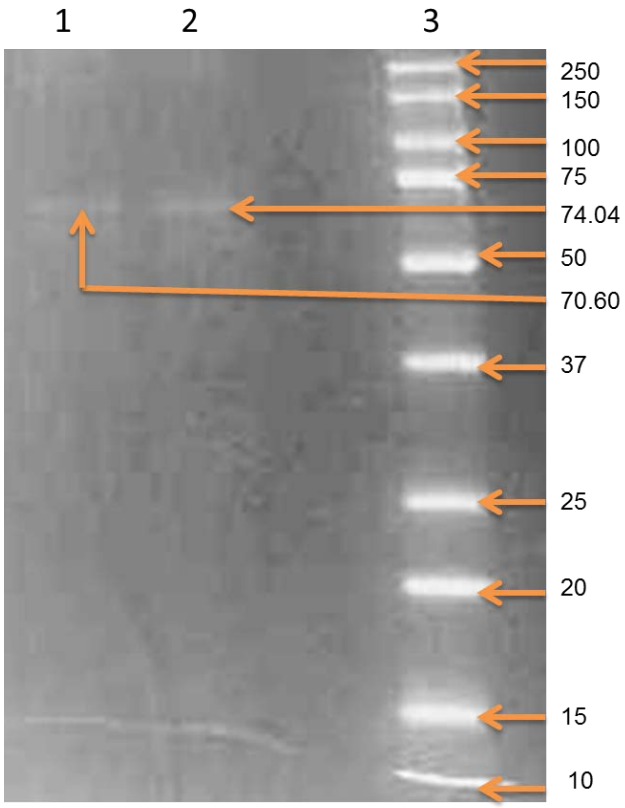

Figure 11: A 12% SDS-PAGE profile of purified HNL from *Sb.* Lane 1, proteins eluted from conA chromatogram with NaCl; whereas Lane 2 indicates the band of proteins that didn't bind to conA column. Lane 3 shows the molecular weight standards in kDa.

Table 6: Peptide mass fingerprint results of proteins

Sample	**Protein identification**	**Protein score**	**% Confidence interval**
Control	BOVALBUMIN NID: - *Bos taurus*	354	100
Ks unbound	Glycogen phosphorylase B (EC 2.4.1.1) (t-state with AMP)	114	100

3.1.6 Absorption spectra of hydroxynitrile lyase from Kalanchoe spp and Senecio spp

Absorption spectra for the purified enzymes at different concentrations were determined by spectrophotometric analysis. The wavelength maxima for HNL enzymes from *Ks* and *Sb* isolated enzymes were found to be 280 nm, 290 nm, 260 nm, and 206 nm for bound-*Ks*HNL, unbound-*Ks*HNL, bound-*Sb*HNL and unbound-*Sb*HNL enzymes, respectively. These spectra show no absorbance at 389 nm and

463 nm which indicates that the proteins do not have a bound FAD molecule. See Figures 12 – 15.

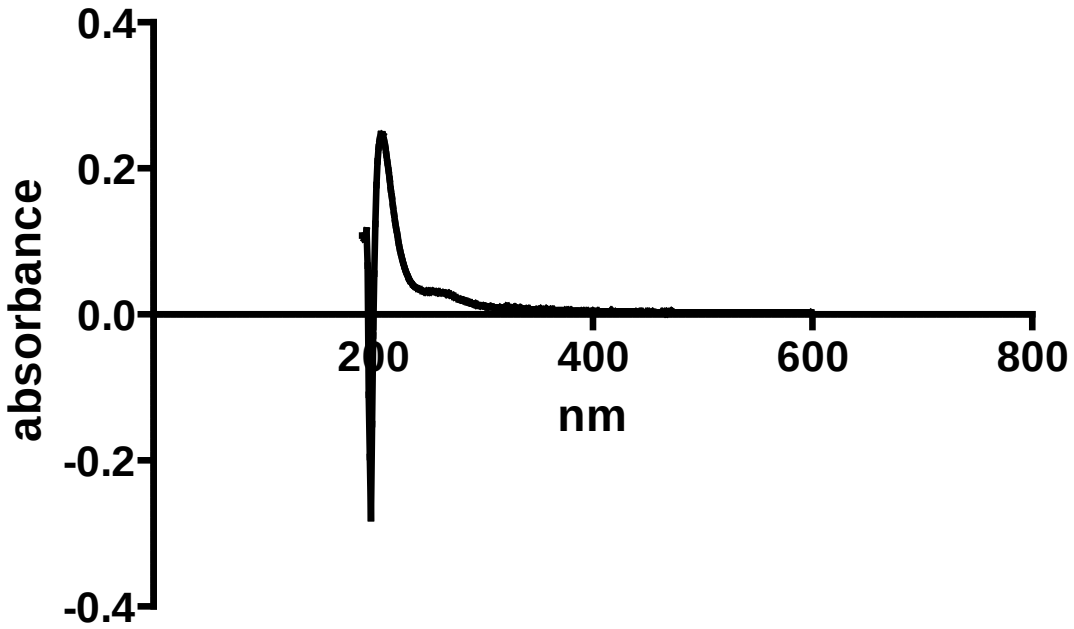

Figure 12: Absorption spectrum of conA bound fraction of *Ks*HNL enzyme dissolved in 10 mM potassium phosphate buffer at pH 6.0 (0.041 mg/ml).

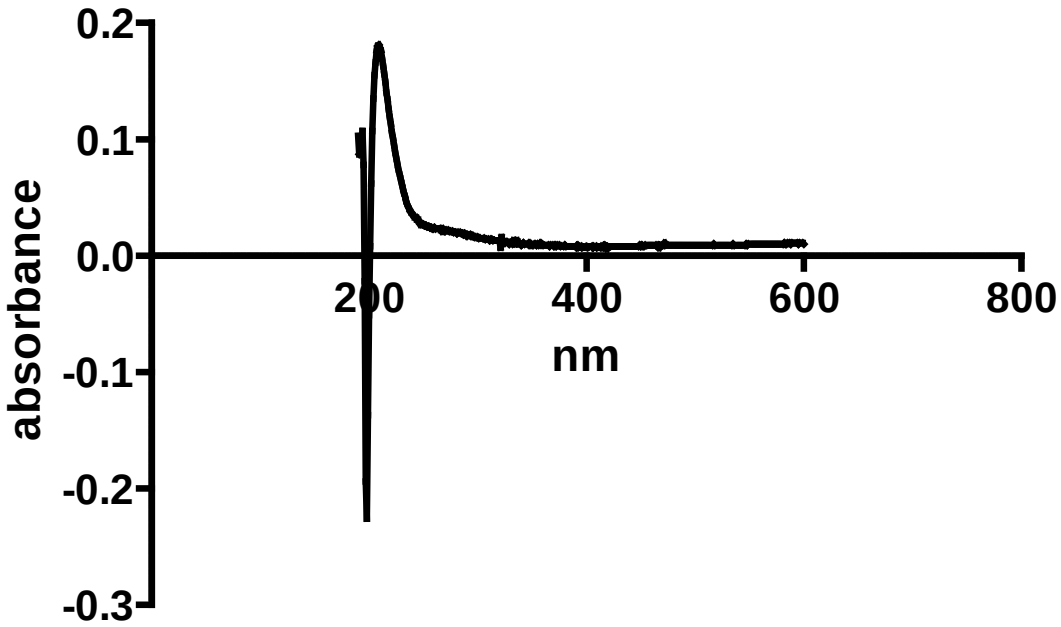

Figure 13: Absorption spectrum of conA unbound fraction of *Ks*HNL enzyme dissolved in 10 mM potassium phosphate buffer at pH 6.0 (0.022 mg/ml).

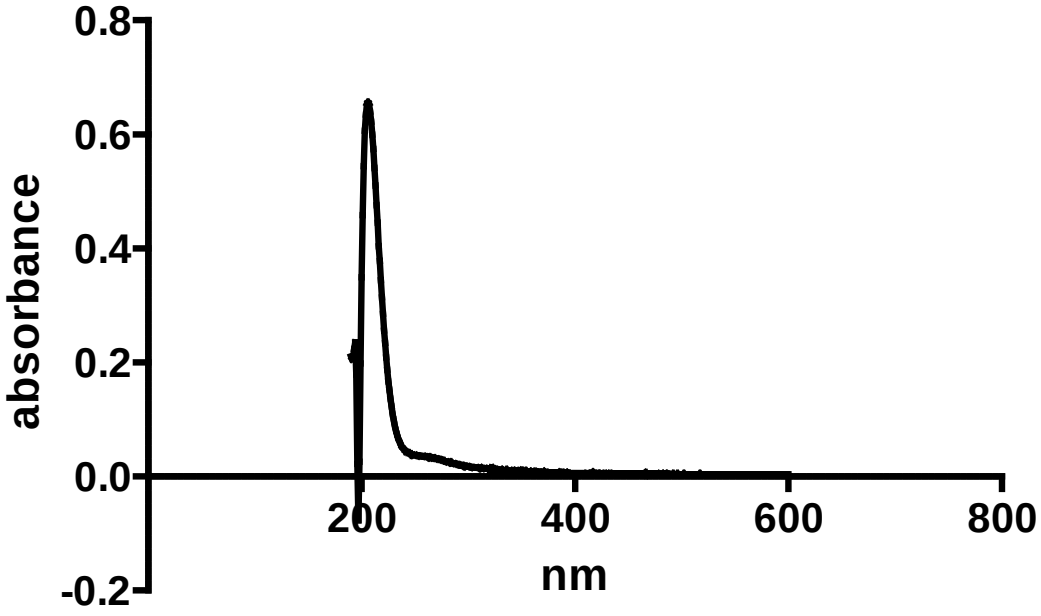

Figure 14: Absorption spectrum of conA bound fraction of *Sb*HNL enzyme dissolved in 10 mM potassium phosphate buffer at pH 6.0 (0.065 mg/ml).

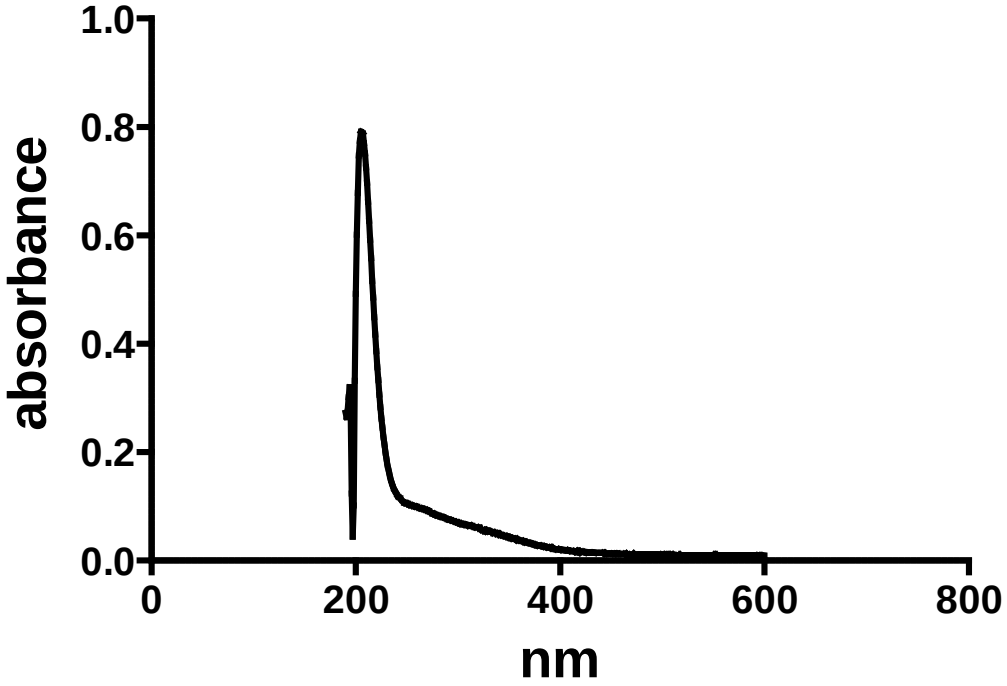

Figure 15: Absorption spectrum of conA unbound fraction of *Sb*HNL enzyme dissolved in 10 mM potassium phosphate buffer at pH 6.0 (0.097 mg/ml).

3.1.7 Optimum pH of hydroxynitrile lyase from Kalanchoe spp and Senecio spp

The pH effect on HNL activity was determined at various pH ranges (3.0–6.5). The optimum pH profiles are shown on Figures 16 – 19. The optimum pH for both *Sb*- and *Ks*HNL enzymes, using benzaldehyde and potassium cyanide as substrates, were found to be pH 5.0. The activity was totally inhibited at pH 4.0, but a rapid increase in activity was clearly observed at the experimental optimum pH. Subsequently, there was a gradual decline in activity at pH values higher than the determined optimum pH (5.5–6.5).

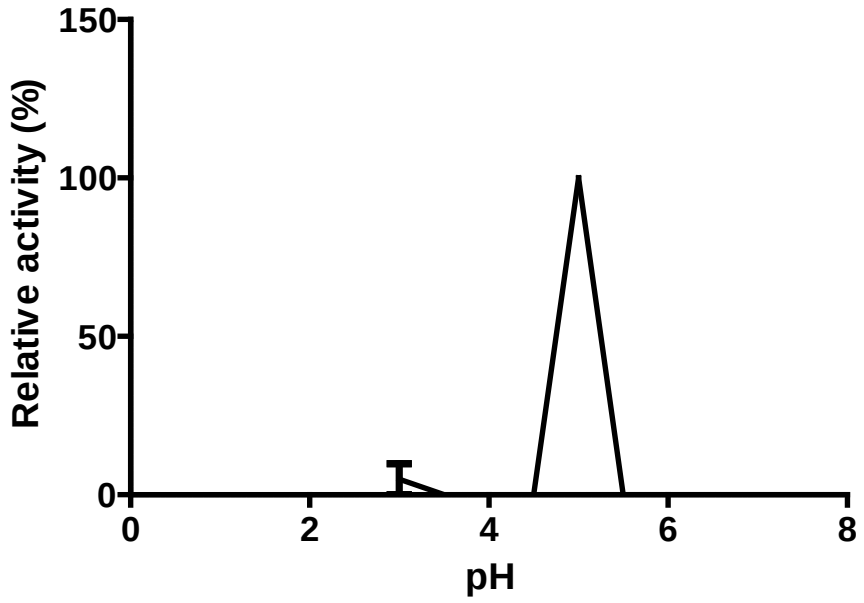

Figure 16: The effect of pH on conA bound-*Ks*HNL enzyme. HNL activity was determined in 400 mM citrate buffer at different pH values (3.0 – 6.5) at 50°C, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

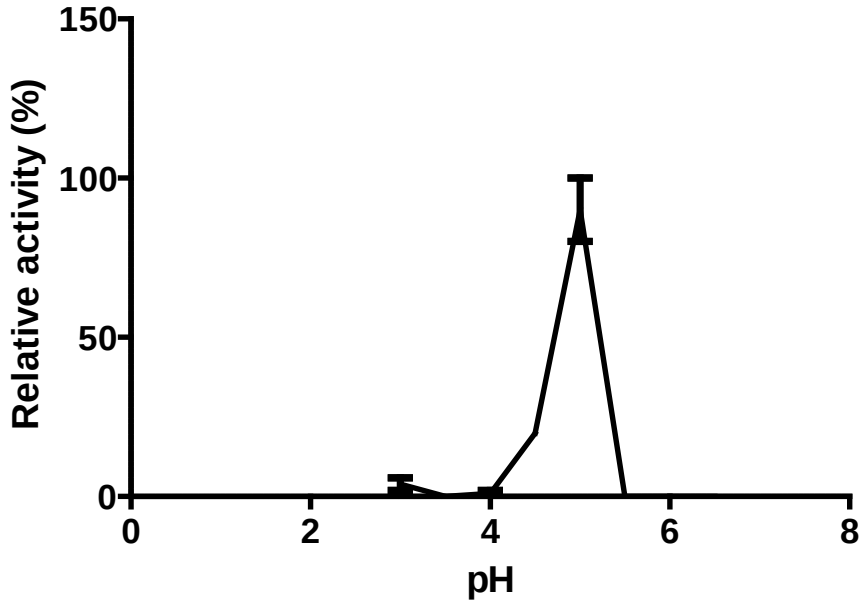

Figure 17: The optimum pH on conA unbound-*Ks*HNL enzyme. HNL activity was determined in 400 mM citrate buffer at different pH values (3.0 – 6.5) at 50°C, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

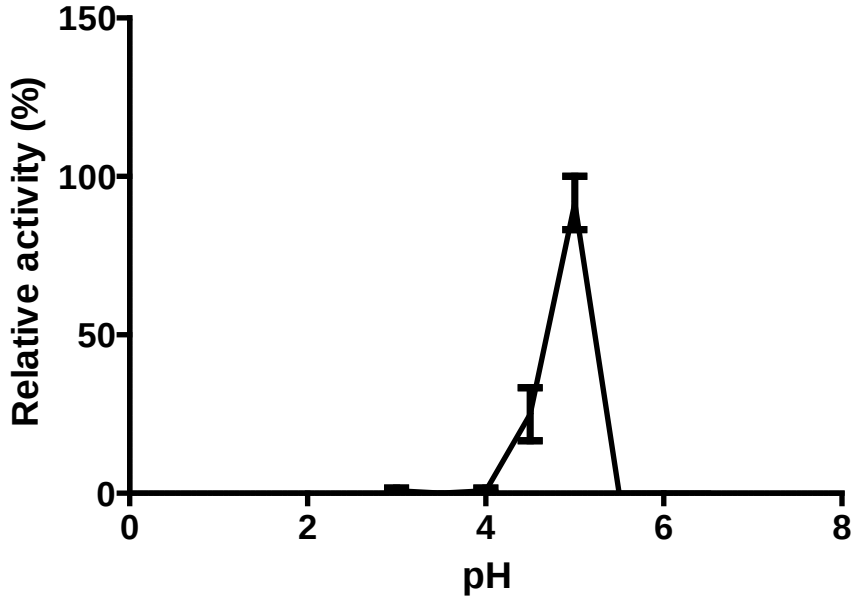

Figure 18: The optimum pH on conA unbound-*Sb*HNL enzyme. HNL activity was determined in 400 mM citrate buffer at different pH values (3.0 – 6.5) at 50°C, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

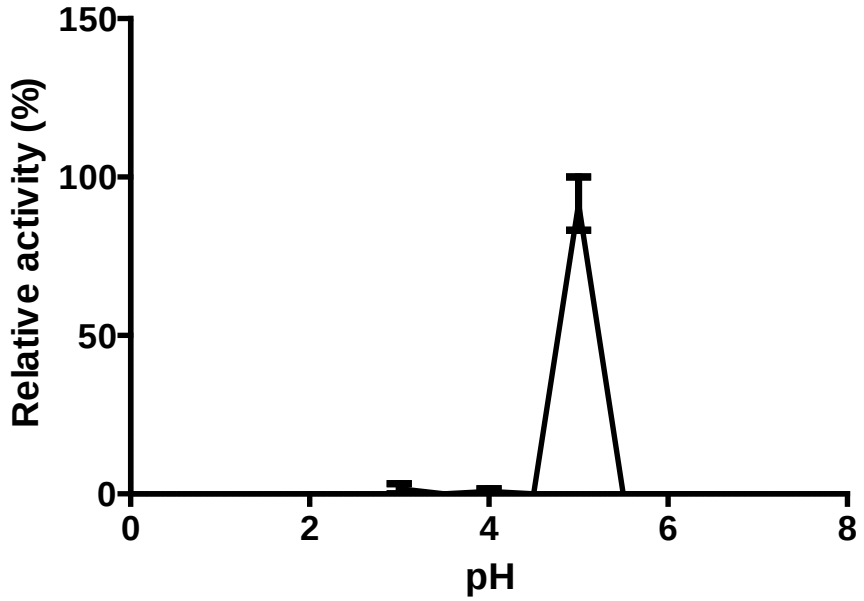

Figure 19: The optimum pH on conA unbound-*Sb*HNL enzyme. HNL activity was determined in 400 mM citrate buffer at different pH values (3.0 – 6.5) at 50°C, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

3.1.8 Optimum temperature of hydroxynitrile lyase from Kalanchoe spp and Senecio spp

The effect of temperature on HNL activity was determined between 25°C and 70°C for all the purified enzymes. The reaction rate of the enzymes was observed to increase with a gradual increase in temperature to an optimum temperature of 50°C for all the proteins, which then declined with a further increase in temperature. There was very little *Sb*HNL and *Ks*HNL activities below the temperature range of 30°C to 40°C for both *Ks*- and *Sb*HNL enzymes. The *Ks*HNL enzyme demonstrated no activity at 70°C whilst the activity of the bound- and unbound-*Sb*HNL enzymes was almost half of that of the optimum temperature (Figures 20 – 23).

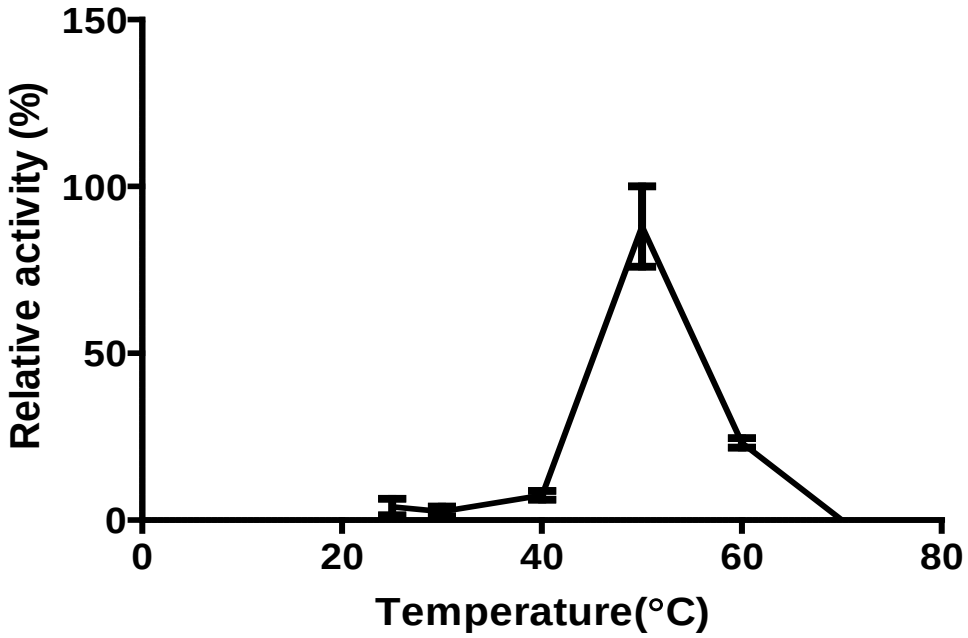

Figure 20: The optimum temperature of conA bound-*Ks*HNL enzyme. The activity of HNL was determined at different temperatures (25 – 70°C) at pH 5.0 of 400 mM citrate buffer, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

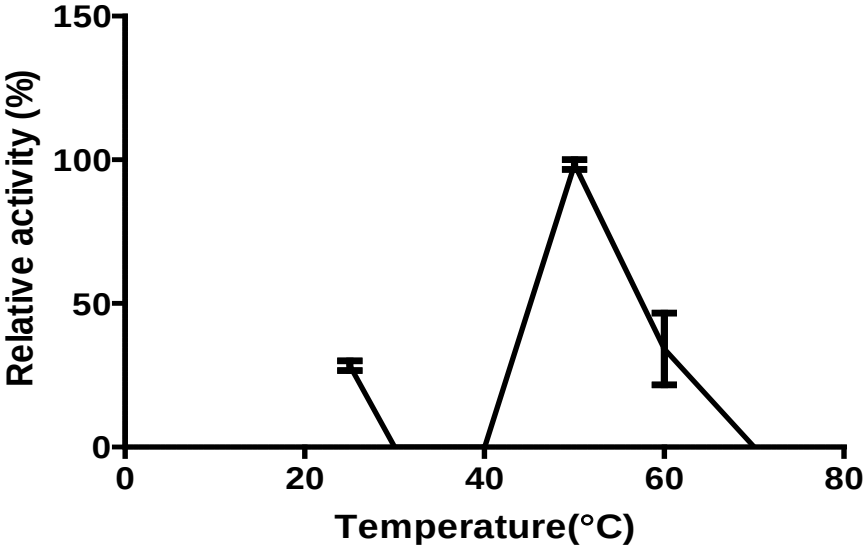

Figure 21: The optimum temperature of conA unbound-*Ks*HNL enzyme. The activity of HNL was determined at different temperatures (25 – 70°C) at pH 5.0 of 400 mM citrate buffer, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

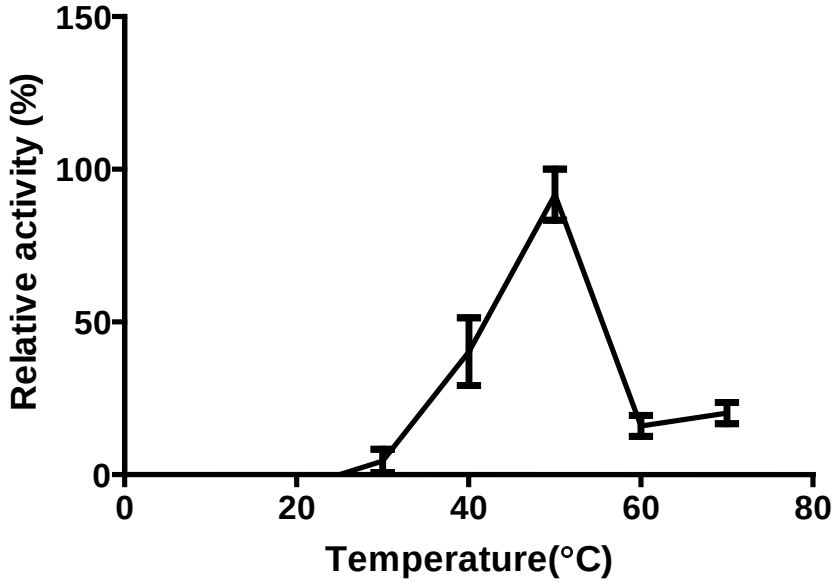

Figure 22: The optimum temperature of conA bound-*Sb*HNL enzyme. The activity of HNL was determined at different temperatures (25 – 70°C) at pH 5.0 of 400 mM citrate buffer, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

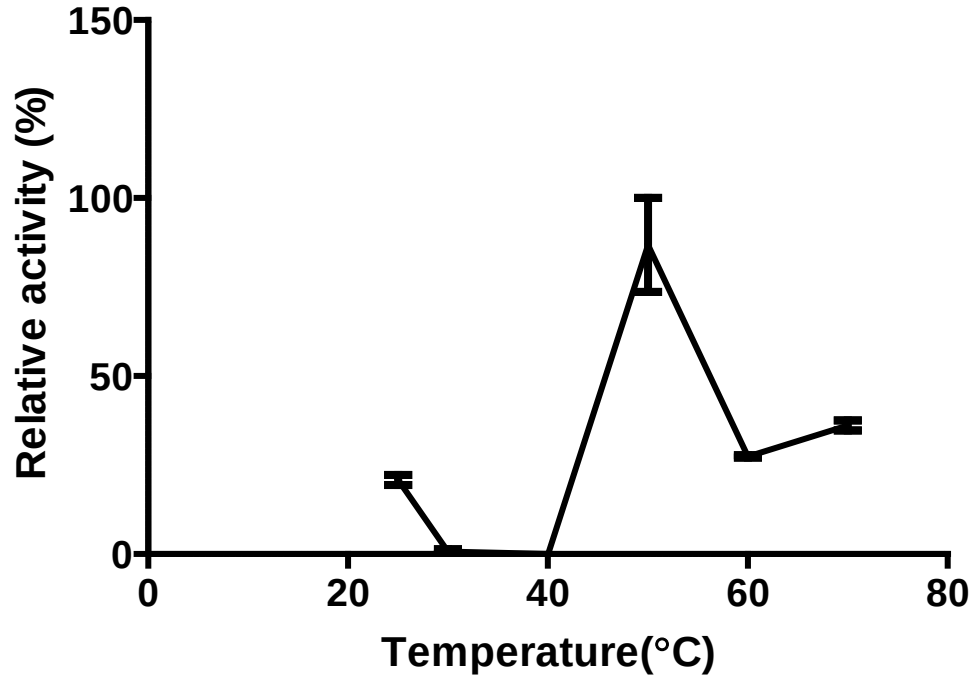

Figure 23: The optimum temperature of conA unbound-*Sb*HNL enzyme. The activity of HNL was determined at different temperatures (25 – 70°C) at pH 5.0 of 400 mM citrate buffer, activity is relative to the highest activity point on the graph. The experiment was repeated twice.

3.1.9 Effect of metal ions and other additives on Hydroxynitrile lyase activity from Kalanchoe spps and Senecio spp

Metal ions and other additives affect the activity of enzymes, for example, by either attaching to an important catalytic side group (e.g., –SH group) of the protein or by affecting the way the protein folds into its tertiary structure. This may result in the structural changes at the active site which will give an indication of the amino acids that play a role in the enzyme reaction. DEP interacts with histidine residue of the protein to inactivate enzymes. DTT attaches to cysteine residues of the protein, resulting in the reduction of the sulfhydryl groups of the protein. $HgCl_2$ attaches to the –SH group of the protein (http://www.chemguide.co.uk/organicprops/aminoacids /enzymes3.html).

The activity of the enzymes was altered by 10 mM DTT, 1 mM of DEP, $ZnCl_2$, $MnCl_2$ and $HgCl_2$. The aforementioned metal ions and additives were found to inhibit both *Ks*HNL enzyme fractions (Figures 24 and 25), even though the unbound-*Ks*HNL fraction was not affected as much as the bound-*Ks*HNL fraction, DEP and $ZnCl_2$ showed no effect on the bound-*Sb*HNL enzyme, whilst $HgCl_2$ enhanced its activity. Both the DTT and $MgCl_2$ inhibited the bound-*Sb*HNL enzyme activity (Figure 26). The unbound-*Sb*HNL enzyme was found to be activated by almost all the metal ions tested, except for $MgCl_2$ that inhibited the activity of the enzyme (Figure 27). A summary of the relative percentage inhibition and activation of the enzyme fractions by the metal ions and additives is presented in Table 7 below.

Table 7: Relative percentages of the effect of metal ion and other additive on isolated enzyme activities relative to the control.

Enzymes	**DTT**	**DEP**	**$ZnCl_2$**	**$MnCl_2$**	**$HgCl_2$**
KsHNL bound	62 ↓	50 ↓	44 ↓	37 ↓	56 ↓
KsHNL unbound	37 ↓	37 ↓	25 ↓	25 ↓	25 ↓
SbHNL bound	58 ↓	0	0	8 ↓	17 ↑
SbHNL unbound	25 ↑	63 ↑	63 ↑	25 ↓	13 ↑

Key: ↑ *%Enzyme activation* ↓ *%Enzyme inhibition*

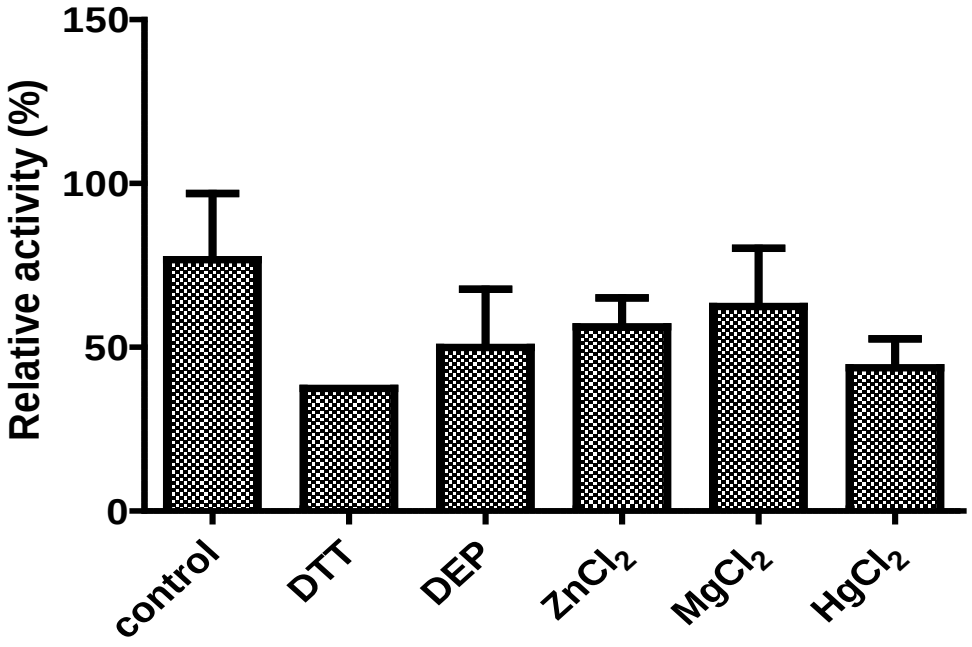

Figure 24: The effect of metal ions and additives on conA bound-*Ks*HNL enzyme. The activity of HNL was determined after incubation of purified enzymes for 30 min in different metal ions. The experiment was repeated twice.

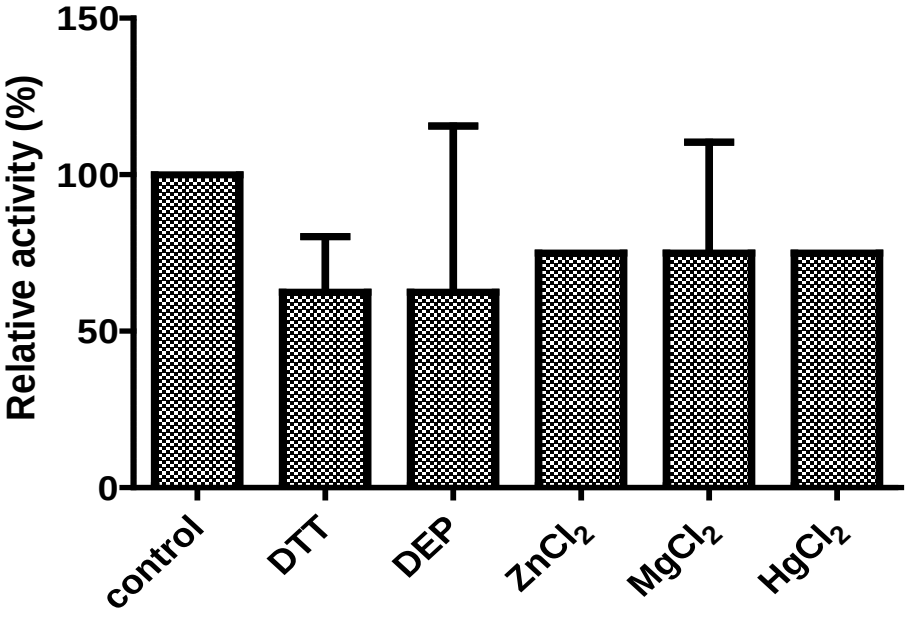

Figure 25: The effect of metal ions and additives on conA unbound-*Ks*HNL enzyme. The activity of HNL was determined after incubation of purified enzymes for 30 min in different metal ions. The experiment was repeated twice.

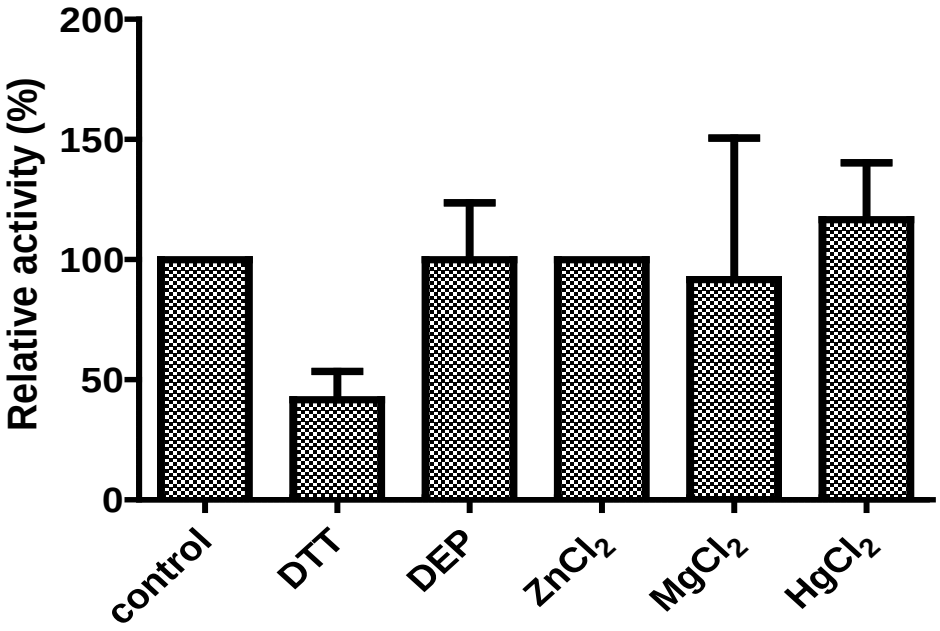

Figure 26: The effect of metal ions and additives on conA bound-*Sb*HNL enzyme. The activity of HNL was determined after incubation of purified enzymes for 30 min in different metal ions. The experiment was repeated twice.

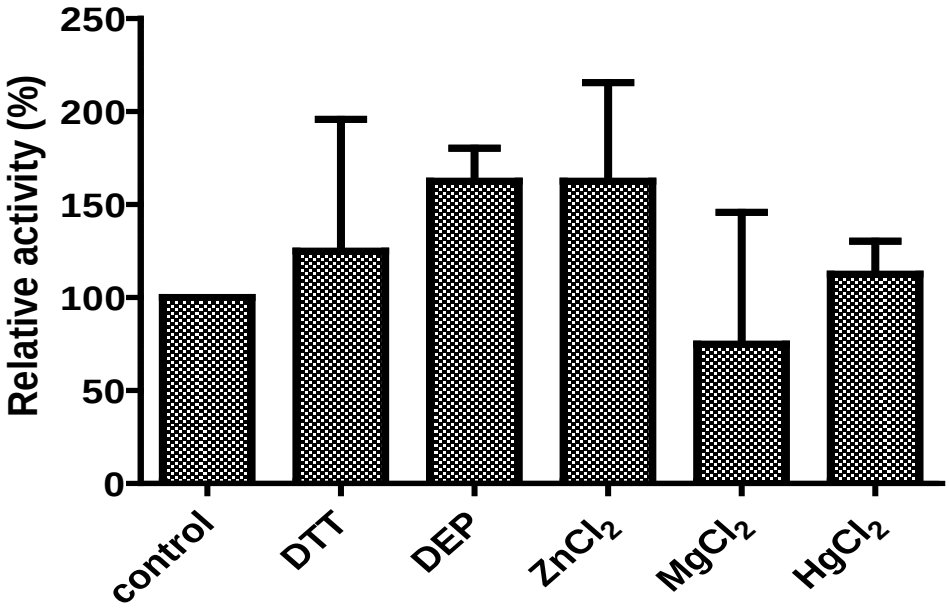

Figure 27: The effect of metal ions and additives on conA unbound-*Sb*HNL enzyme. The activity of HNL was determined after incubation of purified enzymes for 30 min in different metal ions. The experiment was repeated twice.

3.1.10 Kinetic parameters of Hydroxynitrile lyase from Kalanchoe spp and Senecio spp

The Michaelis constant (K_m) is a measure of substrate concentration required for effective catalysis to occur and is often described as the substrate concentration at which the reaction rate is half its maximum speed. Low K_m values often signify that the enzyme has a high affinity for the substrate. The catalytic constant, $k_{cat,}$ on the other hand, is a measure of the number of substrate molecules turned-over per enzyme molecule per second, and thus a large k_{cat} value indicates a rapid turnover of substrate molecules (www.pearsonhighered.com/mathews/ch11/c11kkkk.htm). The enzyme reactions were performed using a pseudo-first order reaction to ensure that the active fractions followed Michaelis-Menten kinetics (Figures 28, 30, 32 and 34). The $V_{max,}$ K_m and k_{cat} values of the isolated enzymes were determined with the use of Hanes-Woolf plots (Figures 29, 31, 33 and 35). The V_{max} of the enzymes were estimated to be 1.238, 1.948 µM/min for the bound- and unbound-*Ks*HNL proteins and 9.741, 1.905 µM/min for bound- and unbound-*Sb*HNL, proteins, respectively. The K_m values, as per Table 8, of all the isolated enzymes demonstrated a high enzyme substrate affinity, and the k_{cat} values of the isolated enzymes showed a slow turnover rate. The k_{cat}/K_m ratios indicated enzyme catalytic efficiency, which should be in the region of $1,60^4 mM^{-1}min^{-1}$ to be effective. As can be seen in Table 8, the catalytic efficiency of the enzyme fractions is much lower than expected.

Table 8: Kinetic parameters of the isolated enzymes

Enzymes	**K_m (mM)**	**$[E]_{total}$ (µM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m ($mM^{-1}min^{-1}$)**
KsHNL bound	0.33	3.242	0.38	1.15
KsHNL unbound	0.73	2.197	0.89	1.22
SbHNL bound	5.86	1.707	5.71	0.97
SbHNL unbound	0.22	0.535	3.56	16.18

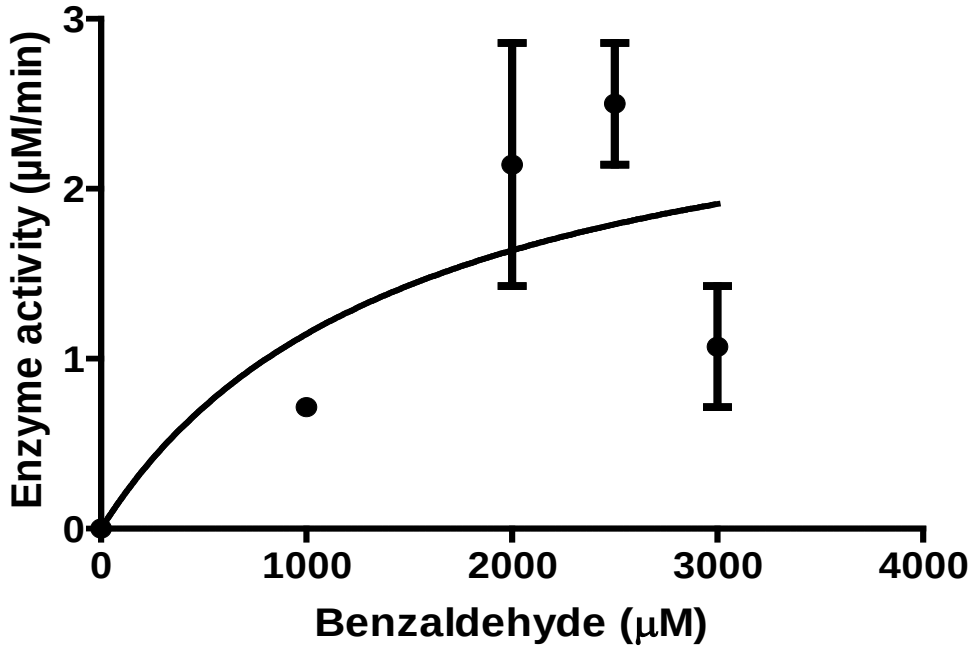

Figure 28: Michaelis-Menten curve of conA bound-*Ks*HNL enzyme. The activity of HNL was determined at different concentrations of benzaldehyde (0 – 3 mM) and 1 M potassium cyanide in 400 mM citrate buffer pH 5.0. The experiment was repeated twice in duplicates.

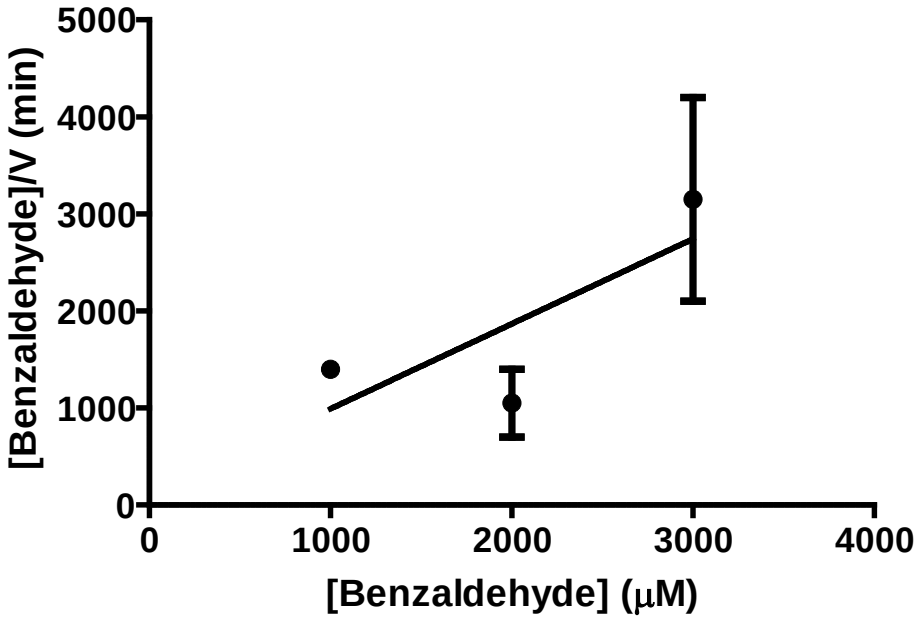

Figure 29: Hanes-Woolf plot of conA bound-*Ks*HNL enzyme. K_m and V_{max} parameters of the enzyme were determined as outlined.

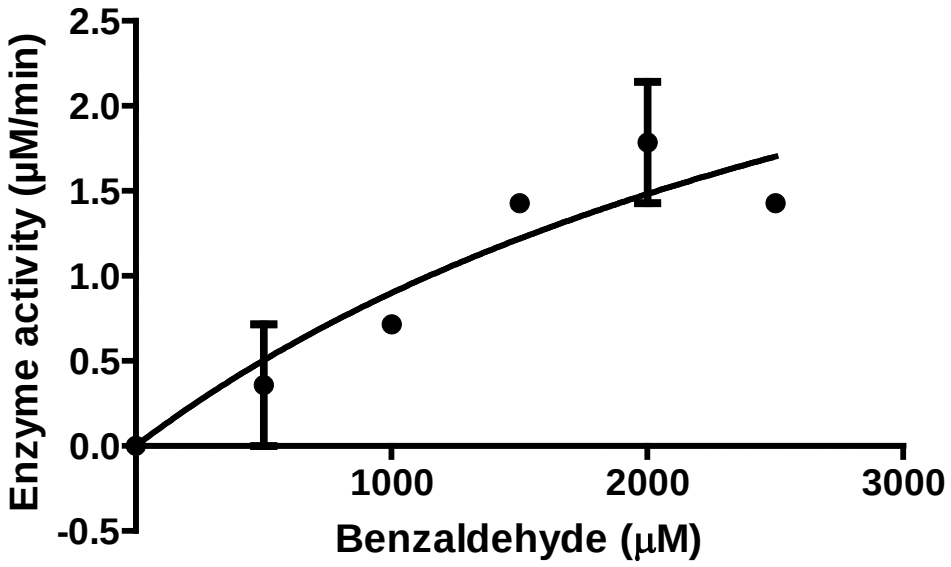

Figure 30: Michaelis-Menten curve of conA unbound-*Ks*HNL enzyme. The activity of HNL was determined at different concentrations of benzaldehyde (0 – 3 mM) and 1 M potassium cyanide in 400 mM citrate buffer pH 5.0. The experiment was repeated twice in duplicates.

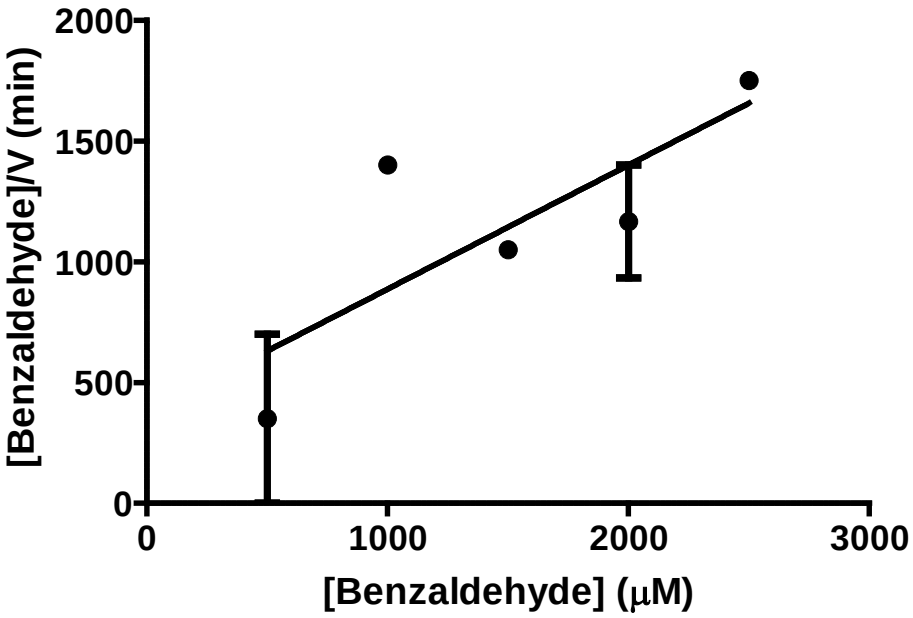

Figure 31: Hanes-Woolf plot of conA unbound-*Ks*HNL enzymes. K_m and V_{max} parameters of the enzyme were determined as outlined.

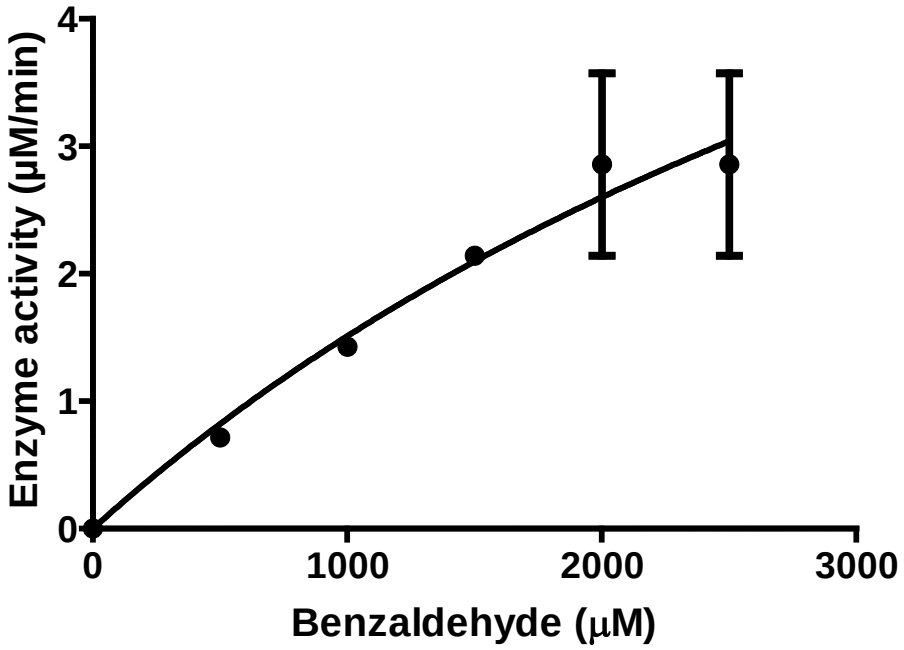

Figure 32: Michaelis-Menten curve of conA bound-*Sb*HNL enzyme.The activity of HNL was determined at different concentrations of benzaldehyde (0 – 3 mM) and 1 M potassium cyanide in 400 mM citrate buffer pH 5.0. The experiment was repeated twice induplicates.

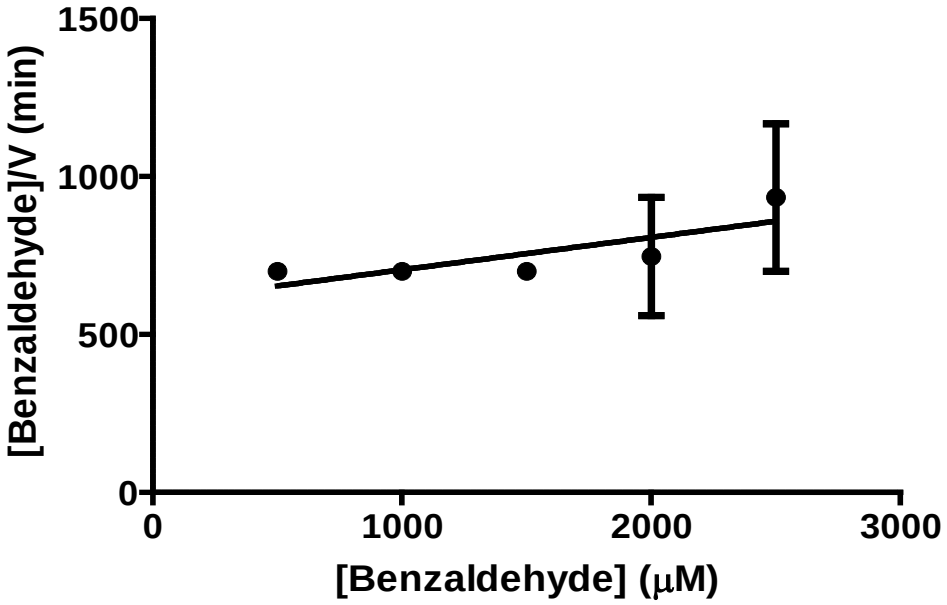

Figure 33: Hanes-Woolf plot of conA bound-*Sb*HNL enzyme. K_m and V_{max} parameters of the enzyme were determined as outlined.

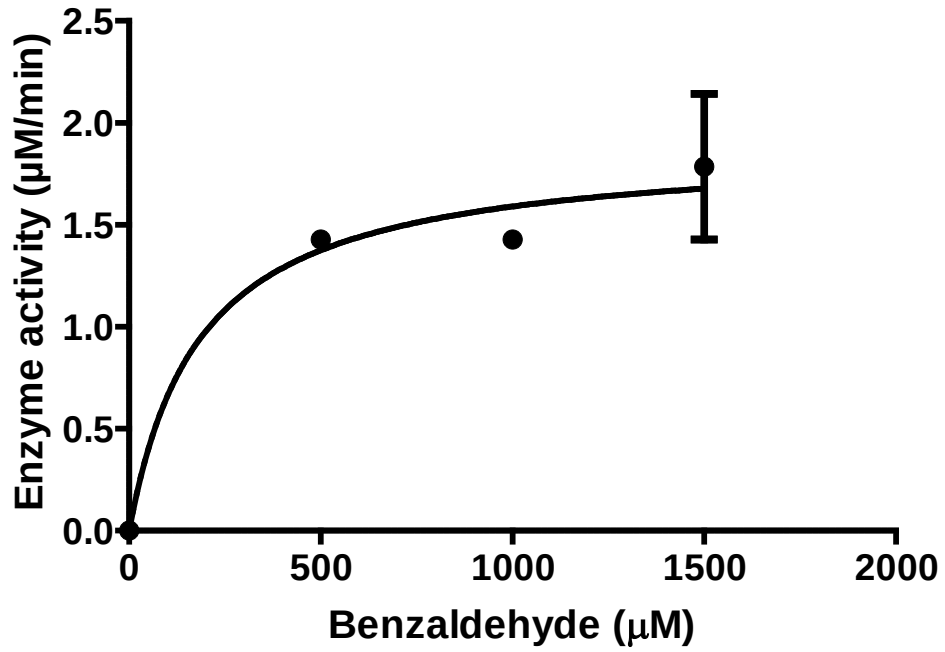

Figure 34: Michaelis-Menten curve of conA unbound-*Sb*HNL enzyme.The activity of HNL was determined at different concentrations of benzaldehyde (0 – 3 mM) and 1 M potassium cyanide in 400 mM citrate buffer pH 5.0. The experiment was repeated twice in duplicates.

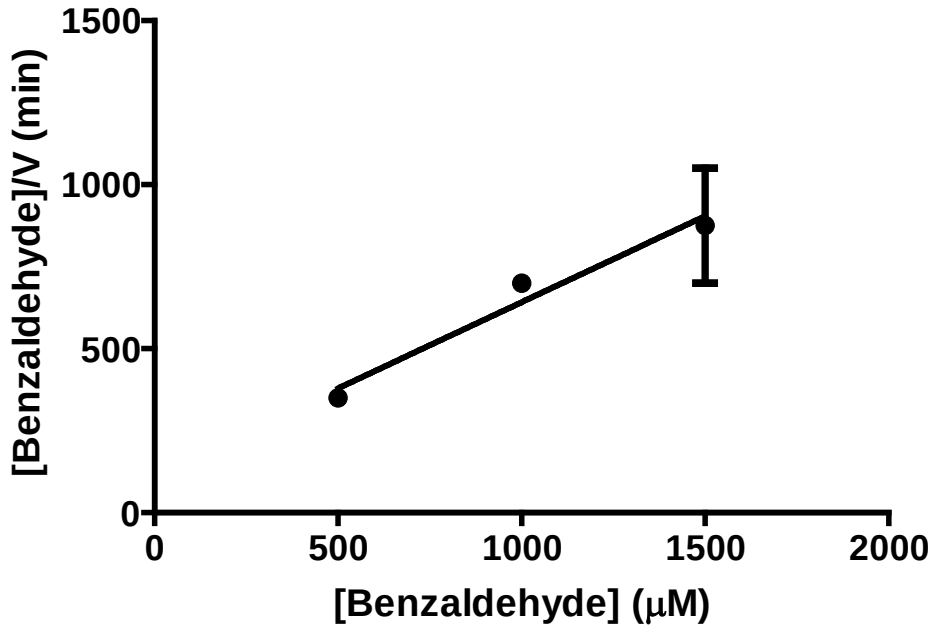

Figure 35: Hanes-Woolf plot of conA unbound-*Sb*HNL enzyme. K_m and V_{max} parameters of the enzyme were determined as outlined.

CHAPTER 4

4.1 DISCUSSION AND CONCLUSION

Hydroxynitrile lyases have attracted interest in biocatalytic applications internationally, and are used predominantly in the chemical industry due to their enantiomeric selectivity. It would be economically important to South Africa to find indigenous plants that contain high HNL activity with characteristics that will enable it to be used in our chemical industry, and also compete on the international market.

Several indigenous plants were selected and screened for HNL activity, based on proximity in locality, as well as due to their potential toxicity as per local knowledge. Six out of thirteen selected plants from the Limpopo province displayed HNL activity, which indicated the potential of South African indigenous plants to contain the HNL enzyme. Two plants with the highest activities, *Kalenchoe sp.* and *Senecio* sp were selected as potential plants to isolate HNL enzymes from. HNL activity was found in both plants extracts, by observing the conversion of benzaldehyde and KCN to the production of mandelonitrile spectrophotometrically. No activity was, however, detected with the substrate *(R)*-mandelonitrile, and hence the forward reaction. This was an interesting observation as, in a similar study, *Baliospermum montanum* HNL (*Bm*HNL) preferred aromatic aldehydes and ketones as a substrate when compared with aliphatic substrates (Dadashipour *et al*., 2011). Also, *Hevea brasiliensis* and *Manihot esculenta* plants known to contain HNLs showed activity in the synthesis of mandelonitrile but not in the reverse reaction (Hickel *et al*.,1997). The result could thus imply that the HNL enzymes isolated in this study may use alternative substrates to mandelonitrile, or that the enzyme has stereoselective activity and prefers S-mandelonitrile as a substrate, which would indicate that the enzymes could possibly be classified within the *(S)*-HNL class. Alternative substrate tests, chiral analysis, as well as the determination of enantiomeric excess (ee) of different substrates, need to be conducted to determine whether the enzymes isolated are indeed enantiomeric.

Another interesting observation was the observation that the enzyme activity of the selected plant extracts only occurred when plants were harvested in winter. Kakes

(1990) in a review article discussed how developmental and environmental factors affect the level of glycosides in plants as well as the levels of their hydrolysing enzymes. According to Wang *et al*. (2003) stress related genes are activated and regulated in plants as molecular control mechanisms for abiotic stress tolerance. According to Hanson (2007) *Trifolium repens* produces cyanogenic glycosides in temperatures above 5°C whilst at temperatures lower than 5°C cyanogenic glycosides are not produced. South African temperatures range between 6 and 20°C in winter and 17 to 30°C in summer. These factors may thus account for the seasonal difference in HNL activity noticed within the plant species tested, however the reason for the difference must be thoroughly investigated.

Purification techniques were tested and used to isolate the enzymes as per previously described literature (Ueatrongchit *et al*., 2010). The final enzyme activities isolated from these extracts, although positive, were quite low if compared with previous studies (Wajant *et al*., 1995). A possible explanation for the low activities could be due to the final protein concentrations of the enzymes isolated. As seen from the purification tables (Tables 4 and 5), small concentrations of proteins (0.42 and 0.2 mg) were extracted. The reason for the low protein yield could be multi-fold in that it might be due to the amount and type of starting material used, or to the techniques used throughout the purification process. In this study, 50 g of the initial frozen plant material was used to obtain the protein amounts mentioned above. The amount of protein extracted in this study is quite low in comparison with the 190 mg total proteins isolated by Wajant *et al*. (1995) who used 20 g of freeze dried plant material to begin with. In addition, both plants used in this study were succulents, and thus most of the plant's weight consisted of water. More protein may have been extracted if the leaves of the plants were freeze dried before the extraction, but a study needs to be conducted to determine whether the enzyme activity will be preserved if this method is used. Alternatively, a scale up of the procedures using more fresh plant material could be used in future to isolated higher enzyme concentrations for further studies.

In the purification of the enzyme extracts 70% of proteins were lost using ammonium sulphate precipitation. This indicated a negative impact on the total protein isolated, the activity, purification fold, and specific activity. The procedure was, however,

deemed necessary in the purification process. There are many plant compounds such as chlorophyll a and b, and other phytocompounds that may absorb ultraviolet light at 280 nm and thus interfere with the enzyme assay by elevating the readings of the crude extracts. Additionally, contaminating compounds may interfere with the protein purification by clogging the column matrixes. Thus, although much enzyme extract activity was lost using the ammonium sulphate precipitation step, it is advisable to use this step when purifying HNL enzymes from leaves. This step did, however, contribute to the small amount of protein being extracted in the purification.

The remaining purification procedures, the DEAE and affinity chromatography, increased the specific activity and purification fold, which meant that the protein of interest was becoming purer, as indicated on the SDS-PAGE where only one protein band was detected for each purified plant protein extract. There was an avoidable loss of enzyme yield in each of the steps but this ultimately led to the isolation of one protein per extract. In order to improve the purification, it may be better to alter the NaCl gradient by lengthening it, or by using a step gradient, in order to ensure less active protein loss during these steps. With the affinity chromatography step, almost 50% of protein, and enzyme activity, was lost for both plants. The reason for this may be an inaccuracy in protein and enzyme activity measurements due to the low protein concentrations that were observed after this step. The conA affinity chromatography step did indicate, however, the presence of unglycosylated (unbound) proteins, or proteins with low affinity to the concanavalin A lectin column, as well as glycosylated (bound) proteins. This is interesting as HNL enzymes have reported as being glycosylated or unglycosylated depending on their electroselectivity classification (Sharma *et al*., 2005).

The K_m and the V_{max} of the enzymes were determined using Michaelis-Menten curves and Hanes-Woolf plots. The Hanes Woolf plot was chosen instead of other linear transformations because Dowd and Riggs (1965) determined that when a Hanes-Woolf plot ([S]/v against [S]) was used assuming an error for v more accurate kinetic parameters were determined in comparison with those obtained using an Eadie-Hofstee plot (v against v/[S]). They also suggested that the Lineweaver-Burk plot (l/v against l/[S]) should be abandoned, because it was the least reliable for the

estimation of V_{max} and K_m for any error that was assumed for v. This suggests that the best linear transformation plot was used in determining the kinetic parameters of the purified enzyme. The Michaelis-Menten constants of the purified enzymes were low using benzaldehyde as a substrate as compared to previously characterised HNLs using the same substrate as seen in Table 9.

Table 9: Comparison of kinetic parameters of HNL using benzaldahyde as substrate, optimum pH and temperature of the HNL enzymes previously isolated to the HNL enzymes isolated in this study.

Enzymes	**K_m (mM)**	**K_{cat} (min^{-1})**	**K_{cat}/K_m ($mM^{-1}min^{-1}$)**	**Optimum pH**	**Optimum temperature (°C)**
*Ks*HNL bound	0.33	0.38	1.15	5	50
*Ks*HNL unbound	0.73	0.89	1.22	5	50
*Sb*HNL bound	5.86	5.71	0.97	5	50
*Sb*HNL unbound	0.22	3.56	16.18	5	50
*Ej*HNL	11.5	1700	148	5.5	40
*Bm*HNL	5.5	26.6	4.8	5	20
*Pm*HNL	5.3	-	-	4.5	-
*Pa*HNL	0.83	-	-	5.3	-

The enzyme substrate affinity of bound-*,* unbound-*Ks*HNL, and unbound-*Sb*HNL was slightly stronger than of the reported *Ej*HNL (Ueatrongchit *et al*., 2008), *Pm*HNL, *Pa*HNL (www.brenda-enzymes.org) and *Bm*HNL (Dadashipour *et al*., 2011), although the enzyme substrate affinity of *Sb*HNL was within the range mentioned for all HNLs. Other *(S)*-hydroxynitrile lyases from www.brenda-enzymes.org using benzaldehyde as a substrate, showed higher enzyme catalytic efficiency; 3.44, 3.48, 3.85, 4.2 and 6.75 $mM^{-1}.s^{-1}$ as compared with the isolated enzymes in this study. These results thus indicate that the *Ks* and *Sb* enzymes isolated in this study prefer benzaldehyde as a substrate as compared to the others. The question still remains, however, as to what the preferred substrate will be in the forward reaction as R-mandelonitrile as a substrate did not yield any enzyme activity.

The reactions had a slow catalytic turnover as indicated by the low calculated k_{cat} values. This implies that the turnover of substrate molecules per enzyme molecule per second was very low in comparison to those obtained previously, although as can be seen from Table 9 the range of k_{cat} determined had a very wide range (1700-26.6 min^{-1}). These results, should however, be re-determined using higher levels of enzyme specific activity to ensure that they did not occur due the low protein concentration and activity. The calculated enzyme catalytic efficiency was also low for both KsHNLs and bound-SbHNL as compared with other reported HNL enzyme catalytic efficiencies, as reported in table 9 above. Interestingly, in most cases the enzyme catalytic efficiency of *(S)*-HNL is lower as compared with *(R)*-HNL enzymes (www.brenda-enzymes.org) which may also give an indication that the enzymes isolated in this study belong to the S-HNL class.

In order for the enzymes to be used industrially they must be capable of functioning in a range of pH and temperature values. The pH and temperature optima of the purified enzymes were within the range of other HNLs that were previously characterised (see Table 9), and in particular, are comparable to the *(S)*-HNL enzymes. If future studies do indicate that these enzymes will indeed be useful for industrial purposes, the enzymes may have to be altered by either using genetic or protein engineering methods, to improve their pH and temperature stabilities to meet the industrial demands.

An essential feature of HNLs in the different classes is the determination of whether the enzymes are FAD-dependent of FAD-independent enzymes. All the enzymes isolated in this study showed no wavelength absorption detection at 389 and 463 nm. FAD-dependent HNLs are single chain protein with *(R)*-mandelonitrile as their natural substrate (Gruber *et al*., 2004) and their maximum absorption is at 389 and 463 nm. This result indicates that the enzymes do not have a FAD group bond to them.

Sharma *et al*. (2005) established that glycosylated HNLs containing an FAD group are grouped in HNL I group, and HNLs lacking FAD and which do not use *(R)*–mandelonitrile as their natural substrate are grouped in HNL II group . Therefore the purified enzymes could possibly be classified within HNL II group. The molecular

weight subunits of FAD independent enzymes are below 50 kDa, and the molecular weight of the isolated enzymes are above 50kDa. (Asano *et al*., 2005). This might possible be an indication that the enzymes without FAD may possibly possess a bigger molecular weight since the extracted enzymes have the characteristics of FAD independent enzymes.

The reaction mechanism of enzymes can be elucidated on the basis of a comprehensive study of structural, kinetic, and on mutagenesis data. In this study, inhibitors and metal ions were used to establish the reaction mechanism of the isolated enzymes. DEP, which is capable of interacting with the imidazole (responsible for acid/base catalysis) moiety of the histidine residue, inhibited *Ks*HNL activity (Sharma *et al*., 2005). This indicates the involvement of histidine in the catalysis by *Ks*HNL enzymes. This effect was not observed for the SbHNLs which indicated no change in the activity of DEP treated conA bound fraction of *Sb*HNL, and increased activity in the unbound fraction. This indicates that histidine may not be involved in the reaction, and that the HNLs isolated from the two plants may have different catalytic mechanisms and thus may be different enzymes.

DTT reduces the disulphide bonds of an enzyme in the presence of cysteine. The activities of all isolated enzyme were affected, meaning that they possibly contained disulphide bonds. The activity of both KsHNL extracts and the bound-SbHNL extract decreased which may imply that once the disulphide bonds were reduced, structural changes may have occurred that affected the activity of the enzyme. The unbound-SbHNL activity was enhanced implying that the DTT enhanced the activity of this enzyme possibly by maintaining the monothiols of the proteins (Lees et al., 1991).

The inhibition effect of Hg^{2+} indicates that a cysteine residue may be involved in the catalysis of the enzyme (Fukuta *et al*., 2011). Hg^{2+} increased the activity of *Sb*HNL, and inhibited *Ks*HNL activity indicating that the enzymes contain a cysteine residue close to its active site. In previous studies, Dreveny *et al*. (2009) established that covalent modification of cysteine abolishes catalytic HNL activity of the *PaHNL* enzyme due to close proximity between cysteine and the substrate binding site (Dreveny *et al*., 2009). In the present study, the enzyme activity was not totally eliminated by Hg^{2+} indicating that the isolated HNL enzymes involve cysteine during

catalysis although it may not be central to the active site. This may indicate that the modification of cysteine may alter the structure of the enzyme in some way, thus altering the rate of the catalytic reaction. Further studies must take place to investigate the reason for the effect of Hg^{2+} on the extracted HNL enzymes.

The general observation of the different effects of metal ions and inhibitors on the HNL activity of the four isolated enzymes is not uncommon as elaborated on by Wajant *et al*. (1995) who clearly compared the differences observed in various HNLs with respect to metal ion and inhibitor studies. These results do indicate that the HNL enzymes isolated from the two plants *Ks* and *Sb* may differ in structure, even though they catalyse the same reaction. This aspect is highlighted further due to the variation of proteins that display HNL activity. These proteins use different substrates, have different structures and different active sites as outlined in the literature review of this study. This aspect is reinforced by the preliminary result obtained for the peptide mass fingerprint study of the unbound-KsHNL protein. The results indicated that the protein isolated presented a similar peptide spectrum as that of glycogen phosphorylase b isolated from rabbit, an enzyme unrelated to HNLs in activity. Glycogen phosphorylase b is the enzyme that catalyses the phosphorolytic cleavage of the α-1,4-glycosidic linkage occurring at the non-reducing ends of glycogen or polysaccharide chains resulting in the formation of glucose-1-P. The variability in activities, structures and catalytic site make the HNL class of enzyme an interesting study, and further studies should be conducted to determine the substrate preferability, the stability of the enzyme under industrial circumstances, modification studies to increase stability, as well as the structure and active site mechanism of the enzymes isolated from the *Kalenchoe* and *Senecio* plant species.

To conclude the study, South African indigenous plants are a source of active HNL enzymes. Of the thirteen plants screened for HNL activity six of the indigenous South African plants showed traces of HNL activity. There could be more plants in South Africa containing this industrially useful HNL enzyme that will assist to place South Africa on the map in enzyme catalysis industrially. Biochemical industries and fine chemicals in South Africa could produce enantiomerically pure products through the use of efficient HNLs. Therefore it is important that the screening of the HNL activity from a variety of indigenous South African plants be continued for future

studies to discover the possibly of HNLs in a more active and robust form, and that the production of these important enzymes be up scaled, possibly by cloning and used locally and perhaps internationally for a wider industrial application.

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