Isolation, identification and characterisation of novel actinobacteria from Zambian hot-springs

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A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Biotechnology, University of the
Western Cape.

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December 2011

DECLARATION

I declare that "Isolation identification and characterisation of novel actinobacteria from Zambian hot-springs" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Natasha Robertha Mavengere 2011

Acknowledgements

I would like to extend my heartfelt gratitude to:

- _ My heavenly Father for providing guidance, strength and wisdom, to God be the glory great things he has done.
- _ My supervisor Professor Don Cowan for providing the opportunity and necessary guidance to complete my project
- _ Dr. B. Kirby for her extreme patience, assistance and support.
- _ My parents and brothers for their love, support and understanding through all my years of study, with a special thanks to my mother-in law for patiently keeping my children safe from me during the difficult times. May God continue to richly and abundantly bless her.
- _ A special thanks to everyone in IMBM, especially Dr. Heidi Goodman for endless assistance.
- _ The National Research Foundation for providing the necessary funding.
- _ Last but most certainly not least, to my best friend and darling husband for keeping me sane. Thank you for being my pillar of strength and supporting me, for carrying me through the tough patches and for dreaming with me. To infinity and beyond.

Abstract

Actinomycetes are ubiquitous in many environments such as soil, activated sludge and water. Besides the genus *Streptomyces*, which has been extensively exploited, members of other genera including *Micromonospora* have been shown to be a promising source of novel secondary metabolites and enzymes.

The biocatalytic conversion of 5-monosubstituted hydantoin derivatives to optically pure amino acids involves two reaction steps. The first step, catalysed by a hydantoinase, yields an N-carbamylamino acid intermediate, which is subsequently broken down by an N-carbamoylase to the amino acid. This process has been successfully applied in industry for the production of optically pure amino acids which are used in the synthesis of pharmaceuticals, insecticides, hormones, and food additives. The need for novel hydantoinases to hydrolyse a wider variety of substrates is increasing.

This thesis describes the search for a novel hydantoinase from environmental isolates obtained from two Zambian hot-springs. The aim of this study was to isolate, characterise and screen novel actinobacteria for industrially relevant enzymes including hydantoinases. Fifty one actinobacteria were isolated. Isolates were characterized by a polyphasic approach using standard methods, combining phylogenetic analysis of the 16S rRNA gene, chemotaxonomic and phenotypic characterization. Results revealed that these sites were dominated by actinobacteria belonging to the family *Micromonosporaceae*, and a potentially novel *Verrucosispora* species was identified. Screening the isolate identified a *Streptomyces* species which has hydantoinase, carbamoylase, amidase and nitrilase activities.

The *Streptomyces* sp. hydantionase was cloned and functionally expressed in E.coli. The recombinant enzyme showed 49 % similarity to a crystallised hydantoinase from a Bacillus species. Homology modelling revealed that the enzyme had the TIM barrel topology which is characteristic of hydantoinases. Amino acid residues predicted to be involved in the catalytic activity as well as substrate orientation were identified. The partially purified hydantoinase was characterised and showed optimally activity at 45 °C and pH 8.

This study revealed that hot springs may represent a previously unexplored source of novel actinobacterial diversity. However, it also revealed that novel secondary metabolites are not only limited to novel organisms but that some of the answers for the challenges we face today maybe found in organisms we have already encountered and characterised.

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List of Abrreviations

Å	angstrom
ACE	Angiotensin converting enzyme
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	bovine serum albumin
Da	Dalton
IPTG	Isopropyl -D-1-thiogalactopyranoside
kDa	kilodalton
NCBI	National Centre for Biotechnology Information
OD600	Optical density at 600nm
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PDB	Protein Data Bank
rpm	Revolutions per minute
RT	Room Temperature
SDS	sodium dodecyl sulphate
sp.	specie
Tris	Tris-2-amino-2-(hydroxymethyl)-1,3-propandiol
v/v	volume per volume

Chapter 1: Literature review

1. Actinobacteria

1.1 Definition

Actinobacteria have long been recognised as a group of microorganisms which are distinct from other eubacteria and fungi. This phylum represents one of the largest taxonomic groups within the bacterial domain, and includes five subclasses and at least 219 genera(Ventura *et al.*, 2007; Zhi *et al*., 2009). Although the criteria used to classify actinobacteria are still evolving, the classical definition is that actinobacteria are Gram-positive bacteria that are characterised by having a high G+C content in their genomic DNA (Stackebrandt *et al.*, 1997), which can be as high as 70% in some *Streptomyces* and *Frankia* species (Ventura *et al*., 2007). The distinctness of Actinobacteria from all other bacteria is strongly supported by the braching positions of 16S rRNA and 23S rRNA gene phylogenetic trees. In addition to these phylogenies, conserved protein (e.g. cytochrome-c oxidase I, CTP synthetase and glutamyl-tRNA synthetase) sequences also support the distinctness of Actinobacteria from all other bacteria (Zhi *et al*., 2009).

1.2 The Occurrence of Actinobacteria in Nature.

Actinobacteria are among the most widely distributed microorganisms in nature and constitute a significant proportion of the microbial population in most soils (Barakate *et al.*, 2002). Although they are primarily found in terrestrial habitats, they are widely distributed in a variety of other habitats such as compost, marine and fresh water habitats including river mud and lake sediments (Alexander, 1977). It has been argued that the actinobacteria isolated from water, particularly from marine sources, are in fact of terrestrial origin, as many species can produce resistant spores which may be transported from the land to sea and remain as dormant yet viable spores for many years (Mincer *et al.*, 2002). However, this hypothesis has been bought into question by the isolation of 'true' marine species including *Rhodococcus*

marinonascens (the first marine actinobacteria to be described) and several *Salinispora* species (Helmke and Weyland, 1984; Mincer *et al.,* 2002; Maldonado *et al.,* 2005). The genus *Salinispora* represents the first actinobacterial taxon to be isolated exclusively from the ocean. Extensive studies have been conducted on this genus and the findings suggest that *Salinispora* species are globally distributed in marine habitats (Mincer *et al.,* 2002; Maldonado *et al.,* 2005).

Actinobacteria have also been shown to be widespread in lacustrine environments (lakes, wetlands and deepwater habitats). Studies have shown that very low numbers of actinobacteria occur in the water column, while relatively high numbers have been recovered from lake sediments (Johnson and Cross, 1976). Consequently, it has been argued that actinobacteria make up a small proportion of the total bacteria in marine habitats and are more abundant in terrestrial environments (Takizawa *et al*., 1993).

While most actinobacterial species are free living, several species are opportunistic pathogens of humans, animals and plants. The genus *Mycobacterium* includes pathogens known to cause serious diseases in humans, including tuberculosis (*Mycobacterium tuberculosis)* and leprosy (*Mycobacterium leprae*). Other pathogenic species include *Corynebacterium diphtheria* which is responsible for diphtheria and *Actinomyces israelii* which is the primary cause of tooth decay in humans (Zaitlin and Susan 2006). Another, often serious, infection in humans is nocardiosis, which can affect either the lungs (pulmonary nocardiosis) or the whole body (systemic nocardiosis). It is commonly caused by either *Nocardia asteroides* or *Nocardia brasiliensis* (Zaitlin and Susan 2006). Fermentative actinobacteria of the genera *Actinomyces, Bifidobacterium* and *Propionibacterium* have also been shown to cause inflammations referred to as actinomycoses (Zaitlin and Susan 2006).

1.3 Life cycle of actinobacteria

Filamentous actinobacteria usually occur as either spores or small fragments of mycelium (Waksman, 1957). Under favourable growth conditions, germination occurs by the formation of branching threads or rods that develop into unicellular mycelia. The hyphae formed are usually nonseptate. Vegetative mycelia grow within the solid media substrate, whilst aerial mycelia protrude from the vegetative growth. Most actinobacteria reproduce either from special sporulating bodies such as in streptomycetes, or from the hyphal tips of the mycelium in the case of non sporulating genera such as *Dietzia*, *Gordonia, Mycobacterium*, *Nocardia* and *Rhodococcus* (Figure 1.1) (Waksman, 1957; Kalakoutskii and Agre 1976; Alexander, 1977).

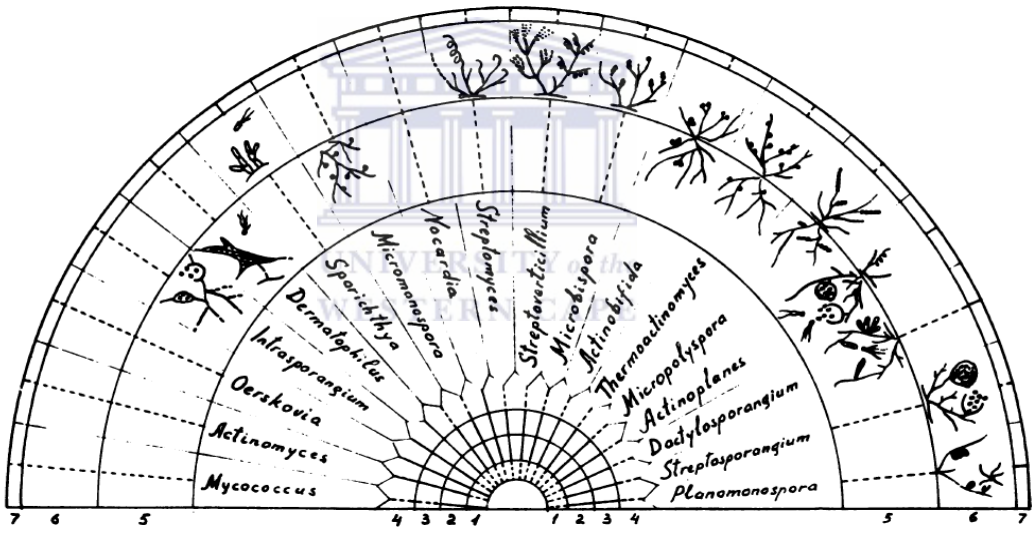

Figure 1.1: Reproduction cycles in selected representatives of the class Actinobacteria.
Numerals refer to certain stages in the life cycles. Solid lines denote the presence of the corresponding stages; dotted lines denote the absence of a stage. (1) Motile cells; (2) nonmotile cells; (3) mycelium; (4) fragmenting mycelium; (5) spores formed on substrate mycelium; (6) spores formed on aerial mycelium; (7) motile spores (figure from Kalakoutskii and Agre, 1976).

1.4 Actinobacterial Taxonomy

1.4.1 Morphological taxonomy

Actinobacteria exhibit a wide variety of morphologies including cocci (e.g. *Micrococcus*) or rod-cocci (e.g. *Arthrobacter*), fragmenting hyphal forms (e.g. *Nocardia*), as well as permanent

and highly differentiated branched mycelium (e.g. *Streptomyces*) and most of these morphologies form the basis of traditional actinobacterial taxonomy. Some of the morphological characteristics considered in actinobacterial taxonomy include the size, shape and colour of colonies on specific media, Gram stain, acid fastness, and the production of diffusible pigments. Other morphological features that are taxonomically important include the colour and morphology of the sporangia, as well as surface arrangement of the conidiospores (Figure 1. 2) (Shirling and Gottlieb, 1966).

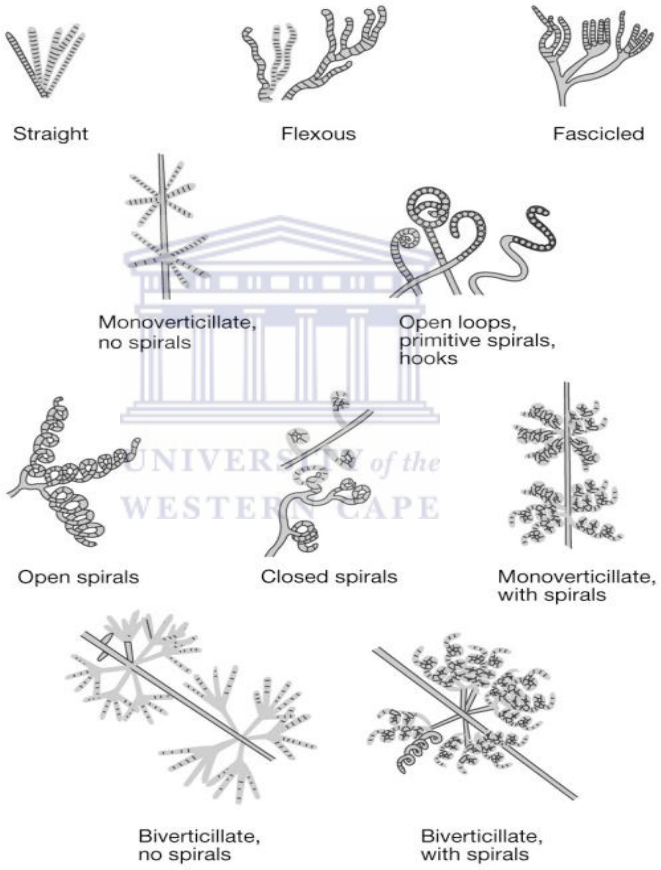

Figure 1.2: Different arrangement patterns of actinobacterial spore chains (Shirling and Gottlieb, 1966).

Physiological attributes such as nutritional requirements (sole carbon and nitrogen source), fermentation products (acid production), growth conditions (oxygen, temperature and inhibitory products) are also important when classifying actinobacteria (Nonomura, 1974).

1.4.2 Chemotaxonomy

Chemotaxonomy refers to the grouping of organisms according to cell chemistry including wall constituents, membrane and quinones (Zaitlin *et al.*, 2006). The composition of the cell wall varies greatly among different groups of actinobacteria. The isomer of diaminopimelic acid (DAP) present is one of the most important cell wall properties of Gram positive bacteria. The 2, 6−DAP is widely distributed as a key amino acid in cell walls and it has three optical isomers (Sasaki *et al.*, 1998). Bacteria generally contain either the LL− form or the *meso* - form, mostly located in the peptidoglycan. Four cell wall types can be distinguished according to three major features of the peptidoglycan composition and structure: i) the amino acid present in tetrapeptide side chain position 3; ii) presence of glycine in the interpeptide bridges; iii) peptidoglycan sugar content (Prescott *et al.*, 1999).

Chemotaxonomy also involves the analysis of other macromolecules such as isoprenoid quinones (e.g. menaquinones and ubiquinones), lipids (lipopolysaccharides and fatty acids including mycolic acids), polysaccharides and related polymers (e.g. methano chondruitin and wall sugars), and proteins (e.g. bacteriochlorophylls, whole organism protein patterns and enzymes) (Ward and Goodfellow, 2004). Although chemotaxonomy is still considered useful in actinobacterial taxonomy, it is not always reliable as several genera may exhibit similar chemical properties. For example, members of the genera *Actinomadura*, *Microbispora*, *Microtetraspora* and *Nonomuri*, cannot be distinguished from each other using chemotaxonomy as they exhibit highly similar chemotaxonomic characteristics (Wang *et al.*, 1999).

1.4.3 Nucleic acid analysis

The techniques used in chemotaxonomy are cumbersome and time consuming. Growth conditions including the media composition may also affect the results obtained, making it difficult to generate reproducible data. Due to these disadvantages bacterial taxonomy can not be based solely on phenotypic properties. This has spurred the development of molecular-based techniques, and recently molecular approaches have become a useful tool in actinobacterial taxonomy.

The molecular phylogenetic approach is useful in determining relatedness at levels ranging from kingdom to species. The comparison of DNA nucleotide sequences between two strains provides a rapid and accurate method for establishing relatedness. Techniques for carrying out the comparisons include DNA-DNA hybridization (whole genome comparison) and PCR-based gene sequence analysis (comparison of single/several gene sequences). The analysis of RNA for bacterial taxonomy focuses on the 16S (~1500 nucleotides) molecule. RNA is an important indicator of relatedness in bacteria because rRNAs are essential elements in protein synthesis and are therefore present in all living organisms (Priest and Austin, 1995). Other factors that make these molecules ideal for the analysis of evolutionary relationships are: i) the lateral/horizontal transfer of rRNAs between different organisms is extremely rare; ii) the longer rRNAs (16S, 18S and 23S) contain distinct regions which are either highly conserved, moderately variable or highly variable. The conserved regions are essential as they provide priming sites for PCR amplification, as well as convenient hybridization targets for the cloning of rRNA genes (Letowski *et al.*, 2004; Gentry *et al.*, 2006). Although 16S rRNA gene sequence analysis has served as a powerful tool for resolving phylogenetic relationships among bacteria, the molecule is too conserved to provide good resolution at the species and subspecies levels (Cho and Tiedje, 2001).

1.4.2.1 16S rRNA gene sequence analysis

16S rRNA is a major component of the small prokaryotic 30S ribosomal subunit, and plays an important role in subunit association and translational accuracy. The 16S rRNA gene, consisting of 1542 base pairs, is highly conserved among microorganisms and is therefore an excellent tool for studying phylogenetic relationships (Sacchi *et al.,* 2002).

The 16S rRNA genes of many phylogenetic groups have characteristic nucleotide sequences called oligonucleotide signatures, which are sequences that occur in most or all members of a particular phylogenetic group (Woese *et al.,* 1985). These oligonucleotide signatures can be used to design primers which are genus or species specific (Bavykin *et al.*, 2004).

The 16S rRNA gene has been extensively used for comparative sequencing studies to determine the taxonomic position of many microorganisms (Wang *et al.,* 1999). Although any one approach used to assess diversity cannot claim to be superior, some are more efficient and 16S rRNA gene sequence analysis allows for the assessment of a broader range of diversity than that obtained from physiological studies (Brambilla *et al.,* 2001).

The 16S rRNA gene can be analyzed by a number of methods which include amplified ribosomal DNA restriction analysis (ARDRA) (Cook and Meyers, 2003), restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and rep-DNA (Gutler and Mayall, 2001). An advantage of some of these PCR based methods is that the amplified DNA can either be sequenced directly or cloned into a phage or plasmid vector prior to sequencing. After the sequences have been generated they can be compared by aligning the corresponding nucleotide sites. This type of simple comparison of sequence positions will provide an estimate of how closely related the organisms are (Priest and Austin, 1995). Analysis of the

16S rRNA gene offers a time saving alternative to the classical methods of identification, such as chemotaxonomy (Alfaresi and Elkosh, 2006).

Despite the exponentially growing number of bacterial 16S rRNA gene sequences available in public databases, it is arguable whether the 16S rRNA gene should be considered to be the 'gold standard' for bacterial phylogeny (Vandamme *et al*., 1996). Some of the biggest drawbacks of employing the 16S rRNA gene for phylogenetic studies are that: (i) in many bacterial genomes the gene is present in multiple copies (Acinas *et al.*, 2004); (ii) in actinobacterial phylogenetics it has been shown to be insufficient in distinguishing between closely related species, notably species within certain *Streptomyces* clades (Liu *et al*., 2005; Guo *et al.*, 2008).

DNA-DNA hybridization (DDH) is one method that provides better resolution when defining species and strains (Stakebrandt and Goebel, 1994), and it is generally required in order to define a novel bacterial species (Garrity and Holt, 2001). DDH measures the degree of similarity between the genomes of different species and is therefore useful for delineating novel species, and for the definitive assignment of a strain with ambiguous phenotypic properties to the correct taxonomic group. However, it has now been argued that DDH has several disadvantages including the high cost because of the need for pair wise cross-hybridizations and the requirement for isotopic or fluorescent dye labelling. In additions, the method it is labour-intensive, results are often not reproducible between laboratories and it is difficult to establish a central database as results are not comparable (Vandamme *et al.*, 1996; Cho and Tiedje, 2001; Coenye *et al.*, 2005).

1.4.2.2 Multilocus sequence analysis (MLSA)

Although DDH cannot be replaced in species delineation, multilocus sequence analysis (MLSA) has been proposed as a more accessible tool for assessing the phylogeny and taxonomy of prokaryotes (Brett *et al.*, 1998; Maiden *et al.*, 1998; Godoy *et al.*, 2003; Cooper and Feil, 2004) in instances where DDH is not possible. MLSA is an unambiguous procedure which characterises bacterial isolates using the sequence of internal fragments of (usually) seven house-keeping genes. A short 450-500 bp internal fragment of each gene is used. Small fragments are used in MLSA as a full length sequence can be obtained with a single Sanger sequencing reaction (Maiden *et al*., 1998). MLSA is fast becoming a useful tool in general actinobacterial taxonomy and the approach has been applied in the study of taxonomic relationships in a number of genera such as *Streptomyces* (Guo *et al.*, 2008; Mignard and Flandrois, 2008) and *Ensifer* (Naser *et al*., 2006; Martens *et al.*, 2007).

House keeping genes that are present as a single copy in a bacterial genome can be used in MLSA. An ideal candidate housekeeping gene should typically be a gene that is constitutively expressed, is required for the maintenance of basic cellular function and is found in all members of a taxonomic group. Some of the house keeping genes that have been used in actinobacterial taxonomy include *atpD, gyrB, recA, rpoB* and *trpB* (Naser *et al.*, 2006; Martens *et al.*, 2007; Guo *et al.*, 2008; Mignard and Flandrois, 2008; Young *et al.*, 2008)

The *gyrB* gene is a single-copy housekeeping gene present in all bacteria and encodes the B-protein subunit of DNA gyrase (Yamamoto and Harayama, 1995; Kasai *et al.*, 2000a, b; Kakinuma *et al.*, 2003; Soler *et al.*, 2004). Bacterial DNA gyrase and topoisomerase IV (topo IV) are type II topoisomerases, which work together in controlling the topological state of bacterial DNA (Ullsperger and Cozzarelli, 1996; Maxwell and Lawson, 2003), relieving

supercoiling by creating nicks in the double helix, introducing negative supercoils into DNA as well as interconverting other topologically isomeric DNA structures, including knotted rings and catenanes (Fukushima *et al*., 2002). The *gyrB* gene has already proved to be a useful tool in phylogenetic studies of several complex taxa such as *Escherichia coli, Micromonospora* (Kasai *et al.,* 2000 a; b), *Mycobacteria, Pseudomonas* (Yamamoto and Harayama, 1995), *Salmonella* and *Shigella* (Fukushima *et al*., 2002).

The *recA* gene, which codes for a DNA repair protein is another housekeeping gene which has been used for phylogenetic studies (Naser *et al.,* 2006; Martens *et al.,* 2007; Guo *et al.,* 2008; Mignard and Flandrois, 2008; Young *et al.,* 2008). RecA has multiple functions, all related to DNA repair. In the bacterial SOS response, it has a co-protease function in the autocatalytic cleavage of the LexA repressor and the λ repressor. The *recA* gene is activated following DNA damage to a form that is able to mediate cleavage of the *lexA* gene product (Bridges and Woodgate, 1985).

The *rpoB* gene has also been used as a potential gene candidate for phylogenetic analyses and the identification of bacteria, especially when studying closely related isolates. The gene codes for RpoB, the β-subunit of the bacterial RNA polymerase, a crucial enzyme in the transcriptional process and is the final target for regulatory pathways controlling gene expression in all living organisms (Borukhov and Nudler, 2003). Morse and collaborators (1996) first reported that the *rpoB* gene was universal in bacteria (Morse *et al*., 1996). The robustness of *rpoB* gene-sequence analyses in phylogenetic studies has been confirmed by the observation that when used in MLSA, phylogenetic trees derived from *rpoB* gene sequences support the trees derived from 16S rRNA gene sequences, with higher bootstrap values and longer branch lengths (Adekambi and Drancourt, 2004; La Scola *et al*., 2006)

Another gene which is a possible candidate for MLSA is the *trpB* gene (Naser *et al*., 2006; Martens *et al*., 2007; Guo *et al*., 2008; Mignard and Flandrois, 2008; Young *et al*., 2008). In many bacteria the *trpB* gene is part of the trp operon which contains five structural genes *trpE, trpD, trpC, trpB*, and *trpA* (Kishan and Hillen, 1990) (Figure 1. 3).

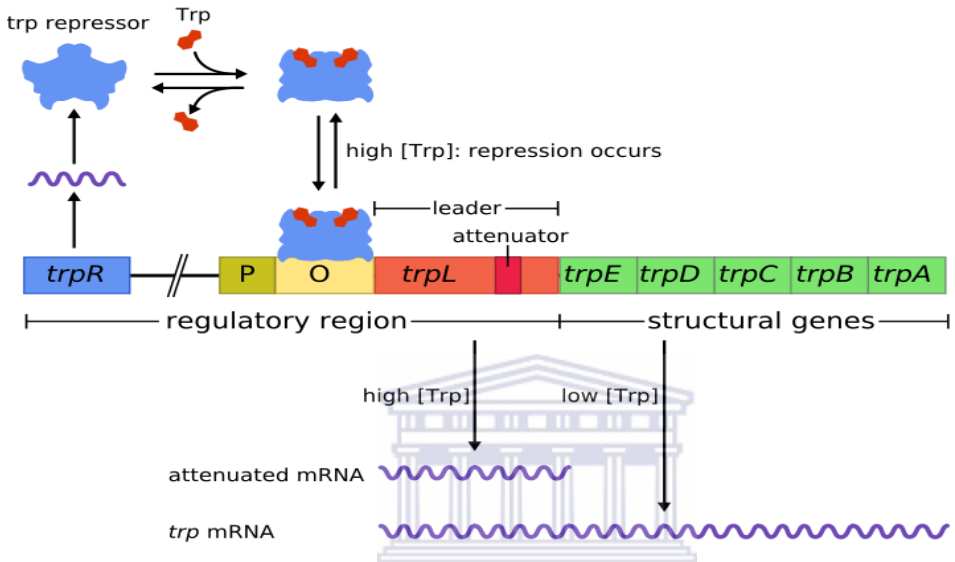

Figure 1.3 Diagrammatic presentation of the trp operon (figure from http://en.wikipedia.org/wiki/File:Trpoperon.svg).

The *trpB* encodes the tryptophan synthase β-subunit. Tryptophan synthase catalyzes the final two steps in the biosynthesis of tryptophan and converts indoleglycerol phosphate to tryptophan. The enzyme contains two functional domains, tryptophan synthase α, for the conversion of indole glycerol phosphate to indole, and tryptophan synthase β, for the conversion of indole to tryptophan (Berlyn *et al.*, 1989).

Another gene routinely used in MLSA is the *atpD* gene which encodes the minor subunit δ of ATP synthase, which catalyses the synthesis of ATP. The ATP synthase enzyme has been remarkably conserved throughout evolution, and shows great structural and functional homology between bacterial enzymes and those from the mitochondria of animals, plants and

fungi, as well as plant chloroplasts (Boyer, 1997). ATP synthase is composed of two regions. The membrane bound F_O, which has three subunits A, B and C, and is involved in proton translocation; and a soluble F_1 which has five subunits, $\alpha 3$, $\beta 3$, λ, δ and ε, which catalyzes ATP hydrolysis. While the α– and β-subunits of ATP synthase enzymes from various sources show strong sequence homology, the minor F_1-ATPase subunits shows more sequence and size variation (Boyer, 1997). For this reason the gene coding for the minor subunit δ is used in MLSA.

1.5 Rare actinobacteria

Streptomyces species are the most abundant actinobacteria in soil and conventional isolation techniques tend to favour their isolation (Seong *et al.*, 2001). Although streptomycetes provide more than half of the naturally occurring antimicrobial compounds discovered to date (Tanaka and Omura, 1990), and continue to be a major source of new bioactive metabolites (Miyadoh, 1993), the rate of discovery of novel secondary metabolites from this genus has declined (Kurtboke and Wildman, 1998). The focus of industrial screening has therefore shifted to the less exploited genera of actinobacteria including the "rare" genera. Typically rare genera are less frequently isolated by conventional isolation methods and include the genera *Actinomadura, Actinoplanes, Dactylosporangium, Kibdelosporangium, Microbispora, Planobispora, Streptosporangium, Planomonospora* and *Verrucosispora*, and to a lesser extent the genera *Amycolatopsis* and *Micromonospora* (Lazzarini *et al.*, 2000).

Several methods have been developed for isolating rare actinobacterial genera from natural habitats (Nonomura and Hayakawa, 1988; Hayakawa, 2003). These methods include a variety of pretreatment and enrichment techniques that are selective for specific rare genera (Table 1.1).

1.5.1 Selective isolation

Actinobacteria, especially the rare genera, grow slowly and hence are at a competitive disadvantage when grown on agar media in association with other soil microorganisms. In order to favour the cultivation of desired actinobacterial genera, selective media have been developed (Table1.1) which limits the growth of competing bacteria, without adversely affecting the growth of desired propagules (Cross, 1982; Williams and Wellington, 1982; Goodfellow and Simpson, 1987; Goodfellow and O'Donnel 1989; Atlas, 1997). Specific compositions of media listed in Table 1.1 are found in Table A1 in the appendix.

Table 1.1 Summary of the selective isolation techniques developed for the isolation of rare actinobacteria from soil (Kurtboke *et al.*, 1992).

Pre-treatment	*Media*	*Genera selected*
Physical:		
Dry heat at 120°C for 1h	AV, MC, MGA-SE agar or salts-starch agar-V with cycloheximide, nystatin, polymixin B and penicillin	*Actinomadura, Kutzneria, Microbispora, Nonomuraea, Saccharomonospora, Streptosporangium, Thermobifida*
Physical and enrichment:		
Dry heat at 120°C for 1h and specific media	SE agar or soil-extract agar with cycloheximide, nystatin, polymixin B and penicillin	*Actinomadura, Microbispora, Microtetraspora, Streptosporangium*
Chemical:		
Phenol, 1.5%	HV agar with nalidixic acid and tunicamycin	*Micromonospora*
Physical and chemical:		
Dry heat at 110°C for 1h and phenol, 1.0%	HV agar with kanamycin, josamycin, lysozyme and nalidixic acid	*Actinomadura (viridis)*
Dry heat at 120°C for 1 h and phenol, 1.5% - Chlorhexidine gluconate 0.01%	HV agar with nalidixic acid	*Microbispora*
Enrichment:		
Chemotaxis (γ-collidine, vanillin)	HV agar with nalidixic acid	*Actinoplanes, Cetenuloplanes, Dactylosporangium,Virgosporangium*
$CaCO_3$, rehydration and centrifugation	HV agar with fradiomycin, kanamycin, nalidixic acid and trimethoprim	*Geodermatophilus, Kineosporia, Sporichthya Actinokineospora*

For composition of media listed in Table 1.1, see Appendix (i)

1.6 Importance of actinobacteria

1.6.1 The role of actinobacteria in the environment

Actinobacteria have many roles in the environment. Streptomycetes are saprophytic bacteria that decompose organic matter, especially complex polymers such as chitin, hemicelluloses, keratin, lignocelluloses, natural rubber, pectin, starch and even some man-made compounds that may reach soil as contaminants (Goodfellow and Williams, 1983; Crawford *et al.*, 1993). Actinobacteria are also important in the rhizosphere, where they may influence plant growth and protect plant roots against invasion by pathogenic fungi (Goodfellow and Williams, 1983). The potential role of actinobacteria as biological control agents of soil-borne root diseases in crop plants has been investigated, predominantly in greenhouse experiments, and several *Streptomyces* species (as well as a few other actinomycete genera) have been shown to protect different plant species against soil borne fungal pathogens. Some genera have also been shown to produce herbicidal and insecticidal compounds (Crawford *et al.*, 1993). Similarly, members of the actinobacterial genus *Frankia* can fix nitrogen. They have a broad host range and have been shown to form root nodule symbioses with more than 200 species of flowering plants (Clawson *et al*., 2004).

1.6.2 Secondary metabolite production

Secondary metabolites are organic compounds that are not thought to be directly involved in normal growth, development or reproduction of the producing organism (Martin *et al.*, 2005). Microbial secondary metabolites have applications as antibiotics, pigments, toxins, enzymes and antitumor agents. It is estimated that approximately 7000 compounds reported in the dictionary of natural products are actinobacterial secondary metabolites (Jensen *et al*., 2005). Therefore, actinobacteria are of great interest to industry because of their ability to produce important secondary metabolites. The genus *Streptomyces* is the largest antibiotic-producing genus, accounting for approximately 80% of the actinobacterial derived natural products

(Goodfellow and Fiedler, 2010)). Although thousands of antibiotics have been described, these are thought to represent only a small fraction of the repertoire of bioactive compounds that members of the genus *Streptomyces* are able to produce (Watve *et al.*, 2001). However, it is becoming increasingly difficult to find new metabolites from common actinobacteria as most screening efforts lead to the costly rediscovery of known compounds. Attempts to address this problem include the selective isolation and screening of rare actinobacteria (Goodfellow and Fiedler, 2010). The discovery of the aminoglycoside gentamicin produced by *Micromonospora purpurea* and *Micromonospora echinospora* stimulated interest in screening non-streptomycete actinobacterial genera for novel antibiotics (Abou-Zeid *et al.*, 1974; 1978). These screening campaigns were fruitful and a vast array of antibacterial compounds has been isolated from non-streptomycete species. Several *Actinomadura* and *Amycolatopsis* species have been found to produce vancomycin-type glycopeptides. Macrolactam and napthacene-quinone antibiotics have been isolated from *Actinomadura* species, whilst *Micromonospora* and *Saccharopolyspora* strains have been found to produce a number of macrolide-type antibiotics (Moncheva *et al.*, 2002). Apart from antibiotics, actinobacteria also produce other economically important compounds including vitamins, immunomodulators and enzymes which are widely used in industry as biocatalyst (Moncheva *et al.*, 2002).

1.7 Biocatalysts

Biocatalysis can be broadly defined as the use of biological molecules (usually enzymes) to catalyse specific chemical reactions. Enzymes have been used as biocatalysts for thousands of years to produce food and beverages, such as cheese, yoghurt, beer and wine, but these processes relied mostly on the use of enzymes produced by spontaneously growing microorganisms. The production of enzymes in fermentative processes has made the large scale manufacture of purified enzymes possible, thereby making biocatalysis available to

chemical and pharmaceutical industries especially for the use in hydrolytic and isomerisation reactions (Schmid *et al.*, 2001). In recent years the most significant development in the chemical industry has been the application of biological systems to chemical reactions (Thomas *et al.*, 2002). Reactions catalyzed by enzymes or enzyme systems often display far greater specificities than more conventional forms of organic reactions. Examples include the use of acylases, hydantoinases, and aminopeptidases in the production of optically pure amino acids, as well as the enzymatic production of acrylamide from acrylonitrile by nitrile hydratases (Figure 1.4) (Schoemaker *et al*., 2003).

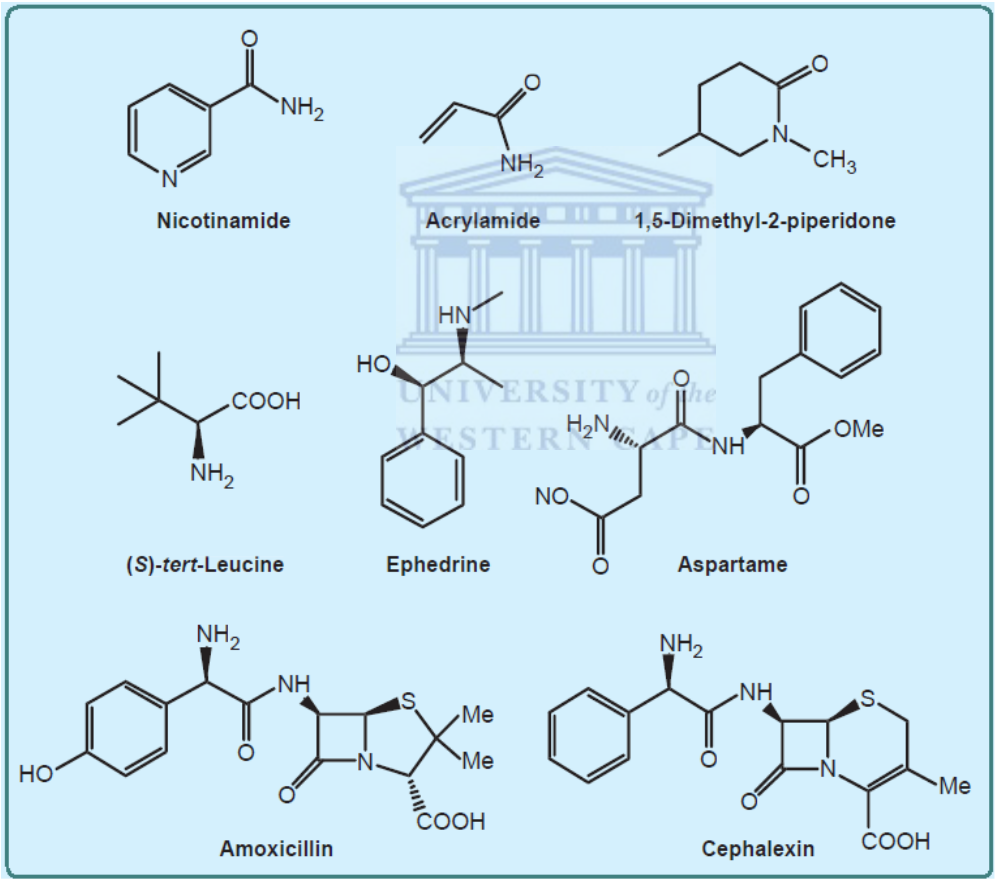

Figure 1.4: Examples of molecules that are manufactured using biocatalysis (Schoemaker *et al.*, 2003).

The use of recombinant gene technology has further improved manufacturing processes and enabled the commercialization of enzymes that could previously not be produced. These advances have made it possible to provide tailor-made enzymes that display new activities or

are adapted to new process conditions (Kirk *et al*., 2002). While the industrial use of enzymes for the production of existing products has rapidly developed, novel enzymes are still required to catalyze several key industrial processes. Microorganisms are still the most prolific source of industrial enzymes and thus screening bacterial isolates for novel enzymes is warranted.

1.7.1 Thermostable biocatalysts

One major disadvantage in the use of biocatalysts is that they are labile and their thermostability can be a limiting factor in biotechnological applications (Illanes, 1999). Thermostable biocatalysts potentially have advantages over mesophilic homologues because they allow for higher operational temperatures, which in turn result in higher reactivies (higher reaction rates and lower diffusional restrictions), higher process yield (increased solubility of substrates and products, and favourable equilibrium displacement in endothermic reactions), lower viscosities and possibly reduced contamination threat (Cowan, 1992; Illanes, 1999; Daniel and Cowan, 2000). Thermostable enzymes can be obtained from different sources including thermophilic microorganisms. A "thermophile" can be defined as an organism that thrives at relatively high temperatures, between 45 and 120°C. A fundamental requirement for survival at such high temperatures is macromolecular stability, and it is therefore reasonable to assume that thermophiles represent an obvious source of thermostable enzymes (Cowan, 1992)

1.8 Biocatalysis and the production of optically pure amino acids

1.8.1 The importance of optically pure amino acids

Natural living systems, such as cells, are intrinsically chiral and as such stereochemistry is a characteristic feature of enzymatic reactions, messenger-receptor interactions and metabolic

processes. Consequently, metabolic and regulatory systems within an organism are sensitive to interactions with chiral compounds and often display different responses to the action of each chiral form of a pair of enantiomers. As such, the stereochemistry of the compounds that are used as xenobiotics, including agrochemicals, food additives, pharmaceuticals, flavourings and fragrances, must be taken into account (Maier *et al*., 2001). Although two enantiomers of an amino acid are identical in most chemical and physical properties, in a chiral environment such as the human body, the enantiomers may display different biological activities (Martens and Bhushan, 1989). For example, the treatment of Parkinson's disease requires L-DOPA (3,4-dihydroxyphenylalanine), whereas the D- form is ineffective. Similarly, the antibiotic D-penicillamine exhibits antimicrobial activity *in vivo*, whereas the L-isomer is toxic (Martens and Bhushan, 1989). Consequently, the pharmaceutical industry requires optically pure amino acids. Examples of optically pure amino acids used in the pharmaceutical industry are presented in Table 1.2.

Table 1.2 Examples of optically pure amino acids and their applications in industry

Amino acid	Industrial application
D-phenylglycine	Cefaclor (antibiotic)
D-hydroxyphenylglycine	Amoxicillin (antibiotic), Cephadroxil (cephalosporins)
D-valine	Fluvanate (pyrethroid insecticide)
D-cysteine	Beta-lactam antibiotics
D-aspartic acid	Beta-lactam antibiotics
D-alanine	Synthetic sweetner
L-homophenylalanine	Benazepril, Lisinopril, Quinapril (angiotensin converting enzyme inhibitor)
L-tert-leucine	Sandoz (antiviral), Abbott (HIV protease inhibitor), Biomega (antiviral), BB-2516 (antitumour), RO-31-9790 (anti-inflammatory), Zeneca (antitumour), Marimastat (anticancer), Captopril (cardiovascular),
L-valine	Valaciclovir (reverse-transcriptase inhibitor)
L-leucine	Ubestatin, Bestatin (immunostimulant), Orlistat (hypolipidemic)
L-methionine	Dorcarpamine Tanadopa (cardiovascular antiallergenic)
Ademethionine	Gumbaral (antiarthritic)

(Bommarius *et al.*, 1998; Yagasaki and Ozaki, 1996; Schulze and Wubbolts, 1999; Drauz, 1997; Liese and Filho, 1999; Hu *et al*., 2003).

One of the methods used by industry to produce optically pure amino acids for pharmaceutical applications is the chemical hydrolysis of hydantoin in a three step process which involves the chemical synthesis of substituted hydantoin substrates, followed by the stereospecific hydrolysis of hydantoins catalysed by hydantoinases, and finally a chemical decarbamoylation (Pozo *et al.*, 2002). This process has several disadvantages which prohibit the large-scale

production of amino acids, including the toxicity of the starting reactants, poor mass yields of the products, and the large amount of energy required for the process (Yamashiro *et al.,* 1988). The use of a fully enzymatic process have numerous advantages compared to chemical processes including higher product yields, higher optical purity and lower environmental impact (Triphathi *et al.,* 2000) as the reaction occurs under mild reaction conditions, which can result in cheaper industrial processes (Polastro, 1989; Hartley *et al*., 1998).

1.9 Hydantoinases

Hydantoinases (dihydropyrimidinase; EC 3.5.2.2) are a diverse group of enantioselective cyclic amidase enzymes which catalyse the opening of the dihydropyrimidine ring and several 5-monosubstituted hydantoins to give N-carbamyl amino acids (Morin *et al.,* 1987). This is the first step in a two step reaction process for the synthesis of D- and L-amino acids.

Interest in hydantoinases as industrial enzymes for the synthesis of D-amino acids started in the 1980s with the discovery of the novel enzyme N-carbamoyl amino acid amidohydrolase (NCAAH) (Olivieri *et al*., 1981). This class of enzymes is now widely used for the industrial production of optically pure amino acids in a two-step enzymatic process (Figure 1.5) involving the hydrolysis of the hydantoin derivative (compound A, Figure 1.5) by an hydantoinase enzyme, and the further hydrolysis of the intermediate N-carbamyl amino acid (compound B) by an N-carbamoylase (Olivieri *et al.,* 1981; Möller *et al*., 1988; Kim and Kim, 1993; Ogawa *et al.,* 1994, Ikenaka *et al*., 1998, May *et al.,* 1998).

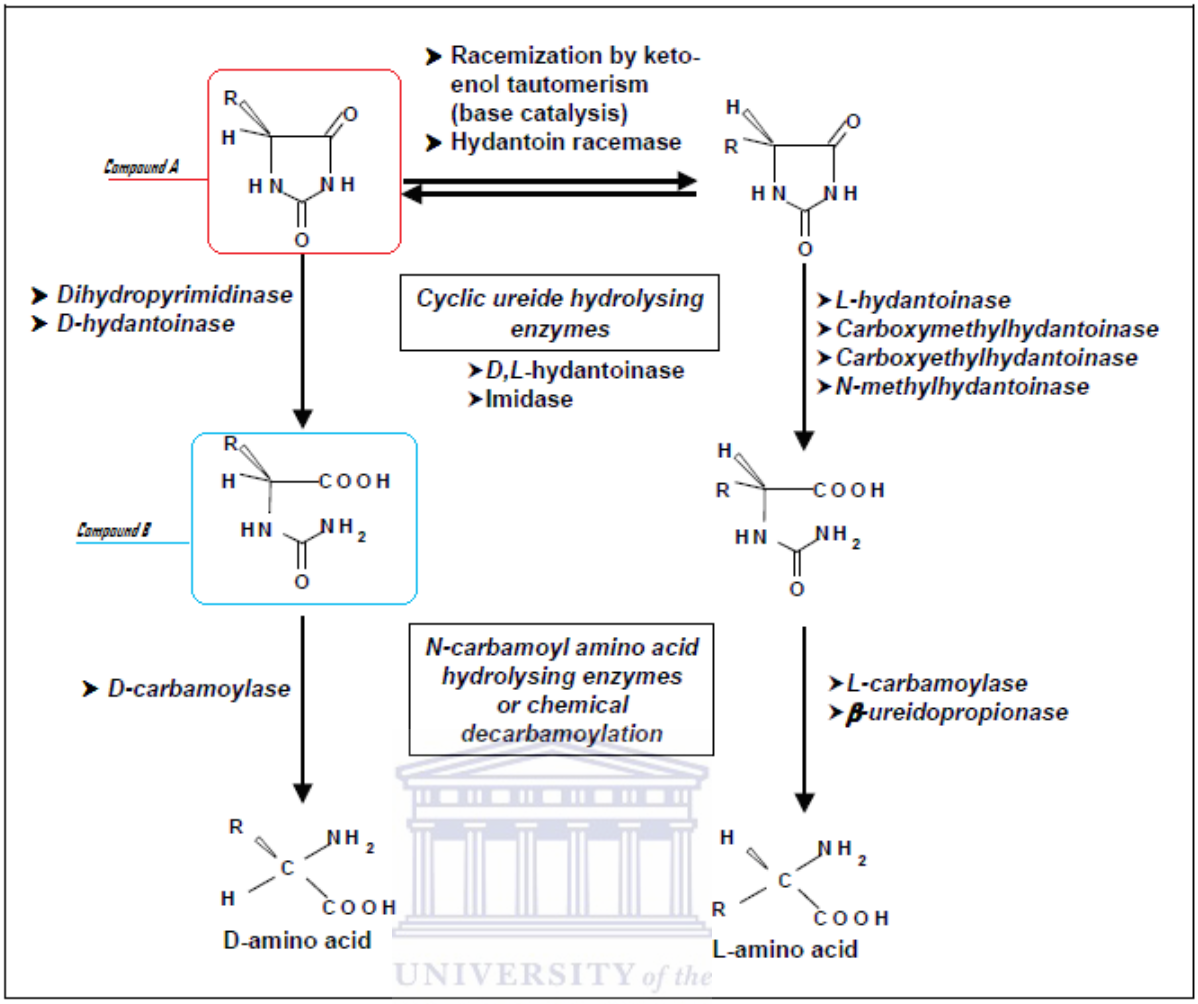

Figure 1.5: Enzymatic hydrolysis of hydantoin to form enantiomerically pure amino acids (modified from Ogawa and Shimizu, 1997).

Hydantoinase stereospecificity may be D-, L-, or non-selective (Siemann *et al.*, 1999). The L- and the non-selective enzymes can be further sub-divided into two groups based on their requirement for ATP for hydrolysis (Figure 1.6) (Hou, 2005).

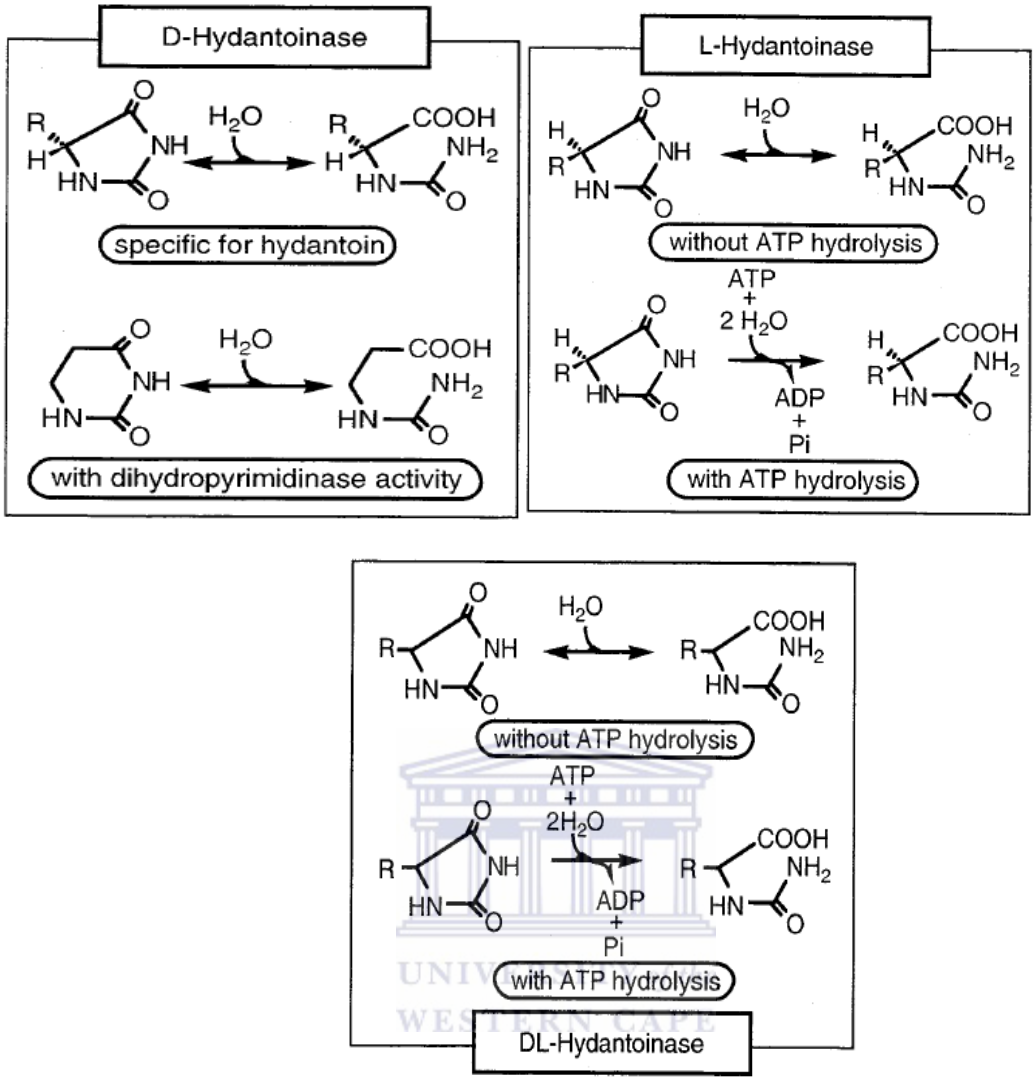

Figure 1.6: Reactions catalysed by typical hydantoinases in the production of amino acids (Syldatk *et al.*, 1990).

1.9.1 D- hydantoinases

The D-specific enzymes are the most widely distributed in nature, having been isolated from several microbial, plant and animal sources (Syldatk *et al*., 1990). These enzymes are often considered synonymous with dihydropyrimidinases (Syldatk *et al.*, 1999) as inducible catabolic enzymes involved in the degradation of pyrimidine nucleotides (Syldatk *et al.*, 1990; Siemann *et al.*, 1999). This suggests that the natural substrates for hydantoinase are 5,6-dihydrouracil and 5,6-dihydrothymine (Siemann *et al.*, 1999). This is due to the finding that dihydropyrimidinases were capable of hydrolysing 5-monsubstituted hydantoin derivatives as

well as their natural substrate dihydropyrimidines (Xu and West, 1994). Similarly, several D-hydantoinases are able to hydrolyse dihydrouracil as a substrate (Durham and Weber, 1995; Lee *et al.*, 1995; Ogawa *et al*., 1995; Sharma and Vohra, 1997, Siemann *et al*., 1999; Sudge *et al*., 1998). However, it has been shown that in some microorganisms the hydantoin-hydrolysing activity is distinct from the pyrimidine hydrolysing activity. Runser and Ohleyer (1990) reported that the substrate dihydrouracil was poorly hydrolysed by the D-hydantoinase of an *Agrobacterium* species. Runser and Meyer (1993) also reported that when dihydrouracil was used as a substrate for resting *Agrobacterium* strain IP I-671 cells, the dihydropyrimidinase activity disappeared on heating, while the hydantoinase activity remained, suggesting that the hydantoinase and dihydropyrimidinase activities observed in this bacterium were catalysed by different enzymes (Runser and Ohleyer, 1990; Runser and Meyer, 1993).

Thus, while the names D-hydantoinase and dihydropyrimidinase are often used interchangeably, these are in fact two separate enzymes with different catalytic capabilities. D-hydantoinase enzymes from a wide variety of bacterial species have been described in literature. With the exception of the D-hydantoinases from *Bacillus stearothermophilus* SD-1 and *Burkholderia pickettii* which are dimeric in structure, most D-hydantoinases analysed were found to form tetramers (Lee *et al*., 1995; Xu *et al*., 2003a).

In all instances where metal ion dependency has been investigated, D-hydantoinases have been observed to require divalent metal ions as co-factors. Consequently, these enzymes are sensitive to metal ion chelators and are stimulated by metal ions such as Mn^{2+} and $Co^{2+.}$ This metal ion dependency was verified by crystallographic analysis of D-hydantoinases from a *Thermus* species. (Abendroth *et al.,* 2002a), *Burkholderia pickettii* (Xu *et al.,* 2003a) and

Bacillus stearothermophilus SD-1 (Cheon *et al.* 2002) in which two metal ions were identified within the catalytic site. In most cases, the preferred co-factor was found to be Zn^{2+} which could be replaced by other divalent transition group metals such as Mn^{2+} or Co^{2+} (Abendroth *et al*., 2002a).

In industry, D-hydantoinases (together with N-carbamoyl-D-amino acid hydrolases) are used in the production of enantiomerically pure D-amino acids which are important building blocks for a variety of biologically active pharmaceuticals like peptides, semisynthetic β-1actam antibiotics (such as ampicillin and amoxicillin), pesticides, hormones, ACE inhibitors as well as nutritional supplements (Keil *et al.*, 1995; Burton *et al.*, 1998; Pozo *et al.*, 2002; Durr *et al.*, 2006).

L-specific hydantoinases also have commercial value and L-amino acids, such as L-methionine, L-leucine, L-proline and L-valine, are used as precursors for the synthesis of antibiotics, cardiovascular drugs and a number of compounds with antiviral, anti-inflammatory and antitumor activities (Pozo *et al.*, 2002).

1.9.2 Regulation of hydantoinase activity in bacterial cells

Several microbial strains belonging to the genera *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Ochrobactrum* and *Pseudomonas* are known to express both a hydantoinase and a carbamylase (Pozo *et al*., 2002). The natural overproduction of D-hydantoinases may be detrimental to the cell because the enzyme hydrolyses dihydropyrimidines which are required for cellular functions. In order to protect cellular functions, microorganisms possess regulatory mechanisms to control the expression of metabolic enzymes (Sanchez and Demain, 2002). For research and industrial applications, deregulation of these mechanisms by classical and genetic

manipulations is required so that the desired enzyme can be over-produced (Sanchez and Demain, 2002).

1.9.3 Induction of hydantoinase activity

Induction ensures that the enzymes are only produced when a suitable substrate is available. In *Agrobacterium tumefaciens,* a wide range of hydantoin and pyrimidine derivatives have been shown to induce expression of hydantoin-hydrolysing enzymes with 5- to 30-fold increases in enzyme production compared to uninduced cells (Hartley *et al.*, 1998; Wiese *et al.*, 2001). Table 1.3 summarises the induction of hydantoinases. Conversely, three microbial species (*Agrobacterium tumefaciens* NRRL B11291, *Pseudomonas fluorescens* DSM84 and *Pseudomonas* species NCIM 5109) are known to constituitively express D-hydantoinases (Morin *et al.* 1986; Deepa *et al*. 1993; Sudge *et al*., 1998).

Induction studies in *Arthrobacter aurescens* DSM 3747 showed a 30-fold increase in hydantoinase activity when N-3-CH_3-IMH was supplied in the growth medium as an inducer (Wiese *et al*., 2001). Similar results were obtained in *Agrobacterium tumefaciens* RU-OR, in which the use of the non-metabolizable 2-thiouracil as an inducer resulted in a 5-fold increase in hydantoinase production (Hartley *et al.,* 1998).

Table 1.3 Inducible hydantoinase activity in several bacterial isolates.

Isolate	Inducer	Enzyme affected	Reference
Agrobacterium tumefaciens RU-OR	2-thiouracil	D-hydantoinase D-carbamoylase	Hartley *et al*. 1998
Arthrobacter aurescens DSM 3747	DL-5-(3-indolylmethyl)-3-*N*-methylhydantoin	Hydantoinase L-carbamoylase	Wiese *et al*. 2001
Arthrobacter crystallopoietes AM2	Hydantoin DL-5-hydroxymethylhydantoin	D-hydantoinase D-carbamoylase	Moller *et al*. 1988
Arthrobacter sp. BH20	DL-5-indolylmethylhydantoin	Hydantoinase	Syldatk *et al.* 1987
Bacillus brevis AJ-12299	DL-5-isopropylhydantoin	D-hydantoinase	Yamashiro *et al*. 1988
Flavobacterium sp.	I-3 DL-5-indolylmethylhydantoin	Hydantoinase L-carbamoylase	Nishida *et al*. 1987
Pseudomonas putida RU-KM1	Hydantoin	Hydantoinase D-carbamoylase	Burton *et al*. 1998
Pseudomonas putida RU-KM3S	Hydantoin	Hydantoinase L-carbamoylase	Burton *et al*. 1998
Pseudomonas sp. AJ-11220	DL-5-methylthioethylhydantoin	D-hydantoinase D-carbamoylase	Yokozeki *et al.*1987

It has also been reported that a substitution at the chiral centre of an inducer may affect its efficiency. Runser and Meyer (1993) showed that the hydantoinase of *Agrobacterium tumefaciens* strain 47C was induced to a greater extent by *p*-hydroxyphenylhydantoin than by hydantoin. However, when Möller *et al.* (1988) investigated the effect of the different hydantoin derivatives as inducers of *Arthrobacter crystallopoietes* hydantoinase they found that maximum D,L-5-methylhydantoin transformation was achieved when hydantoin was used as the inducer (Möller *et al.,* 1988).

1.9.4 Catabolite repression

Catabolite repression is a regulatory mechanism which ensures that nutrients are utilised in an organised and sequential manner. This is usually achieved through the inhibition of all enzymes involved in the catabolism of one nutrient source in favour of those of an alternate source. Hydantoin derivatives are poor nitrogen- and carbon sources, and the expression of hydantoin-hydrolysing genes are likely to be subject to carbon and/or nitrogen catabolic repression mechanisms. Studies have shown that supplementation of the growth medium with various carbon and nitrogen sources affects the production of both hydantoinase and N-carbamoylase (Morin *et al.,* 1986; Nishida et al., 1987; Yamashiro *et al.,* 1988; Ishikawa *et al.,* 1994; Sudge *et al.,* 1998; Syldatk *et al.,* 1990). This may account for the fact that the production of hydantoinase and N-carbamoylase enzymes in microorganisms occurs only in late logarithmic to stationary growth phase (Nishida *et al.,* 1987; Syldatk *et al.,* 1990; Gokhale *et al*., 1996; Hartley *et al.,* 1998; Sudge *et al*., 1998).

1.9.5 Cloning the genes coding for hydantoinase

The molecular cloning of hydantoinase genes is an important objective. In industrial applications, a recombinant strain producing large amounts of enantioselective hydantoinases would decrease the production costs of optically pure amino acids (Chien *et al*., 1998). From an enzyme engineering perspective, a comparative study of enzymes that catalyze the same type of reactions, but have different substrate and stereospecificities is a useful approach to understanding the molecular mechanism of the enzyme reaction and the evolutionary relationships between the enzymes (Kim *et al.,* 1997). This knowledge would in turn aid in the engineering of an enzyme to invert enantioselectivity, increase total enzyme activity, broaden substrate specificity and improve thermostability of the biocatalyst (Lee *et al.,* 1996b; Lee *et al.,* 2009).

The first hydantoin-hydrolysing genes were cloned from *Pseudomonas* sp. NS671 by Watabe and co-workers, who cloned the hydantoinase, the N-carbamoylase and the racemase from this organism (Watabe *et al.*, 1992). To date several other hydantoinases that exhibit both the D- and L-stereospecificities have been cloned. Many of these genes have been sequenced and modified. Some of the modifications include the fusion of hydantoinase and N-carbamoylase genes to other proteins in order to facilitate purification and immobilisation (Kim *et al.*, 2000a; Pietszch *et al.*, 2000).

The activity of the hydantoinase enzyme from *Pseudomonas putida* CCRC 12857 was found to be 20-fold higher when the gene was cloned into *Escherichia coli* compared to the native wild type enzyme in *P. putida* (Chien *et al.*, 1998). When the gene coding for the thermostable D-hydantoinase from the thermophilic bacterium *Bacillus stearothermophilus* SD1 was cloned and overexpressed in *E. coli*, the recombinant strain showed a 30-fold increase in enzyme activity compared to the wild-type (Lee *et al.*, 1996a).

Genes coding for hydantoinases from several bacterial sources have been successfully cloned and expressed in *E. coli.* For example, when the D-hydantoinase from *B. stearothermophilus* was expressed in *E. coli* under its own promoter specific activities 30 times higher than that of the wild type strain were observed (Lee *et al.*, 1996b). The heterologous expression of the gene coding for the D-hydantoinase from *Agrobacterium radiobacter*, under the control of its own promoter, resulted in the conversion of D,L-hydroxyphenylhydantoin to D-hydroxyphenylglycine with a conversion yield of 97% and a productivity five times higher than that obtained from the gene donor strain (Chao *et al*,. 1999).

The expression of hydantoinases in *E. coli* is, however, not always successful. Several studies have reported that the increased levels of expression of hydantoinase in *E. coli* resulted in the formation of insoluble aggregates of inactive proteins (inclusion bodies) due to incorrectly folded proteins (Buson *et al.,* 1996; Chien *et al.,* 1998; Baneyx, 1999; Chao et al., 2000; Hils *et al*., 2001; Wilms *et al.,* 2001b). In addition, growth rates were also retarded, which may be attributed to the unregulated degradation of pyrimidines in the cell by the overproduced hydantoinases (Mukohara *et al.,* 1994; Chien *et al*., 1998).

1.9.6 Hydantoinase structures

Analysis of the crystal structures of hydantoinases can shed light on the catalytic properties and reaction mechanisms, as well as factors affecting substrate specificity, enantioselectivity and thermostability of the enzymes (Cheon *et al*., 2002; Xu *et al.,* 2003a). 3D-crystal structures for the D-hydantoinases of *Thermus* sp. (Abendroth *et al.,* 2000b, 2002a), *Bacillus stearothermophilus* SD-1 (Cheon *et al.,* 2002), and *Burkholderia pickettii* (Xu *et al.,* 2003a), and the L-hydantoinase from *Arthrobacter aurescens* DSM 3745 (Abendroth *et al.,* 2002b) have been reported. Elucidation of the crystal structures revealed that the monomeric subunits of hydantoinases always assemble as tetramers when crystallized (Abendroth *et al*., 2002a; Cheon *et al.,* 2002; Xu *et al.,* 2003a). Hydantoinases and dihydropyrimidinases all exhibit highly homologous structures, with a classic triose phosphate isomerise (TIM) barrel topology (Abendroth *et al.,* 2002a; Cheon *et al.,* 2002, Xu *et al.,* 2003a). The TIM-barrel domain is an eightfold repetition of a β/α-motif wrapped in a circular fashion to make a central β-barrel. Each β-strand (B1 to B8) is connected to an α-helix (H1 to H8) through a front loop (FL1 to FL8), and each helix is connected to a strand by a back loop (BL1 to BL7) (Figure 1.7)

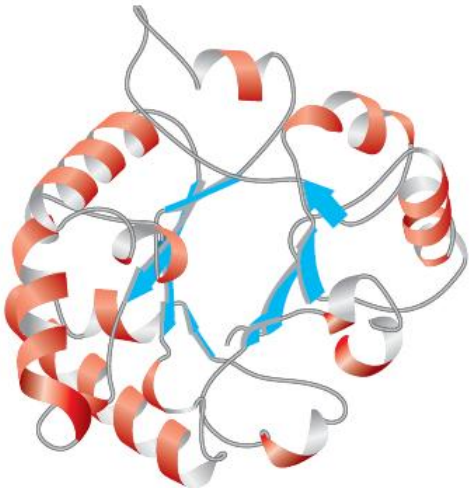

Figure 1.7 Illustration of a TIM barrel domain. β Sheets are coloured blue, α helices are red.

Holm and Sander (1997) confirmed that the hydantoinase active site was situated in a deep hydrophobic pocket at one end of the TIM barrel on the C-terminal end of the β-strands. The metal-binding amino acid residues were found to be completely conserved (Holme and Sander, 1997). The active site of the D-hydantoinase produced by *Thermus* sp. consists of six amino acids involved in coordination of the metal ions. The first zinc ion is coordinated by a carboxylated lysine residue (Lys^{150}), His^{183}, His^{239}, the oxygen atoms of the hydroxide ion and water, while the second zinc ion is coordinated by Lys^{150}, His^{59}, His^{61} and Asp^{315} (Figure 1.8) (Abendroth *et al.*, 2002a).

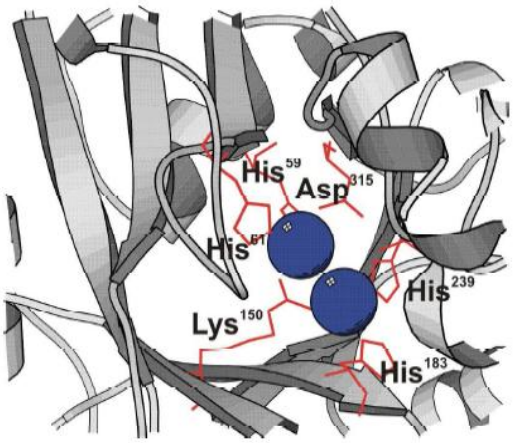

Figure 1.8: Ribbon representation of the Thermus sp. D-hydantoinase active site. Catalytic residues are depicted in red and the zinc atoms as blue spheres (Abendroth et al., 2002a).

In all hydantoinases analysed, the presence of these key amino acids in the active site was strictly conserved, irrespective of the enzyme's entantioselectivity (Figure 1.9), implying a common mechanism of hydantoin hydrolysis (Abendroth *et al.*, 2002a, b; Cheon *et al.*, 2002).

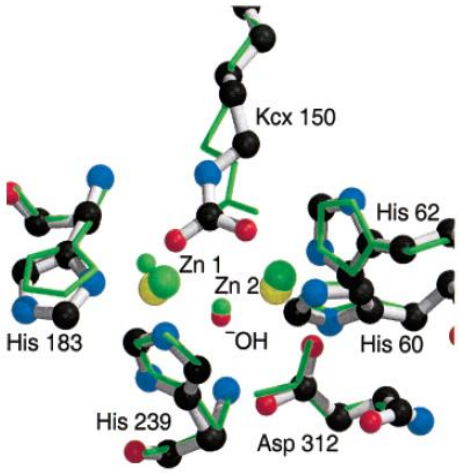

Figure 1.9: Active sites of L-hydantoinase and D-hydantoinase. Alignment of the active sites of L-hydantoinase and D-hydantoinase showing the coordination of the active site zincs. The residues of L-hydantoinase are represented as a ball-and-stick-model, the residues of D-hydantoinase as thin sticks in green (Abendroth et al., 2002b).

This observation is interesting because enzymes acting on different enantiomers of a substrate often possess completely different structures (Abendroth *et al.*, 2002a ;b). The understanding of substrate and enantiospecificity requires the generation of substrate bound crystals, which has not been possible thus far due to the lack of a potent inhibitor for the hydantoinase enzyme. In consequence, homology modelling of hydantoinases using structurally similar enzymes such as urease and dihydroorotase has been used to propose reaction mechanisms (Abendroth *et al.*, 2002a; b).

The interaction of a substrate with the hydantoinase active site is dependent on the recognition of the functional amide group which controls the orientation of the substrate, and on the recognition of the exocyclic substituents which depends on the stereochemistry of the substrate (Cheon *et al*., 2002). Substrate interaction with the hydantoinase is thought to occur

through the formation of several hydrogen bonds between the hydantoin ring and specific amino acids in the hydantoinase. Two hydrogen bonds occur with the backbone atoms Ser288, while Tyr155, zinc and His183 are thought to be responsible for the stabilization of the oxygen atoms in the tetrahedral transition state (Abendroth *et al.,* 2002a). Comparing the residues involved in substrate interaction in the D-hydantoinase from *B. pickettii* to those in the D-hydantoinase from *Thermus* sp. showed only a single amino acid substitution (Thr 286 in *Thermus* sp. to Ser 286 in *B. pickettii).* This substitution is conservative and does not affect the hydrogen bonding interaction with the substrate (Xu *et al*., 2003a).

The D-hydantoinase from *Thermus* sp. readily hydrolyses 5-phenylic substituted hydantoins but hydantoin derivatives with benzylic or polar substituents were found to be hydrolysed with significantly lower efficiency (Abendroth *et al.,* 2002a). The hydrophobic pocket formed by the amino acid residues Leu64, Cys95, Phe152, Tyr155 and Phe159 was thought to be responsible for the enatio- and substrate-specificity exhibited by this enzyme (Abendroth *et al.,* 2002a).

Although the active sites of D- and L-hydantoinases appeared to be similar (Figure 1. 9) the L-hydantoinase from *A. aurescens* contains no hydrophobic pocket. However, it was observed that a V154A point mutation of this enzyme resulted in increased D-enantiospecificity with respect to 5-methylthioethyl-hydantoin (May *et al.,* 2000). This is probably due to the mutation creating more space in the active site (Abendroth *et al.,* 2002b). A I95F point mutation resulted in increased L-enantiospecificity of the enzyme (May *et al.,* 2000) and this was attributed to increased hydrophobicity close to the side chain of L-5-methylthioethyl hydantoin resulting in increased cleavage of the L-enantiomer (Abendroth *et al.,* 2002b).

A comparison of the crystal structures of mesophilic and thermophilic hydantoinases was used to identify factors that might contribute to the thermostability of hydantoinases (Xu *et al.,* 2003a). Some of the factors that were hypothesized to be responsible for the increased thermal stability of the D-hydantoinase from *Thermus* sp. include reduced presence of methionine and cysteine residues on the surface of the enzyme. These residues can be readily oxidized at high temperatures, resulting in decreased thermostability. Ion pairs and hydrogen-bonding were also implicated in stabilisation. *Thermus* sp. D-hydantoinase was found to contain 44 salt bridges and 777 hydrogen-bonding interactions, compared to the 38 salt bridges and 714 hydrogen bonds in the mesophilic D-hydantoinase. The amino acid composition of enzymes also appears to affect thermostability. Proline, for example, has a rigid conformation and thus imposes constraints on the flexibility of proteins. Enzymes with high proline content, such as the D-hydantoinase from *Thermus sp*., are therefore capable of resisting unfolding at high temperatures (Xu *et al.,* 2003a).

1.10 Research proposal

The aim of this project is to isolate and characterise novel rare actinobacteria from Zambian hot springs by employing enrichment and pre-treatment techniques which have been found to selectively promote the isolation of rare genera of actinobacteria. The biocatalytic production of enantiomerically pure amino acids from hydantoin derivatives is finding increasing application in industry requiring enzyme systems with improved or novel biocatalytic activities. Novel isolates and isolates from previously uncharacterised environments present great potential for novel enzymes. As such, isolates will be screened for enzymes with industrial importance, including hydantoinases.

The specific objectives of this study are to:

- Isolate and characterise novel actinobacteria from Zambian hotsprings.
- Screen isolates for hydantoinase and/ or carbamoylase activity.
- Identify the genes responsible for the enzyme activity. Clone, express and purify the enzymes.

Chapter 2: Isolation, identification and characterisation of actinobacteria.

2.1 Introduction

2.1.1 Extremophiles

Microbial life is not limited to temperate environments. Dynamic microbial communities have been found thriving in the most diverse conditions, including extremes of temperature (thermophiles and psychrophiles), pressure (piezophiles), salinity (halophiles) and pH (acidophiles and alkaliphiles) (Horikoshi and Grant, 2010). These extremophilic microorganisms are of great interest to scientists for several reasons. As extremophiles are adapted to their respective extreme habitats, they are assumed to produce biocatalysts that are functional under these extreme conditions, thereby possibly making them more industrially applicable. Microorganisms are a rich and versatile source of new compounds with pharmaceutical relevance. It is suggested that the collection of currently described secondary metabolites represents a small fraction of the repertoire of bioactive compounds that microorganisms are able to produce (Watve *et al.*, 2001; Koehn and Carter 2005; Baltz, 2006; Palaez, 2006). Until recently natural product screens focused almost entirely on mesophilic terrestrial habitats. As extremophilic environments have not been investigated in such detail, they are a potential source of both novel microorganisms and novel secondary metabolites.

2.1.2 Hot springs as a source of novelty

Streptomycetes are the most abundant actinobacterial genus and thus have been extensively isolated since 1940. As such, the chance of rediscovering a known species which produces already characterised compounds is very high (Baltz, 2006). While this genus is likely to remain an invaluable source of secondary metabolites, the focus of screening programs must shift to include underexploited, rare actinobacterial genera.

The discovery of gentamicin (an aminoglycoside produced by *Micromonospora purpurea* and *Micromonospora echinospora*) greatly increased interest in the screening of rare non-streptomycete genera for novel compounds (Abou-Zeid *et al.*, 1978). Another avenue for the discovery of novel bioactive compounds is the exploration of new microbial habitats such as extreme environmental niches that have not been extensively studied, which may harbour novel micro-organisms.

Bacteria found within some aquatic environments exhibit unique physiological and structural characteristics that enable them to survive in extremes of pressure, salinity and temperature, and give the bacteria the potential to produce novel secondary metabolites not observed in terrestrial microorganisms (Cross, 1981; Kurtböke, 2000; Eccleston *et al.*, 2008).

2.1.3 The family Micromonosporaceae

The family *Micromonosporaceae* in the suborder *Micromonosporineae* (order, *Actinomycetales*) was first described by Krasil'nikov in 1938, and its description was subsequently emended by Koch *et al*. (1996) and Stackebrandt *et al*. (1997), on the basis of chemotaxonomic characteristics and phylogenetic positions inferred from 16S rRNA gene sequences and signature nucleotides. Orginally, the family comprised of seven genera, namely *Micromonospora, Actinoplanes, Dactylosporangium, Catellatospora, Catenuloplanes, Couchioplanes and Pilimelia* (Koch *et al*., 1996; Stackebrandt *et al.*, 1997). Currently, the family consists of 28 genera, including the recently described genus *Jishengella* (Xie *et al*., 2011).

Members of the family *Micromonosporaceae* are an important source of novel metabolites and the genus *Micromonospora* is particularly important, being second only to the genus *Streptomyces* as a source of metabolites. *Micromonospora* are Gram-positive, chemoorganotrophic aerobes (Vobis, 1992). They exhibit several unique physiological and biochemical characteristics, such as carbon source utilisation (pentoses and hexoses, as well as organic acids) and mycelial pigments which can all be used to differentiate them from other actinobacteria. *Micromonospora* are also distinct from other actinobacterial genera in that their peptidoglycan contains *meso*-DAP, and/or its 3-hydroxy derivative and glycine, and their cell wall hydrolysates contain arabinose and xylose (Kawamoto, 1989).

Micromonospora are widely distributed in aquatic environments and have been dominantly (compared to other genera of actinobacteria) isolated from water samples from streams, rivers, lakes, beach sands, coastal sediments (Zhao *et al.*, 2004), marine sediments (Mincer *et al*., 2002; Maldonado *et al*., 2005), peat swamp forests (Thawai *et al*., 2005), and floodplain meadows (Zenova and Zviagintsev, 2002).

2.1.4 The genus *Verrucosispora*

Since the discovery of abyssomicin C, the genus *Verrucosispora* is considered a potential source of novel bioactive secondary metabolites (Fiedler *et al*., 2005). Abyssomicin C is a polycyclic polyketide antibiotic which inhibits the folic acid biosynthesis pathways of Gram-positive bacteria (Lam, 2006) and was originally isolated from a marine *Verrucoscispora* strain (Riedlinger *et al*., 2004). *Verrucosispora* is a recently described genus which was established by Rheims *et al.*, (1998) as a member of the family *Micromonosporaceae* based on the unique combination of morphological, chemotaxonomic and phylogenetic properties. At

the time of writing there were four described species within the genus; *Verrucosispora gifhornensis* (Rheims *et al.*, 1998), *Verrucosispora lutea* (Liao *et al.*, 2009), *Verrucosispora sediminis* (Dai *et al.*, 2010) and *Verucosispora qiuiae* (Xi *et al.*, in press 2012). The type strain of this genus, *Verrucosispora gifhornensis*, was described as a Gram-positive, aerobic, non-acid-fast actinobacterium with branching hyphae that form well developed septate mycelium and non-motile spores, which are borne singly (either sessile or on short or long sporophores) (Rheims *et al.*, 1998). The genus *Verrucosispora* is characterised by branching hyphae that form well-developed septate mycelium, non-motile warty spores with smooth surfaces. The warty spore surface might change to become hairy over time. Aerial mycelium is absent.

The reclassification of actinobacteria at the family level by Stackebrandt *et al.* based on 16S rRNA gene sequence analysis revealed that there were taxon specific signature nucleotides (Stackendrandt *et al.*, 1997). Members of the genus *Verrucosispora* have the *Micromonosporaceae* specific signature sequence (5'CAAUUCGGUUG3') between positions 1132 and 1143 (*E. coli* numbering). A 16S rRNA gene sequence-based study on the taxonomy of *Micromonosporaceae* by Koch *et al.* (1996a) showed that the 16S rDNA-based phylogeny of the genus *Micromonospora* (the closest genus to *Verrucosispora*) did not always agree with other taxonomic characteristics. The use of protein-encoding genes such as *gyrβ* has since been suggested for the phylogenetic classification and identification of closely related bacteria (Yamamoto and Harayama, 1995). Studies have shown that *gyrβ* genes sequence analysis is a useful additional phylogenetic tool to classify members of the family *Micromonosporaceae* (Kasai *et al.*, 2000, Menezes *et al.*, 2008).

Morphological and chemotaxonomical properties are important in describing new members of a genus. Although phylogenetic characterisation of isolates on the basis of 16S rRNA gene sequences and DNA-DNA homology are considered by many microbial taxonomists to be the most robust approaches to microbial identification at the species level (Zhao *et al.*, 2004), members of the family *Micromonosporaceae* are morphologically and physiologically similar, hence phenotypic traits alone are insufficient to classify members (Rheims *et al.,* 1998). Characteristics that are important in describing members of the genus *Verrucosispora* include spore surface ornamentation, peptidoglycan, major whole cell sugars, phospholipids, menaquinone, major fatty acids and G+C content of genomic DNA (Rheims *et al.,* 1999; Dai *et al.,* 2009; Lio *et al.,* 2009). The genus is characterised by having little or absent aerial mycelium and well developed septate mycelium formed by branching hyphae (Rheims *et al.,* 1998; Dai *et al.,* 2009; Lio *et al.*, 2009).

2.2 Materials and methods

2.2.1 Sampling sites

Actinobacteria were isolated from soil samples collected from two Zambian hotsprings (Fig 2.1) by Professor D.A. Cowan in June 2008. Bwanda and Gwisho hot springs lie 3 km apart in Lochinvar National Park (16-00 S, 27-15 E), at 1000 m above sea level.

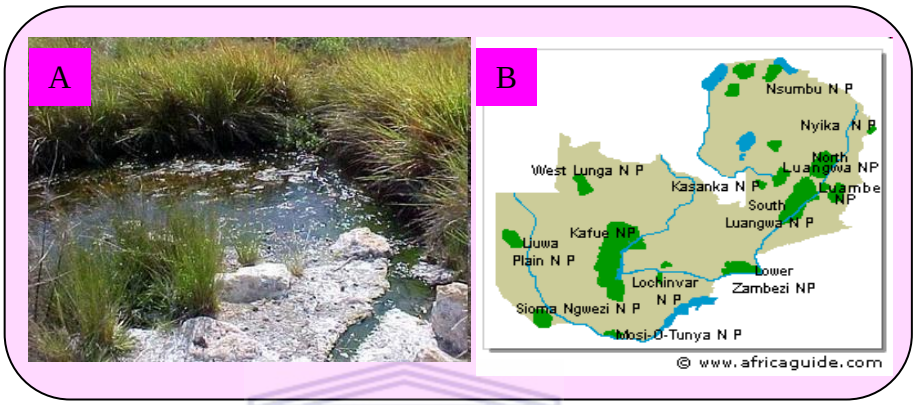

Figure 2.1: Gwisho hot spring (A) located in the Lochnivar National park, situated in the Monze District of Zambia's Southern province (B).

2.2.2 Sample collection

Soil samples were collected from both sites in sterile 50 ml Sterilin® tubes under aseptic conditions. Individual samples were mixed thoroughly and re-sampled before storage. Samples were stored at <0 °C during transport and at -80 °C in the laboratory.

Table 2.1: Gwisho Hot Springs (GS) Minor site

Sample Code	Description
GS1a	Black sediment with high organic content from overlying rushes Temperature 41 ^{o}C. Elevation 995 m. GPS S15 59.526 E27 14.520
GS1b	Similar to GS1a but with higher organic content
GS2	Major thermal pool – dead matter indicates higher past temperatures Temperature 67.2 ^{o}C. Elevation 1003 m. GPS S15 59.231 E27 14.549

Table 2.2: Bwanda Hot Springs (BS)

Sample Code	Description
BS1	Temperature 68.3 ^{o}C. Elevation 1003 m. GPS S16 00.741 E27 13.151
BS2	Outflow 1m downstream from water source Temperature 47.2 ^{o}C
BS3	Dry salty sand in run off area from hot pool GPS S16 00.518 E27 31.395

2.2.3 Isolation from soil samples

Soil samples were pre-treated by drying at 55 °C for 48 hours followed by heating at 90 °C for 1 hour. One gram (1 g) of soil was suspended in 9 ml of sterile distilled water, shaken for one hour and serially diluted (10 fold dilutions) with sterile water. Aliquots of 100 µl were spread on isolation agar supplemented with cycloheximide (50 µg/ml). Six different selective media were used. glycerol asparagine agar (International *Streptomyces* Project ISP5), starch casein nitrate agar, modified Emerson agar (Pferferle *et al.*, 2000), modified 172F agar (composition g/L; 10 g glucose, 5 g yeast extract, 10 g starch, 5 g casitone, 2.5 g $MgSO_4.7H_2O$, 2 g $CaSO_4.2H_2O$), Bennets agar (composition g/L; 10 g glucose, 2 g casitone, 1 g yeast extract, 1 g beef extract) and humic acid vitamin agar (Hayakawa and Nonomura, 1987a). Each dilution was plated on four replicate plates. Plates were incubated at 28 °C, 37 °C, 45 °C and 55 °C for 14 to 21 days. Actinobacterial colonies were picked with sterile toothpicks and re-streaked to obtain single colonies on the same media (cycloheximide omitted) from which they were isolated.

2.2.4 Cultivation of isolates

Pure colonies were inoculated into the broth medium from which they were isolated and incubated at isolation temperature with shaking (110 rpm) for 7 to 14 days. Cell mycelia were harvested by centrifugation at 4000 x *g* for 10 minutes.

2.2.5 Identification of isolates

2.2.5.1 Genomic DNA isolation

Genomic DNA was extracted from all cultures using the protocol described by Wang *et al.* (1996) with minor modifications. Cell mass was harvested by centrifugation at 13000 x *g* for 2 minutes until a pellet of approximately 200 µl was obtained. The cell pellets were resuspended in 500 µl of lysozyme buffer (25 mM Tris-HCl pH 8, 50 mM glucose, 10 mM EDTA, 25 mg/ml lysozyme) and incubated 37 °C. SDS was added to a final concentration of 1 % and the tubes were incubated at 65°C for 30 minutes. An equal volume of phenol was added and the samples were mixed by gently inverting the tubes. Samples were centrifuged at 10000 x *g* for 30 seconds. The phenol extraction was repeated twice. The aqueous layer was transferred to a clean 1.5 ml eppendorf tube and an equal volume of chloroform: isoamyl alcohol (24:1; v/v) was added. The samples were centrifuged as above and the aqueous phase was transferred to a clean tube. DNA was precipitated with one volume isopropanol at room temperature and the DNA was pelleted by centrifugation at 15000 x *g* for 2 minutes. The supernatant was discarded and the pellet was air dried for 30 minutes. DNA was re-suspended in 100 µl 1 x TE (10 mM Tris, 1 mM EDTA) buffer pH 8.

2.2.5.2 DNA purification

Genomic DNA contaminated with humic acids was further purified using polyvinylpolypyrrolidone (PVPP) (Sigma product number P 6755) minicolumns (Berthlet *et al.*, 1996). The columns were constructed in 20 µl filter tips housed in 0.6 ml tubes. The

column was loaded with 200 µl PVPP suspension and centrifuged at 150 x *g* for 2 min. The flow-through was discarded and the loading step was repeated. The column was washed twice with 150 µl 1 x TE pH 8 and dried by centrifugation (600 x *g*, 10 min). The column was placed in a new 1.5 ml eppendorf tube and 100 µl DNA was applied to the column and incubated for 1 min at RT. The DNA was eluted by centrifugation at 600 x *g* for 5 min then at 1700 x *g* for 10 min.

Polysaccharides were removed from DNA samples using the method proposed by Fang *et al.* (1992). The polysaccharide contaminated DNA was dissolved in TE supplemented with 2 M NaCl. DNA was precipitated with two volumes of absolute ethanol followed by two 70 % ethanol washes to remove all the residual salt.

DNA was quantified with a Nanodrop ND-1000 spectrophotometer (Delaware-USA) and absorbance at 280 nm, 260 nm and 230 nm was recorded (Sambrook *et al.*, 1989). DNA solutions were considered to be pure if the $A_{260\ nm}$ / $A_{230\ nm}$ ratio was between 1.8 and 2.3 and the $A_{260\ nm}$ / $A_{280\ nm}$ ratio was between 1.5 and 2.0 (Marmur, 1961). Genomic DNA was stored at -20 °C.

2.2.5.3 16S rRNA gene PCR

Two sets of universal eubacterial primers (i) E9F 5'GAGTTTGATCCTGGCTCAG 3' (Farelly *et al.*, 1995) /U1510R 5'GGTTACCTTGTTGTTACACTT 3' (Reysenbach and Pace, 1995) and (ii) F1 5'AGAGTTTGATCITGGCTCAG3' / R5 5'ACGGITACCTTGTTACGACTT3' (modified from primers fD1 and rP2, respectively of Weisburg *et al.*, 1991) were used to amplify an approximately 1.4 kb DNA fragment. Each 50 µl PCR reaction contained a final concentration of 1 X buffer, 2 mM $MgCl_2$, 150 µM of each

dNTP, 0.5 µM of each primer, 2 µl DNA template and 0.25 U of Dream*Taq* polymerase (Fermentas). DNA was amplified in an automated thermal cycler (Thermo Hybaid system). The PCR conditions were an initial denaturation at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C for 60 s, followed by a final extension step at 72 °C for 10 minutes. Negative controls with no DNA template were included in all PCR experiments.

Agarose gel electrophoresis was performed according to standard methods (Sambrook *et al*., 1989). Horizontal 1 % (w/v) TAE agarose gels supplemented with 0.5 µg/ml ethidium bromide were cast and run at 100 V in 0.5 × TAE buffer (Tris-acetate-EDTA). DNA was visualized on a UV transilluminator Gel Doc. The DNA fragments were sized according to their migration in the gel as compared to that of the DNA molecular marker, Lamda DNA restricted with *Pst*I.

2.2.5.4 ARDRA

16S rRNA gene amplicons were digested singly with two restriction endonucleases. Each 20 µl restriction digest contained 10 µl of the 16S rDNA PCR amplicons, 5 U of restriction enzymes *Alu*I (Fermentas; sequence: AG↓CT) and *Rsa*1 (Fermentas; sequence: GT↓AC), and 1 x restriction buffer (Fermentas). Restriction digestions were viewed on 1% agarose gels.

2.2.5.5 DNA sequencing

PCR amplicons were visualised by electrophoresis and the correct sized DNA fragments were excised from the gel and purified using a GFX™ PCR and Gel Band Purification Kit (Amersham Biosciences), according to the manufacture's specifications. Sequencing reactions were carried out by the DNA sequencing facility at the Department of Molecular and Cell

Biology at the University of Cape Town. Two forward and two reverse overlapping sequencing primers were used to sequence the entire length of the double stranded DNA.

2.2.5.6 Phylogenetic analysis

Sequences were analysed using the software packages, BioEdit Version 7.0 (Hall, 1999) and DNAMAN Version 4.13 (Lynnon Biosoft). The GenBank database was used for analysis of DNA sequences and homology searches. The Basic Local Alignment Search Tool (BLASTn) programmes (Altschul *et al.*, 1997) was used to determine sequence similarity and identity to known species in the GenBank database using software from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/). Sequences were aligned using the Clustal W multiple alignment featured in Bioedit Sequence Alignment Editor version 6.0.5 (Tom Hall Isis Pharmaceuticals Inc). Phylogenetic and molecular evolutionary analyses were conducted and phylogenetic trees constructed using MEGA 4 (Tamura *et al.*, 2007). The trees were based on the Maximum Composite Likelihood method and substitution model using Neighbour-Joining Bootstrap analysis using PHYLIP for 100 replicates was performed with pair-wise deletion of gaps to attach confidence estimates for the tree topologies. (Felsenstein, 1985; Saitou and Nei, 1987; Tamura *et al.*, 2004)

2.2.6 Storage of isolates and DNA samples

All isolates were stored in glycerol (15 % final concentration) at -80 °C. DNA was stored in TE buffer at 4 °C for short term storage and at -20 °C for long term storage.

2.2.7 Characterisation of isolates

2.2.7.1 Morphological characterisation

Phenotypic characterisation was carried out on ISP (International *Streptomyces* Project) media (ISP2- Yeast extract- malt extract agar; ISP3 Oatmeal agar; ISP4 Inorganic salts- starch agar;

ISP5 Glycerol asparagine agar; ISP6 Peptone yeast extract iron agar; ISP7 L-Tyrosine agar) as outlined by Shirling and Gottlieb (Shirling and Gottlieb, 1966). For morphological characteristics, isolates were cultivated on either modified 172F agar or tryptic soy agar (TSA) at 28 °C for 21 days. Sporophores, spore chains, and spore surfaces were observed using light (Olympus CX21) and scanning electron (Leica S440) microscopy.

2.2.7.2 Physiological characterisation

Unless otherwise specified, all physiological characterisation was conducted at 28 °C. The utilisation of different sole carbon sources was carried out on ISP9 supplemented with filter sterilised carbon sources at a final concentration of 1 % (Shirling and Gottlieb, 1966). NaCl tolerance was determined using Bennet media (BM) supplemented with different concentrations of NaCl (0, 1, 3, 5, 10, 13 %). Degradation of adenine, casein, starch, hypoxanthine, tyrosine and xanthine, as well as the liquefaction of gelatin was tested using BM as a basal medium, in a modification of the method described by Williams and Wellington,. (1982). Growth in the presence of inhibitory compounds was tested on MBA supplemented with the various compounds; crystal violet (0.001 %), phenol (0.1 %), sodium azide (NaN_3) (0.01 and 0.02 %), Gentamicin (100 µg/ml), Streptomycin (100 µg/ml).

2.2.7.3 Production of antimicrobial compounds

Isolates were stab inoculated into either M172F or TS agar using sterile toothpicks. Plates were incubated at 28 °C for 7, 14 and 21 days. Test organisms included *Micrococcus luteus, Staphylococcus aureus, Streptococcus* sp., *Bacillus megaterium, Citrobacter braaki* strain 90, *Enterococcus faecium, Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* K11, *Proteus mirabilis* strain 87 and *Pseudomonas aeruginosa* ATCC 27853. Test strains were cultured in 5 ml volumes of Luria Bertani (LB) media at 37 °C for 24 hours and these starter cultures were

used to seed sloppy agar (Luria Bertani agar prepared with 0.7 % bacteriological agar). The appropriate volume of test culture to be used was calculated using the formula OD_{600} x µl = 160 for all the bacteria except *E. coli*, for which the formula was OD_{600} x µl = 4. The seeded sloppy agar was poured over the actinobacterial colonies and the plates were incubated at 37 °C for 24 h. Plates which did not have bacterial lawns after the 24 hour incubation were incubated for a further 24 hours.

2.2.7.4 Chemotaxonomy

Isomers of diaminopimelic acid (DAP) and whole cell sugar pattern analyses of whole cell hydrolysates was performed as described by Hasegawa *et al.* (1983). Predominant fatty acids patterns were analysed by gas liquid chromatography (Chou *et al.*, 1998). Menaquinones and phospholipids were extracted and analysed according to the method of Minnikin *et al.* (1984) and Komagata *et al.* (1987).

2.2.7.5 Determination of optimum growth conditions

The temperature and pH ranges for growth of isolates were determined on modified Bennet's media. For temperature optima, liquid cultures were grown in 10 ml volumes at 20, 25, 30, 37, 45 and 55 °C and the cell dry mass of the 10 ml cultures was measured at 3 day intervals.

2.3 Results and Discussion

2.3.1 Isolation of actinobacteria.

The successful isolation of organisms from environmental samples requires an understanding of the environmental factors affecting their growth. Nutritional conditions found in natural ecosystems are not easy to recreate *in vitro* and the development of growth medium which mimic these conditions is challenging. In the present study factors such as carbon source, mineral ions, temperature and pH were varied to facilitate the isolation of a wide range of actinobacteria with different growth requirements. Initially it was found that fungi were the most abundant contaminating microorganisms, making it impossible to pick any actinobacterial colonies. Therefore, all isolation media was supplemented with cycloheximide to suppress fungal growth. Cycloheximide is a protein synthesis inhibitor that acts specifically on the 60S subunit of the eukaryotic ribosome (Obrig *et al.*, 1971).

Initially, soil samples were plated without pre- treatment. However non-actinomycete bacteria were found to dominate the isolation plates and thus heat pre-treatment was used to select against actively growing bacteria in the soil samples. Dry heat methods favour the selection of heat-resistant spores thereby reducing the number of non spore forming bacterial colonies.

On all isolation plates (Figure 2.2), colonies showing streptomycete-like morphologies appeared first and in most cases they were more abundant than other actinobacterial genera.

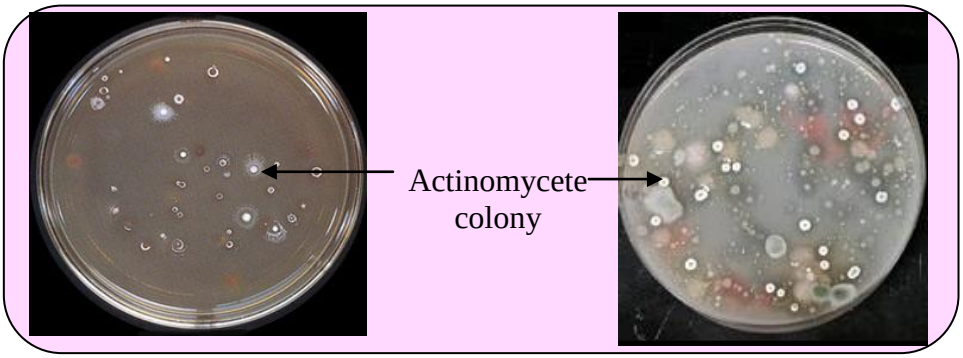

Figure 2.2: Bacterial and fungal colonies growing on Bennet (left) and ISP5 (right) isolation plates. Arrows point to actinobacterial colonies

In the present study non-streptomycete actinobacteria were dominant on humic acid vitamin agar (HV). This medium contains humic acid as the sole carbon and nitrogen source. Humic acids are extremely heterogeneous cross-linked polymers (Kumada, 1975) and while ubiquitously present in soil are generally resistant to biological decomposition. However, actinobacteria have been shown to utilise humic acids and it has been demonstrated that HV agar is useful for the efficient recovery of streptomycetes and various rare genera, while restricting the growth of non-filamentous bacterial species (Hayakawa and Nonomura, 1987a, b).

Although the presence of contaminating bacteria, fungi and yeast was minimal on humic acid vitamin agar, non- streptomycete actinobacterial colonies were difficult to observe on the dark HV agar plates, especially smaller colonies bearing no aerial mycelium, which are typical culture characteristics of the rare actinobacteria genera. In general, actinobacterial colonies were selected on the basis of their unique morphological features (Figure 2.2). The colonies of aerial mycelium-bearing strains appeared powdery (Figure 2.2 and Figure 2.3) assisting in identification. In this study a total of 51 actinobacterial colonies were selected and sub-cultured (Figure 2.3 and Table 2.3)

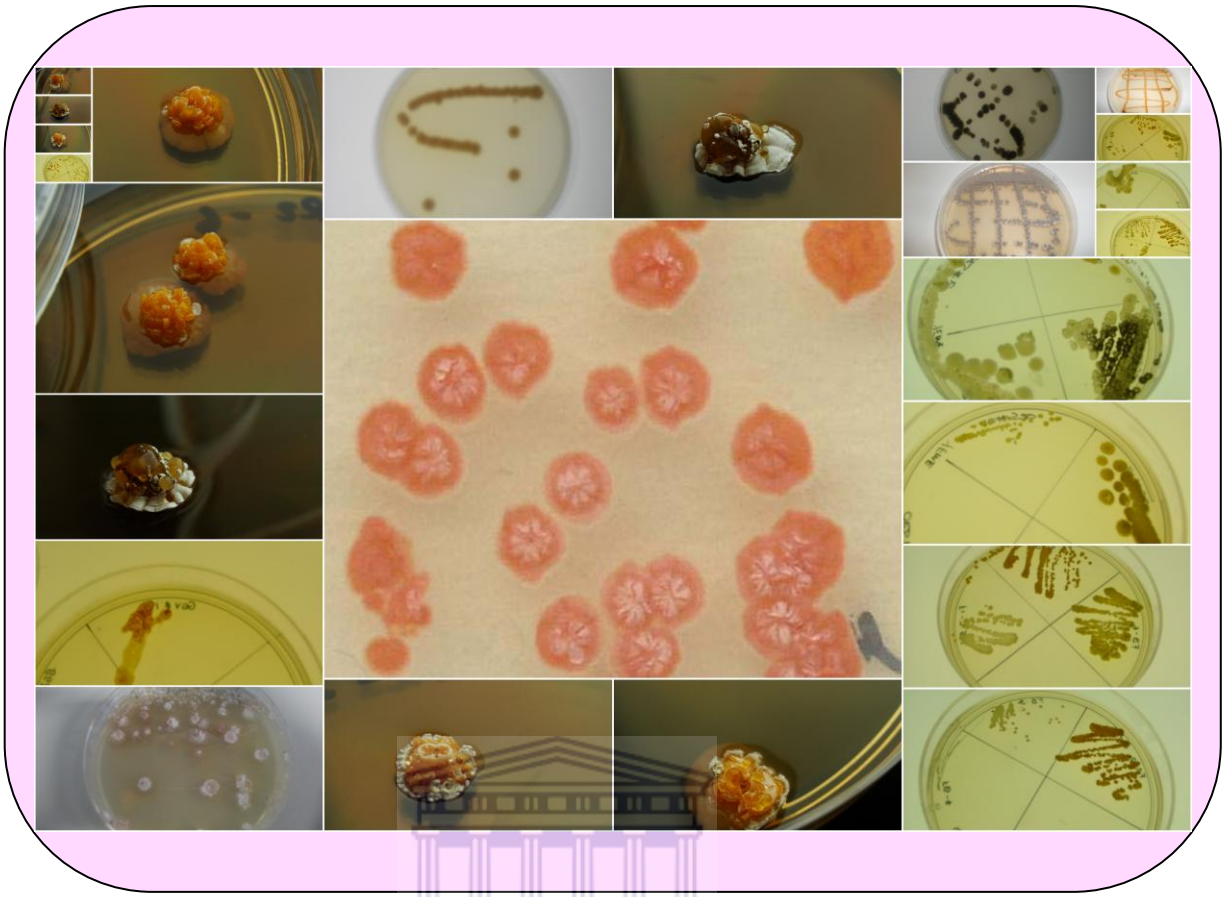

Figure 2.3: A selection of actinobacterial strains isolated in this study

Table 2.3: Strains isolated on the different media

Media	Total number of actinobacterial colonies	Number of colonies with non-streptomycete morphology
Glycerol asparagine agar	21	9 (42.9%)
Bennet's agar	39	12 (30.8%)
Humic acid vitamin agar	13	8 (61.6%)
Modified 172F agar	42	16 (38.1%)
Modified Emerson agar	26	11 (42.3%)
Starch casein nitrate agar	18	6 (33.3%)

The greatest number of actinobacteria grew on the isolation plates incubated at 28 °C. Isolation plates incubated at 37 °C were dominated by colonies with streptomycete-like

morphologies. Plates incubated at 45 °C were dominated by actinobacteria with micromonospora-like growth characteristics. However, these isolates grew very slowly and in some cases colonies were only visible after 14 days incubation.

The highest numbers of isolates were obtained on modified 172F agar and Bennett's agar, while fewer isolates were obtained from humic acid vitamin agar (Table 2.3). Modified 172F medium was chosen for routine maintenance of isolates. Only six isolates, all originally isolated on humic acid vitamin agar, were unable to grow on M172F. However, these isolates did not necessarily require humic acid for growth as could be cultured on minimal salt agar without humic acid. The poor growth exhibited by these isolates on relatively rich media might imply that they are obligate oligotrophs. Oligotrophs are characterized by slow growth, low rates of metabolism, and overall low population densities (Semenov, 1991).

The results obtained in this study were consistent with previous findings. Based on numerous studies investigating the ecological distribution of actinobacteria, the most common actinobacterial genera isolated from soil are *Streptomyces*, *Micromonospora* and *Nocardia* species (in the order of abundance) (Nonomura and Ohara, 1969; Hayakawa *et al.*, 1988; Xu *et al.*, 1996).

. 2.3.2 Identification of isolates

Pure isolates were cultured in liquid media to obtain cell mass for DNA isolation. Prior to DNA extraction purity of cultures was routinely verified using Gram staining (Figure 2.4)

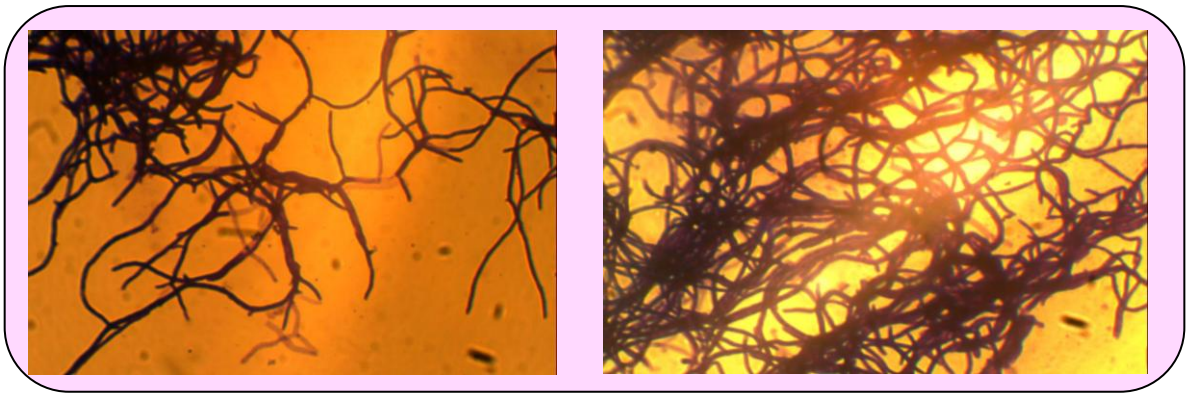

Figure 2.4: Isolate BSII3 (Gram stained) visualised by light microscopy (magnification 1000x / 1.25 oil)

DNA was extracted from pure cultures and the 16S rRNA gene was amplified using universal primers E9F and U1510R. PCR amplification was unsuccessful when DNA was extracted from cells cultured in humic acid (HA) vitamin broth. HA inhibits PCR by directly inhibiting *Taq* polymerase, decreasing the efficiency of primer binding or chelating Mg^{2+} ions (Tsai and Olson, 1992). Self constructed polyvinylpyrrolidone (PVP) mini columns were used to purify the genomic DNA. In addition, DNA isolated from some *Streptomyces* strains contained high levels of polysaccharide contamination which reduced the quality of DNA preparations. Fang *et al.* (1992) have shown that high concentrations of NaCl (0.5- 3 M) can be used to remove polysaccharides from DNA. Following purification employing a modified protocol the quality of the DNA improved and PCR amplification was successful.

Many of the isolates had very similar colony morphologies on the different isolation media and all isolation plates appeared to be dominated by *Streptomyces* and *Micromonospora* species. Therefore, isolates were dereplicated using amplified ribosomal DNA restriction analysis (ARDRA) (Figures 2.5 and 2.6). Digestion with *Alu*I resulted in twelve different banding patterns (shown as different coloured boxes in Figure 2.5) and *Rsa*I yielded seven different patterns (Figure 2.6). Isolates with identical ARDRA patterns were grouped together, resulting in twelve different clusters, of which four contained only one member.

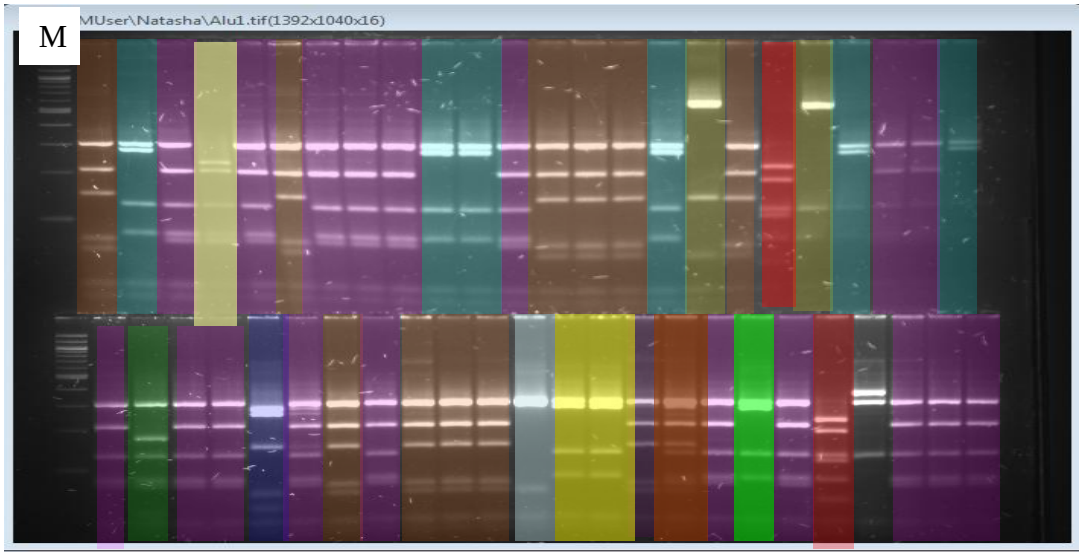

Figure 2.5: ARDRA patterns obtained with AluI. M, λ PstI molecular weight marker.

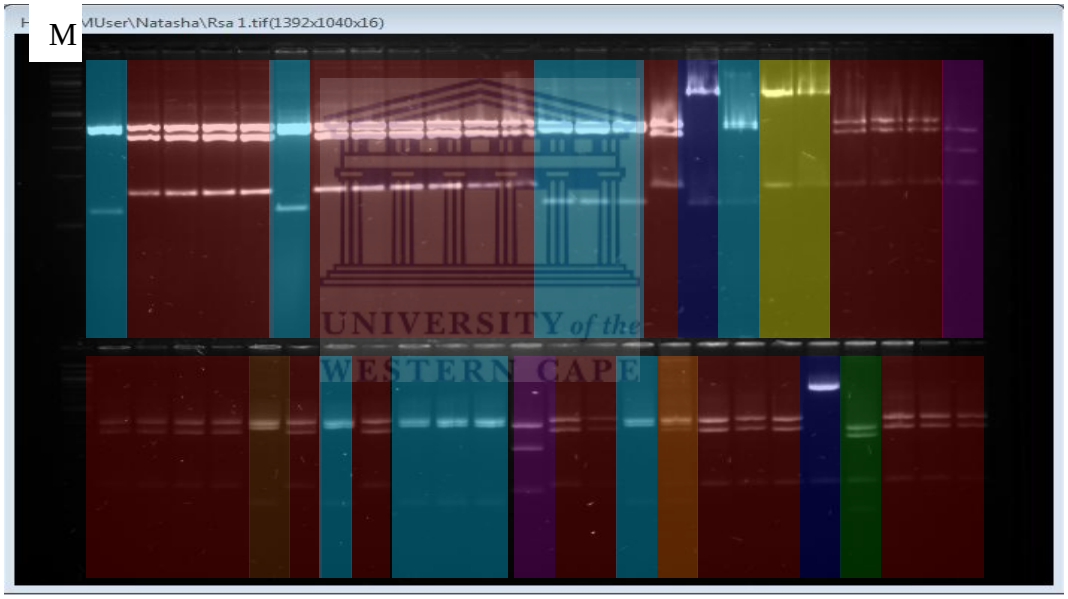

Figure 2.6: ARDRA patterns obtained with RsaI. M, λ PstI molecular weight marker.

A single isolate from each *Alu*1 cluster was selected for 16S rRNA gene sequencing. Table 2.4 shows the results from the BLAST analysis for the twelve representative isolates. From these results it can be concluded that *Micromonospora* species were the dominant isolates. Other studies have also reported that *Micromonospora* species are the dominant actinobacterial group in a range of aquatic environments. Micromonospora have been isolated from streams, rivers and lakes, as well as from lake mud, river sediments, beach sands, littoral sediments and

deep marine sediments (Johnston and Cross, 1976; Goodfellow and Williams, 1983; Cross, 1981; Jiang and Xu, 1995; Mincer *at al*., 2002; Rusnak *et al.,* 2002; Jensen *et al.*, 2005; Gullo *et al*., 2006; Eccleston *et al.*, 2008).

Table 2.4: Phylogenetic analysis of isolates.

Isolate	Closest relative	%Homology
BSI 4	*Streptomyces sampsonii* AB184299.1	100
BSII 7	*Streptomyces albidoflavus* EF620360.1	100
BSII 1	*Micromonospora carbonacea* EF583904.1	98
BSII 9	*Streptomyces greseorubens* JN180193.1	100
GSI 2	*Verrucosispora gifhornensis* AB546292.1	98
GSI 3	*Micromonospora inyonensis* NR_044893.1	99
GSIII 2	*Micromonospora coxensis* FJ486500.1	99
GSV 6	*Micromonospora pattaloongensis* NR_041532.1	99
GSV 13	*Micromonospora halophytica* NR_026278.1	99
GSV 15	*Micromonospora eburnea* AB159778.2	98
GSV 18	*Micromonospora chaiyamphumensis* EU841636.1	100
GSV 20	*Micromonospora floridensis* EU274366.1	99

Sequencing results identified one of the isolates, GSI2, as being most closely related to *Verrocosispora gifhornensis*. At the time of writing, there were only four described species in this genus and none have been isolated from fresh water environments. As this isolate could potentially be novel, further full taxonomic characterisation was undertaken.

2.3.3 Characterisation of isolate GSI2

2.3.3.1 Morphology and physiology

Isolate GSI2 produced well developed substrate mycelia on all media tested but aerial mycelia were absent (Figure 2.7). This is consistent with the finding that all members of the family *Micromonosporacea* do not form aerial mycelium at any stage of their development. Colonies were orange/red with a tough leathery colony texture. The colonies darkened with time forming a brown-black mucoid film as seen in Figure 2.7 –photo 3. When colonies were kept for longer than 35 days the substrate mycelia disappeared, leaving only the brown-black mucoid film (photo 4).

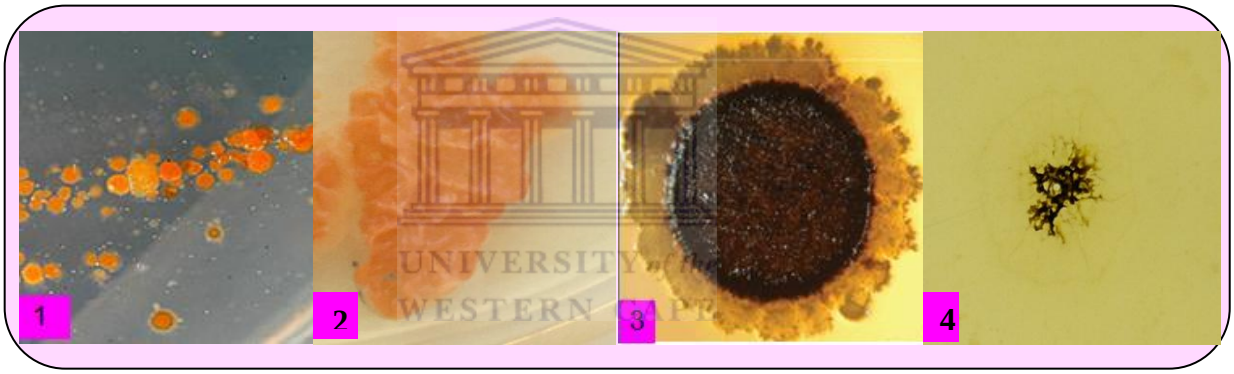

Figure 2.7: GSI 2 colonies at days 7 (1), 14 (2), 21 (3) and 35 (4).

Isolate GSI2 grew well on yeast extract- malt extract agar (ISP2). Good growth was observed on peptone-yeast extract iron agar and tyrosine agar (ISP6 and 7, respectively), moderate to poor growth was observed on inorganic salt-starch agar and glycerol-asparagine agar (ISP4 and 5, respectively). The isolate did not grow on ISP3. No production of melanin or diffusible pigments was noted. Spore surface ornamentation observed under the scanning electron microscope was smooth (Figure 2.8).

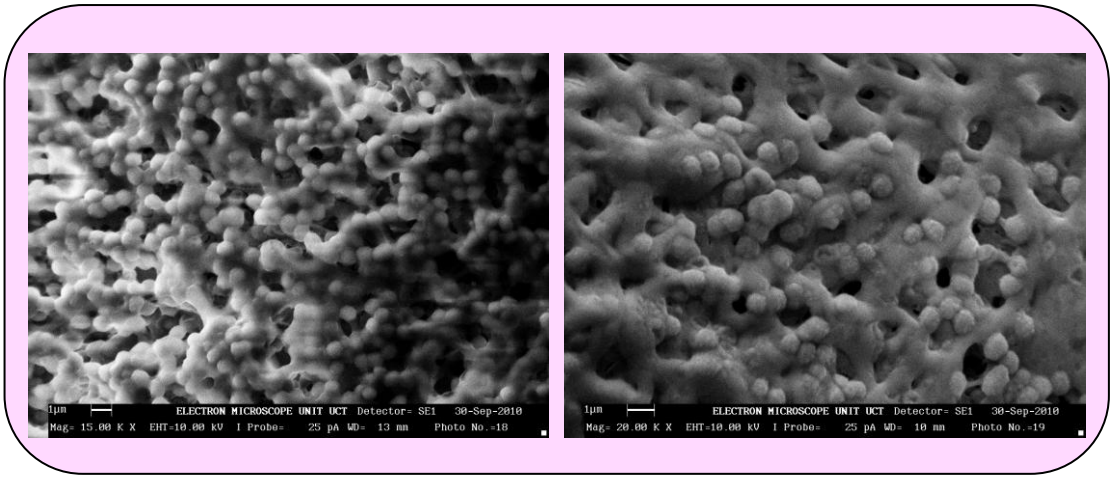

Figure 2.8: Isolate GSI2 spores on M172F media under scanning electron microscope.

2.4.3.2 Growth in the presence of inhibitory substances

Isolate GSI2 was unable to grow on all the inhibitory substances tested except for Crystal violet (CV). Very good growth was observed on Bennett's medium supplemented with 0.001% CV.

2.3.3.3. Production of antibiotic substances

Isolate GSI2 did not produce antibiotic compounds under the conditions tested in this study. Within the genus *Verrucosispora* the species *V. sediminis* and the *V. mais* have been reported to produce antibacterial compounds such as abyssomicins and proxamicins.

2.3.3.4 Determination of optimum growth conditions

The optimum growth temperature of isolate GSI2 was determined from growth curves constructed over 21 days (Figure 2.9). The optimum temperature for growth, estimated from exponential growth profiles was 30 °C (Figure 2.10). GS12 was isolated from a site in Gwisho hotspring (Table 2.1) were the temperature at the time of sampling was 41 °C. The estimated optimum temperature for growth is therefore consistent with the source temperature. The observed optimum temperature for growth is also consistent with other *Verrucisispora* species

which have optimum growth temperatures in the range of 28 to 37 °C for (Rheims *et al.*, 1998; Dai *et al.*, 2009; Liao *et al.*, 2009; Xi *et al.*, 2011).

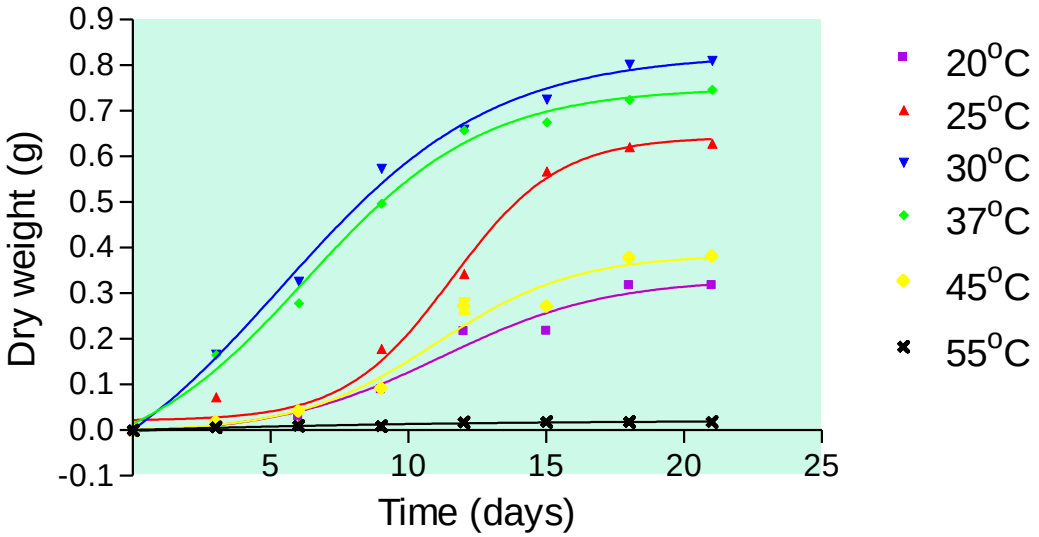

Figure 2.9: Effect of temperature on the growth of isolate GSI2 in tryptic soy broth.

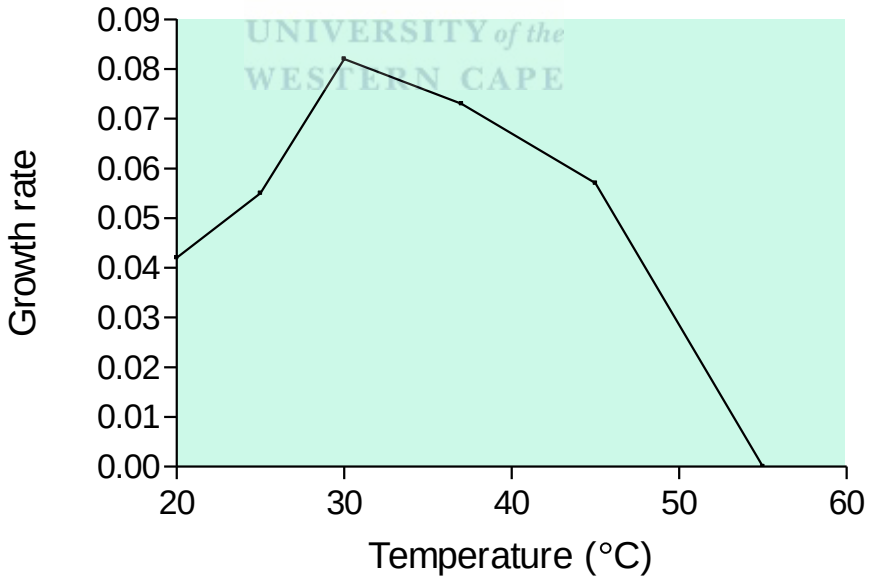

Figure 2.10: Exponential growth profile of isolate GSI2.

The optimum pH for growth based on radial growth on TSB agar was 9.0 (Figure 2.11).

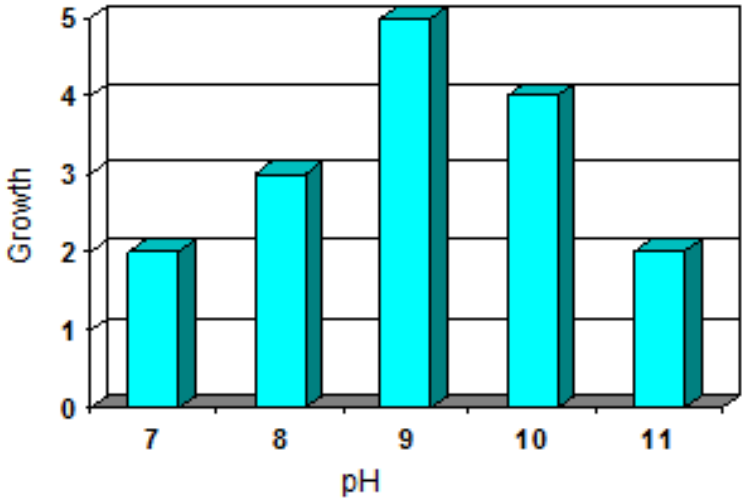

Figure 2.11: pH growth profile for isolate GSI2 cultured on TSB agar. Growth was scored on an arbitrary scale ranging from zero (no growth at all) to five (very good growth).

2.3.3.5 Phylogenetic analysis

An almost complete 16S rRNA gene sequence (1344 nucleotides) of isolate GSI2 was compared to sequences in GenBank. Phylogenetic analysis (Fig 2.12) indicated that isolate GSI2 clustered with the other three members of the genus *Verrucosispora* confirming that it belonged to this genus. The newly published *Verrucosispora qiuiae* did not cluster with the other *Verrucosispora* species, forming a separate clade with the species *Micromonospora olivasterospora*. This was consistent with the species description of *V. qiuiae* which reported that phylogenetic analysis based on the 16S rRNA gene showed that the strain formed a distinct monophyletic line at the periphery of branch containing the recognized members of the genus *Verrucosispora* (Xi *et al*., 2011). Although analysis of the 16S rRNA gene sequences would imply that *V. qiuiae* does not belong to the genus *Verrucosispora*, other characteristics confirmed its status as a novel member of this genus (Xi *et al*., 2011)

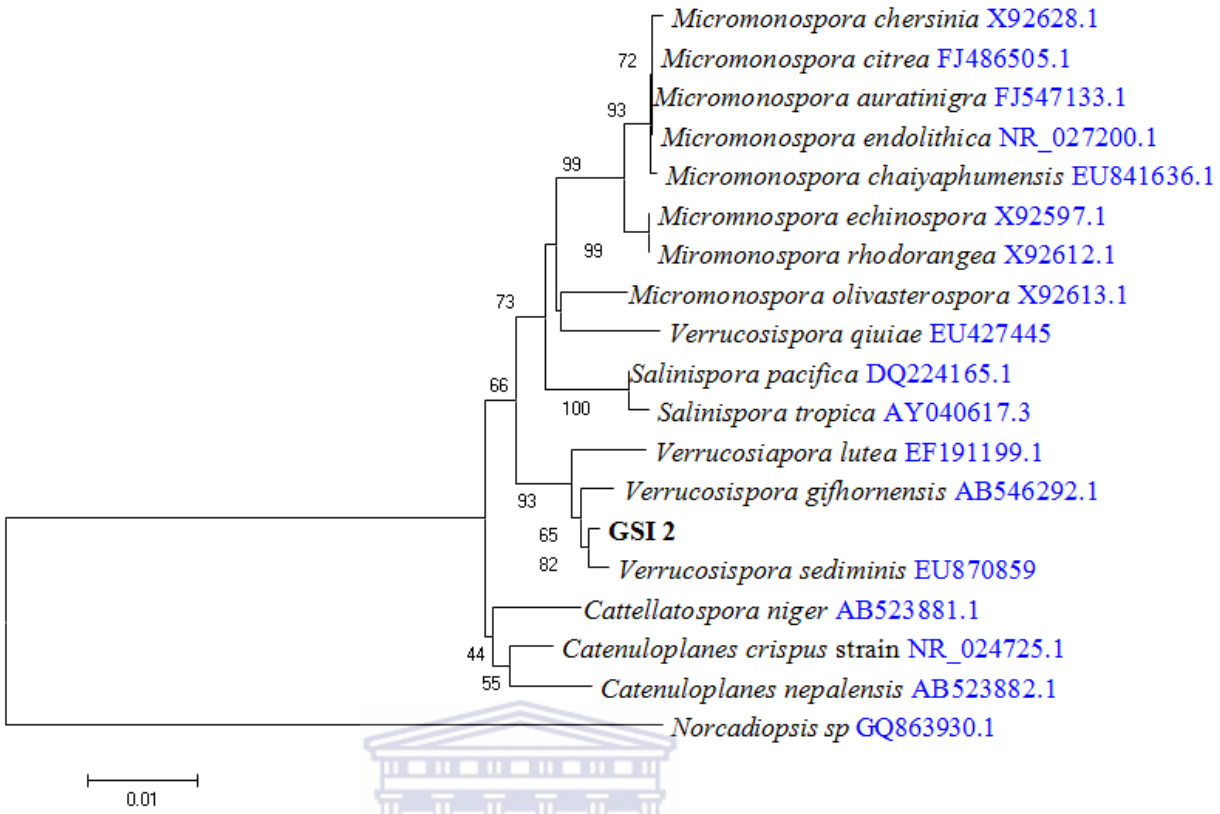

Figure 2.12: Neighbour joining phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between isolate GSI2, recognized species of the genus *Verrucosispora* and other closely related representatives of the family *Micromonosporaceae*.

Morphological, physiological and chemotaxonomic characteristics of GSI2 were compared to those published for the other 3 species in this genus. The isolate GSI2 showed some differences from its closest relatives and these are listed in Table 2. 5. One of the most distinct differences between isolate GSI2 and the other *Verrucosispora* species is the pH range for growth.

V. gifhorensis was reported to grow within a narrow pH range (6.4 to 8.2), with optimum growth at pH 7.5. *Verrucosispora lutea* was reported to grow within a wider pH range, from 5 to 10. Isolate GSI2 showed no growth below pH 7.

Table 2.5: Physiological and chemotaxonomic properties that distinguish strain GSI2 from its closest relatives.

Characteristic	Isolate GSI2	*V.gifhorensis* DSM 44337T(a)	*V. lutea* KCTC19195T(b)	*V. sediminis* MS426T(c)
•Spore surface ornamentation	Smooth	Smooth, warty, hairy	Smooth	Warty
•Areial mycelia	Absent	Absent	Sparse	Sparse
•Temperature range (optimum)	20-45 (30)	20-40 (35)	20-45 (28)	20-45 (28-37)
•pH range (optimum)	7-11 (9)	6.5-8.2 (7.5)	6-8 (7)	3.5-10 (5-7.5)
•NaCl concentration for growth (% w/v)	0 - 4	0 - 4	0 – 7	0-6
•Antibiotic susceptibily	Susceptible to erythromycin	Resistant to erythromycin	ND	Resistant to erythromycin
•Utilisation as sole carbon source :				
D- Fructose	+	-	+	-
Lactose	+	-	+	+
Glycerol	+	-	+	+
Trehalose	+	+	-	+
•Utilisation as nitrogen source :				
Alanine	+	W	-	+
L-glutamic acid	+	+	-	-
L- phenylalanine	+	+	-	-
Methionine	+	-	+	-
Valine	+	W	-	-
•Liquifaction of gelatin	+	+	-	ND
•Major cell wall sugars*	Xyl, Glc, Gal	Man,Xyl	Xyl, Glc	GlcN, Glu, Man
•Phospholipids §	PI, PE	PE, DPG, PIM, PS, PL	PE, DPG, PIM, PI, PL	PE, DPG, PIM, PI, PL

Table index

* Glc, Glucose; GlcN, glucosamine; Man, mannose; Xyl, xylose;
§ PE, phosphatidylethanolamine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PIM, phosphatidylinositol mannoside; PS, phosphatidylserine; PL, unknown phospholipid
+ positive growth observed; - no growth observed; w weak growth; ND not determined
(a) (Rheims *et al.*, 1998) ; (b) (Liao *et al.*, 2009); (c) (Dai *et al.*, 2009).

The cell wall sugars of isolate GSI2 differed from those of the other *Verrucosispora* isolates. Cell wall sugar composition is an important characteristic in actinobacterial chemotaxonomy. Xi *et al.* (2011) amended the description of the genus *Verrucosispora* by stipulating that mannose is the diagnostic sugar in cell walls. The results in this study as well as in the species description of *V. lutea* (Liao *et al.*, 2009) do not support this amendment. In *V. lutea* the major wall cell sugars are xylose and glucose, while those in GSI2 are xylose, glucose and galactose (Figure 2.13). Although minor amounts of mannose were detected in *V. lutea*, the use of mannose as a diagnostic sugar is not justified.

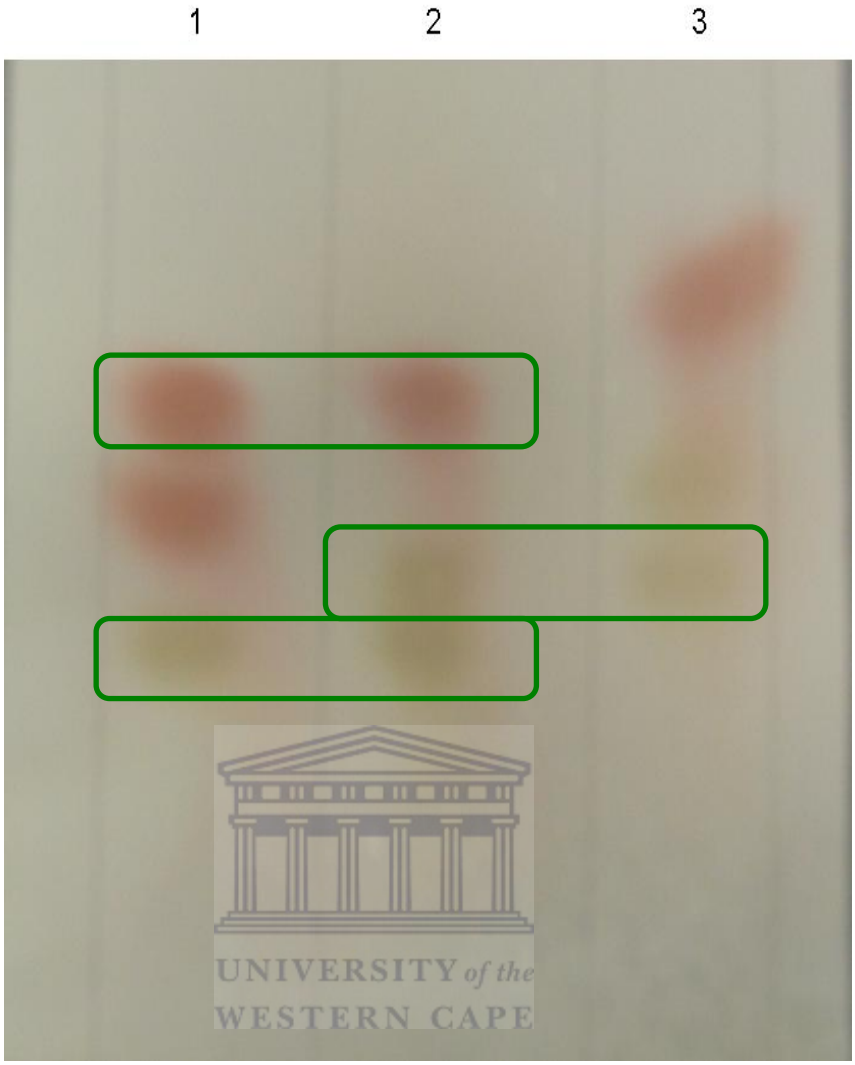

Figure 2.13: Separation of whole cell hydrosylate of GSI2 by thin layer chromatography. Lane 1: 1 % galactose, arabinose, xylose standards (ascending order), Lane 2: whole cell hydrosylate of GSI 2 prepared from freeze dried cells, Lane 3: 1 % glucose, mannose, ribose standards (ascending order)

GSI2 was distinguishable from the type strains of the three described *Verrucosispora* species by using a combination of chemical and morphological markers. We suggest that GSI2 should be placed in the genus *Verrucosispora* as the type strain of a novel species, for which we propose the name *Verrucosispora africana* sp. nov.

2.3.4 Characterisation of isolate BSII9

Preliminary enzyme screening of isolates (Chapter 3) identified isolate BSII 9 as a potential source of industrially important enzymes. Identification and partial characterisation of this isolate was therefore warranted.

2.3.4.1 Morphology

Isolate BSII9 grew well on most of the media tested but grew poorly on ISP4 and ISP5 media. The isolate produced well defined substrate mycelium and white/grey aerial mycelium (Figure 2.14 a and b). The spore chain morphology, observed by light microscopy, was straight (Figure 2.14 c)

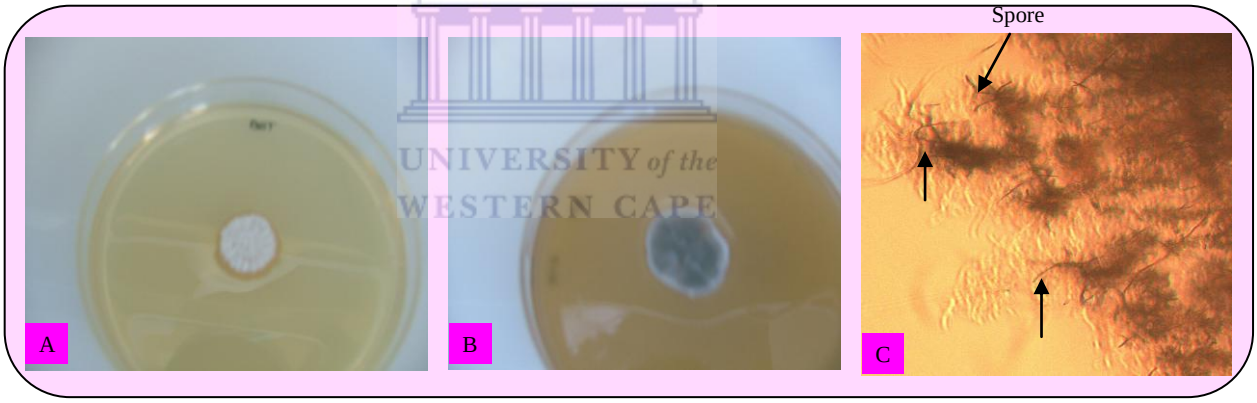

Figure 2.14: Isolate BSII 9 on tryptic soy agar (a) and Emerson agar (b) after incubation at 37 °C for 7 days. Spore chain morphology as seen by light microscopy at x 100 magnification (c).

2.3.4.2 Optimum growth temperature

Isolate BSII 9 grew over a temperature range of 20 °C to 45 °C and optimum growth was observed at 37 °C (Figure 2.15), and is therefore a mesophile. Therefore in all subsequent experiments isolate BSII9 was cultivated at 37 °C.

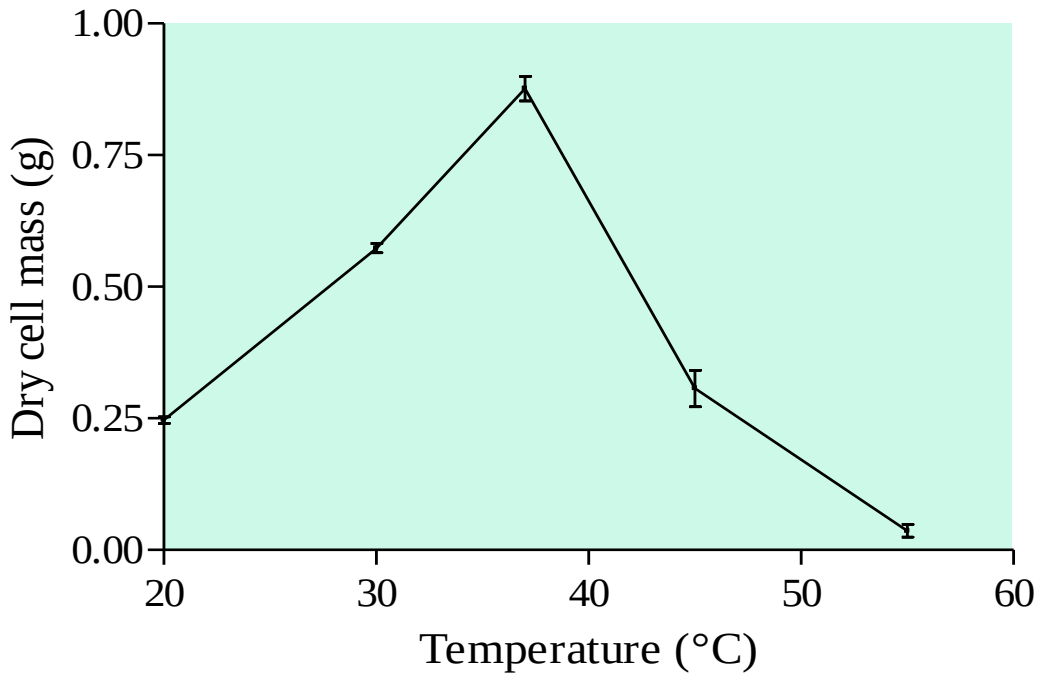

Figure 2.15: Growth profile of BSII9 in tryptic soy broth at different temperatures.

2.3.4.3 Optimum growth pH

On solid agar, isolate BSII 9 was able to grow over a wide pH range of 5 to 11 (initial pH). The optimum pH for growth in a buffered system in liquid medium was 9. Interestingly, growth in unbuffered media (initial pH 7.2) was optimal and resembled growth at pH 9. However when the media was buffered at pH 7 growth was noticeably reduced. Monitoring of pH values during growth in unbuffered media showed a rapid increase to 8, but with a three day lag phase observed in the growth curve (Figure 2.16). When isolate BSII9 was grown in a buffer system at pH 9 the lag phase was reduced to 1 day (Figure 2.17)

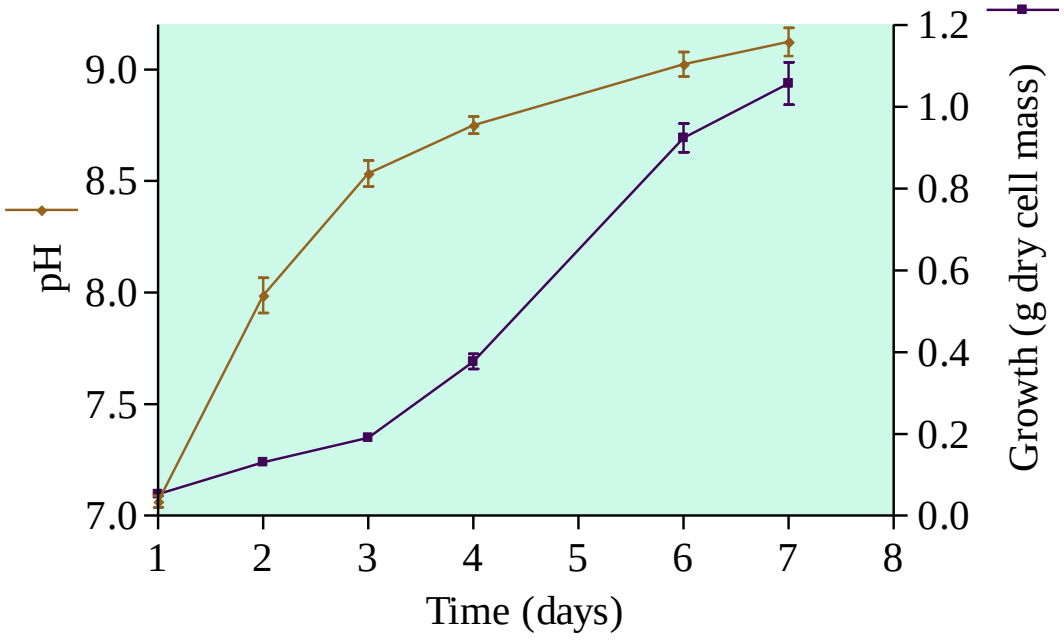

Figure 2.16: pH changes observed in unbuffered tryptic soy broth during growth of isolate BSII 9.

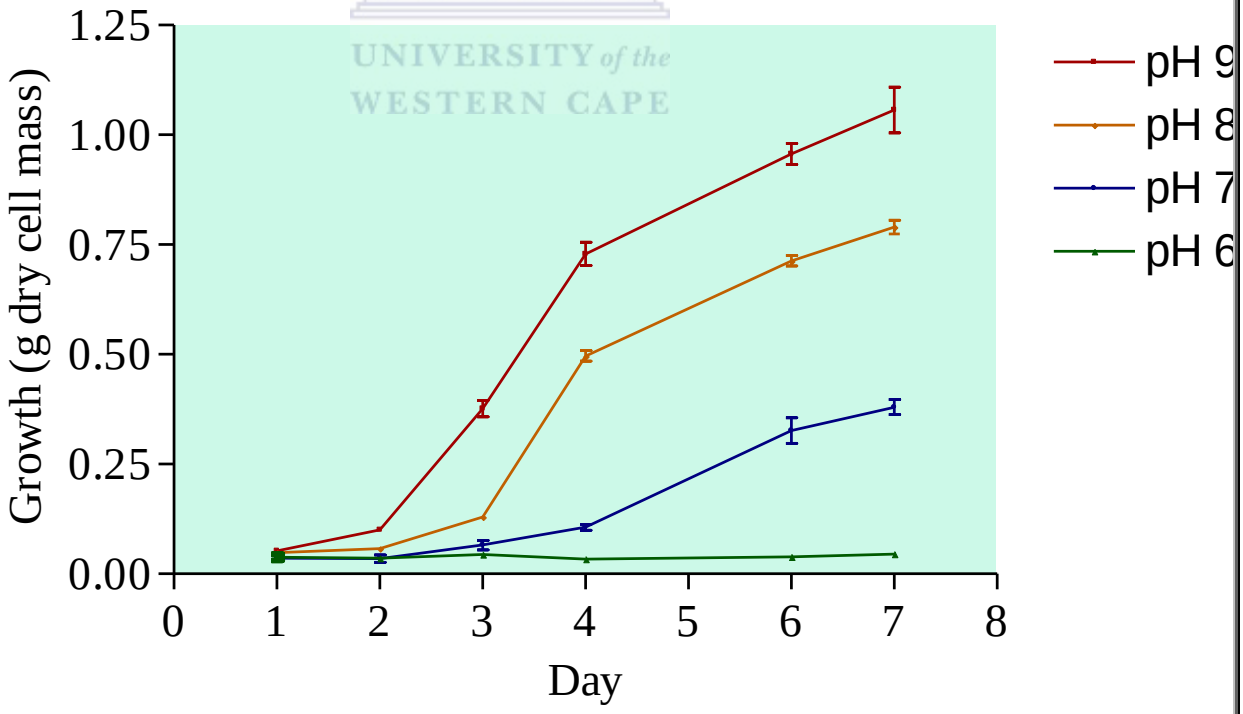

Figure 2.17: Growth curves for isolate BSII 9 grown in tryptic soy broth buffered at different pH values.

In most organisms, the ability to tolerate a wide pH range is due to (i) an efficient pH homeostatic system, whereby cells maintain a relatively constant intracellular pH (pHi) over a broad range of external pH (pHo) values and (ii) regulatory mechanisms which ensure that molecules exposed to the extracellualar environment (such as certain permeases, small metabolites and extracellular enzymes) are only produced under specific pH conditions (Booth, 1985; Russell,1991). The alteration of environmental pH during growth has been well studied in *Lactobacillus* stains (Messaoudi *et al.*, 2005). This phenomenon, if proven to occur in isolate BSII 9, would be very interesting to investigate further as it might give some insight into some of the competitive mechanisms used by *Streptomyces* species to dominate in soil microbial communities.

2.3.4.4 Physiology

Isolate BSII9 degraded starch and hypoxanthine and did not degrade gelatine, L- tyrosine, cellulose and xylan. Sucrose, lactose and glucose were the preferred carbon sources for growth, and phenylalanine, L- arginine and tyrosine were the preferred nitrogen sources. BSII9 tolerated NaCl up to concentrations of 10 %. BSII 9 was susceptible to erythromycin (10 µg/ml), gentamicin (100 µg/ml), kanamycin (50 µg/ml) and streptomycin (10 µg/ml), but was resistant to ampicillin (100 µg/ml). The isolate did not produce any antibiotics under the conditions tested in this study. Table 2.6 is a summary of the morphological and physiological characteristics of isolate BSII9.

Table 2.6: Physiological characteristics of BSII9.

Characteristic	Result
Utilisation as sole carbon source :	
Arabinose	++
Glucose	+++
Inositol	++
Lactose	+++
Maltose	+++
Raffinose	+
Ribose	++
Sucrose	+++
Xylose	++
Utilisation as nitrogen source :	
Alanine	++
L-arginine	+++
DL-DOPA	+
Leucine	+++
Methionine	++
Milk powder	+++
Phenylalanine	+++
Proline	++
Tyrosine	+++
Degradation of :	
Hypoxanthine	P
Starch	P
Gelatin	N
L-Tyrosine	N
Cellulose	N
Xylan	N

Key: P- positive, N- negative. + poor growth, ++ fair growth, +++ very good growth

2.3.4.5 Phylogenetic analysis

The 16S rRNA gene sequence (1344 nucleotides) of isolate BSII9 was compared to sequences on GenBank (Fig 2.18).

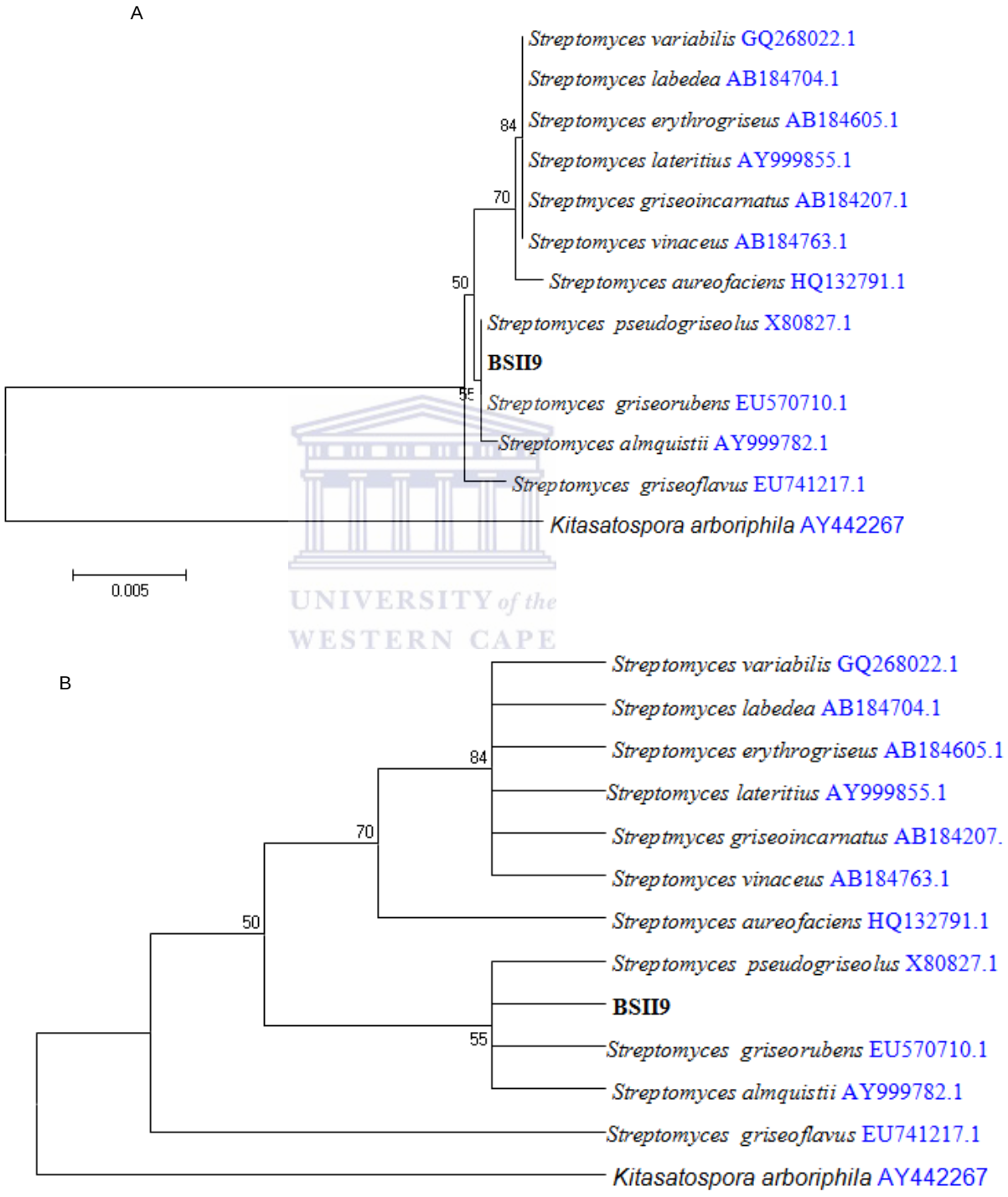

Figure 2.18: Neighbour joining phylogenetic trees derived from 16S rRNA gene sequences showing the relationship between isolate BSII9 and the closely related species (A). Topolgy of the tree is indicated in B.

Isolate BSII9 clustered with three *Streptomyces* species. However, this clade was supported by a low bootstrap value of 55, showing that the sequences analysed were highly similar and hence could not accurately distinguish the different nodes. This was consistent with the sequence identity matrix which showed that the compared sequences had identities between 0.999 and 1. Although 16S rRNA gene sequencing is useful in routine bacterial classification, it has been shown to poorly distinguish between species in some genera (Fox *et al*., 1992; Janda and Abbott, 2007). The inability of 16S rRNA gene sequence analysis to distinguish between closely related species within *Streptomyces* clades is widely acknowledged (Liu *et al.,* 2005; Guo *et al.,* 2008). Some of the most well studied *Streptomyces* clades such as the *Streptomyces griseus* and the *Streptomyces albidoflavus* clades are taxonomically complex and the majority of members of these groups share highly similar phenotypes and 16S rRNA gene sequences (Rong et al., 2009; Rong and Huang, 2010). Despite the importance placed on DNA-DNA hybridization in describing novel bacterial species (Stackebrandt and Goebel, 1994; Rosselló-Móra and Amann, 2001), the technique is not easily accessible to all researchers. Moreover, results obtained from the method have been reported to have low reproducibility and the hybridization similarity values are influenced by physical and chemical factors (Stackenbrandt *et al.,* 2002). Apart from DNA-DNA hybridization, multilocus sequence analysis of several house-keeping genes such as *atpD, gyrB, recA, rpoB and trpB* has been suggested (Kampfer and Glaeser, 2011). In this study, the sequences for these house-keeping genes were obtained from contigs assembled from the genome sequence and were included in the phylogenetic classification of isolate BSII9. Neighbour joining bootstrap tests of phylogeny were constructed and in all phylogenetic trees, bootstrap values lower than 40 were excluded.

An 830 bp fragment of the *gyrB* gene from BSII9 was compared to sequences in the database (NCBI GeneBank). Isolate BSII9 was closely related to *Streptomyces labedae* and *Streptomyces erythrogriseus* with a sequence similarity of 99% in both cases. Isolate BSII9 clustered with *Streptomyces labedae* and *Streptomyces erythrogriseus* in a clade supported by a bootstrap value of 99 (Figure 2.19).

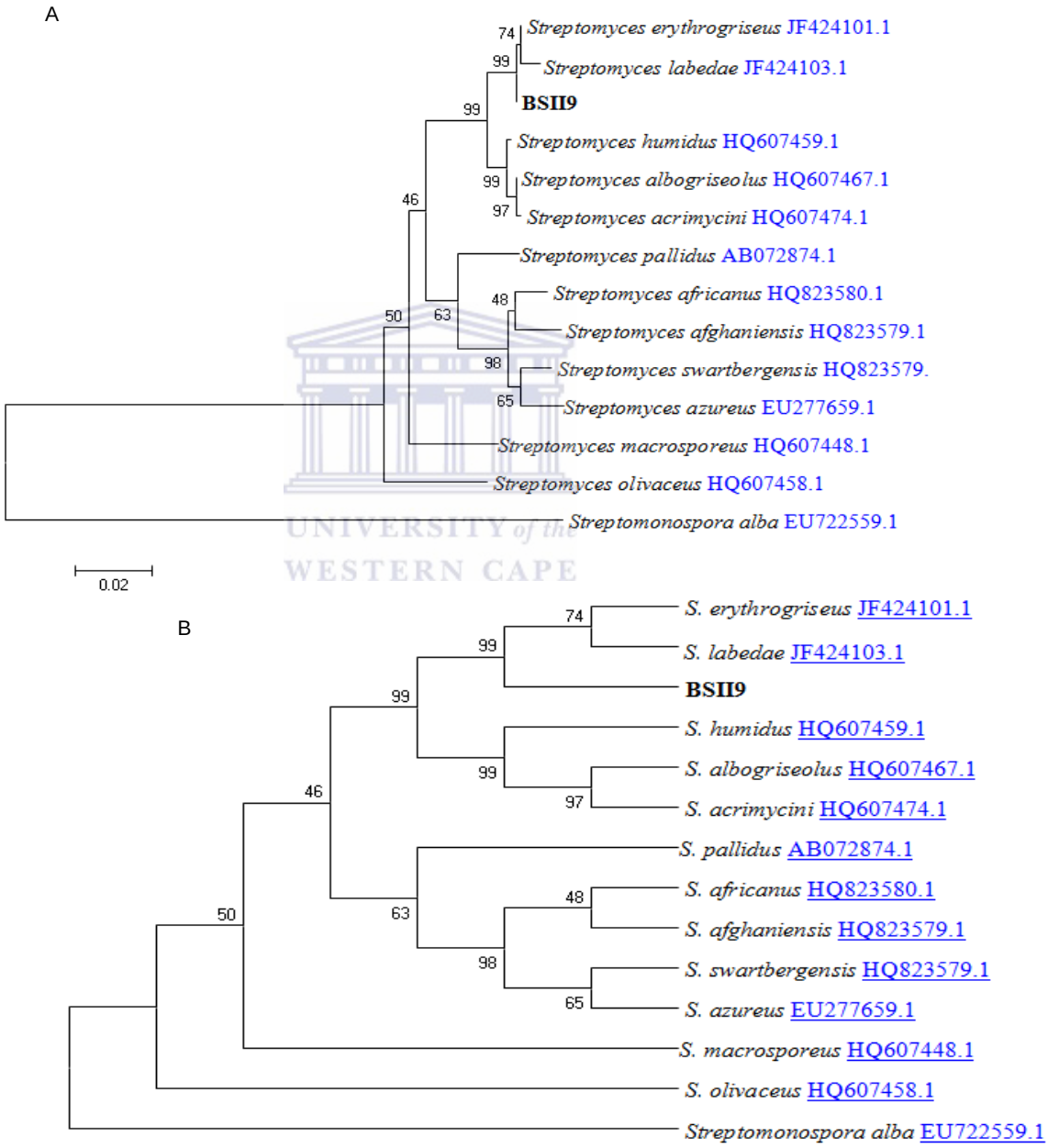

Figure 2.19: Neighbour joining phylogenetic tree derived from gyrB gene sequences. The topology of the tree is highlighted in B.

However, the isolate formed a distinct monophyletic branch which was supported by a bootstrap value of 99, showing that although it was closely related to *S. labedae* and *S. erythrogriseus*, according to the *gyrB* sequence analysis it was distinguishable at the species level. This trend was also observed for the *recA* tree (Figure 2.20) and the *rpoB* tree (Figure 2.21).

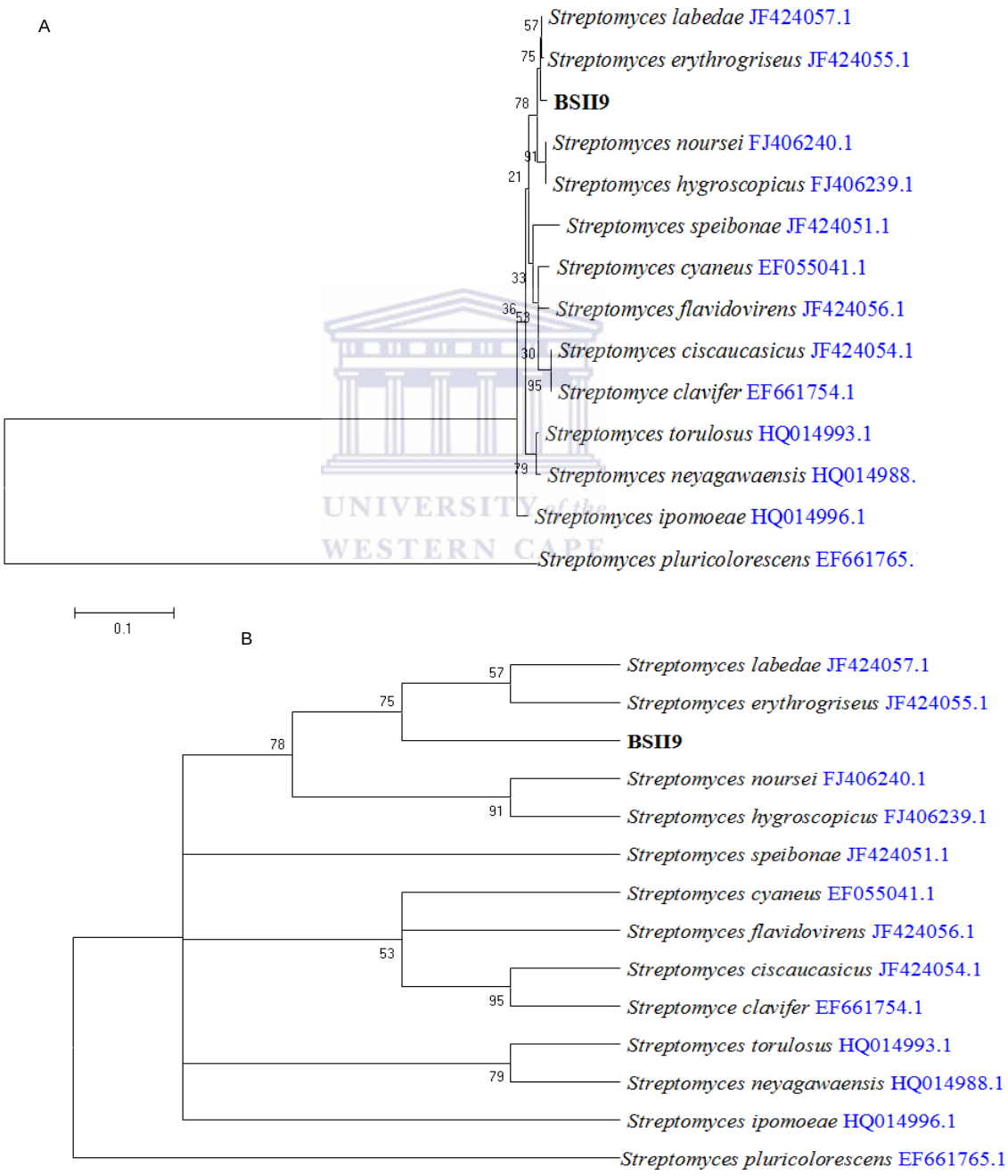

Figure 2.20: Neighbour joining phylogenetic tree derived from recA gene sequences. The topology of the tree is highlighted in B.

A 497 bp fragment of the BSII9 *recA* gene sequence was compared to related sequences in the NCBI database. Sequence identity was 99 % to both the *S. erythrogriseus and S. labedae* genes.

A 993 bp fragment of the BSII9 *rpoB* gene sequence showed 99% similarity to both *S. erythrogriseus* and *S. labedae* sequences. The three strains formed a distinct clade separate from the other related *Streptomyces* species (Figure 2.21). This clade was supported by a bootstrap value of 75. The separation of isolate BSII 9 into a distinct monophyletic branch was also supported by a very high bootstrap value of 98.

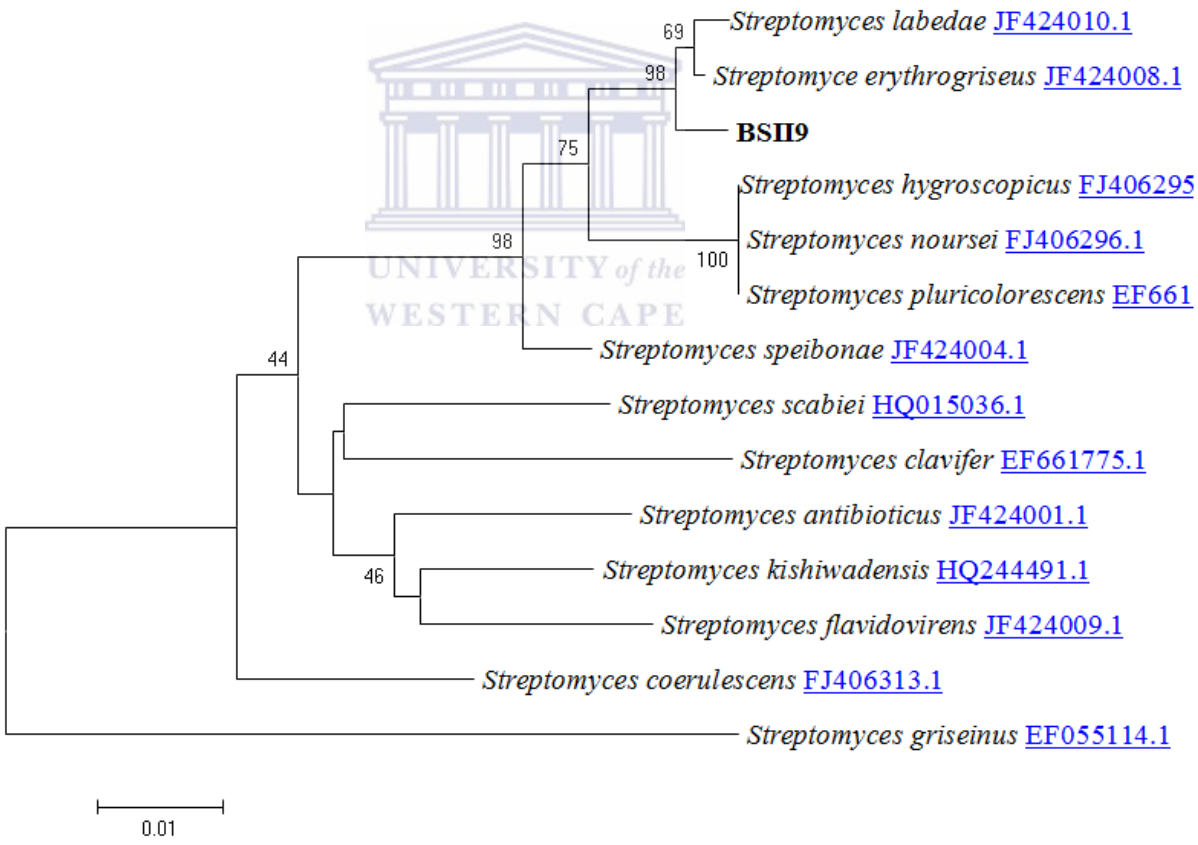

Figure 2.21: Neighbour joining phylogenetic tree derived from rpoB gene sequences.

The 500 bp *trpB* gene sequence fragment of BSII9 only showed 95% similarity to *S. labedae* and 93% similarity to *S. erythrogriseus*. When the closely related sequences were analysed in

a neighbour joining tree (Figure 2.22), the observed trend was slightly different from that of the previous trees. The three strains BSII9, *S. labedae* and *S. erythrogriseus* formed a distinct clade separate from the other *Streptomyces* species. However, branching within this clade differed from that observed in the previous three trees in that isolate BSII9 did not form a monophyletic branch but formed a distinct branch with *S. labedae*. The separation of *S. erythrogriseus* from the other two isolates was supported by a bootstrap value of 100 and the BSII 9 - *S. labedae* pairing was supported by a bootstrap value of 83.

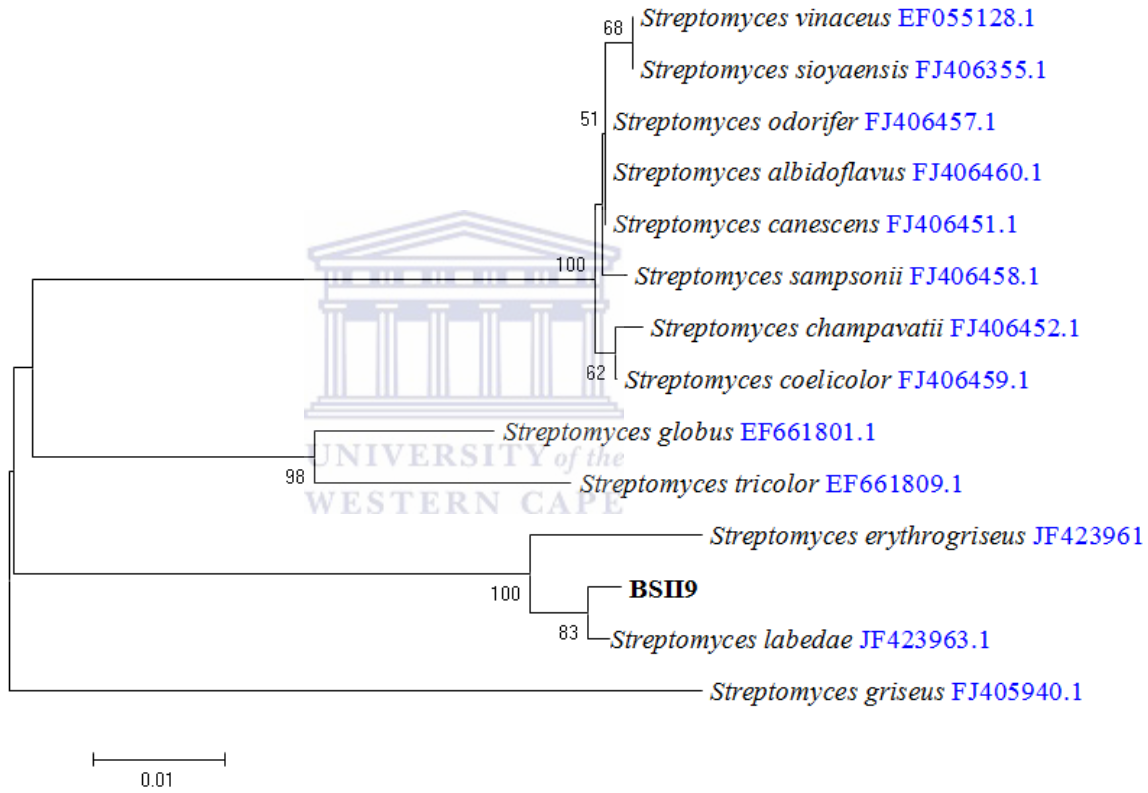

Figure 2.22: Phylogenetic tree derived from trpB gene sequences showing the relationship between isolate BSII9 and the closely related species.

When a 399 bp fragment of the BSII9 *atpD* was compared to those in the database, the sequence showed 100% similarity to both the *S. erythrogriseus* and the *S. labedae* sequences. The clade containing isolate BSII9, *S. erythrogriseus* and *S. labedae* was supported by a bootstrap value of 93 (Figure 2.23).

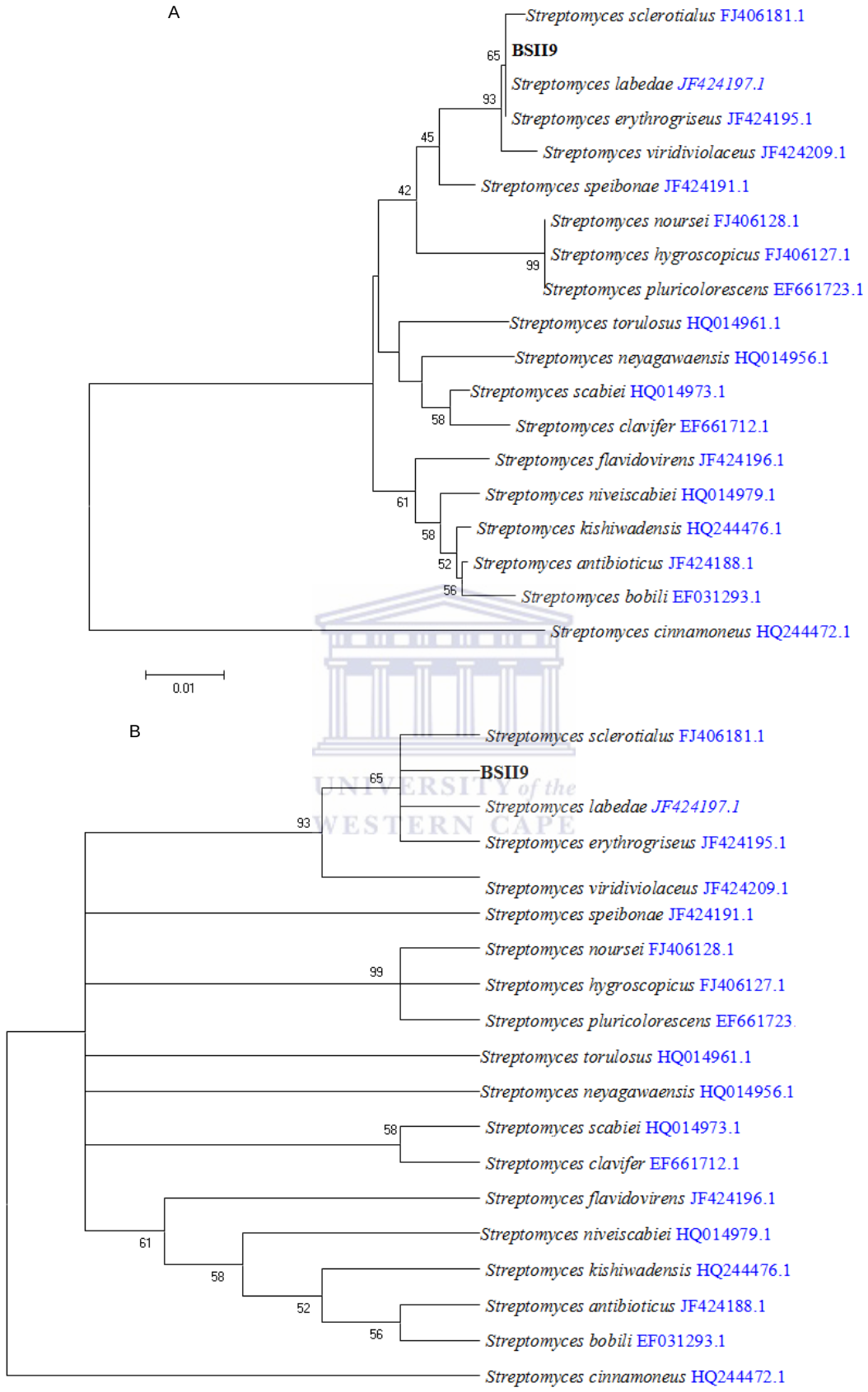

Figure 2.23: Phylogenetic tree derived from atpD gene sequences showing the relationship between isolate BSII9 and the closely related species.

However, unlike in previous trees, there was no distinct branching to separate nodes of the sequences in this clade. Isolate BSII 9 was therefore not distinguishable from *S. erythrogriseus* and *S. labedae* on the basis of *atpD* gene sequence analysis.

Results of the trees constructed from the housekeeping genes were comparable and revealed that isolate BSII9 was closely related to *S. labedae* and *S. erythrogriseus*. Although the 16S rRNA gene sequence of isolate BSII9 showed 100% similarity to that of *S. labedae* and *S. erythrogriseus*, BSII9 fell into a different clade on the neighbour joining tree (Figure 2.18). Isolate BSII9 was positioned in the same clade as *Streptomyces griseorubens, Streptomyces pseudogriseolus* and *Streptomyces almiquistii*. However, the house-keeping genes from these three species have not been analysed and therefore could not be included in this study. Four of the five gene sequences indicated that isolate BSII9 was closely related to *S. labedae* and *S. erythrogriseus* but could clearly be distinguished as a separate species. From the observations made in this study, MLSA is a good tool for phylogenetic analysis of closely related species. However, the biggest drawback of the technique is the lack of sufficient sequence data for comparison. The technique is clearly useful as a complement were 16S rRNA gene sequence analysis alone is insufficient.

Chapter 3: Screening actinobacterial isolates for industrially relevant nitrile metabolising enzymes

3.1 Introduction

Since *Bacillus* proteases were first introduced into detergents around 1965, enzymes have found many applications in industry including in organic synthesis, clinical analysis, pharmaceuticals, food production and fermentation. Enzyme transformations are considered as practical alternatives to chemical synthesis processes (Ogawa and Shimuzu, 1997). Although reactions catalyzed by enzymes or enzyme systems display far greater specificities compared to chemical processes, in many cases the substrates used in industrial processes are artificial compounds and enzymes required to transform these complex substrates are still unknown (Schmid *et al.,* 2002). Previously undiscovered microbial enzymes which catalyse novel synthetic processes have the potential to replace existing chemical catalysts (Koeller, 2001). Typical industrial enzyme discovery programs screen a large number of microorganisms. While cumbersome, this method has proved to be highly successful in obtaining novel biocatalysts (Shimuzu *et al.,* 1997).

Nitriles are organic compound that have a -C≡N functional group. They are widely used in industry because they are important intermediates in the chemical synthesis of various products such as super-glue® (methyl cyanoacrylate), pesticides (dichlobenil, bromoxynil, ioxynil, buctril), as well as nitrile-containing drugs used for the medical treatment of various conditions such as diabetes, breast cancer, HIV and clinical depression (Figure 3.1.) (Banerjee *et al.,* 2002; Fleming *et al.,* 2010).

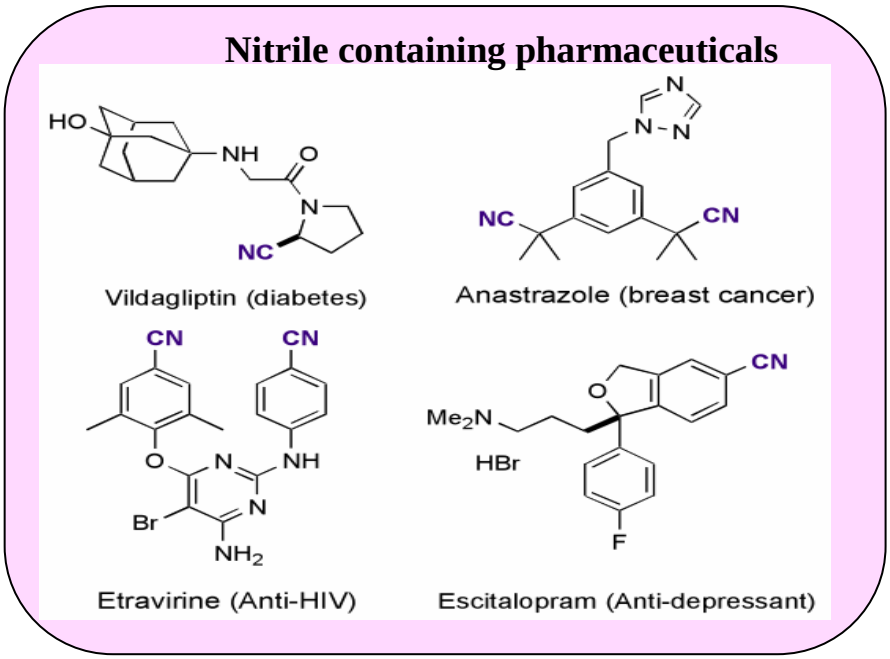

Figure 3.1: Examples of structurally diverse nitrile containing pharmaceutical compounds and the respective conditions they are prescribed for (Fleming *et al.*, 2010)

Wastewater containing nitrile compounds are potentially a source of environmental contamination as some of these nitriles are toxic, carcinogenic and mutagenic (Kobayashi *et al.*, 1998; Nagasawa *et al.*, 2000; Brandao and Bull, 2003). As such the metabolism of nitriles by nitrile-converting biocatalysts is of great importance to the chemical industry. These biocatalysts can be used to treat toxic nitrile- and cyanide-containing waste.

Nitrile-hydrolyzing enzymes such as nitrile hydratases (NHases) and nitrilases are also used in the chemical conversion of nitriles to the corresponding amides or acids which are high value products. These enzymes are currently widely used in the industrial production of high purity acrylamide (Yamada and Kobayashi, 1996). Most industrialized NHases with high activity such as the NHases from *Rhodococcus* sp. N-774 and *Pseudomonas chlororaphils* B23 (Nagasawa *et al.*, 1993) are not thermally stable (Liu *et al.*, 2008). Therefore, bioreactors have to be refrigerated in order to maintain a low reaction temperature to stabilize the NHases, which ultimately results in unwanted energy costs.

Another important group of related enzymes are hydantoinases, which are cyclic amidases that can catalyse the hydrolysis of hydantoins to amino acids. Amino acids can be chemically synthesised using alkaline hydrolysis of hydantoins (Syldatk *et al.,* 1990; Watabe *et al.,* 1992; Sudge *et al.,* 1998), yielding a racemic mixture of the amino acids. However, the racemic mixture requires further chemical resolution to produce optically active amino acid (Syldatk *et al.,* 1990; Sudge *et al.,* 1998). Currently an alternative three step process is used in the production of optically pure amino acids (Figure 3. 2). Step 1 (1 in Figure 3.2) involves the chemical synthesis of substituted hydantoin substrates, followed by the stereo-specific hydrolysis of hydantoins catalysed by hydantoinases (2). The final step (3) is a chemical decarbamoylation which produces the optically pure amino acids.

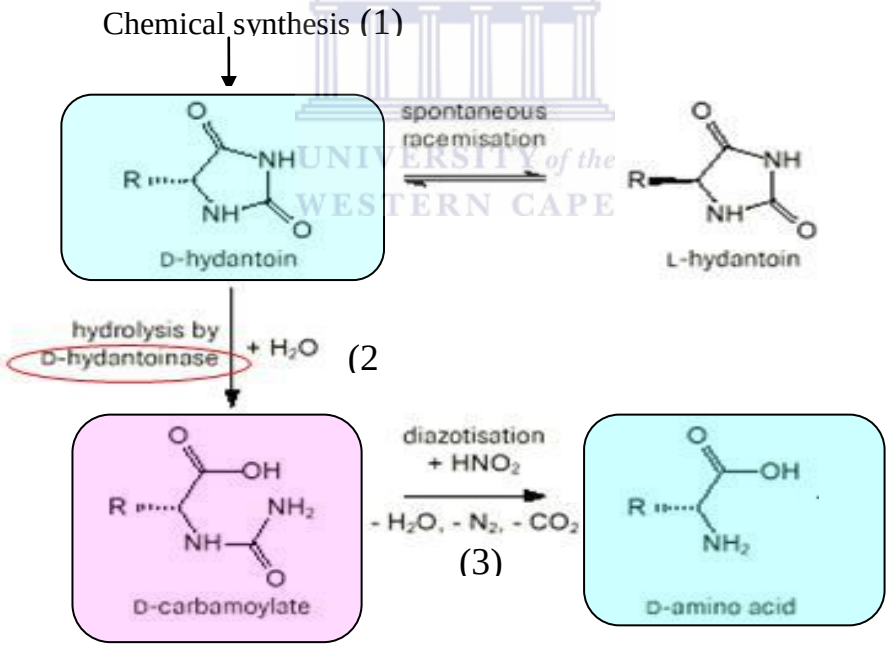

Figure 3.2: The chemo-enzymatic production of D-amino acid. The compounds in blue boxes are synthesised chemically while the compound in the pink box is synthesised enzymatically by D-hydantoinase (red circle) Adapted from May *et al.* (1998).

This process has several disadvantages which includes the toxicity of the starting reactants, poor mass yields of the product and the large amount of energy required (the reaction is carried out at temperatures as high as 170 °C) (Yamashiro *et al.,* 1988). These disadvantages

prohibit the development of large-scale production of optically pure amino acids. The use of a full enzymatic process would offer several advantages which include higher product yield, higher optical purity, and lower environmental impact (Triphathi *et al.,* 2000). Several micro-organisms that express both a hydantoinase and a carbamylase have been isolated from genera such as *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Ochrobactrum* and *Pseudomonas* (Pozo *et al.,* 2002). Interestingly, no streptomycete hydantoinases or carbamoylases have been characterised thus far.

Secondary metabolites include antibiotics, pigments, toxins, enzymes and antitumor agents which are not directly involved in normal growth, development or reproduction of the producing organism. Microorganisms that produce secondary metabolites do so during the stationary phase of the growth cycle (Martin *et al.,* 2005). Secondary metabolite production is believed to be triggered by fermentation conditions including the depletion of nutrients, the biosynthesis of an inducer or a decrease in growth rate (Bibb, 2005). In response to these conditions the microorganism produces signals which trigger a cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphological differentiation (morphogenesis) (Demain, 1998).

In order to optimise the production of the secondary metabolite of interest, it is important to understand the growth cycle and requirements for optimum growth of the producing organism. The life cycle of actinobacteria has been well studied in the genus *Streptomyces*. This genus is the largest producer of actinobacterial secondary metabolites and as such are of great industrial importance (Jensen *et al.,* 2005).The life cycle of streptomycetes is complex and is classified as being neither unicellular nor multicellular (Maguelez *et al.,* 2000).

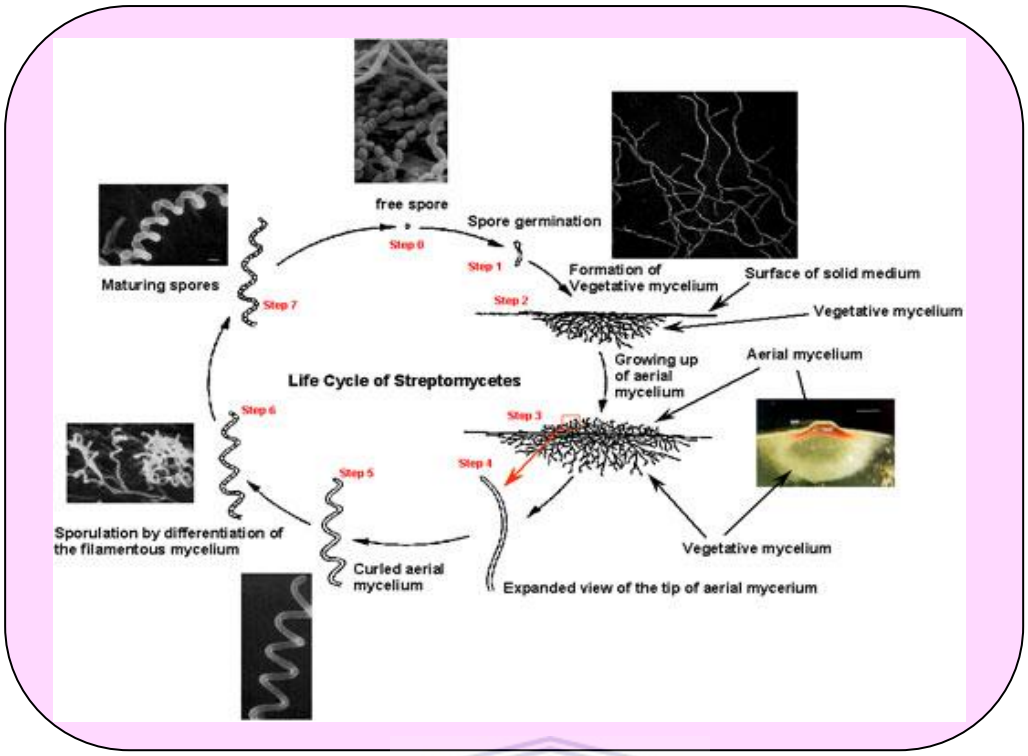

Figure 3.3: Streptomycete life cycle (http://home.hiroshima_u.ac.jp/mbiotech/hosenkin_lab/Strepto-E.html)

On solid media a streptomycetes life cycle begins with the germination of spores. Filaments spread into the solid medium to form vegetative mycelium (substrate mycelium) (Figure 3.3 steps 0-2). After several days, the mature substrate mycelium forms sporophores which extend vertically toward the surface, above the colony and these are collectively known as "aerial mycelium" (step 3-4). Both substrate and aerial mycelia are multinucleated. The aerial mycelium undergo septation to form polynucleated cells (step 5 and 6), which later become spores (step 7). On solid media, secondary metabolites are thought to be secreted during the generation of aerial hyphae from the vegetative mycelium (Maguelez *et al.*, 2000). Although most industrial secondary metabolite production processes are in liquid cultures, most *Streptomyces* strains do not sporulate under submerged conditions and the differentiation in liquid cultures is poorly understood (Manteca and Sanchez, 2009).

3.2 Materials and Methods.

3.2.1 Screening of isolates for enzyme activity

3.2.1.1 Preparation of whole cell biotransformation reactions and crude cell free extract

Cells were grown in modified 172F (M172F) broth and harvested after seven days by centrifugation (10 min, 8000 x *g*, 4 °C). Cells were washed twice by re-suspending in 100 mM potassium phosphate buffer (pH 8) and centrifuging (10 min, 8000 x *g*, 4°C). Resting cell suspensions for use in the biotransformation reactions were prepared by re-suspending 0.5 g (wet mass) of cells in 100 mM potassium phosphate buffer (pH 8) to a final concentration of 50 mg/ml. Crude cell free extracts were prepared by first digesting the cell walls overnight using lysozyme (25 mg/ml) in 100 mM Tris-HCl pH 8 at 4 °C, followed by disruption using 5 cycles of sonication at 50 % power for 30 seconds (Bandelin Sonopuls HD2070 sonicator). Cell free extracts were obtained by centrifugation (10 min, 8000 x *g*, 4 °C) followed by filtration of the supernatant through a 0.22 µm syringe filter.

3.2.1.2 Determining protein concentration in crude cell free extract

Bovine serum albumin (BSA) protein standards were prepared over the range 0 to 2 mg/ml from a 10 mg/ml stock. A Bradford assay was perfomed using 20 µl aliquots of protein standard or sample and 980 µl of filtered protein assay reagent (BioRad). The solutions were mixed thoroughly and incubated for at least five minutes at room temperature before measuring the absorbance at 595 nm. Protein sample concentrations were calculated by interpolation of absorbance values against the standard curve (Appendix i) determined from the BSA protein standards.

3.2.1.3 Amidase and nitrile hydratase activities

Hydrolysis of amides and nitriles was investigated using acetamide, acrylamide, benzamide, acetonitrile, acrylonitrile and benzonitrile as substrates. The final reaction volume of 1.5 ml contained 675 µl buffer (100 mM potassium phosphate buffer pH 8), 75 µl crude cell free extract and 25 mM substrate. The enzyme reaction was stopped by the addition of 3.5 volumes reagent A (0.59 M phenol/ 1 mM sodium nitropruside). Samples were developed by the addition of an equal volume of reagent B (0.11 M sodium hypochlorite/ 2.0 M sodium hydroxide). Results were quantified spectrophotometrically at 600 nm. The amount of ammonia liberated was determined using an NH_4Cl standard curve (Appendix ii) and the relative activities were calculated. Units of specific activity were defined as µmols of ammonia liberated from one mg of protein per minute.

3.2.1.4. Hydantoinase and carbamoylase activity

To screen bacterial isolates for hydantoinase activity, assays were conducted using resting cells as prepared in section 3.2.1.1. The substrates (25 mM hydantoin, 4 mM benzyl hydantoin, 25 mM dihydrouracil and 5 mM phenyhydantoin) were prepared separately in 100 mM potassium phosphate buffer (pH 8). The reaction was initiated by the addition of 1.5 ml resting cells to 1.5 ml pre-incubated substrate solution in sterile 15 ml falcon tubes and incubated at 37 °C for 1 hour. To terminate the reaction cells were pelleted by centrifugation at 16 000 x *g* for 10 min.

To analyse the reaction products, 300 µl of the assay supernatant was added to 800 µl Ehrlich Reagent (1 g 4-dimethylaminobenzaldehyde, 5 ml H_2O, 5 ml 32 % HCl) and 900 µl distilled water. The quantification of the yellow product was performed spectrophotometrically at 430 nm. The biotransformation of phenyl hydantoin and benzyl hydantoin was determined by

HPLC analysis (Merck Hitachi, La Chrom), using a Nucleodex β-PM-column (Macherey-Nagel, Germany). The mobile phase contained 20 % MeOH/ 80 % 0.1 % H_3PO_4 (v/v) solution. The amount of amino acid produced was calculated from standard curves of the expected products (Table 3.1).

Table 3.1: Substrate/product pairs and the respective amino acids expected from their enzymatic hydrolysis.

Substrate	Expected N-carbamoyl amino acid	Expected amino acid
Hydantoin	N-carbamoyl glycine	Glycine
Dihydrouracil	N-carbamoyl β-alanine	β-alanine
Benzyl hydantoin	N-carbamoyl phenyl alanine	Phenyl alanine
Phenyl hydantoin	N-carbamoyl phenyl glycine	Phenyl glycine

To investigate carbamoylase activities carbamoyl amino acids were used as substrates at a concentration of 10 mM and the reactions were carried out as described above. The products of the N- carbamoyl amino acid hydrolysis were measured using the ninhydrin assay. A suitable dilution (1:10 to 1:50) of the sample was made with 0.1 M Tris-HCl buffer pH 8.0, to a final volume of 1 ml in acid-washed test tubes. An equal volume of ninhydrin reagent was added and the mixture was mixed thoroughly by vortexing. Samples were incubated in a boiling water bath (100 °C) for 15 min. The tubes were allowed to cool to room temperature and diluted with 3 ml 50 % ethanol, mixed thoroughly and incubated at room temperature for a further 10 min. The coloured products were measured spectrophotometrically at 570 nm and the amount of amino acid produced was quantified using glycine and alanine standard curves (Appendix iii and iv, respectively). Hydantoinase activity was calculated as the amount of N-carbamoyl glycine produced from 1 ml (50 mg/ml) of cells per hour and carbamoylase activity was calculated as the amount of glycine produced from 1 ml (50 mg/ml) of cells per hour.

3.2.2 Optimisation of growth and hydantoinase production

3.2.2.1 Effect of media on growth

The growth of hydantoinase producing isolates was optimised by investigating the effects of different media. The isolates were grown in 7H9 (Difco™ Middlebrook 7H9 Broth supplemented with 100 mM glucose, ADC supplement omitted), Yeast extract malt extract (ISP2) (Shirling and Gottlieb, 1966), Tryptic soy broth (composition g/L: casein peptone (pancreatic) 17 g, dipotassium hydrogen phosphate 2.5 g, glucose 2.5 g, sodium chloride 5 g, soya peptone (papain digest) 3 g, modified 172F (composition g/L, 10 g glucose, 5 g yeast extract, 10 g starch, 5 g casitone, 2.5 g $MgSO_4.7H_2O$, 2 g $CaSO_4.2H_2O$), Bennet's medium (composition g/L: 10 g glucose, 2 g casitone, 1 g yeast extract, 1 g beef extract), ISP3, ISP6 and ISP7. The pH of all media was adjusted to pH 7.3±0.2. All flasks were inoculated with the same volume of starter culture (1 ml starter to 10 ml of medium) and cultures were incubated at 37°C with shaking (100 rpm) for seven days. Cells were harvested by filtering the growth cultures onto pre-weighed 0.2um filter papers using a vacuum pump. Cells were dried in closed Petri dishes in a 55°C oven for 24 hours. Growth was recorded as dry cell mass.

3.2.2.2 Effect of temperature on growth

The effect of temperature on growth was investigated by inoculating an equal volume of starter culture into 10 ml volumes of M172F broth as outlined in 3.2.2.1. Duplicate flasks were inoculated for each temperature tested. Flasks were incubated at 20 °C, 30 °C, 37 °C, 45 °C and 55 °C with shaking (100 rpm) for 5 days. Cells were harvested and growth was recorded as described in 3.2.2.1.

3.2.2.3 Effect of pH on growth

The ability of the isolate to grow at different initial pH was tested on solid media. The pH of the media was adjusted to 5, 6, 7, 8, 9, 10 or 11 using either HCl or NaOH. Plates were

incubated at 37 °C for 7 days. Growth was scored on an arbitrary scale from zero, meaning no growth to five, meaning very good growth. To determine the optimal pH for growth cells were grown in M172F broth buffered at pH 6.0. 7.0 8.0 or 9.0 using either 25 mM potassium phosphate buffer or 25 mM Tris-HCl buffer (Table 3.2).

Table 3.2: Buffers used to maintain pH during growth of isolates

Buffer	pH used	pKa	Buffering range
Potassium phosphate	6, 7	7.2	5.8 to 8.5
Tris- HCl	8, 9	8.06	7.5 to 9

Growth in liquid cultures was determined in duplicate 10 ml cultures. Fourteen 10 ml flasks were inoculated for each pH and growth was measured from two duplicate flasks. Flasks were incubated at 37 °C with shaking (100 rpm) for 7 days. Growth was observed by measuring the dry weight of a 10 ml culture at 24 hour intervals. Purity of the isolate was determined at every stage by observing the culture under a light microscope (Olympus CX21).

3.2.3 Optimisation of nitrile metabolising enzyme production

3.2.3.1 Optimal growth parameters for enzyme production

In order to obtain the optimal parameters for enzyme production, activity was monitored in different media, at different temperature as well as different pH. Enzyme activity was monitored during the course of growth at 24 hour intervals to determine if the enzyme was constitutively expressed or induced at a specific point during the growth phase.

3.2.3.2 Induction

Eight substrates (indol meth-3-methyl hydantoin, pNH2 benzyl hydantoin, tert-butyl hydantoin, phenyl hydantoin, 3-naphthyl-1-methyl hydantoin, hydroxyphenyl hydantoin, benzyl hydantoin, 4-nitrobenzyl hydantoin,) were tested for their potential to induce hydantoinase activity in isolate BSII9. A final concentration of 4 mM of each substrate (filter sterilised) was added to tryptic soy broth at inoculation. Induction was also tested in growing cells by adding the filtered substrates after four days of growth. Enzyme activity was then compared to that of un-induced cells.

3.2.3.3 Effect of divalent ions on enzyme activity

The effect of divalent ions (calcium, cobalt, copper, iron, magnesium, manganese, nickel and zinc) on hydantoinase activity was investigated. Metal salts were prepared at a concentration of 500 mM and filter sterilized (0.2 µm syringe filter). Each ion was added to a final concentration of 0.5 mM in 50 ml culture volumes of M172F media after autoclaving. Enzyme activity was observed as outlined in 3.2.1.4 using dihydrouracil as a substrate. The effect of different ions on enzyme activity was determined by comparing the activity to that of cells grown in M172F without the addition of metal ions.

3.2.4 Protein purification

All chromatographic procedures were performed on an ÄKTA FPLC liquid chromatography system (Amersham Biosciences) controlled via a Unicorn graphic user interface (Version 4.10). Unless otherwise indicated, default flow rates and pressure limits for each column were used. Samples from all stages of purification were analysed by SDS-PAGE (section 3.2.4.3). Protein concentration was determined using the Bradford assay with BSA as a standard

(section 3.2.1.2). Fractions were tested for hydantoinase activity after each purification step (section3.2.1.4).

3.2.4.1 Preparation of cell-free extracts and ammonium sulphate precipitation

One litre culture volumes were inoculated from 10 ml starter cultures and incubated at 37 °C with shaking (100 rpm) for seven days. Cell free extracts were prepared in 100 ml potassium phosphate buffer, pH 7.2 (section 3.2.1.1) and an ammonium sulphate precipitation titration was used as the first purification step. Ammonium sulphate crystals were added to the cell free extract to achieve 10 % saturation (Appendix v) and left on ice for 1 hour. Precipitated proteins were removed by centrifugation at 7000 x g for 30 minutes at 4 °C. Ammonium sulphate was added to the supernatant (Appendix v) to achieve 20 % saturation and precipitated proteins were removed as outlined above. This was repeated at 30 and 40 % ammonium sulphate saturation. All pellets were re-dissolved in phosphate buffer and dialysed against 25 mM phosphate buffer pH 7.2 at 4 °C overnight. Fractions were assayed for hydantoinase activity and proteins were separated on 12 % SDS PAGE gels (section 3.2.4.5).

3.2.4.2 Ion exchange chromatography

The active fraction from ammonium sulphate precipitation was dialysed against 25 mM potassium phosphate buffer, pH 7.2 and loaded onto a HiLoad 26/10 Q Sepharose column (Amersham Biosciences) equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of increasing sodium chloride concentrations generated with buffer containing 1 M sodium chloride, 25 mM potassium phosphate, pH 7.2 (5 column volumes, 0 - 1 M sodium chloride). Fractions containing hydantoinase were pooled.

3.2.4.3 SDS-polyacrylamide gel electrophoresis

Separation of proteins by SDS PAGE was performed using a Mighty Small vertical Slab Unit SE 280 (Hoefer). Gels (1 mm and 1.5 mm thick) containing 12 % acrylamide resolving (Table 3.3) and 4 % stacking (Table 3.4) were prepared using the Hoefer SE 245 dual gel caster. Protein samples were prepared by adding 5 µl 2 x SDS PAGE gel loading buffer to 5 µl protein sample followed by incubation at 90 °C for 5 minutes (Sambrook and Russel, 2001). Protein samples were electrophoresed at a constant voltage of 100 V. Following electrophoresis, gels were stained with Coomassie Blue and destained overnight using PAGE destaining solution (Sambook and Russel, 2001).

Table 3.3: SDS PAGE (12 %) separating gel components.

Components	Volume (ml)
ddH_2O	6.6
1.5 M Tris-HCl, pH 8.8	5
20 % (w/v) SDS	0.2
30 % Acrylamide/0.8% Bisacrylamide (w/v)	8
10 % (w/v) ammonium persulfate	0.2
TEMED	0.01

Table 3.4: SDS PAGE stacking gel components.

Components	Volume (ml)
dd H_2O	2.8
0.5 M Tris-HCl, pH 6.8	1.25
20 % (w/v) SDS	0.05
30 % Acrylamide/0.8% Bisacrylamide (w/v)	0.85
10 % (w/v) ammonium persulfate	0.05
TEMED	0.005

3.3 Results and Discussion

3.3.1 Preparation of whole cell bio-tranformations and crude cell free extract

The initial screening for enzyme activities was carried out using whole cells. However, it was found that resting cells released ammonia which interfered with the assay. In order to reduce background ammonia levels, cell free extracts were used to assay for amidase and nitrile hydratase activity. Vegetative cells of BSII 7 (most similar to *Streptomyces albidoflavus)* and BSII 9 (*Streptomyces griseorubens*) were found to be resistant to sonication. In preference, cells were incubated with lysozyme at 37 °C for 2 hours prior to sonication.

While amidase and hydantoinase activity was successfully detected with crude cell free extracts, carbamoylase activity was only detected when whole cell reactions were used. As the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) was used to slow down the enzymatic degradation of proteins by serine proteases, the observed enzyme inactivation was therefore attributed to other factors. Several studies have found N-carbamoylase to be more susceptible to oxidation and thermal inactivation than D-hydantoinase (Kim and Kim, 1995; Kim *et al.*, 2000 a, Park *et al.*, 2000). Cellular disruption exposes cell contents, including the proteins, to oxidative stress. The reducing agent DTT was added to the purification buffer in an attempt to reduce oxidative damage to proteins. This, however, did not improve the carbamoylase activity after sonication. The addition of glycerol (at a final concentration of 5 %), Mg^{2+} and ATP to the buffer also failed to improve the carbamoylase activity after sonication. Heavy metal ion contamination is known to contribute to the loss of carbomylase activity, as these proteins contain a free thiol group (Martínez-Rodríguez *et al.*, 2010). While metal chelators such as EDTA are often used to solve this problem, this strategy could not be employed in this study as hydantoinases are metal-dependant and EDTA would inactivate the enzyme. Whole cell biotransformation reactions were therefore routinely used for screening isolates for hydantoinase and carbamoylase activity. Total hydantoinase activity was therefore

calculated as the sum of both hydantoinase and carbamoylase activity, and for consistency both reactions were assayed using resting whole cells.

3.3.2 Amidase activity

Seven isolates (Table 3.5) showed amidase activity with at least one of the substrates used. A representative graph for isolate GSV 6 (*Micromonospora pattaloongensis)* is presented in Figure 3. 4. As all seven isolates showed activity with acetamide, activities were calculated as amount of ammonia liberated from acetamide per minute (Table 3. 5). None of the isolates tested were able to use benzamide as a substrate.

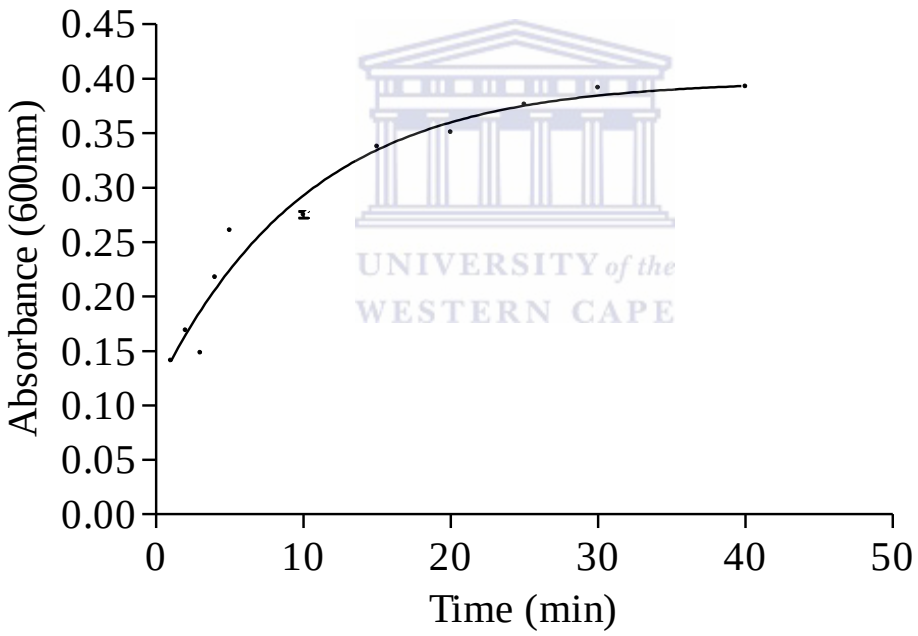

Figure 3.4: Ammonia production from acetamide breakdown indicating amidase activity in isolate GSV 6. Data points are mean values of triplicate assays.

Table 3.5: Amidase activity of different isolates with acetamide

Isolate	Amidase activity ($\mu Mmin^{-1}$)
BSII 1	13
BSI 4	34
BSII7	58
BSII9	65
GSI 2	53
GSIII 2	11
GSV6	28

3.3.3 Hydantoinase activity

Hydrolysis of hydantoins was determined using the Erhlich's test for amino acids hydantoin and dihydrouracil, and HPLC analysis for benzyl and phenyl hydantoin. Six isolates were found to be capable of degrading hydantoin to N-carbamoyl glycine (Figure 3.5).

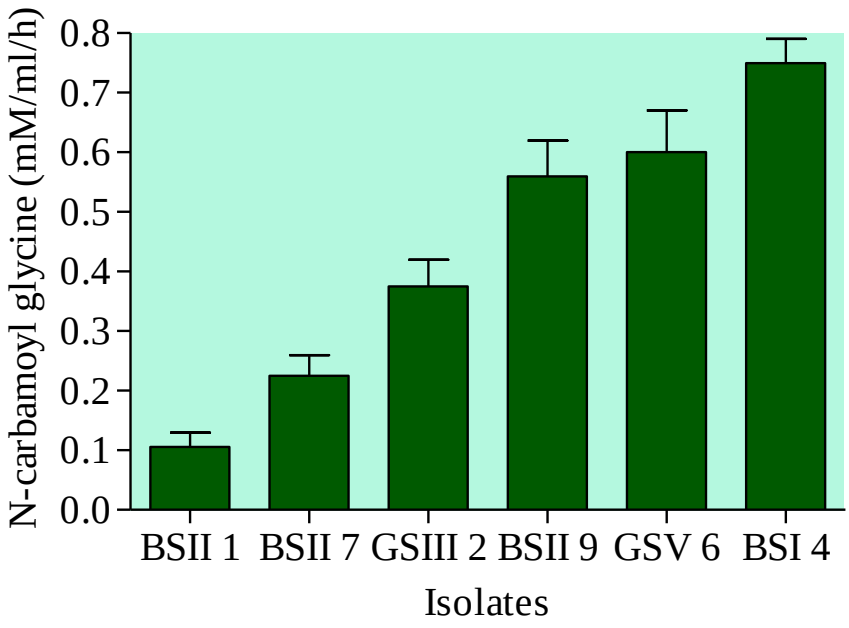

Figure 3.5: Hydantoin hydrolysis by different isolates.

The reaction for isolate BSI 4 (*Streptomyces sampsonii*) produced much higher titres of N-carbamoyl glycine than other isolates. It is suggested that isolate BSI 4 lacks N- carbamoylase activity, and that the low N-carbamoyl glycine observed in other isolates was a result of the sequential degradation of N- carbamoyl glycine to glycine. This was verified by using N-carbamoyl glycine as a substrate and monitoring the production of glycine. Glycine was only detected for isolates BSII 7 (0.62 mM ±0.01) and BSII 9 (0.93 mM ±0.2). It was concluded that although isolate BSI 4 showed the highest amount of N- carbamoyl glycine from hydantoin degradation, the isolate did not show the highest hydantoinase activity. Therefore, isolate BSII9 showed the highest total activity with hydantoin.

Comparison of the results of hydantoin degradation (Figure 3.5) to those of dihydrouracil degradation (Figure 3.6) indicated that not all the isolates which could hydrolyse hydantoin were capable of hydrolysing dihydrouracil.

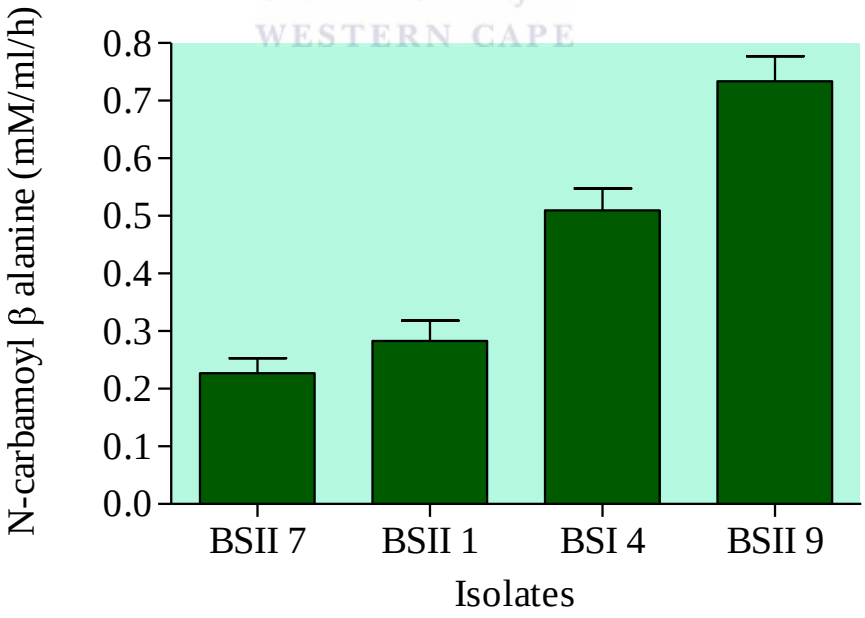

Figure 3.6: Dihydrouracil hydrolysis by different isolates.

These results suggest that the two reactions may not be catalysed by the same enzyme. Although EC nomenclature and most relevant literature use the names hydantoinase and

dihydropyrimidase interchangeably (Hydantoinases, dihydropyrimidinase; EC 3.5.2.2), several studies have shown that hydantoinases and dihydropyrimidases are not the same enzyme (Runser and Ohleyer, 1990; Runser and Meyer, 1993; Syldakt *et al.*, 1999). *Agrobacterium* sp. hydantoinase (Runser and Ohleyer, 1990) hydrolysed hydantoin and 5'-monosubstituted hydantoin derivatives but not dihydropyrimidines. Runser and Meyer (1993) also reported that when dihydrouracil was used as a substrate for resting *Agrobacterium* IP I-671 cells, dihydropyrimidinase activity disappeared on heating while hydantoinase activity was unchanged. A similar observation was noted in the hydantoinase activity of isolate BSII9. In whole cell reactions, the isolate hydrolysed both hydantoin and dihydrouracil with equal efficiency as estimated from the amount of product released. However, when a cell free extract was used, dihydrouracil was poorly hydrolysed but the efficiency of hydrolysis of hydantoin was unchanged, supporting the hypothesis that the hydantoinase and dihydropyrimidase activities were derived from two different enzymes. Although the other three isolates (BSII 1, BSI 4. BSII7) showed activity with both hydantoinase and dihydrouracil, the activities could potentially be from different enzymes.

Activity with phenyl hydantoin and benzyl hydantoin substrates was determined by comparing the peak profiles of the reaction mixture to those of the substrate, the expected product and the negative control (Figure 3.7).

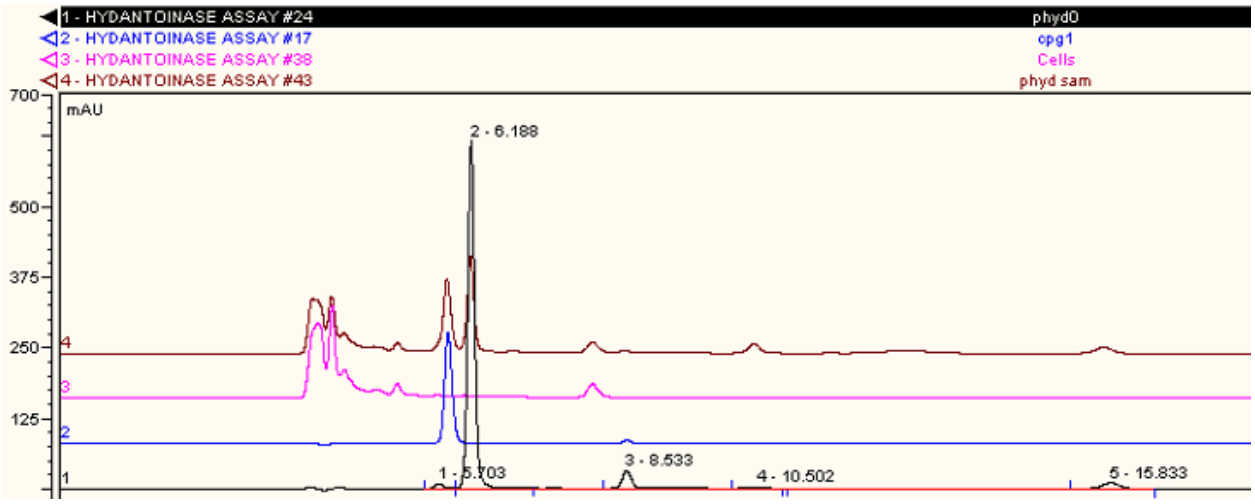

Figure 3.7: Analysis of phenyl hydantoin (phy0 in black) hydrolysis by isolate BSII9. The product of the hydrolysis, N- carbamoyl phenyl glycine (cpg1 in blue), the cell free reaction mixture (phyd sam in brown) as well as the negative control reaction excluding substrate (cells in pink), are over-layed on each other.

Isolates BSI 4, BSII7 and BSII9 were capable of hydrolysing both benzyl hydantoin and phenyl hydantoin to their respective N-carbamoyl amino acids. However, none of the isolates were capable of further hydrolysing the N-carbamoyl amino acids to their respective amino acids.

The preliminary results of the biotransformation reactions using BSII9 suggested that the hydantoinase might be stereo-specific. From the chromatograms and estimations from standard curves it was clearly seen that irrespective of the cell volumes or the duration of incubation, the substrate was never fully degraded. Since the substrate was a mixture of both D and L forma, it is suggested that only one enantiomer was hydrolysed. As the HPLC column used in this study was not chiral the strereospecificity of the enzyme could not be verified. Other methods to verify enantioselectivity of the enzyme would include either the use of enantiopure susbstrates or the separation of the resultant amino acids on a chiral TLC plate.

3.3.4 Carbamoylase activity

Although it was initially speculated that the low concentrations of N-carbamoyl glycine observed from the hydrolysis of hydantoin could be indicative of carbamoylase activity, this result was only observed for two isolate, BSII7 and BSII9. Carbamoylase activity was confirmed in these isolates by using N-carbamoyl glycine as the substrate. The ability to degrade N-carbamoyl phenyl glycine to phenyl glycine (determined by HPLC) was not observed in any of the isolates.

Isolate BSII9 showed good hydantoinase activity with all four substrates tested (Table 3.6). For hydantoinase activity, the total activity was calculated as the sum of the hydantoinase and carbamoylase activity. It must be noted that the activities reported in this chapter are not specific activities as whole cell biotransformation reactions were used. It was observed that during the screening for hydantoinase and carbamoylase activity, when cells were sonicated there was a noticeable decrease in the hydrolysis of hydantoin and the carbamoylase activity observed in isolate BSII 9 disappeared.

Table 3.6: Relative hydantoinase activities of different isolates with hydantoin and N-carbamoyl glycine

Isolate	**Activity**		
	Hydantoinase ($mM.h^{-1}.ml^{-1}$) (±SE)	**Carbamoylase ($mM.h^{-1}.ml^{-1}$)** (±SE)	**Total ($mM.h^{-1}.ml^{-1}$)**
BSII 1	0.27 (±0.01)	0	0.27
BSI 4	2.53 (±0.64)	0	2.53
BSII7	0.71 (±0.22)	8.33 (±1.2)	9.04
BSII9	1.57 (±0.25)	9.21 (±1.7)	10.78
GSIII2	0.47 (±0.06)	0	0.47
GSV6	0.53 (±0.18)	0	0.53

3. 4 Selection of an enzyme for further study

Table 3.7 summarises the enzyme activities observed for the different isolates screened in this study.

Table 3.7: Summary of the enzyme activities observed in different isolates.

Isolate	Enzyme activity			
	Amidase	**Carbamoylase**	**Nitrile hydratase**	**Hyadntoinase**
BSII 1	+	-	+	+
BSI 4	+	-	-	+
BSII7	+	+	+	+
BSII9	+	+	+	+
GSIII2	+	-	-	+
GSI2	+	-	-	-
GSV6	+	-	-	+

Key: + activity present with at least one substrate tested
– no activity with any of the substrates tested

Isolates requiring complex media for growth, as well as those that required long incubation periods were omitted from the screening procedures. On the basis of the observed activities (Table 3.7), ease of cultivation and potential novelty of enzymes, isolate BSII9 was chosen for further study. The partial characterisation of BSII9 is reported in Chapter 2 where preliminary 16S rRNA gene analysis identified the isolate as belonging to the genus *Streptomyces*. Despite streptomycetes having been extensively screened for secondary metabolites, no streptomycete hydantoinases have been characterised. Therefore, the hydantoinase activity of this isolate was investigated further.

3.5 Optimisation of BSII9 growth and hydantoinase production

3.5.1 Optimum media for growth and enzyme production

In order to achieve the optimal growth and hydantoinase production in BSII9 several media containing different carbon and nitrogen sources were tested.

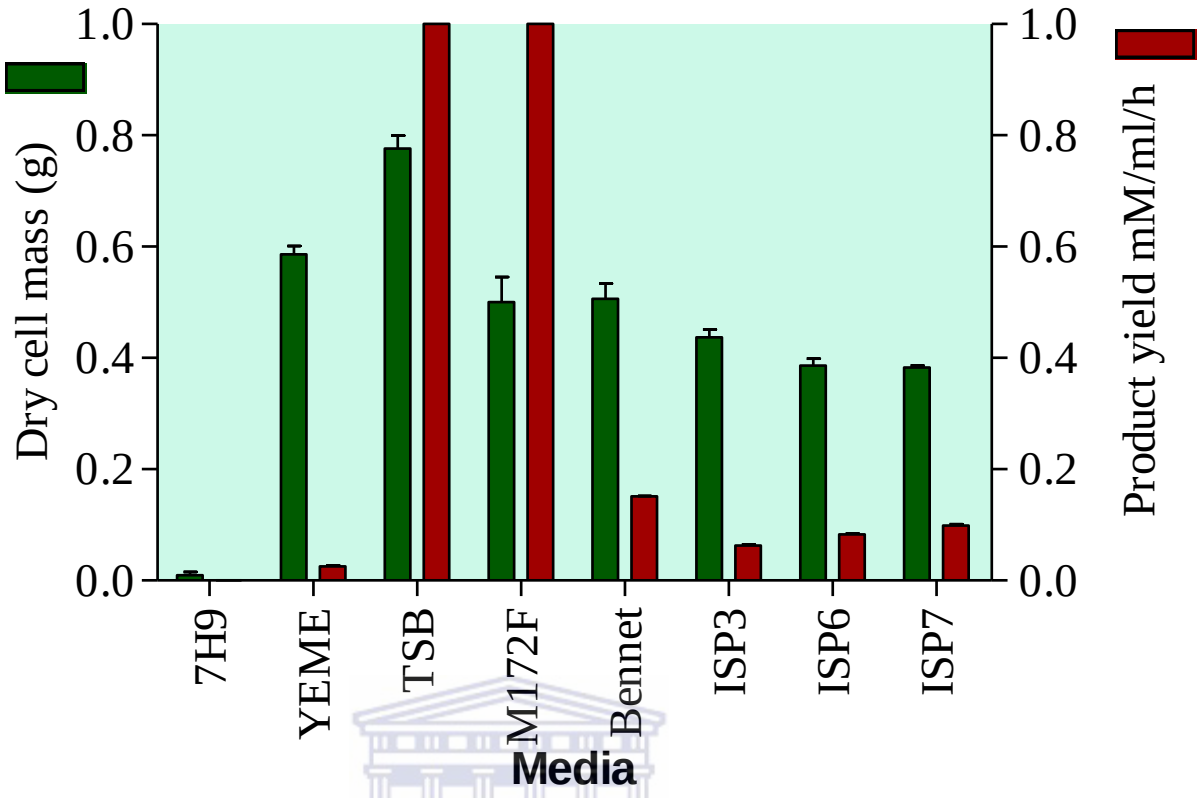

Figure 3.8: Relative growth and enzyme production of isolate BSII9 in different media

Optimum growth was observed in tryptic soy broth (TSB). The isolate grew fairly well in all other media tested, except 7H9. The highest enzyme activity was observed in TSB and M172F media. Therefore TSB was chosen as the standard medium for further cultivation of BSII9. The large difference observed in activity between cells grown in TSB, modified 172F and those grown in the YEME was attributed to several components in the media composition. Media containing meat or beef extract (such as M172F and Bennett's) have been implicated in optimal hydantoinase production. Lee *et al.* (1996) reported that the use of meat and beef extract as a nitrogen source enhanced D-hydantoinase production in *Peudomonas putida* and *Bacillus* SD1 strains. This was attributed to the high pyrimidine and purine content in these complex meat-derived nitrogen sources (Lee *et al.*, 1996). However, it was interesting to note that cells grown in TSB (which do not contain meat or beef extract) showed higher activity compared to those grown in Bennet's medium (containing 1g/L beef extract).

3.5.2 Effect of divalent ions on enzyme activity.

According to our observation, the presence of divalent ions (K^+ in TSB and Mg^{2+}, Ca^{2+} in M172F) could have played a more significant role in enhancing hydantoinase activity compared to carbon or nitrogen source in growth media. To investigate the role of divalent ions on enzyme activity, isolate BSII9 was grown in YEME broth supplemented individually with eight different divalent ions. The metal ions were added as the selected salt compounds to a final concentration of 0.5 mM. Cells grown in un-supplemented YEME were used as a negative control. No significant change was observed in cell yields. However, a significant difference was observed in enzyme titres from cells grown in supplemented and un-supplemented media (Figure 3.9)

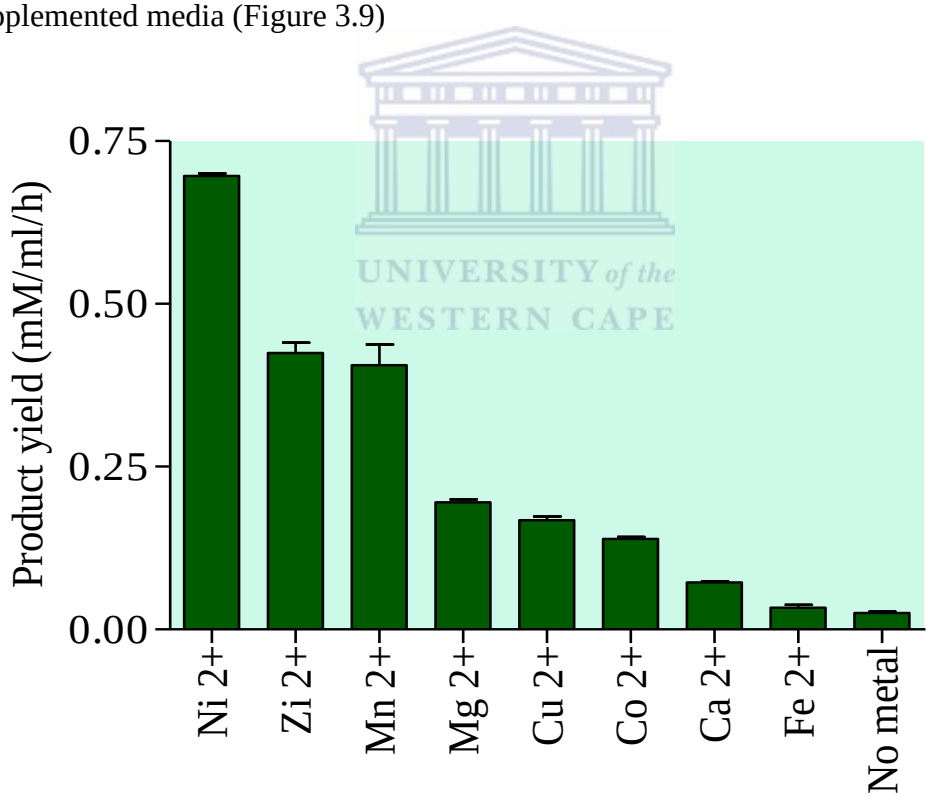

Figure 3.9: Graph showing the effect of different divalent ions on enzyme activity of BSII9 when supplemented into YEME media.

This observation suggested that the BSII9 hydantoinase required divalent ions for activation. When nickel was supplemented in YEME the amount of N-carbamoyl β-alanine released from

hydrolysis of hydantoin increased 30 fold. The metallo-dependence of hydantoinases has been reported elsewhere. Chevalier *et al.* (1989) reported that the hydantoinase from *Pseudomonas* sp. required Fe^{+3} ions for activation, while Lee *et al.* (1995) reported that the D-hydantoinase from *Agrobacterium tumefaciens* required Ni^{+2}, while the hydantoinases from *Bacillus stearothermophilus* SD1 and *Bacillus circulans* required Mn^{+2} ions for activation.

3.5.3 Dermination of hydantoinase activity onset in BSII 9.

To determine whether enzyme expression in BSII9 was consituitively expressed or induced, the hydantoinase activity of BSII 9 was correlated to the growth profile. Enzyme activity was investigated using both un-substituted hydantoins (hydantoin and dihyrouracil), as well as 5 substituted hydantoins (phenyl hydantoin and benzyl hydantion).

Enzyme activity was observed in cells at day 5 and older (Figure 3.10). Cells harvested at day four showed very little activity while those harvested at day three and earlier showed no activity at all.

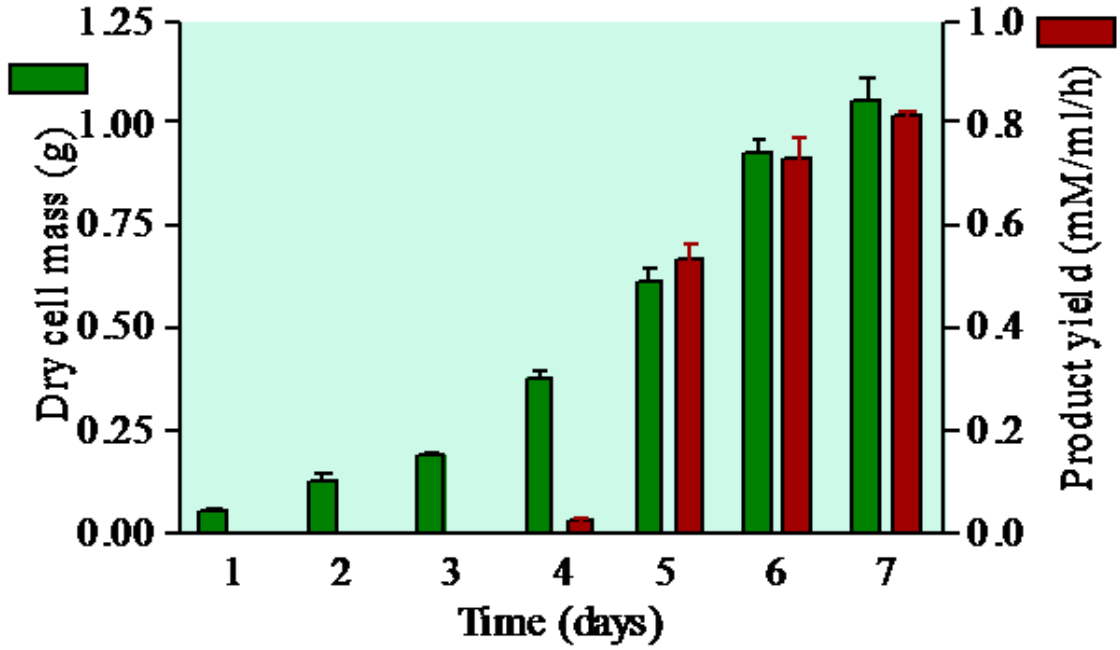

Figure 3.10: Enzyme activities observed in BSII9 cells harvested at different points of the growth curve.

The onset of the hydantoinase activity in BSII9 after 4 days incubation indicates that the enzyme is not constitutively expressed.

3.5.4 Induction of hydantoinase expression

Growth of isolate BSII9 was inhibited by the presence of all substrates, other than 4-nitrobenzyl hydantoin. However, enzyme activity in cells grown in the presence of 4-nitrobenzylhydantoin were not significantly higher, thus suggesting that 4 nitrobenzylhydantoin did not induce hydantoinase production. When substrates were added at day four, no significant increase in product yield was observed either. It was concluded that none of the substrates used in this study was able to induce hydantoinase activity in isolate BSII9.

3.5.5 Enzyme purification

3.5.5.1 Ammonium sulphate precipitation

Soluble cell proteins from isolate BSII9 were initially separated by an ammonium sulphate precipitation gradient. It was difficult to assess either qualitatively or quantitatively the extent of this purification, as the high concentration of ammonium sulphate present after resolubilisation of pellets severely interfered with SDS-PAGE. Protein pellets were dialysed to remove ammonium sulphate before hydantoinase activity assays. The highest activity was observed in fractions collected after precipitation with 20 % ammonium sulphate saturation (Figure 3.11).

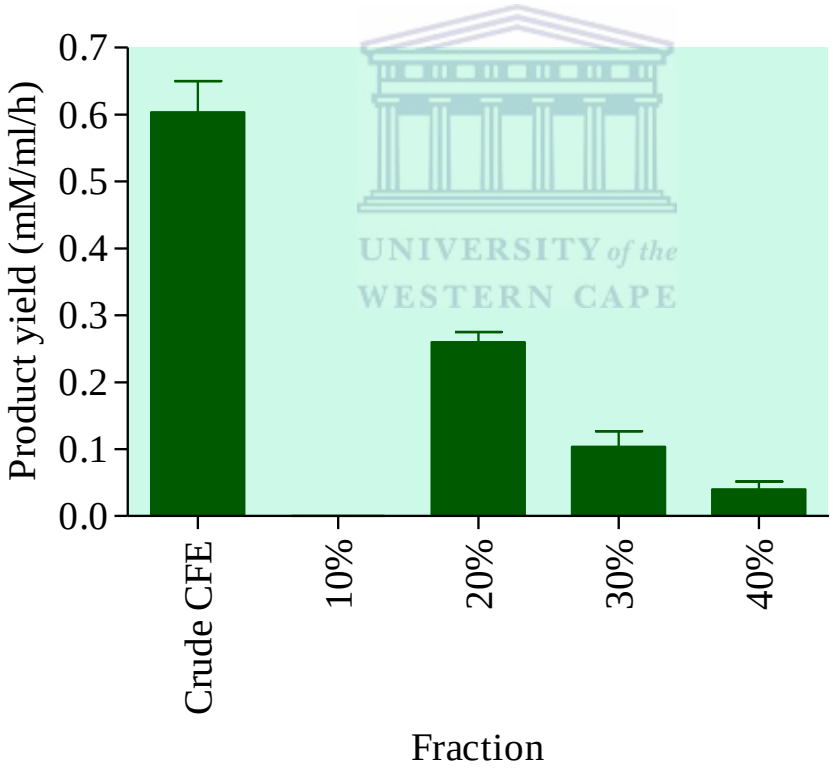

Figure 3.11: Hydantoinase activity detected in different fractions after salting out with ammonium sulphate.

3.5.5.2 Ion exchange chromatography

The 20 % ammonium sulphate fraction was dialysed and further purified using ion exchange chromatography. Initial assays indicated that none of the eluted fractions were positive for hydantoinase activity. However, after incubation with Ni^{2+} for 2 hours, activity was observed in four fractions (Figure 3.12). Qualitative analysis by SDS-PAGE showed that although ion exchange chromatography was able to separate the hydantoinase from several other cellular proteins, the level of purity achieved was very low as numerous contaminating proteins were co-eluted with the hydantoinase (Figure 3.13).

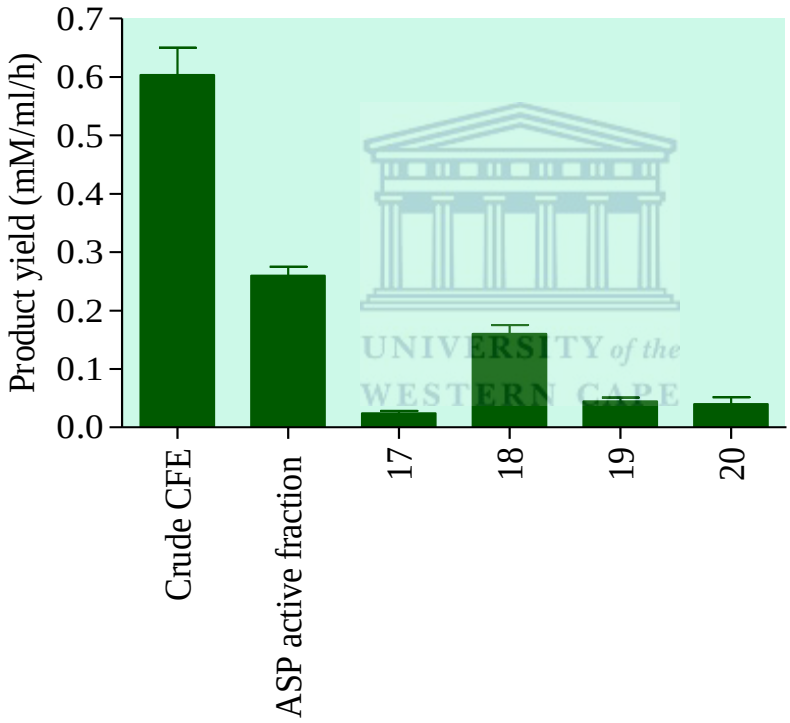

Figure 3.12: Hydantoinase activity observed in different fractions after purification of the 20% ammonium sulphate precipitation (ASP) active fraction using ion exchange chromatography.

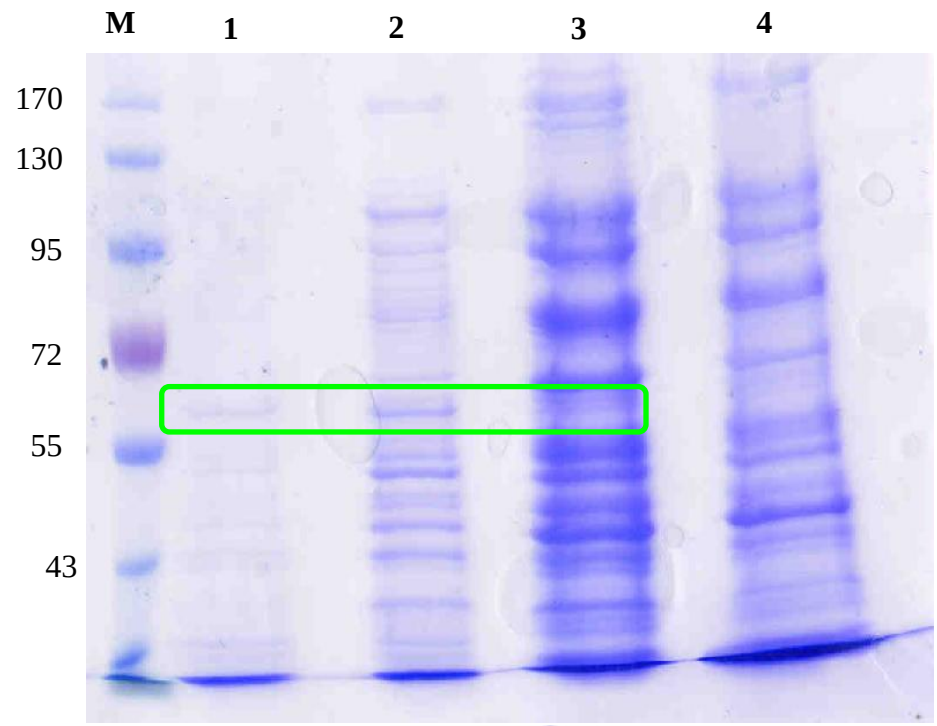

Figure 3.13: SDS-PAGE analysis of the active fractions from ion exchange chromatography. Lane M: Molecular weight marker, lanes 1 to 4: fraction 17 to 20, respectively. Green box highlights the correct size bands.

Table 3.8: Purification table

Sample	Total protein (mg)	Total units (µmol/ml/h)	Specific activity (U/mg)	Purification fold	Yield (%)
BSII9 CFE	602	60900	101	1	100
ASP	486	26000	55	0.5	43
17	31	2400	77	0.07	4
18	114	16000	140	1.4	26
19	182	4400	24	0.02	7
20	143	4240	29	0.02	7

Chapter 4: Cloning and expressing the BSII9 hydantoinase gene

4.1 Introduction

In order to understand the biochemistry and kinetics of enzymes, they need to be purified in sufficient quantities. However, this is difficult to achieve when the enzyme is produced by the native host. Very few proteins are naturally abundant *in vivo* as cells only produce the quantities required for physiological functions (Hartley, 2006). Molecular gene cloning is one of the most powerful techniques of modern biotechnology and it enables the production of proteins of interest in high yields. In theory, protein over-expression in a heterologous host requires three components: a gene, a vector that contains the gene and an expression host that maximizes the quantity and quality of the protein produced by the vector–gene combination (Hartley, 2006).

Hydantoinases and dihydropyrimidinases have been cloned and sequenced from various animal and microbial sources. The D-hydantoinase gene from *Pseudomonas* sp. NS 671 was the first hydantoinase gene to be cloned (Watabe *et al.*, 1992). To date several hydantoinase genes have been cloned from various microorganisms (La Pointe *et al.*, 1994; Chien *et al*., 1998; Wiese *et al*., 2001; Xu *et al*., 2003a), including a thermophilic hydantoinase from *Bacillus stearothermophilus* SD1 (Kim *et al*., 1997). According to literature searches carried out in this study, no hydantoinases or dihydropyrimidases have been cloned and expressed from a *Streptomyces* species thus far. However, the completion of several streptomycete genome sequencing projects has revealed putative hydantoinase genes in various *Streptomyces* genomes including *Streptomyces avermitilis*, *Streptomyces bingchengensis*, *Streptomyces coelicolor*, *Streptomyces flavogriseus* and *Streptomyces griseus*. In this chapter the cloning and expression of the hydantoinase gene obtained from the genome sequencing of isolate BSII9 is presented.

4.2 Materials and methods

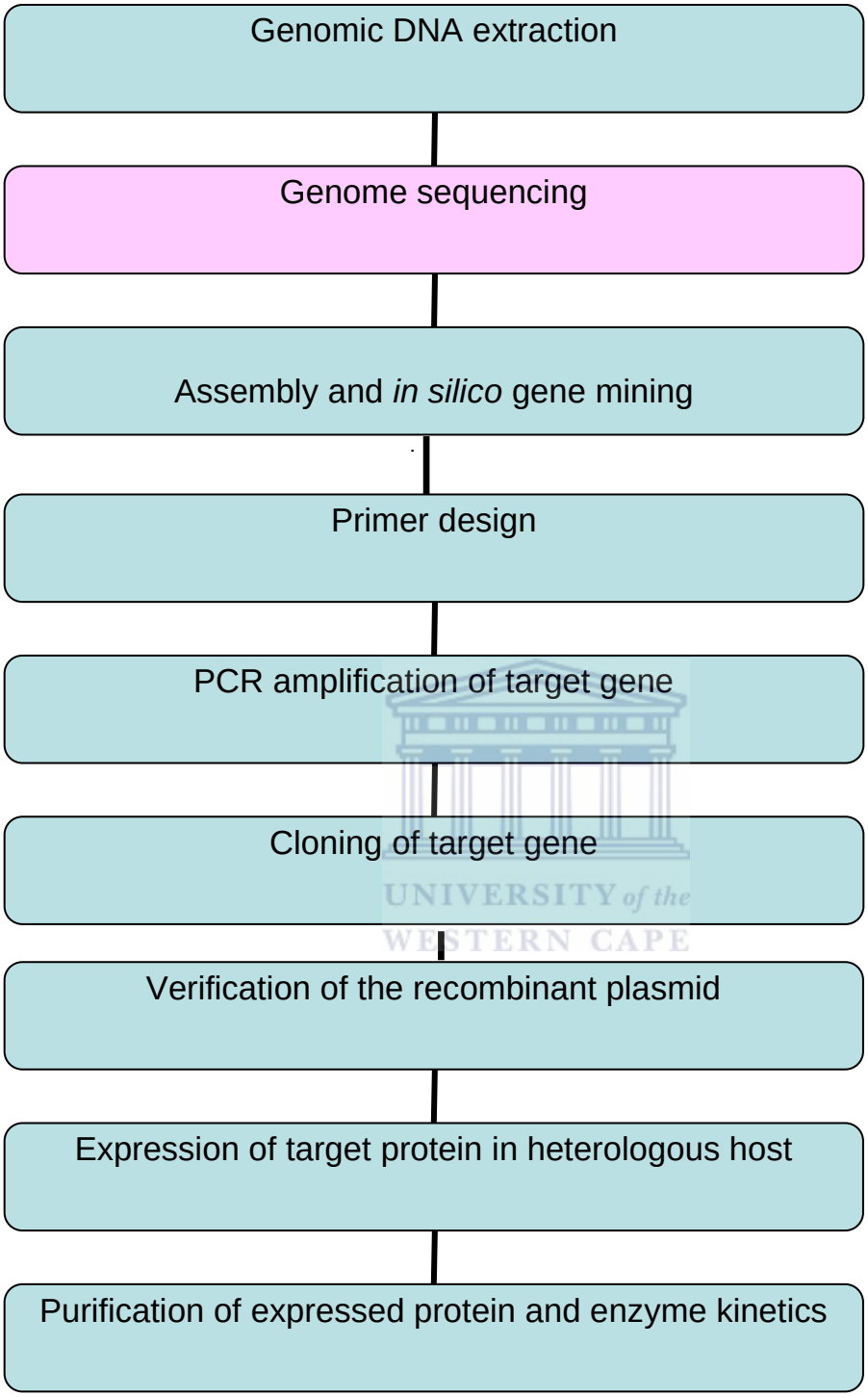

Figure 4.1: Flowchart of the work undertaken in cloning the hydantoinase gene from BSII9. The pink box denotes work done as a service by an industrial laboratory. Protocols are outlined in the following section.

4.2.1 Genome sequencing and in silico gene mining

Genomic DNA for sequencing was isolated using the method described in section 2.2.5.1 and purified as described in section 2.2.5.2. The genome of isolate BSII9 was sequenced using Illumina HiSeq 2000 DNA sequencing technology at the GATC Biotech (Konstanz, Germany). Contigs were generated by the *de novo* assembly of reads using the CLC bio genomics workbench 4.

The nucleotide sequences of semi-aligned contigs were used as the database for a BLAST search. Protein sequences for the different hydantoinase gene sequences retrieved from NCBI were used as queries in BLAST with a cut off expectation value greater than 0.0001. Nucleotide sequences with significant hits were aligned with the identified contigs in multiple sequence alignment using DNAman.

4.2.2 PCR amplification of the hydantoinase gene

Genomic DNA was extracted and purified from BSII9 cells as outlined in sections 2. 2.5.1 and 2.2.5.2, respectively. Gene specific primers (Table 4.1) were designed by aligning the hydantoinase gene from BSII9 with the putative hydantoinase genes from S. *avermitilis, S. bingchengensis, S. coelicolor, S. flavogriseus* and *S. griseus* using CLUSTAL W.

Primers Thydfor and Thydrev were used to amplify the full length hydantoinase gene from BSII9. To amplify the hydantoinase gene 20 µl PCR reaction contained the following reagents: 1 x GC buffer, 50 ng genomic DNA template, 0.5 µM each of the

forward and reverse primers, 200 µM of each dNTP (dATPs, dCTPs, dGTPs and dTTPs) and 0.5 U of Dream*Taq* polymerase (Fermentas).

Table 4.1: Primers used for cloning the hydantoinase gene form BSII9.

Primer	Sequence	Comment
Thydfor	5'AGCACCCGCACAGTCATCC3'	BSII 9 hydantoinase specific forward primer
Thydrev	5'CTAGTTGAGGTACTGACAGGTG3'	BSII 9 hydantoinase specific reverse primer
Forhydsp	5' TCGAG CATA TGA GCA CCC GCA CAG TCATC3'	Forward cloning primer with *Nde*I site
Revhydsp	5'CTAGTTGAGGTACTGACAGGTGAAGCT T3'	Reverse cloning primer with *Hind*III site

A master cycler gradient Epperndorf machine was used and the cycling conditions were: Initial denaturation 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The final elongation step was performed at 72 °C for 5 minute.

PCR amplicons were visualised and the size of the bands estimated using agarose gel electrophoresis. Correctly sized bands were purified using a GFX gel purification kit (Amersham Biosciences) according to manufacture's specifications and sequenced. Once the Thydfor and Thydrev primers were verified to amplify the full length hydantoinase gene, primers including restriction cut sites (Table 4.2) were designed to facilitate directional cloning of the hydantoinase gene. Primers were optimised by following the Taguchi method using the array for four variables (Cobb and Clarkson, 1994). The parameters that were varied are Mg^{2+} concentration, primer concentration, template DNA concentration as well as annealing temperature.

4.2.3.1 Cloning the hydantoinase genes

The PCR product was purified using the GFX PCR and gel purification kit (Amersham Biosciences) according to manufacture's specifications. Both the PCR product and pET28 vector were digested with *Nde*I and *Hind*III. Restriction enzyme digestions were performed in sterile eppendorf tubes in a final reaction volume of 50 µl. The reactions contained the appropriate volume of 10 × or 2 × buffer (supplied by Fermentas), 10 U of enzyme per µg of plasmid DNA or PCR product. Reactions were incubated overnight at 37 °C. The digestion products were analysed and purified by gel electrophoresis on 1 % (w/v) agarose gels. Purification of the excised DNA bands from agarose gels was performed using the GFX™ PCR and Gel Band Purification Kit (Amersham Biosciences) according to manufacture's specifications.

A 10 µl ligation reaction mixture was prepared with appropriate concentrations of vector and insert at a 1:3 molar ratio (ng insert required = (ng vector x kb insert/kb vector) x 3), reaction buffer at 1 x final concentration, 1 U of T4 DNA ligase and water to a final volume of 10 µl. The ligation reaction was incubated at 4 °C overnight. Three microliters of the ligation mix was dialysed on a 0.2 um nitrocellulose filter (Millipore) and one microliter was used to transform competent cells.

4.2.3.2 Preparation of Electro-Competent Cells

Electro-competent Genehog and BL21 (DE3) pLysS *E. coli* cells were prepared as outlined in Sambrook and Russell (2001) with slight modification. All glassware was thoroughly washed with 70 % EtOH, rinsed and autoclaved prior to use. A single colony of the *E. coli* strain was inoculated into 5 ml LB-broth and incubated at 37 °C with shaking overnight. An aliquot of the overnight culture (2.5 ml) was transferred to 250 ml

LB-broth and incubated at 37 °C until mid-logarithmic phase (OD_{600} of 0.4). The flask was immediately placed on ice and cooled for 20 minutes. Cells were collected in polypropylene tubes by centrifugation at 3000 x *g* for 10 minutes in an Eppendorf 5810R fixed rotor centrifuge. The supernatant was decanted and the cells were re-suspended in an equal volume of 10 % sterile glycerol. The cells were harvested as above. The glycerol wash step was repeated 6 times. The resultant pellet was re-suspended in 1 ml of 15 % glycerol, 2 % sorbitol. The cells were stored as 50 µl aliquots at -80 °C.

4.2.3.3 Preparation of Chemically Competent Cells

Chemically competent Genehog, BL21 (DE3) and Rosetta2 (DE3) pLysS *E.coli* cells (Table 4.2) were prepared according to the method in Sambrook and Russell (2001) with slight modifications.

Table 4.2: Bacterial strains used for cloning in this study

Bacterial Strains	Features	Growth requirements	Supplier
E.coli Gene Hog	Cloning strain prepared for cloning of large insert DNA.		Invitrogen (USA)
E.coli BL21(DE3)	Contains T7 polymerase. IPTG inducible. Suitable for the expression of non-toxic genes.	No tight control over expression.	Stratagen (USA)
E.coli BL21(DE3)pLysS	pLysS plasmid produces T7 lysozyme to reduce basal level expression of gene of interest. Suitable for expression of toxic genes.	Chloramphenicol 34µg/ml	Invitrogen (USA)
E.coli Rosetta2(DE3)pLysS	Rare tRNA genes are present on the same plasmids that carry the T7 lysozyme.	Chloramphenicol 34µg/ml	Novagen (USA)

All glassware was thoroughly washed with 70 % EtOH, rinsed and autoclaved prior to use. A single *E. coli* colony was inoculated into 5 ml LB-broth and incubated overnight at 37 °C with shaking. A 2.5 ml aliquot of the overnight culture was aseptically transferred into 250 ml of LB broth and incubated at 37 °C until mid logarithmic phase (OD_{600} of 0.4). The flask was rapidly placed on ice and cooled for 20 minutes. The cells were harvested in polypropylene tubes by centrifugation at 3000 x *g* for 10 minutes in an Eppendorf 5810R fixed rotor centrifuge. The supernatant was decanted and the cells were re-suspended in 30 ml of 0.1 M $CaCl_2$ and incubated on ice for 20 minutes. The cells were harvested as above and the pellet was re-suspended in 2 ml of 0.1M $CaCl_2$ and 15 % sterile glycerol. The cells were aliquoted into 100 µl volumes and stored at -80 °C.

4.2.3.4 Transformation of electro-competent cells.

The electro-competent cells were transformed with the recombinant DNA from section 4.2.3.1 An Eppendorf tube containing 50 µl of electro competent cells was thawed on ice. For each ligation reaction, 2 µl of the ligation mixture was added to the thawed cells and gently mixed. The mixture was returned to ice for ~ 1 min and transferred to a pre-cooled 0.1 cm sterile electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Electroporation was performed using the following conditions: 1.25 – 1.8 kV, 25 µF, 200 Ω on the BioRad Micropulser machine. Immediately following electroporation, 1 ml SOB broth was added to the cuvette, the cells transferred to a 15 ml Falcon tube and incubated at 37 °C for 1 h with agitation. One hundred microlitres (100 µl) of the cells were plated onto LB-agar plates supplemented with the appropriate selection antibiotic(s) (Table 4.3)

4.2.3.5 Transformation of chemically competent cells

A 1.5 ml tube containing 100 µl of chemically competent cells was removed from -80 °C and allowed to thaw on ice. Five microlitres (5 µl) of the ligation mix was added to the thawed cells and incubated on ice for 20 minutes. The cells were heat shocked at 42 °C for 90 s in water bath, then immediately returned to ice with addition of 1 ml of SOC broth. The cells were then incubated at 37 °C for 1 hr with agitation. A 100 µl aliquot of cells was plated out onto LB agar plates supplemented with the appropriate antibiotic(s) and incubated overnight at 37 °C.

4.2.4 Confirmation of positive transformants

Colony PCR using gene specific primers (Forhydsp and Revhydsp) were used to confirm the presence of the desired insert in randomly selected clones. Glycerol stocks of clones containing the correct insert were prepared and stored at -80 °C.

4.2.5 Plasmid extraction by alkaline lysis method

Positive clones were re-streaked on LB agar containing the relevant antibiotics. A single colony was picked from each plate and inoculated into 5 ml LB broth supplemented with the appropriate antibiotic(s). The culture was incubated overnight at 37 °C with agitation. Plasmid DNA was isolated from the overnight cultures by alkaline lysis method (Sambrook and Russell, 2001), with slight modifications. Two millilitres (2 ml) of the overnight culture was transferred into 2 ml tubes and the cells were harvested by centrifugation at 10000 x *g* for 1 minute at room temperature. The supernatant was discarded and the pellet was re-suspended in 200 µl solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). Two hundred microlitres (200 µl) solution 2

(1 % w/v SDS, 0.2 M NaOH) was added to the mixture and the contents were mixed well by inversion and incubated for 5 minutes on ice. Following the addition of 300 µl 7.5 M ammonium acetate (pH 7.6), the tubes were incubated on ice for 10 minutes, centrifuged at 13000 x *g* for 15 minutes at room temperature. The supernatant was transferred to a new tube, 500 µl of chloroform: isoamylalcohol (24:1 v/v) was added and the samples were centrifuged at 13000 x *g* for 10 minutes at 4 °C. The supernatant was transferred to a new tube and the plasmid DNA was precipitated by the addition of an equal volume of isopropanol. The tubes were incubated at -80 °C for 15 minutes and centrifuged at 13,000 x g for 10 minutes at 4 °C. The pellet was air dried and the DNA was resuspended in TE containing RNAse A to the final concentration of 20 µg/ ml.

4.2.6 Sequencing

Sequencing of cloned insert DNA was performed using a MegaBACE 500 Automated Capillary DNA sequencing System (Amersham Biosciences) by Stellenbosch University using the T7 vector sequencing primers.

4.2.7 Optimisation of expression of the hydantoinase gene in E.coli

The recombinant pET28 plasmid containing the hydantoinase gene was designated pET28-hyd and was used to transform various competent *E.coli* cell lines (BL21, BL21 pLysS and Rossetta; Table 4.3). Random transformants were selected from these plates for a small scale expression study. Colonies were grown overnight in 5 ml LB supplemented with chloramphenicol (34 µg/ ml) and kanamycin (50 µg/ ml). Five hundred microliters (500 µl) of the starter culture was used to inoculate 10 ml LB

chloramphenicol (34 µg / ml) and kanamycin (50 µg/ ml) and grown at 37 °C until an OD_{600} of 0.6. One millilitre (1 ml) was transferred to a sterile eppendorf tube and centrifuged at 10000 × *g* for 15 minutes. Cell pellets were air-dried and stored at -20 °C. The remaining culture was split in two equal parts and one was induced with 1 mM IPTG. Both the induced and un-induced cultures were further split into three portions each which were incubated for 24 hours at 25, 30 and 37 °C. After 24 hours OD_{600} measurement were recorded and 1 ml from each culture was centrifuged, dried and stored at -20 °C. Effects of incubation temperature after induction, amount of IPTG used (0.5 to 5 mM final concentration) and addition of metal ions (5 to 50 mM final concentration Mn^{2+}:Ni^{2+}: Zn^{2+}) at induction on expression were investigated. Expression was monitored by SDS PAGE.

4.2.8 Large scale expression of the hydantoinase gene

A single Rosetta (DE3) pLysS transformed with pET28-hyd colony was inoculated into 10 ml LB broth supplemented with chloramphenicol (34 µg/ml) and grown overnight at 37 °C, with shaking (150 rpm). The culture was transferred to 1 L LB broth and grown at 30 °C until an OD_{600} of 0.6 was obtained. IPTG was added to a final concentration of 0.5 mM and cells were incubated at 20 °C for 2 days with shaking. Cells were harvested by centrifugation (15 minutes 6000 x *g* at 4 °C). The cell pellet was washed with 25 mM phosphate buffer pH 7 before final resuspension in 50 ml 1 x binding buffer (Table 4.4). Cell free extracts were prepared by sonication using a Bandelin sonoplus HD2070 sonicator in cycles of 30 s pulse – 30 s stops for 5 min. All cultures were maintained on ice during sonication. Soluble and insoluble protein fractions were separated by

centrifugation (30 minutes 15000 x *g* at 4 °C). The resulting cell pellet, cell-free extract and cell lysate fractions were analysed by SDS-PAGE. The recovered fractions was stored at 4 °C and used in a subsequent preliminary enzyme assays, as well as His-tag purification.

4.2.9 His-tag purification of recombinant hydantoinase

His-Bind resin (Novagen, USA) was completely resuspended by gentle inversion. Two millilitres (2 ml) of the slurry was transferred to a purification column and packed by gravitational flow to a final bed volume of 1 ml. The column was charged and equilibrated according to the manufacture's specifications using the respective buffers (Table 4.3)

Table 4.3 Buffers used for purification of his tagged recombinant proteins

Buffer	Composition
8 x Binding buffer	4 M NaCl, 160 mM Tris-HCl, 40mM Imidazole, pH 7.9
8 x Wash buffer	4 M NaCl, 160 mM Tris-HCl, 480 mM Imidazole, pH 7.9
4 x Elution Buffer	2 M NaCl, 400 mM EDTA, 80 mM Tris-HCl, 4 M Imidazole, pH 7.9
4 x Strip Buffer	2 M NaCl, 400 mM EDTA, 80 mM Tris-HCl, pH 7.9
8 x Charge Buffer	400 mM $NiSO_4$

The fractions were collected and tested for hydantionase activity using the method described in section 3.2.1.4. The units of activity were measured as µmol/ml of product and expressed as an average of 3 replicates.

4.3 Results and Discussion

4.3.1 Genomic DNA extraction

High molecular weight DNA was isolated from a pure culture of BSII9. The DNA isolated was of high quality and quantity (Figure 4.2) as required sequencing.

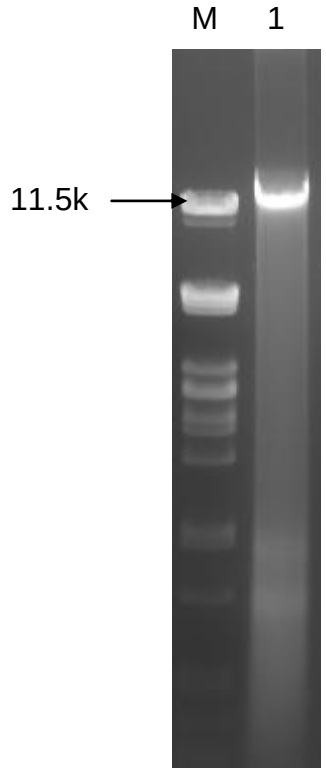

Figure 4.2: BSII9 genomic DNA for genome sequencing.

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4.3.2 In silico gene mining

The genomic DNA was sequenced by GATC Biotech using Illumina pyrosequencing technology (Konstanz, Germany). Raw sequence data was assembled into contigs using the *de novo* assembly algorithm of CLC Genomics Workbench and a summary of the assembly is given in Table 4.4. The mean read length was 51 bp and a total of 40 244 contigs were assembled, of which 37466 contigs had a length between 100 and 1244 bp and 2778 contigs were longer than 1244 bp.

Table 4.4: Summary of statistics of the *de novo* assembly

Contig count	40,244
Type	De novo assembly
Total read count	68,151,118
Mean read length	51.00
Total read length	3,475,707,018
Mean contig length	421
Total contig length	16,948,439
GC contents in %	65.82

.

The efficiency of the contig assembly was analysed by mapping the reads. Mapping results are summarised in Table 4.5

Table 4.5: Mapping data statistics

All mapped reads

Read count	68,151,118
Mean read length	51.00
Total read length	3,475,707,018

Non specific matches

Read count	31,078
Mean read length	51.00
Total read length	1,584,978

Non perfect matches

Read count	9,292,784
Mean read length	51.00
Total read length	473,931,984

Several actinobacterial putative D-hydantoinase gene sequences were obtained from the NCBI database and were used to search for contigs containing the hydantoinase gene. The full hydantoinase gene sequence was obtained within two contigs (circled in green in Figure 4.3). The sequence aligned well with the putative hydantoinase genes from *S avermitilis, S. bingchengensis, S. coelicolor, S. flavogriseus* and *S. griseus* (Figure 4.3)

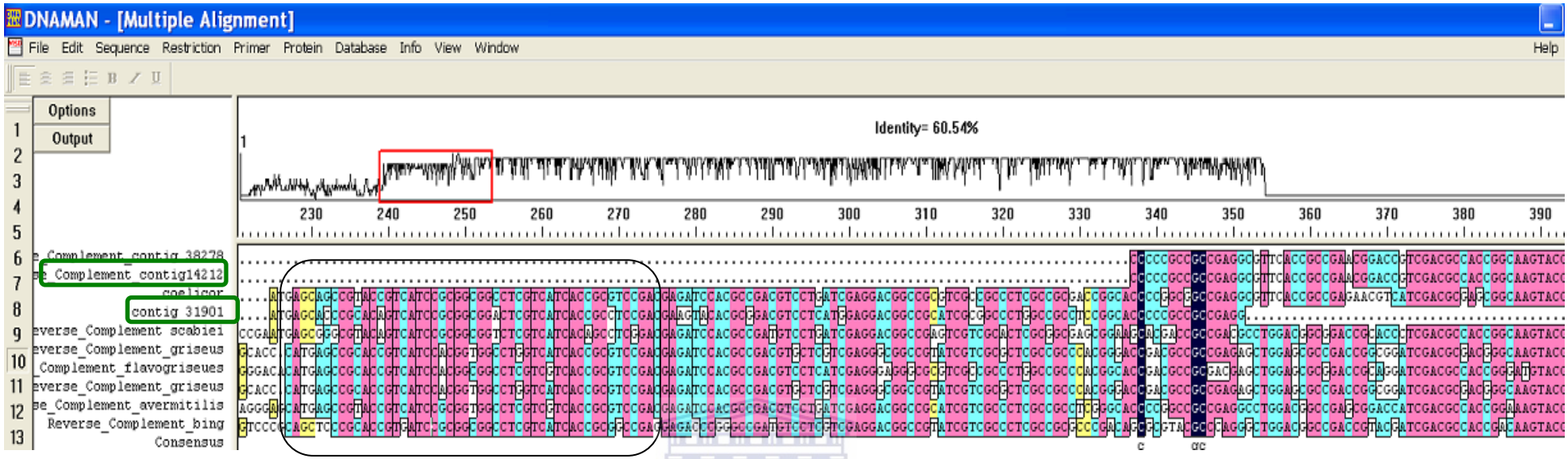

Figure 4.3: Multiple sequence analysis of the D-hydantoinase and dihydropyrimidinase gene sequences. Several *Streptomyces* species including the contigs (circled in green boxes) predicted to contain the hydantoinase gene were aligned. Black box indicates primer designing site.

Several bioinformatics tools were used to analyse the hydantoinase sequence (Figure 4.4). The EXPASY translate tool (www.expasy.org) was used to translate the nucleotide sequence to an amino acid sequence and one ORF was predicted in the plus strand of the 1464 bp sequence (Figure 4.5). The predicted putative protein consists of 394 amino acids and has a theoretical molecular weight of 43.25 kDa (Figure 4.5). A 3D homology model was built and the putative protein showed 49.76 % similarity to the D-hyadntoinase from *Bacillus* sp. AR9 (Figure 4.6).

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCAT
ATGAGCACCCGCACAGTCATCCGCGGCGGACTCGTCATCACCGCCTCCGACGAAGTACAC
GCGGACGTCCTCATGGAGGACGGCCGCATCGCGGCCCTGGCCGCCTCCGGCACCCCCGCC
GCCGAGGCGTTCACCGCCGAACGGACCGTCGACGCCACCGGCAAGTACGTCATCCCGGGC
GGCGTGGACGCGCACACCCACATGGAGCTTCCCTTCGGCGGCACCTTCGCCTCCGACACC
TTCGAGACCGGCACCCGCGCCGCCGCCTGGGGCGGCACCACCACGATCGTCGACTTCGCC
GTGCAGAGCGTCGGGCACACCCTGCGCGAGGGCCTGGACGCCTGGCACGCGAAGGCGGAG
GGCACCTGTGCGATCGACTACGCGTTCCACATGATCGTCTCGGACGTGAACCAGGAGACC
CTGAAGGAGATGGACCTCCTCGTGGAAGAGGGCGTCACCTCCTTCAAGCAGTTCATGGCC
TACCCCGGAGTTTTCTACTCCGACGACGGACAGATCCTCCGCGCCATGCAACGCTCCGCC
GACAACGGCGGCCTCATCATGATGCACGCCGAGAACGGCATCGCCATCGACGTCCTCGTC
GAGCAGGCCCTCGCCCGCGGCGAGACCGACCCCCGCTTCCACGGCGAGGTCCGCAAGGCT
CTCCTGGAGGCGGAGGCCACCCACCGCGCCATCCGCCTCGCGCAGGTCGCCGGCGCTCCG
CTCTACGTCGTGCACGTCTCCGCCACCGAGGCGGTCGCCGAACTCGCCCGGGCCCGTGAC
GACGGCCTGCCCGTCTTCGGTGAGACCTGTCCGCAGTACCTGTTCCTGTCCACCGACAAC
CTCGCCGAGCCCGGATTCGAGGGCGCCAAGTACGTGTGCAGCACGCCCCTGCGCCCCAAG
GAGCACCAGGCGGCGCTCTGGCGGGGGCTGCGCACCAACGACCTCCAGGTGGTCTCCACC
GACCACTGCCCCTTCTGCTTCAGCGGCCAGAAGGAACTCGGCCGCGGGGACTTCTCCAAG
ATCCCCAACGGGCTGCCGGGCGTGGAGAACCGCATGGACCTCCTCCACCAGGCCGTCCTC
GACGGGCACATCAGCCGCCGCCGCTGGATCGAGATCGCCTGCGCCACCCCGGCGCGGATG

Figure 4.4: Full length sequence of the hydantoinase gene from isolate BSII9. The start codon is highlighted in green.

```
Translation of BSII 9 hydantoinase (1-1200) Universal code
Total amino acid number: 400, MW=43179
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Max ORF: 1-1200, 400 AA, MW=43179

1          ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCAT
1           M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H

61         ATGAGCACCCGCACAGTCATCCGCGGCGGACTCGTCATCACCGCCTCCGACGAAGTACAC
21          M  S  T  R  T  V  I  R  G  G  L  V  I  T  A  S  D  E  V  H

121        GCGGACGTCCTCATGGAGGACGGCCGCATCGCGGCCCTGGCCGCCTCCGGCACCCCCGCC
41          A  D  V  L  M  E  D  G  R  I  A  A  L  A  A  S  G  T  P  A

181        GCCGAGGCGTTCACCGCCGAACGGACCGTCGACGCCACCGGCAAGTACGTCATCCCGGGC
61          A  E  A  F  T  A  E  R  T  V  D  A  T  G  K  Y  V  I  P  G

241        GGCGTGGACGCGCACACCCACATGGAGCTTCCCTTCGGCGGCACCTTCGCCTCCGACACC
81          G  V  D  A  H  T  H  M  E  L  P  F  G  G  T  F  A  S  D  T

301        TTCGAGACCGGCACCCGCGCCGCCGCCTGGGGCGGCACCACCACGATCGTCGACTTCGCC
101         F  E  T  G  T  R  A  A  A  W  G  G  T  T  T  I  V  D  F  A

361        GTGCAGAGCGTCGGGCACACCCTGCGCGAGGGCCTGGACGCCTGGCACGCGAAGGCGGAG
121         V  Q  S  V  G  H  T  L  R  E  G  L  D  A  W  H  A  K  A  E

421        GGCACCTGTGCGATCGACTACGCGTTCCACATGATCGTCTCGGACGTGAACCAGGAGACC
141         G  T  C  A  I  D  Y  A  F  H  M  I  V  S  D  V  N  Q  E  T

481        CTGAAGGAGATGGACCTCCTCGTGGAAGAGGGCGTCACCTCCTTCAAGCAGTTCATGGCC
161         L  K  E  M  D  L  L  V  E  E  G  V  T  S  F  K  Q  F  M  A

541        TACCCCGGAGTTTTCTACTCCGACGACGGACAGATCCTCCGCGCCATGCAACGCTCCGCC
181         Y  P  G  V  F  Y  S  D  D  G  Q  I  L  R  A  M  Q  R  S  A

601        GACAACGGCGGCCTCATCATGATGCACGCCGAGAACGGCATCGCCATCGACGTCCTCGTC
201         D  N  G  G  L  I  M  M  H  A  E  N  G  I  A  I  D  V  L  V

661        GAGCAGGCCCTCGCCCGCGGCGAGACCGACCCCCGCTTCCACGGCGAGGTCCGCAAGGCT
221         E  Q  A  L  A  R  G  E  T  D  P  R  F  H  G  E  V  R  K  A

721        CTCCTGGAGGCGGAGGCCACCCACCGCGCCATCCGCCTCGCGCAGGTCGCCGGCGCTCCG
241         L  L  E  A  E  A  T  H  R  A  I  R  L  A  Q  V  A  G  A  P

781        CTCTACGTCGTGCACGTCTCCGCCACCGAGGCGGTCGCCGAACTCGCCCGGGCCCGTGAC
261         L  Y  V  V  H  V  S  A  T  E  A  V  A  E  L  A  R  A  R  D

841        GACGGCCTGCCCGTCTTCGGTGAGACCTGTCCGCAGTACCTGTTCCTGTCCACCGACAAC
281         D  G  L  P  V  F  G  E  T  C  P  Q  Y  L  F  L  S  T  D  N

901        CTCGCCGAGCCCGGATTCGAGGGCGCCAAGTACGTGTGCAGCACGCCCCTGCGCCCCAAG
301         L  A  E  P  G  F  E  G  A  K  Y  V  C  S  T  P  L  R  P  K

961        GAGCACCAGGCGGCGCTCTGGCGGGGGCTGCGCACCAACGACCTCCAGGTGGTCTCCACC
321         E  H  Q  A  A  L  W  R  G  L  R  T  N  D  L  Q  V  V  S  T

1021       GACCACTGCCCCTTCTGCTTCAGCGGCCAGAAGGAACTCGGCCGCGGGGACTTCTCCAAG
341         D  H  C  P  F  C  F  S  G  Q  K  E  L  G  R  G  D  F  S  K

1081       ATCCCCAACGGGCTGCCGGGCGTGGAGAACCGCATGGACCTCCTCCACCAGGCCGTCCTC
361         I  P  N  G  L  P  G  V  E  N  R  M  D  L  L  H  Q  A  V  L

1141       GACGGGCACATCAGCCGCCGCCGCTGGATCGAGATCGCCTGCGCCACCCCGGCGCGGATG
381         D  G  H  I  S  R  R  R  W  I  E  I  A  C  A  T  P  A  R  M
```


Figure 4.5: Translation of the BSII9 hyadantoinase sequence.

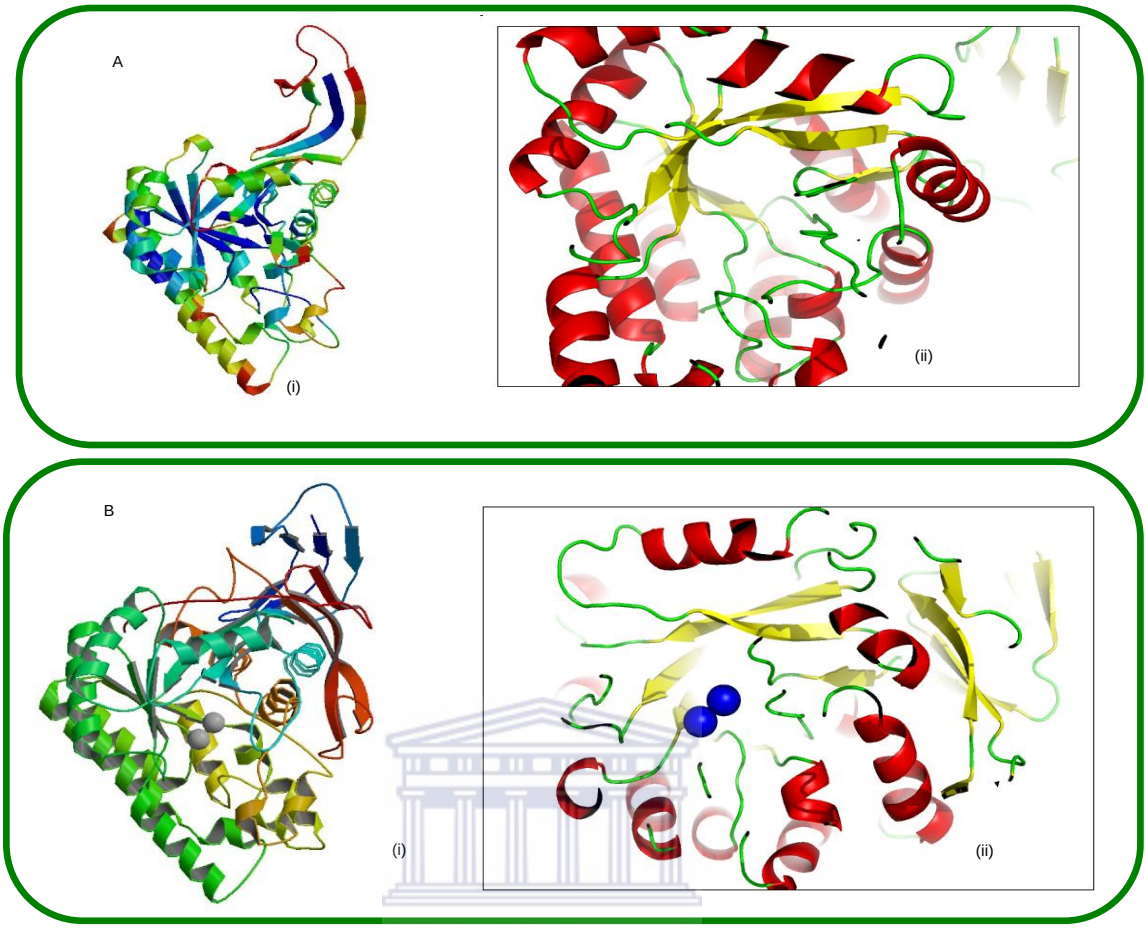


Figure 4.6: (Ai) Homology model built by the Swiss model server using amino acids 5 to 426 of the predicted protein from the cloned hydantoinase gene. Aii is a closeup on the TIM barrel motif characteristic of hydantoinases. (Bi) The 1ynyB chain from the molecular structure of the D- hydantoinase from *Bacillus* sp. AR9 was used as the template. Bii the corresponding closeup sowing the motif.

The homology models of both the BSII9 hydantoianse (Fifure 4.6 A) and the *Bacillus* sp. template (Figure 4.6 B) showed good structural conservation of the triose phosphate isomerise (TIM) barrel topology. The TIM-barrel domain is an eightfold repetition of a β/α-motif wrapped in a circular fashion to make a central β-barrel, which is evident in the models (Figure 4.6 A (ii) and B (ii)). Hydantoinases and dihydropyrimidinases all exhibit this highly homologous TIM barrel topology (Abendroth *et al.*, 2002 a; Cheon *et al.*, 2002, Xu *et al.*, 2003 a).

The accuracy of the homology model was assessed using a RAMPAGE Ramachandrian plot (Figure 4.7). For a model to be accurate, 98% of residues are expected in the favoured region and 2% of residues in the allowed region.

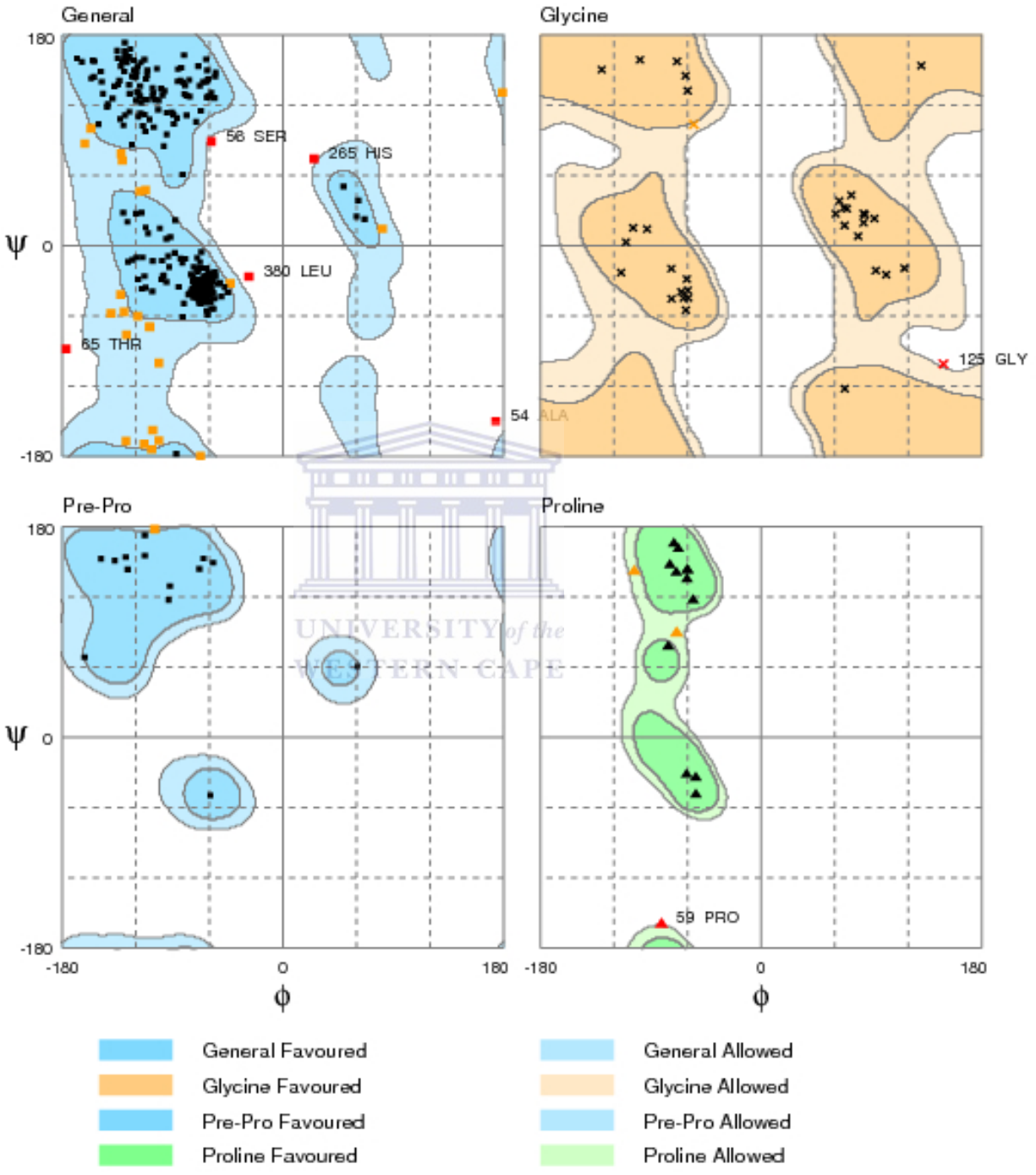


Figure 4.7: Ramachandran plot for the BSII9 hydantoinase model built by the Swiss model server.

For the model in Figure 4.6, 90.9 % of the residues were in the favoured region, 7.2 % residues were in the allowed region and 1.9 % of the residues were in outlier regions. Although the accuracy of the homology model was considered to be low, the putative protein was confirmed to be a hydantoinase.

Comparison of the novel amino acid sequence to sequences of closely related hydantoinases from the Uniport database showed that the putative hydantoinase from BSII 9 aligned very well with other actinobacterial dihypropyrimidinase and hydantoinase sequences (Figure 4.8).

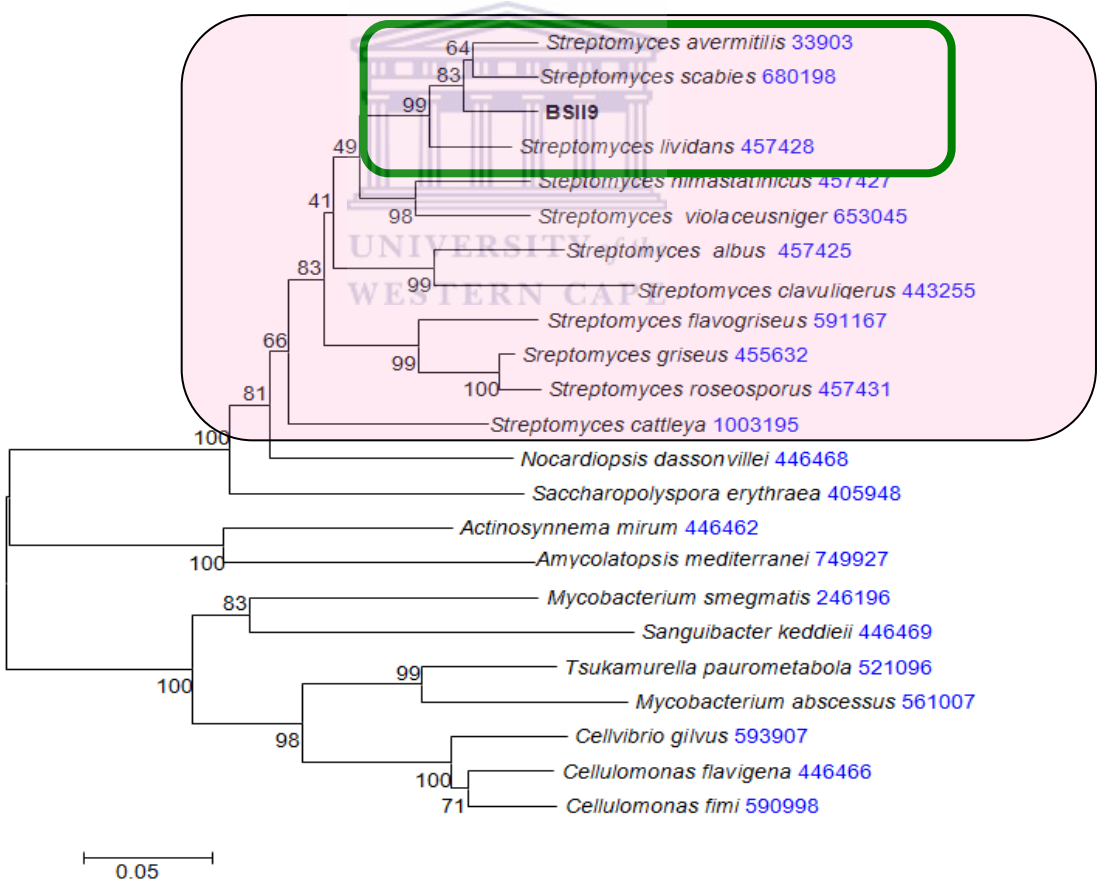


Figure 4.8: Neighbour joining phylogenetic tree of BSII 9 and closely related hydantoinases. *Streptomyces* hydantoinases are highlighted in the pink box. The numbers highlighted in blue are protein identity numbers on NCBI.

It was noted that all *Streptomyces* hydantoinase sequences clustered together in one clade supported by a bootstrap value of 66 (pink box in Figure 4.8). The hydantoinase from BSII 9 formed a separate group in the *Streptomyces lividans* clade (green box).

Previous studies have identified amino acid residues involved in the stereospecific recognition, substrate binding and catalytic cleavage of 5-monosubstituted hydantoin derivatives based on studies of hydantoinase crystal structures (Abrendroth *et al.*, 2002a; b; Cheon *et al.*, 2002). The catalytic site of the hydantoinase enzyme consists of six strictly conserved amino acids involved in coordination of the metal ions. The first metal ion is coordinated by Lys150, His183, His 239. The second metal ion is coordinated by Lys150, His59, His61, and Asp315 (Abendroth *et al.*, 2002b). These amino acids are strictly conserved, implying that the mechanism of hydantoin hydrolysis is similar regardless of the enantiospecificity of the enzyme (Cheon *et al.*, 2002).

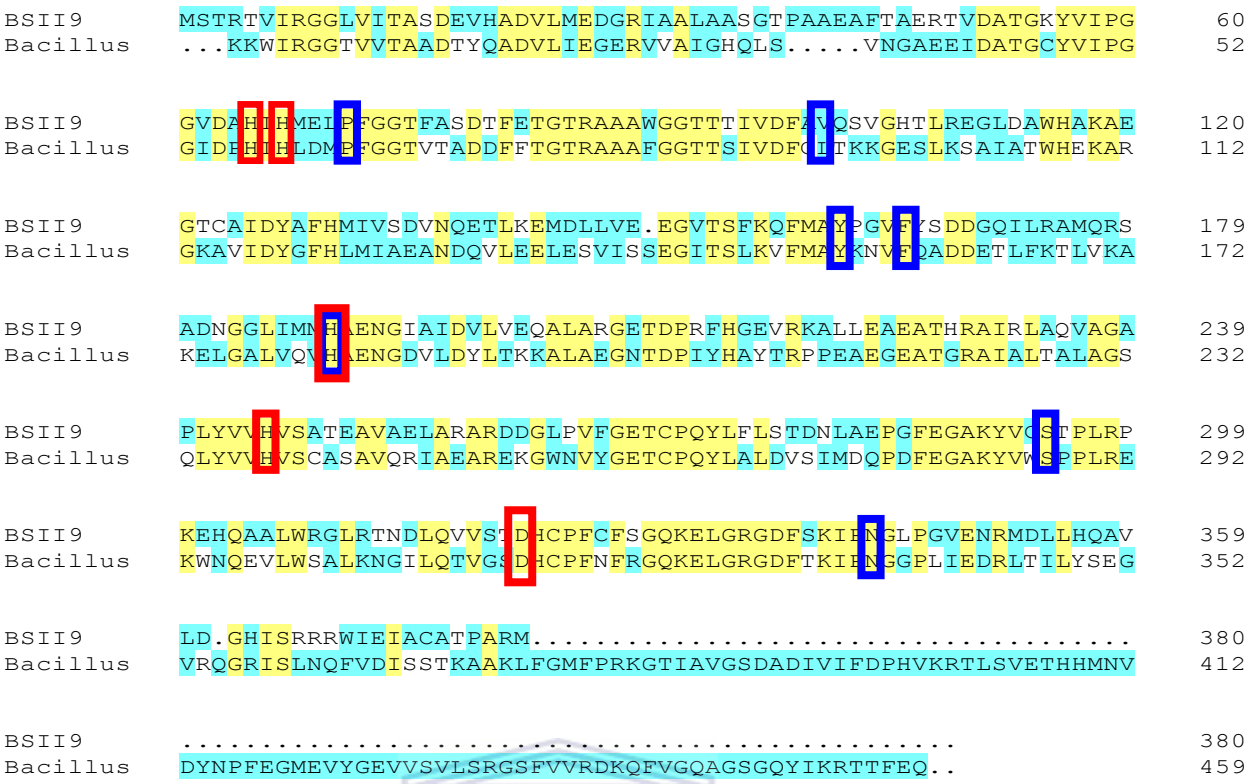


Figure 4.9: Amino acid sequence alignment of Bacillus sp. and BSII9 hydantoinases.
Putative catalytic residues deduced from the crystal structure of *Bacillus* sp. are highlighted in red and residues involved in formation of the hydrophopic pocket are highlighted in blue.

The catalytic site amino acid residues were identified in BSII9 (highlighted in red in Figure 4.9) by alignment and comparison of the amino acid sequence to that of the *Bacillus* sp. hydantoinase (Radha Kishan *et al.*, 2005). The catalytic site of the BSII9 hydantoinase was predicted to consist of the residues His 65, His 67, Lys 157, His 189, His 245, and Asp 321 (Figure 4.10).

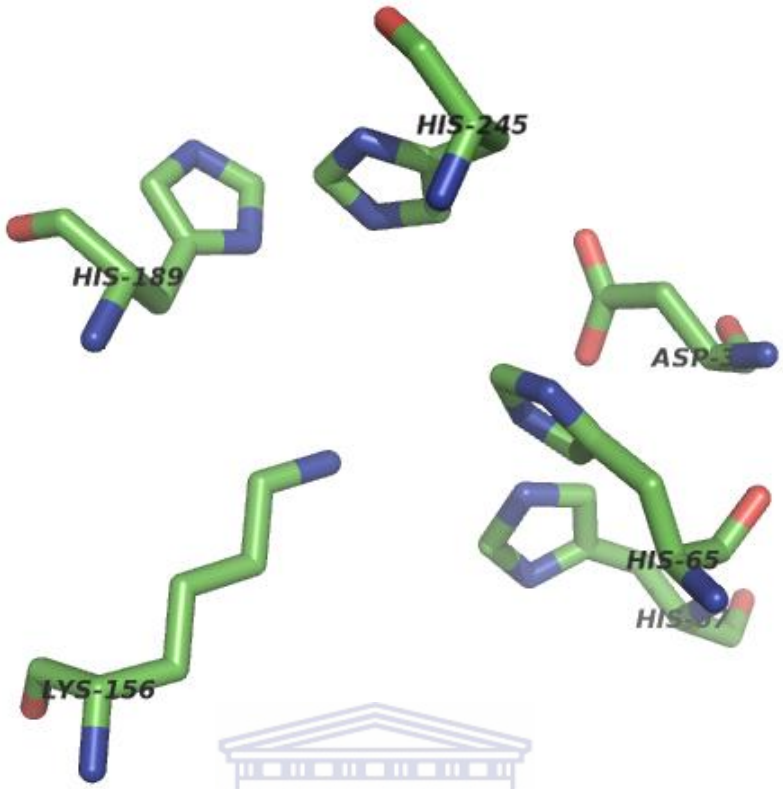


Figure 4.10: Model of the BSII9 hydantoinase catalytic site.

Although the overall amino acid sequence homology between the BSII9 hydantoinase and the crystallised *Bacillus* sp. hydantoinase was only 49 %, the active sites were identical and the catalytic residues could be perfectly superimposed (results not shown). These results are consistent with the high degree of active site conservation between hydantoinases (Abendroth *et al*., 2002 a; Cheon *et al.*, 2002; Xu *et al.*, 2003 a).

The interaction of hydantoinase and their substrates involves two components, the recognition of the functional amide groups which controls the orientation of the substrate, and recognition of the exocyclic side chains which in turn determines the stereo-specificity of the enzyme (Abendroth *et al.,* 2002a; b). The residues proposed to be involved in both these mechanisms are also highly conserved throughout hydantoinases

(Abrendroth *et al*., 2002a; b). These residues were identified in the BSII9 hydantoinase and are highlighted in Figure 4.9 by blue boxes.

The recognition of the exocyclic side chain of the hydantoin substrate occurs in a buried hydrophobic pocket. The residues forming this pocket have been found to vary between the hydantoinases studied thus far (Cheon *et al.*, 2002). Residues that have been reported to form this pocket include Met63, Leu65, Phe152, Phe159 and Tyr155 and are located in three loops called stereochemistry gate loops or SGLs. These loops, SGL-1 (residues 62-72) SGL-2 (residues 93-100) and SGL-3 (residues 153-162) are highly conserved (Cheon *et al*., 2002; Xu *et al.*, 2003). The residues proposed to form the hydrophobic pocket in the BSII9 hydantoinase were identified by aligning the BSII9 and *Bacillus* sp. hydantoinase amino acid sequence. The residues Pro 71, Val 100, Tyr 161, Phe 165, His 189, Ser 294 and Asn 343 are proposed to form the hydrophobic pocket in BSII9 (Figure 4.11).

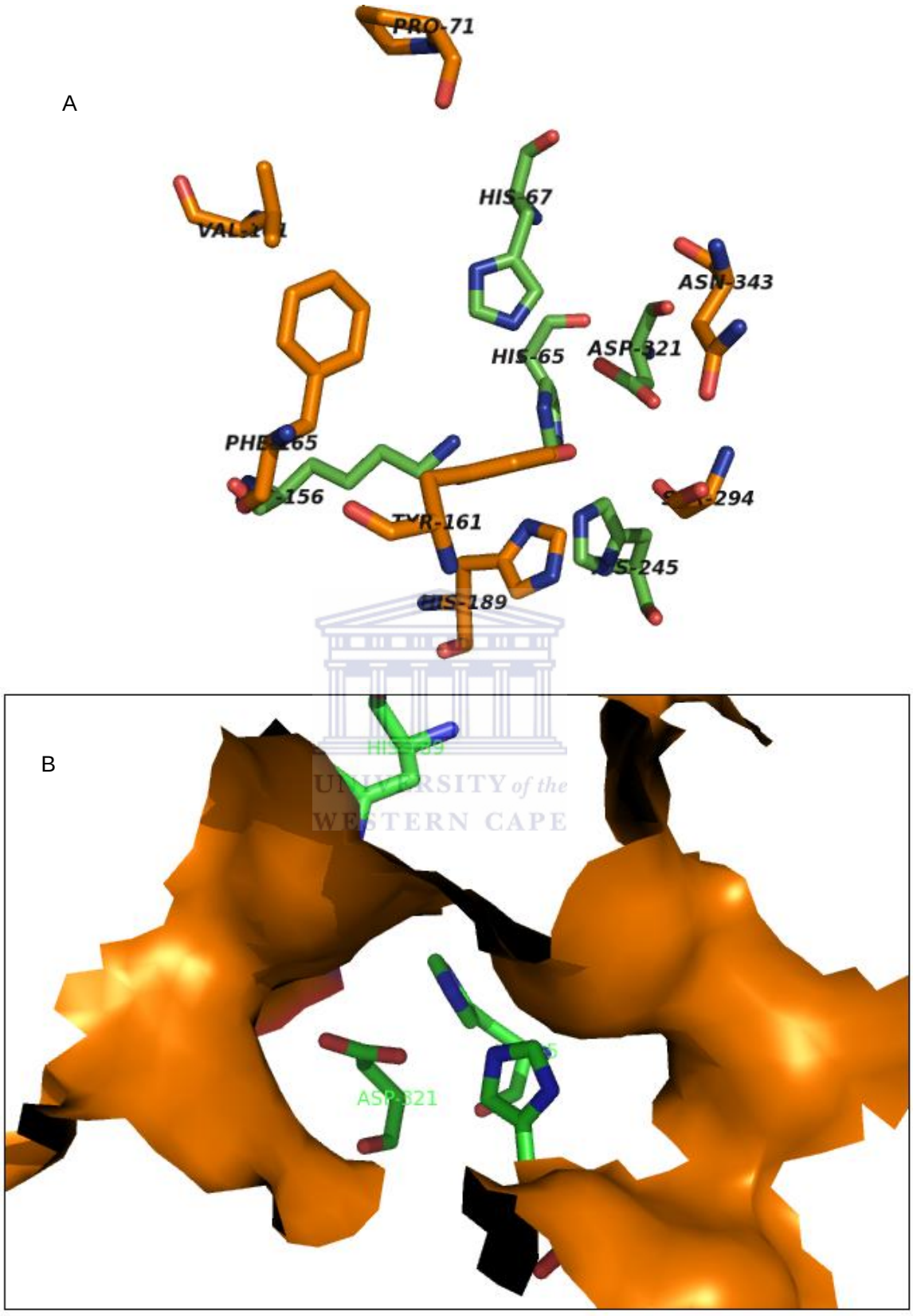


Figure 4.11: Model of the BSII9 predicted hydrophobic pocket (orange residues) around the catalytic site (green residues). Image B indicates the pocket formed by the residues around the active site.

Interestingly, these residues formed what could be perceived as a hydrophobic pocket around the predicted catalytic site (Figure 4.11 B), consisting of four hydrophobic residues and three uncharged residues.

4.3.3 Cloning of the hydantoinase gene sequence

It is noteworthy that all the *Streptomyces* hydantoinase sequences available on public databases are of predicted proteins and to our knowledge none of these proteins have been characterised. As such, the cloning, expression and characterisation of the BSII9 hydantoinase had a high degree of novelty. Hydantoinase gene specific primers were designed and these specifically amplified an approximately 1.5 kb fragment (Figure 4.12).

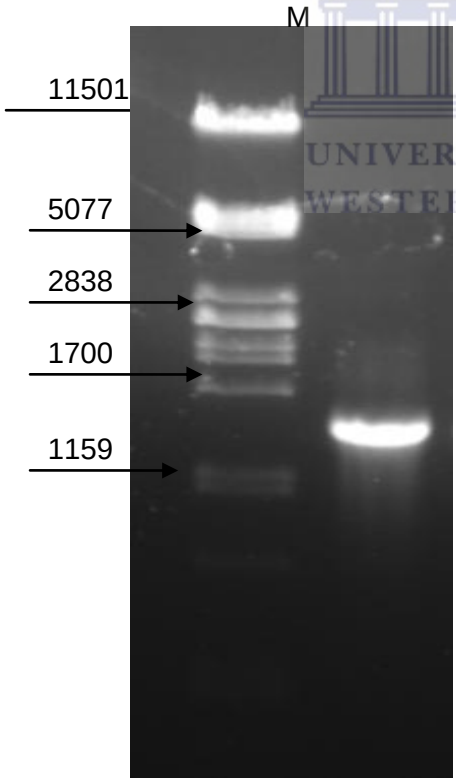


Figure 4.12: PCR amplification of the BSII9 hydantoinase gene using gene specific primers Thydfor and Thydrev. M, lambda DNA digested with *Pst*I. Lane 1, BSII9 hydantoinase gene amplicon.

The PCR product was sequenced to confirm that the fragment was the hydantoinase gene. NEBcutter v 2.0 was used to predict restriction enzyme recognition sites occurring within the hydantoinase gene sequence. It was established that *Nde*I and *Hind*III did not cut within the gene sequence and a primer pair (Forhydsp and Revhydsp) (Table 4.2) was designed which included these restriction sites. It was noted that although the gene specific primers worked well and amplified the hydantoinase gene, once restriction endonuclease sites had been added to the primers PCR amplification of the gene was unsuccessful. A modification of the Taguchi method was used to optimise the PCR using the array for four variables as described by Cobb and Clarkson (1994). Optimal amplification was achieved when the final concentration of $MgCl_2$ in PCR reactions was 4 mM, and the template DNA concentration was 2 ng/µl. The use of the Taguchi method to optimise PCR was significantly easier than the classical method of changing one variable at a time. The array for four variables developed by Cobb and Clarkson (1994) results in the use of nine reactions, compared to twelve reactions used in the classical gradient PCR reaction. The Taguchi method was considered as the standard method for optimisation of PCR in all subsequent experiments.

The amplified fragment was purified and cloned into the pET28a vector. Successful cloning was confirmed by colony PCR using Forhydsp and Revhydsp primers, restriction digestion using *Nde*I and *Hind*III (Figure 4.13), and sequencing using the T7 promoter and terminator primers

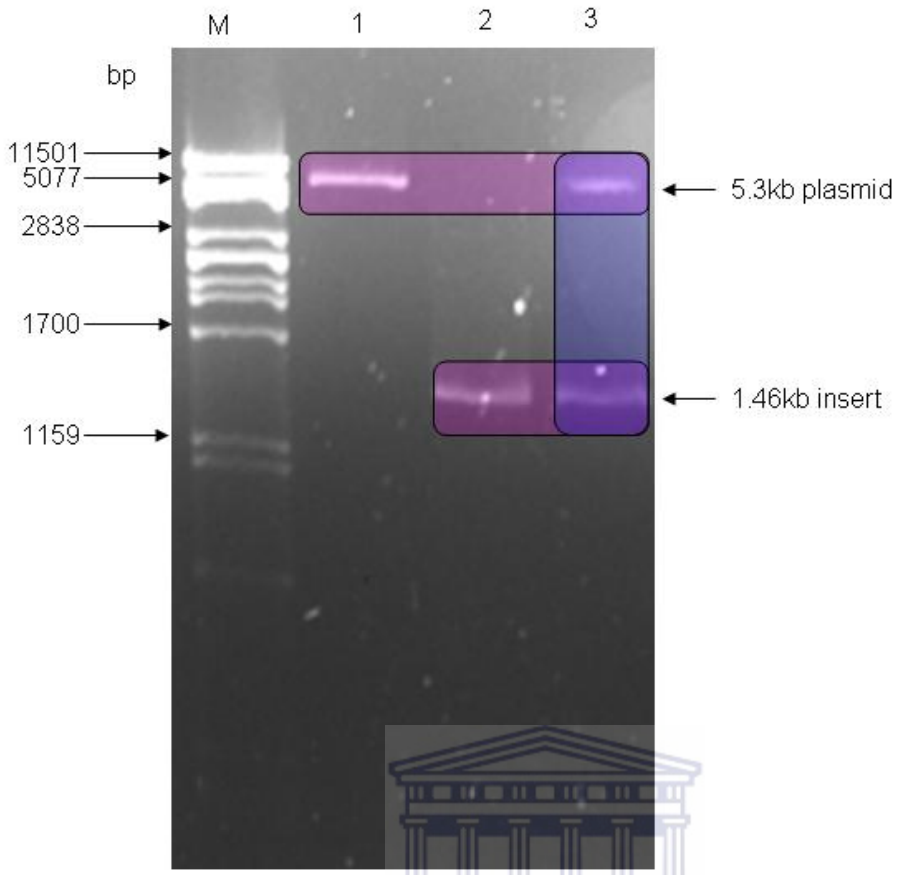


Figure 4.13: Restriction enzyme digestion of a positive clone. Lane M: DNA molecular marker, lambda-*Pst*I digested DNA. Lane 1: Linearised pET28a vector DNA. Lane 2: PCR amplified hydantoinase gene digested with *Nde*I and *Hind*III. Lane 3: Plasmid extracted from a positive clone and digested with *Nde*I and *Hind*III.

4.3.4 Expression of the hyadntoinase gene in E.coli

The recombinant plasmid pET28hyd was transformed into the *E. coli* expression strains BL21 (DE3) and Rosetta (DE3) pLysS. Small-scale expression experiments were performed at 25, 30 and 37 °C for 24 or 36 hours and were analysed by SDS-PAGE. In both BL21 pLysS and Rosetta2 pLysS, no over-expression of the hydantoinase was evident in the soluble cell protein fraction. Subsequent experiments to optimise the expression of hydantoinase were carried out with Rosetta cells. Expression was confirmed by analysis of the protein profiles using SDS PAGE (Figure 4.14). A 55 kDa protein was observed in all small scale expression experiments (circle in black in Figure

4.14). No corresponding band was observed in the negative control containing cells transformed with pET 28a vector without the hydntoinse gene (lane 1 in Figure 4.14).

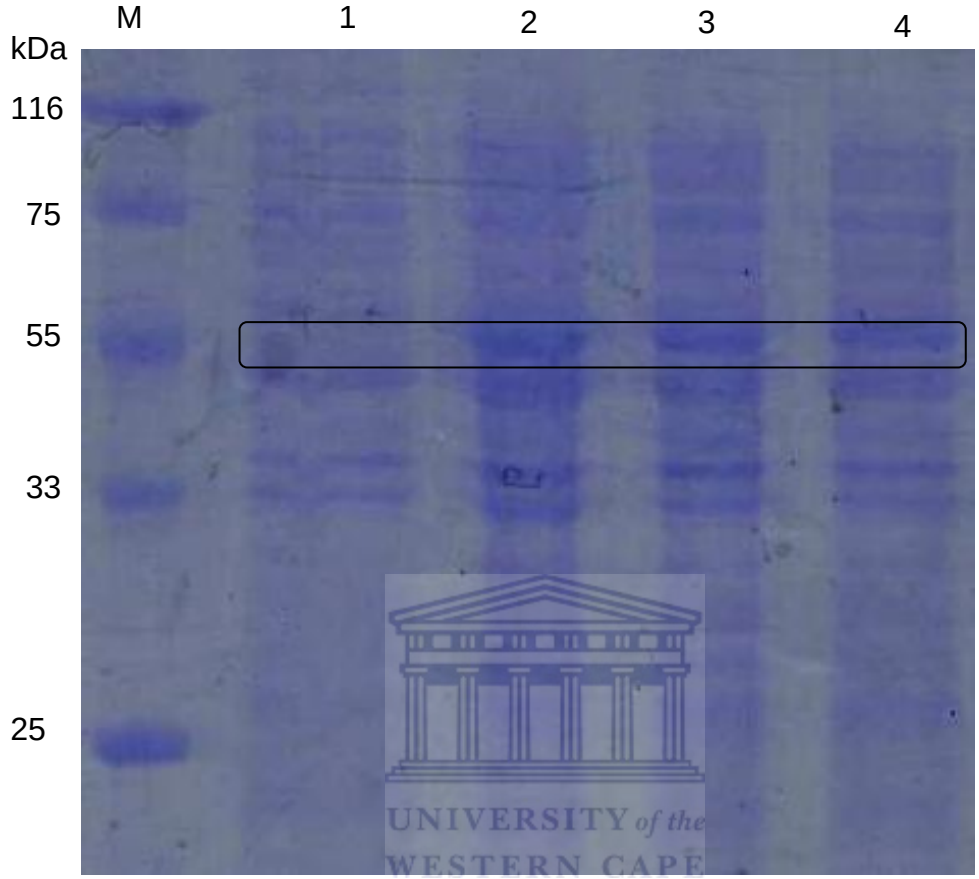


Figure 4.14: Expression of BSII9 hydantoinase in E.coli Rosetta (DE3) pLysS analysed by SDS PAGE. M: Protein Ladder (Fermentas unstained), Lane 1: Negative control cells transformed with pET28a vector, Lanes 2, 3 and 4: Expression trial at 25, 30 and 37 °C, respectively.

Although the expression of the hydantoinase protein was generally low, expression at 25 °C was slightly higher than at 30 and 37 °C. All subsequent expression experiments were conducted at 25 °C. Analysis of the insoluble fraction revealed a band corresponding to the size of the expected protein (Figure 4.15). This suggested that a large quantity of the expressed protein was insoluble, possibly in inclusion bodies.

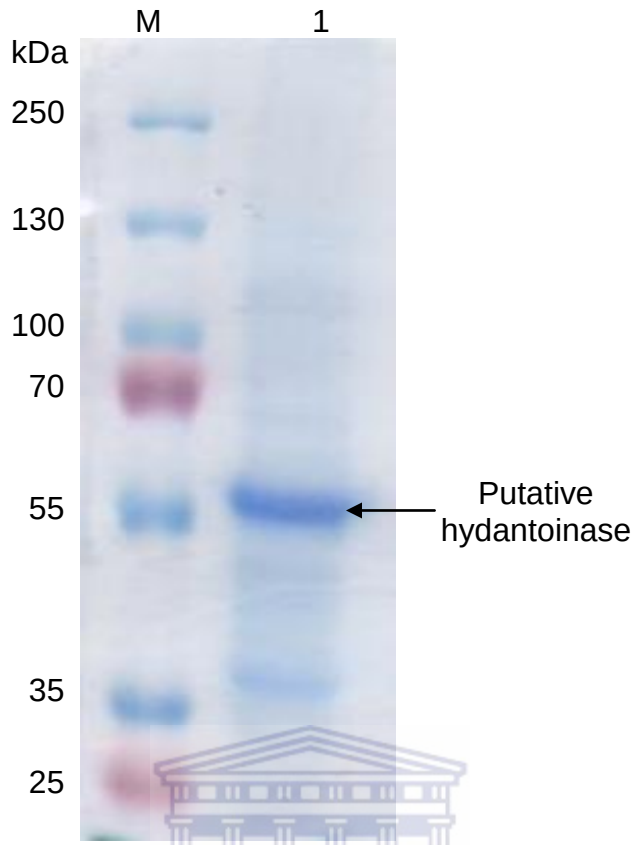


Figure 4.15: SDS PAGE analysis of the insoluble protein fraction from the expression of hydantoinase in E.coli Rosetta (DE3) pLysS.

Similar observations have been reported elsewhere (Chao *et al.*, 2000, Chiang *et al.*, 2007, Liu *et al*., 2008). Attempts to increase the solubility of the over-expressed protein included varying the amount of IPTG used for induction, varying the incubation temperature after induction as well as varying the length of incubation after induction.

A slight improvement was observed (as determined by an increase in band intensity) in solubility of the expressed protein when the amount of IPTG used for induction was increased from a final concentration of 0.5 mM to 1 mM (Figure 4.16).

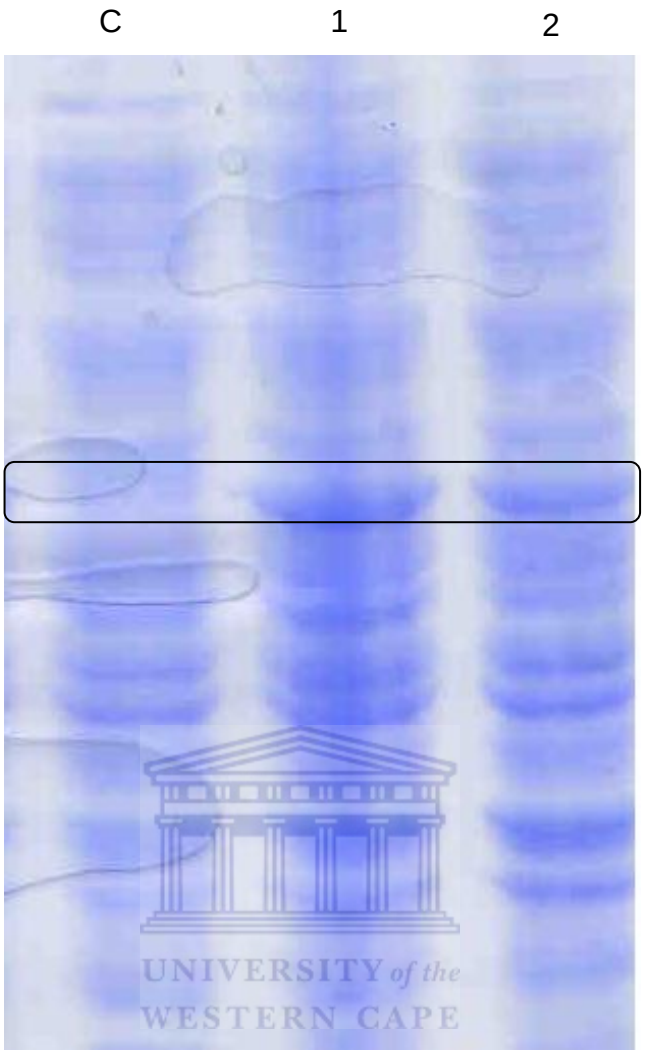


Figure 4.16: Optimisation of BSII9 hydantoinase expression under the induction of different concentrations of IPTG. Lane C: Negative control, no IPTG added, Lane 1: 1 mM IPTG, Lane 2: 0.5 mM IPTG (final concentrations).

IPTG concentrations higher than 1 mM did not improve expression as no increase in the intensity of the expected band was observed (results not shown). A final IPTG concentration of 1 mM was considered as the optimal amount of IPTG for induction of the hydantoinase gene. The use of lactose instead of IPTG for induction has been reported to improve the solubility of the hydantoinases in *E. coli* expression systems (Chien *et al*., 1998). However, no significant difference was noted when lactose was used for induction in this study (data not shown).

Previous studies have shown that many proteins do not fold properly in the absence of their co-factors, resulting in mis-folded proteins that aggregate into inclusion bodies (d'Abusco *et al.*, 2001; Pabarcus and Casida, 2002). The addition of the divalent metal ions Mn^{2+}, Ni^{2+}, and Zn^{2+} to the growth media during induction appeared to yield a slight improvement in the solubility of the expressed protein as judged from the increase in intensity of the expected band in the soluble cell fraction (Figure 4.17). However, increasing the amount of metal ions did not significantly increase the solubility of the expressed protein.

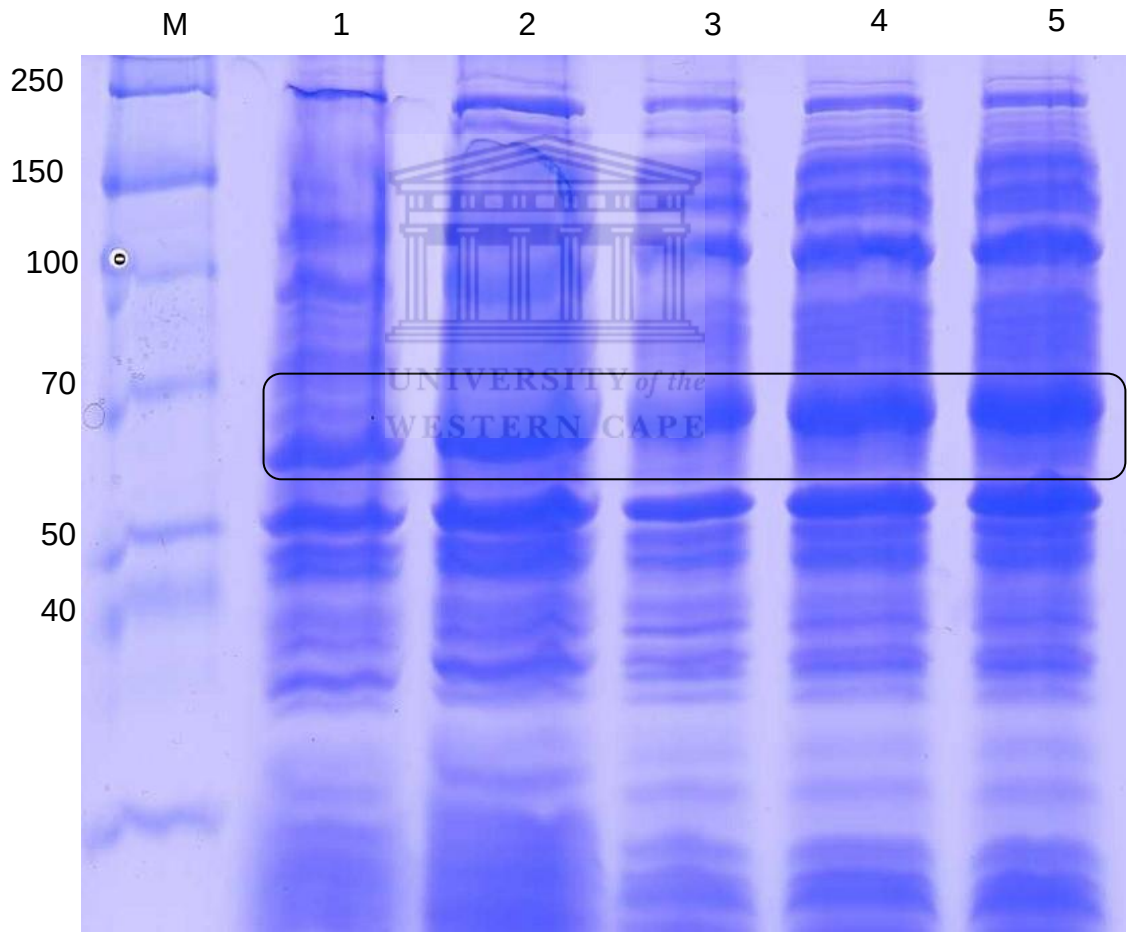


Figure 4.17: Effect of divalent metal ions on expression of the hydantoinase gene in *E. coli*. M: Page ruler protein ladder, Lane 1: No metal ions supplemented at induction. Lane 2, 3, 4,: Expression reactions supplemented with 5 mM Mn^{2+}, Ni^{2+}, and Zn^{2+}, respectively. Lane 5, 10 mM Ni^{2+}.

Although the solubility of the expressed hydantoinase was improved by varying different expression parameters such as incubation temperature, amount of IPTG used for induction and addition of metal ions to growth media, a large proportion of the expressed protein remained insoluble (Figure 4.18).

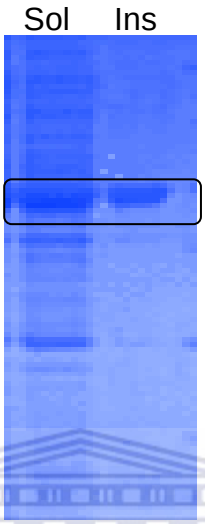


Figure 4.18: Analysis of the soluble and insoluble protein fractions after optimisation of expression (25 °C, 1 mM IPTG, 5 mM divalent metal ions. Sol: soluble fraction, Ins: insoluble fraction. Band predicted as hydantoinase circled in black box.

Apart from experimental conditions, successful expression of a recombinant protein is affected by several other factors, including the use of rare codons in the heterologous host (Baneyx, 1999). The success of high level expression of a cloned gene can be correlated to the codon adaptation index (CAI), a statistical measure of the codon usage bias in an organism (Sharp and Li, 1987). The index uses a reference set of highly expressed genes from a species to assess the relative merits of each codon. A cloned gene can be analysed using bioinformatics tools and a score for the gene is calculated from the frequency of use of all codons in that gene. A CAI of 1.0 is considered to be ideal, while a CAI of <0.8 is undesirable as it might indicate unsuccessful expression in the heterologous host (Sharp and Li, 1987). GenScript was used to calculate the CAI of the hydantoinase gene using *E. coli* as an expression host and the calculated CAI was 0.7. This could have account to the low efficiency of expression of the soluble protein observed in this study.

Codon preference and codon usage have also been implicated as factors affecting the efficient expression of proteins. The amount of protein expressed in *E. coli* has been shown to be strongly dependent on the occurrence of rarely used codons on the gene to be expressed (Welch *et al.*, 2009). The hydantoinase gene was analysed for the *E. coli* preferred codons using GenScript.

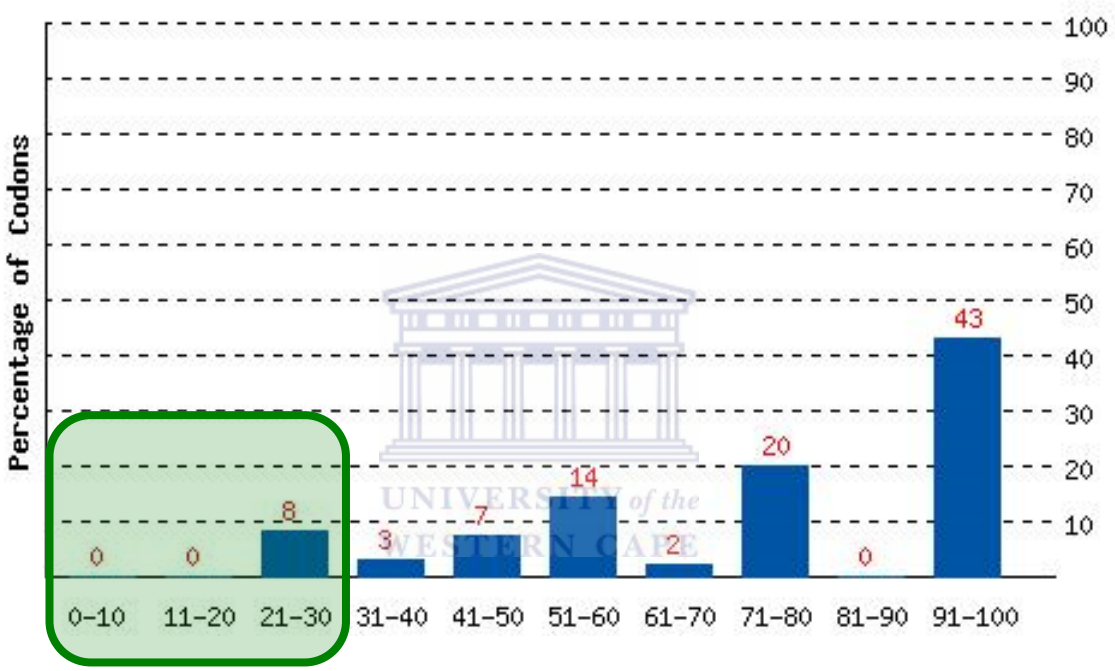


Figure 4.19: The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in *E. coli*. Codons with values lower than 30 (highlighted in the green box) are likely to lower the expression efficiency.

The codon frequency distribution of the hydantoinase gene (Figure 4.19) showed that approximately 92 % of the codons had a frequency value of higher than 30, with 43% of the codons having a value between 91 and 100 and only 8% of the codons having values lower than 30. This finding suggests that codon preference might not be a factor in the expression of this gene in *E. coli*.

The GC content of the gene of interest has also been implicated in lowering the efficiency of expression in a heterologous host. Genes with high GC contents, for example actinobacterial genes, have been shown to have low expression efficiencies in *E. coli*. This has been attributed to the instability of the mRNA at the translation initiation region. A high GC content in the 5'-end of the gene of interest usually leads to the formation of secondary structure in the mRNA (Gu *et al.*, 2010). This could result in interrupted translation and lower levels of expression. The average GC content of the cloned hydantoinase gene was calculated to be 69.7%.

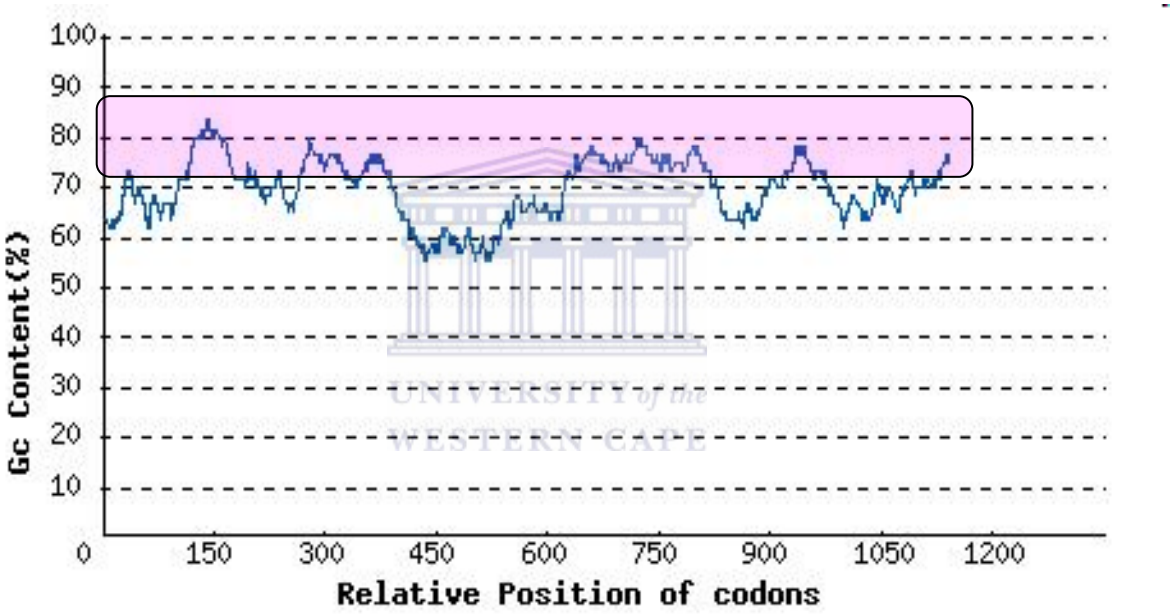


Figure 4.20: Predicated GC curve for the hydantoinase gene. Peaks highlighted in the pink box are above the 70% GC content range and could potentially lower the efficiency of protein expression in *E. coli*.

A plot of the codon GC contents along the length of the gene revealed several regions with an average GC content higher than 70% (Figure 4.20). The ideal GC percentage range of a cloned gene for successful expression in *E. coli* is between 30% and 70% (Gu *et al.*, 2010). Any peaks outside of this range might adversely affect transcriptional and translational efficiency.

4.3.4.1 Effect of the his-tag on solubility of expressed proteins

The most widely used tag to purify and detect recombinant expressed proteins is the polyhistidine tag (Yip *et al.,* 1989) which incorporates six histidine residues either on the N or C terminal end of the expressed protein. The location of this tag within a particular protein has been reported to influence the expression, solubility, and bioactivity of the protein (Busso *et al.,* 2003; Woestenenk *et al.,* 2004). During cloning of the hydantoinase in this study, a 6 x His tag was incorporated at the N terminal end (Figure 4.21).

MGSSHHHHHHSSGLVPRGSHMSTRTVIRGGLVITASDEVHADVLMEDGRIAALAASGTP
AAEAFTAERTVDATGKYVIPGGVDAHTHMELPFGGTFASDTFETGTRAAAWGGTTTIVD
FAVQSVGHTLREGLDAWHAKAEGTCAIDYAFHMIVSDVNQETLKEMDLLVEEGVTSFK
QFMAYPGVFYSDDGQILRAMQRSADNGGLIMMHAENGIAIDVLVEQALARGETDPRFH
GEVRKALLEAEATHRAIRLAQVAGAPLYVVHVSATEAVAELARARDDGLPVFGETCPQ
YLFLSTDNLAEPGFEGAKYVCSTPLRPKEHQAALWRGLRTNDLQVVSTDHCPFCFSGQK
ELGRGDFSKIPNGLPGVENRMDLLHQAVLDGHISRRRWIEIACATPARM

Figure 4.21: Deduced amino acid sequences of the BSII 9 hydantoinase expressed in E. coli with the His-tag highlighted in red. Total amino acid number: 400, MW: 43179, ORF: 1-1200.

To determine whether the His tag significantly influenced the solubility of the hydantoinase, the gene was sub-cloned into pET17b to remove the His-Tag from the protein. However, the removal of the His-Tag did not increase the solubility of the hydantoinase, as most of the expressed protein was still observed in the insoluble fraction (data not shown).

4.3.5 Protein purification

The soluble fraction of the expressed hydantoinase was subjected to His-Tag affinity chromatography. Although high level purification was achieved the expressed protein was not purified to homogeneity. Several contaminating bands co-eluted with the target protein (Figure 4.22).

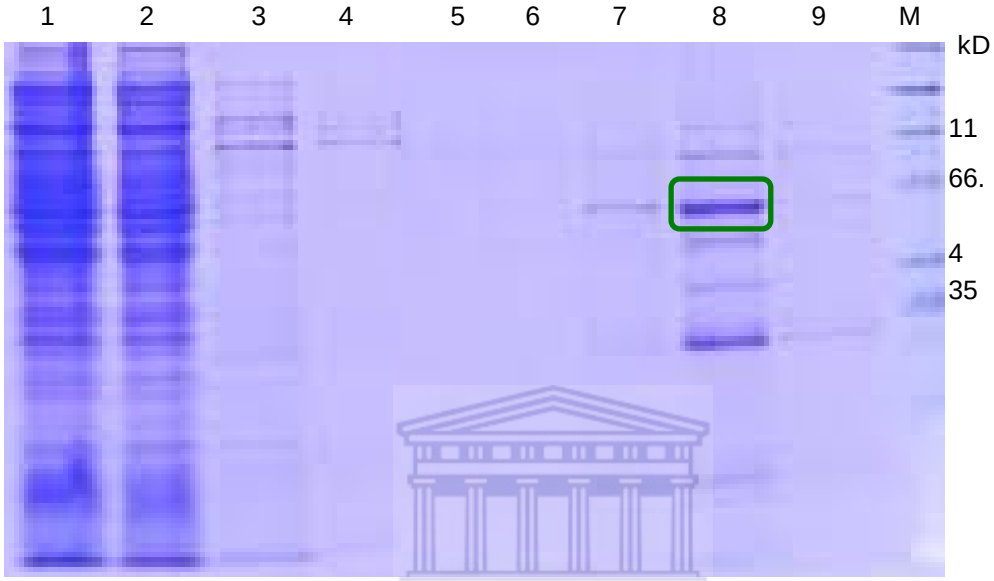


Figure 4.22: SDS-PAGE analysis of His-tag purification of BSII9 hydantoinase expressed in Rosetta (DE3). The expected protein band corresponding to a size of ~55 kDa in the elution fraction is indicated by the green box. Lanes 1: Crude cell free extract, Lane 2: flow through fraction, Lane 3: first wash with binding buffer, Lane 4 second wash with binding buffer Lane 5: first was with 1 x wash buffer, Lane 6 second wash with wash 1 x wash buffer, Lane 7: wash with 2 x wash buffer, Lane 8: elution fraction, Lane 9: post elution wash fraction, M: Protein molecular weight ladder.

Attempts to reduce the number of contaminating proteins included using higher imidazole concentrations during the washing steps. This however resulted in the subsequent loss of the target protein with no significant improvement in purity.

4.3.6 Activity of the purified hydantoinase

Positive *E. coli* clones transformed with pET28-hyd displayed hydantoinase activity thereby confirming the functional expression of the cloned gene. Units of activity were measured as µmol/ml/h of N-carbamoylglycine produced from the hydrolysis of hydantoin. All fractions collected during the purification steps were dialysed and tested for hydantoinase activity (Figure 4.23).

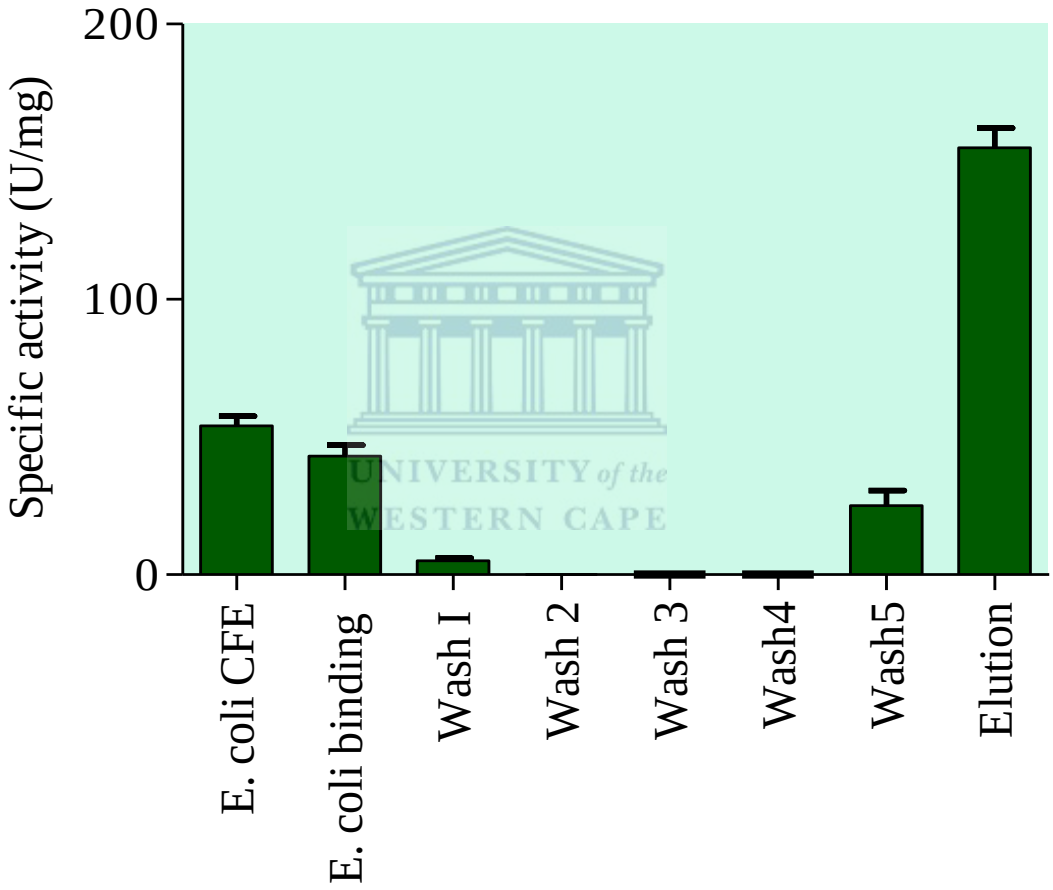


Figure 4.23: Hydantoinase activities observed in different fractions collected during His-tag purification steps. CFE = cell free extract.

Specific enzyme activities of the crude and partially purified recombinant hydantoinase were compared to those of the wild type enzyme.

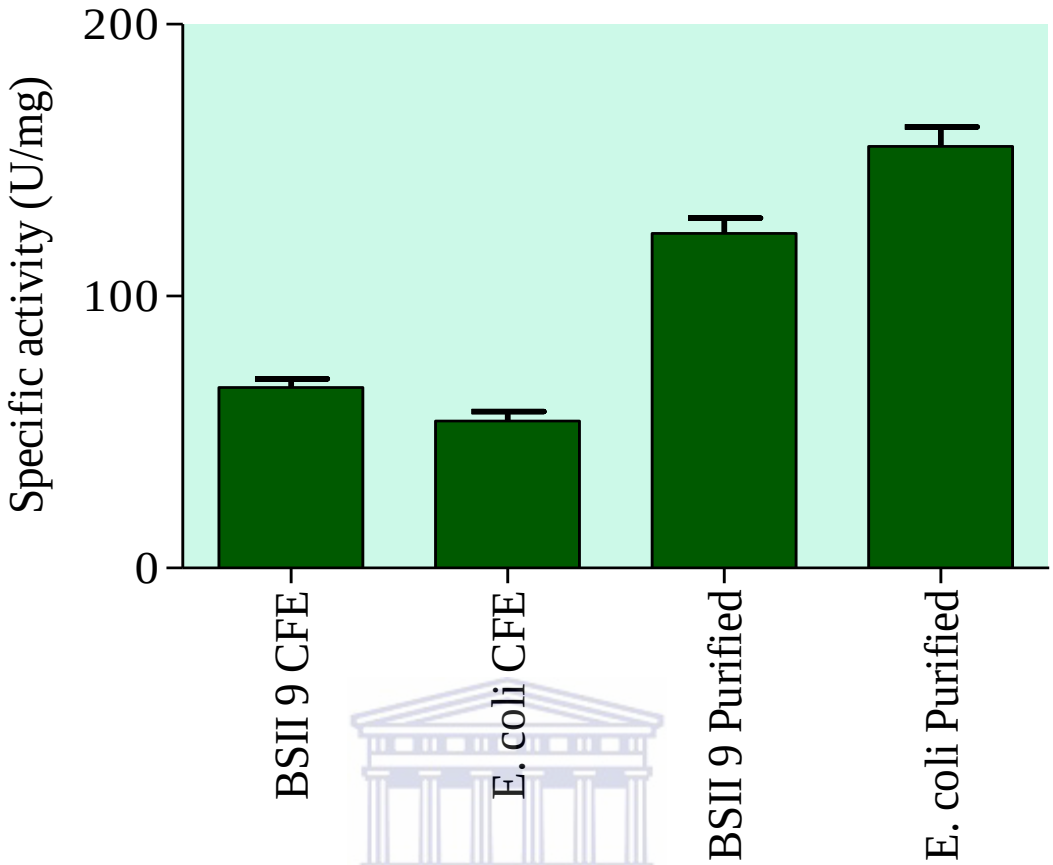


Figure 4.24: Comparison of enzyme activities of wild type and cloned hydantoinase in both crude and purified states.

There were no major differences in activities observed between the wildtype enzyme purified from BSII9 and the recombinant enzyme purified from *E. coli* (Figure 4.24). Although the activity of the recombinant enzyme purified from *E. coli* was slightly higher, this was attributed to the higher level of purity (Table 4.6).

Table 4.6: Purification table

Sample	Total protein (mg)	Total units (umol/ml/h)	Specific activity (U/mg)	Purification fold	Yield (%)
BSII9 CFE	602	60900	101	1	100
E. coli CFE	378	20412	54	1	100
BSII9 Purified	114	16000	140	1.4	26
E. coli Purified	46	7130	155	2.9	35

Although the His-tag purification method resulted in a significantly lower yield, the recovered protein showed a two fold increase in purity compared to the protein purified from BSII9.

4.3.7 Characterisation of partially purified enzyme

4.3.7.1 Optimal temperature.

Optimal activity of the partially purified enzyme was observed at 45 °C (Figure 4.25).

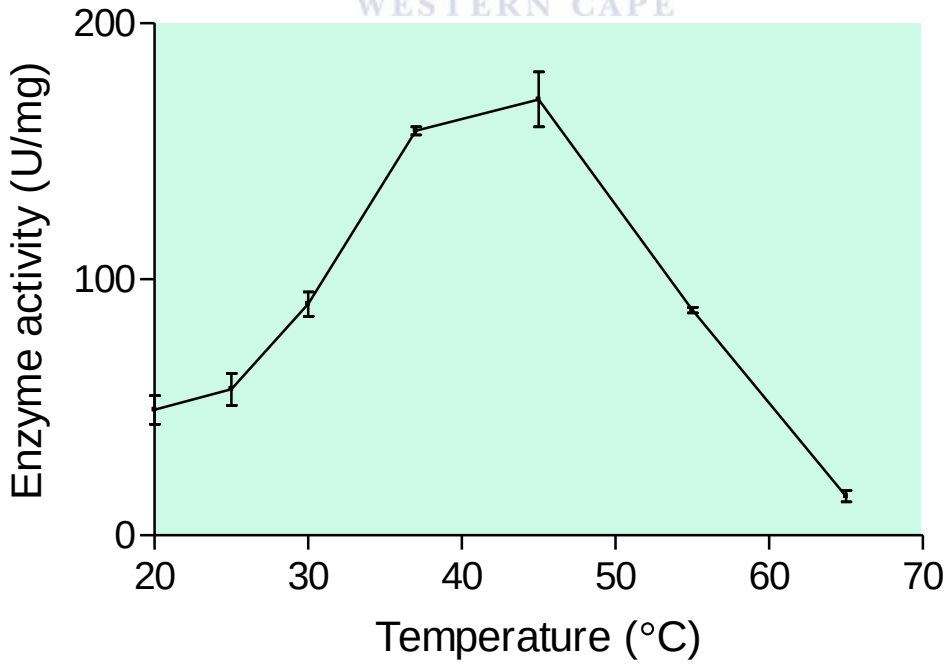


Figure 4.25: Effect of temperature on activity of the purified hydantoinase

This result was consistent with the optimal temperatures reported for other hydantoinases cloned from *Pseudomonas* species (Takahashi *et al*., 1978; Morin *et al*., 1986). At temperatures above 45 °C enzyme activity dropped rapidly.

4.3.7.2 Thermal stability

Thermal stability results (Figure 4.26) indicated that when purified enzyme samples were incubated above 55 °C, approximately 50 % of activity was lost within 30 minutes.

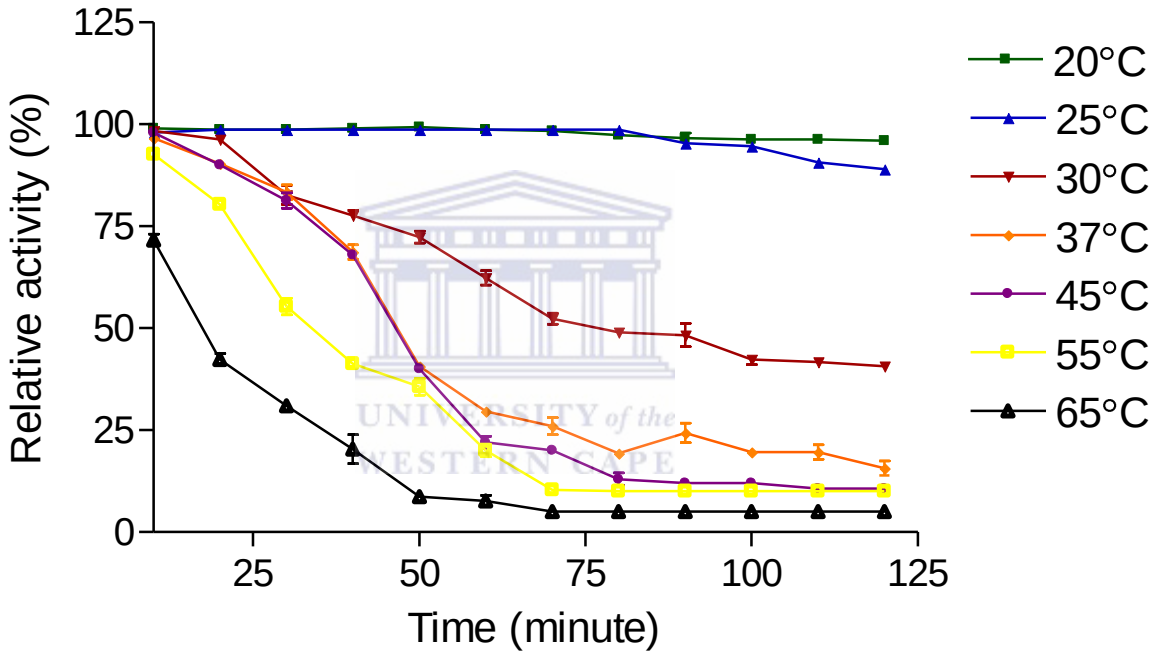


Figure 4.26: Effect of temperature on the stability of the expressed hydantoinase. Activity was measured by performing enzyme assay at optimal temperature after initial incubation at different temperatures for the specified time.

The recombinant enzyme was stable at 20 °C, maintaining approximately 99 % activity after two hours. Stability at was also maintained at 25 °C, with less than 15 % loss in activity after a two hour incubation period. When protein samples were incubated at 65 C more than half the activity was lost after 20 minutes. Incubation for an hour resulted in more than 90 % loss of activity.

4.3.7.3 Optimal pH

The effect of pH on activity of the purified hydantoinase was investigated. Maximum activity was observed at pH 8 and the pH range for activity was 6 to 10 (Figure 4.27).

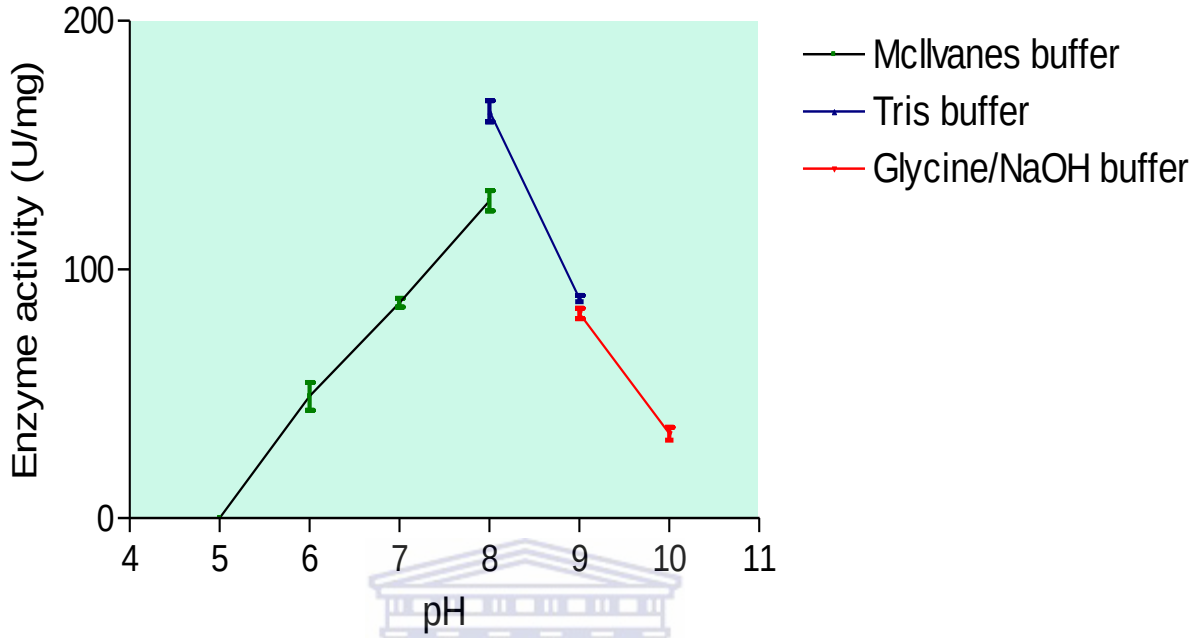


Figure 4.27: Effects of pH on activity of the purified hydantoinase.

4.3.7.4 Stability during storage

Stability assessment of crude and purified enzyme purifications during storage at 4 °C that samples stored in Tris buffer, pH 8 had an estimated half-life of 12 to 15 days (Figure 4.28).

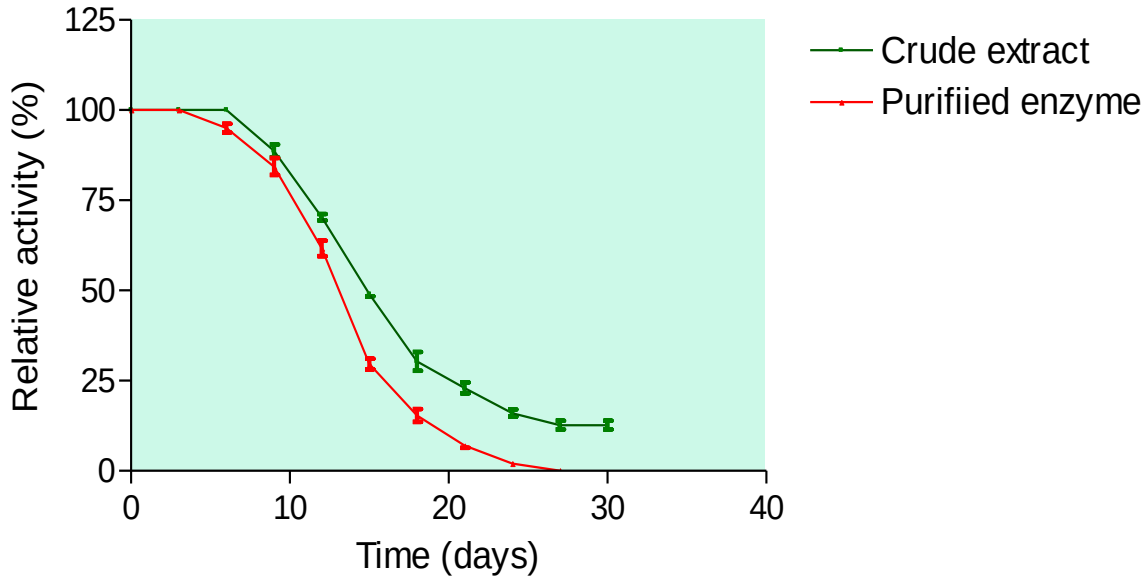


Figure 4.28: Enzyme stability during storage at 4 °C.

Chapter 5: General conclusions

The search for novel microbial secondary metabolites for potential agricultural, pharmaceutical and industrial applications is very important. Actinobacteria are of considerable importance in industry as they are a rich source of secondary metabolites. The objectives of this study were to isolate, characterise and screen actinobacteria for industrially relevant enzymes.

Isolation and characterisation of novel actinobacetria

During a screen for actinobacteria from Zambian hot-spring soil samples, 51 actinobacterial isolates were obtained. ARDRA was used to de-replicate the isolates into twelve phylotypes. Analysis of the 16S rRNA genes indicated that eight isolates were *Micromonospora* species, three were *Streptomyces* species and one was a *Verrucosispora* species. Phylogenetic analysis showed the isolate GSI2 belonged to a distinct clade occupied only by members of the genus *Verrucosispora* and was most similar to *Verrucosispora sediminis* in a distinct cluster. GSI2 was distinguishable from the type strains of the three described *Verrucosispora* species by using a combination of chemical and morphological markers. We suggest that GSI2 should be placed in the genus *Verrucosispora* as the type strain of a novel species, for which we propose the name *Verrucosispora africana* sp. nov.

Although the *Micromonospora* and the *Streptomyces* species identified in this study were not novel species, as these two genera may still be potential sources of novel secondary metabolites (Lazzarini et al., 2001; Seong et al., 2001;), the inclusion of these isolates in the screening for enzymes was warranted.

Screening actinobacterial isolates for industrially relevant enzymes.

Isolates were screened for amidase, carbamoylase, hydantoinase, and nitrile hydratase activity. Screening of isolates for these industrially imporatant enzymes was successful. The hydantoinase activity observed in isolate BSII 9 was chosen for further study. Analysis of 16S rRNA gene sequence of this isolate identified it as a non novel Streptomyces species with 99 % sequence similarity to *Streptomyces erythrogriseus* and *Streptomyces labedae*. To date, hyantoinase and carbamoylase activities have not been characterised from streptomycetes. The discovery of these activities in this genus might indicate potential novelty of the enzymes or other enzyme characteristics such as substrate specificity.

Growth and enzyme production was optimised for the isolate and optimal growth was observed at 37°C in tryptic soy broth. The addition of different hydantoin substrates did not result in a significant increase in enzyme activity. Attempts to fully purify the hydantoinase from BSII 9 were not successful. The biggest challenge in protein purification is to separate the protein of interest from all other contaminating proteins, without significantly affecting the yield. In this study this goal was not met as enzyme activity was lost when a three step purification protocol (ammonium sulphate precipitation, hydrophobic interaction and ion exchange chromatography) was used. The use of a two step protocol (ammonium sulphate precipitation and ion exchange chromatography) did not yield reasonable purity. The addition of a third purification step resulted in a relative loss of 90 % of the activity without a significant increase in purity. The full characterisation of the hydantoinase from BSII9 required large quantities of enzymes which even after optimisation was not attainable in the natural host. Therefore a

molecular based approach involving cloning and over-expression in a heterologous host was undertaken.

Cloning and expression of htydantoinase from BSII 9

During this study, the BSII 9 genome was sequenced. *In silico* gene mining of the partially assembled BSII9 genome led to the identification of the hydantoinase gene. The hydantoinase gene from *Streptomyces* isolate BSII 9 was successfully cloned and functionally expressed in *E. coli*. To our knowledge, this is the first reported *Streptomyces* hydantoinase gene to be cloned and functionally expressed. Cloning of the hydantoinase did not alter its activity as specific activity of the wild type and the cloned enzyme were similar. Activity was confirmed in the partially purified protein. The purified hydantoinase showed maximum activity at 45 °C and at a pH of 8, consistent with the hydantoinase cloned from *Arthrobacter aurescens* (Slydakt *et al*., 1992). Stability of the enzyme during storage at 4 °C was slightly diminished by purification.

The amino acid residues that have been identified as forming the catalytic site and the hydrophobic pocket (Abendroth *et al*., 2002a; Cheon *et al*., 2002; Xu *et al*., 2003a), are conserved in the BSII 9 hydantoinase. According to Cheon *et al*. (2002), the residues which form the hydrophobic pocket are responsible for recognition and binding of the exocyclic side chain of the hydantoin derivatives, and thus control substrate selectivity and enantioselectivity. Future studies involving the site-directed mutagenesis of these residues in the BSII 9 gene would conclusively determine the role these residues play in substrate selectivity and in turn, it might provide a platform for engineering the enzyme to increase substrate specificity.

Expression of the enzyme in a soluble state still requires optimisation. Investigations into various strategies including the use of a different expression host would contribute greatly not only to the expression of this particular gene but in the expression of actinobacterial genes in general. A streptomycete expression system could be a possible alternative.

Other studies have shown that the genes responsible for complete hydrolysis of hydantoin derivatives to optically pure amino acids occur as gene clusters or operons (Stover et al. 2000, Nelson et al. 2002). The annotation of the genome sequenced during this study is still underway. Completion of the annotation project would make available the complete genetic information and thus facilitate identification of gene clusters and operons. The cloning of the whole hydantoin hydrolysisng operon, under its own promoter might prove advantageous for the expression of the hydantoinase in a soluble state.

In conclusion, this research identified a potentially novel *Verrucosispora* species. Members of this genus have recently been considered as a potential source of anti-tumor. The identification and partial characterisation of a hydantoinase from a *Streptomyces* species has the potential to yield novel enzyme characteristics such as wider substrate specificity as no hydantoinases have been characterised from this genus before.

This study revealed that hot springs may represent a previously unexplored source of novel actinobacterial diversity. However, it also revealed that novel secondary metabolites are not only limited to novel organisms but that some of the answers for the challenges we face today maybe found in organisms we have already encountered and characterised.

Appendices

Appendix (i)
Table A1: Composition of media useful for the isolation of rare actinobacteria

Ingredients (g/liter)	AV agar	MC agar	MGA-SE agar	GAC agar	HV agar	LSV-SE agar
Glucose	1.0	2.0	2.0	2.0	-	-
Glycerol	1.0	-	-	-	-	-
Humic acid	-	-	-	-	1.0	-
Lignin	-	-	-	-	-	1.0
Soil extract	-	-	200ml	-	-	100ml
L-Arginine	0.3	-	-	-	-	-
L-Asparagine	-	-	1.0	1.0	-	-
Soy bean flour	-	-	-	-	-	0.2
$NaNO_3$	-	0.5	-	-	-	-
$CaCO_3$	-	-	-	-	0.02	0.02
K_2HPO_4	0.3	0.3	0.5	0.3	-	-
NaH_2PO_4	-	-	-	-	0.5	0.5
KCl	-	0.3	-	0.3	1.7	1.7
NaCl	0.3	-	-	-	-	-
$MgSO_4.7H_20$	0.2	0.3	0.5	0.3	0.5	0.5
$Fe_2(SO_4)_3$	0.01	-	-	-	-	-
$FeSO_4.7H_2O$	-	0.01	0.01	0.01	0.01	0.01
$CuSO_4.5H_2O$	0.001	0.001	0.001	0.001	-	-
$ZnSO_4.7H_2O$	0.001	0.001	0.001	0.001	-	-
$MnSO_4.7H_20$	0.001	0.001	0.001	0.001	-	-
B-vitamins	*	-	-	-	*	*
Agar	15	20	20	20	18	18
pH	6.4	7.4	7.4	7.4	7.2	7.2

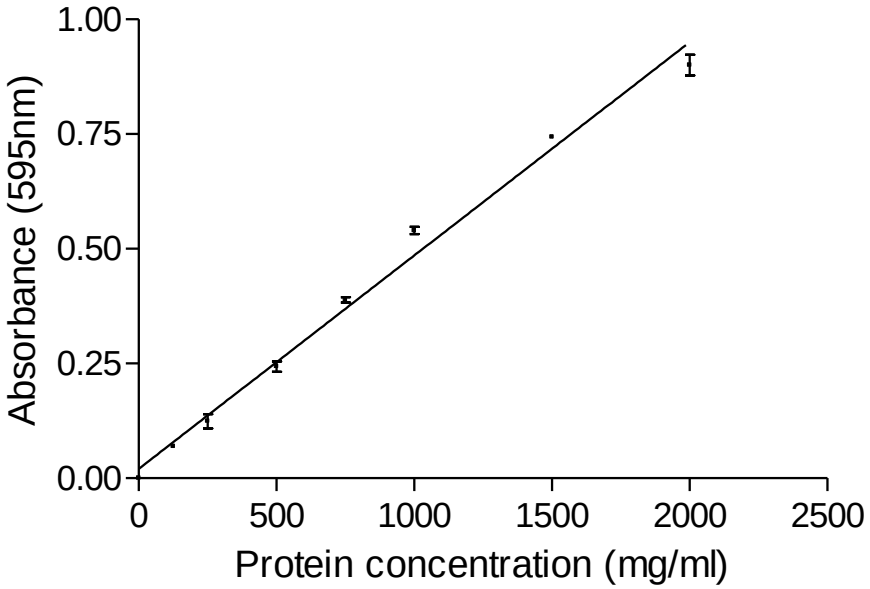


Figure A (i): BioRad Bradford assay standard curve from BSA standards. y = 1.102x + 0.011. R^2 = 0.9878

Appendix (ii)

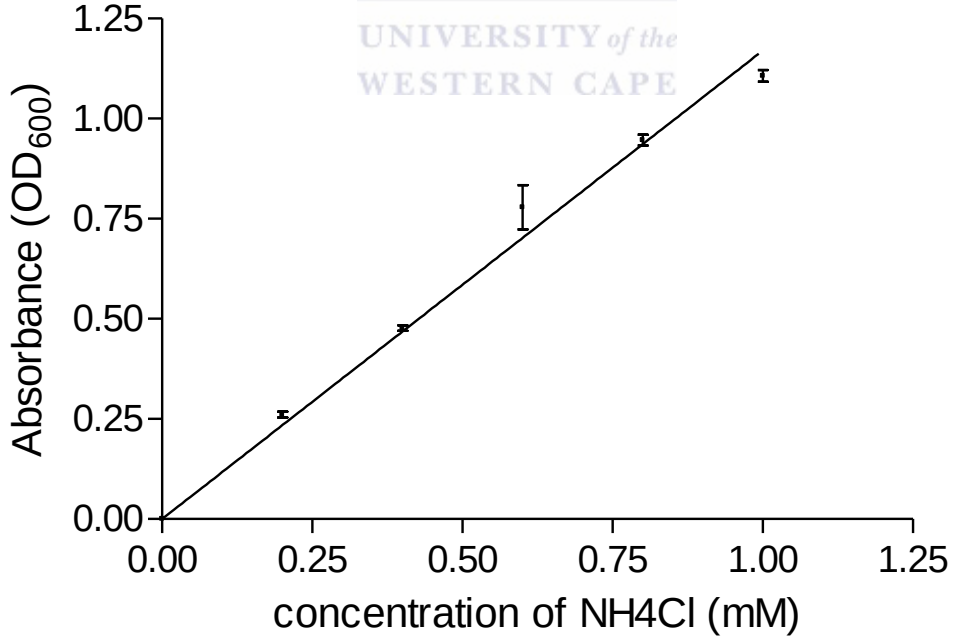
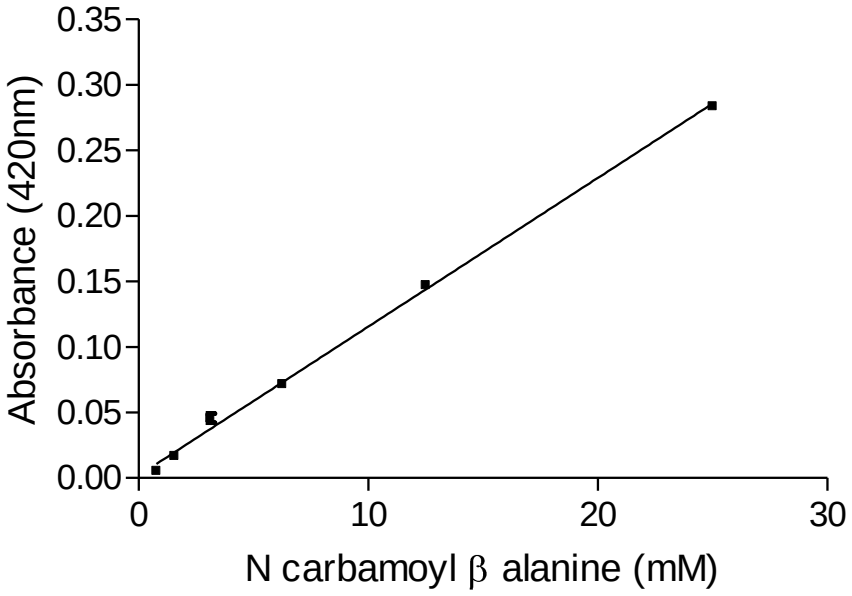


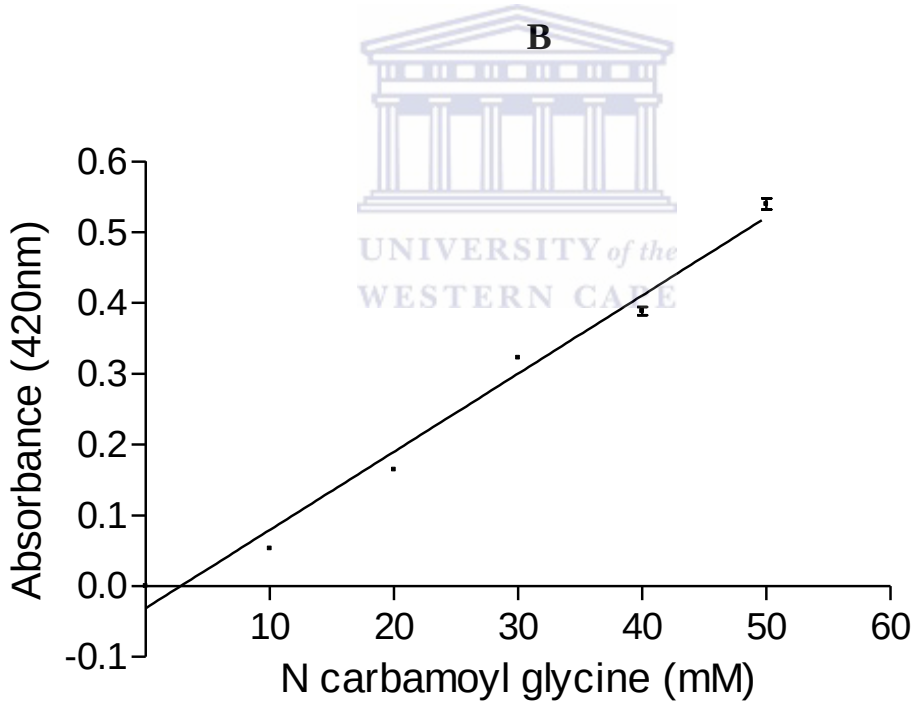
Figure A (ii): Ammonia assay standard curve. y = 1.124 x, R^2= 0.98.

Appendix (iii)

A



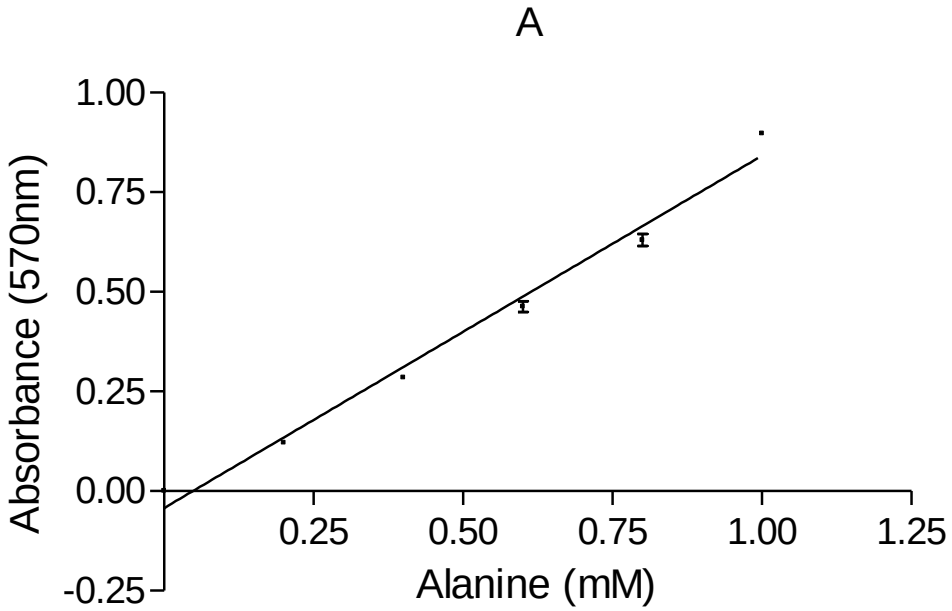
y= 0.01135 x + 0.000189, R^2 = 0.9967



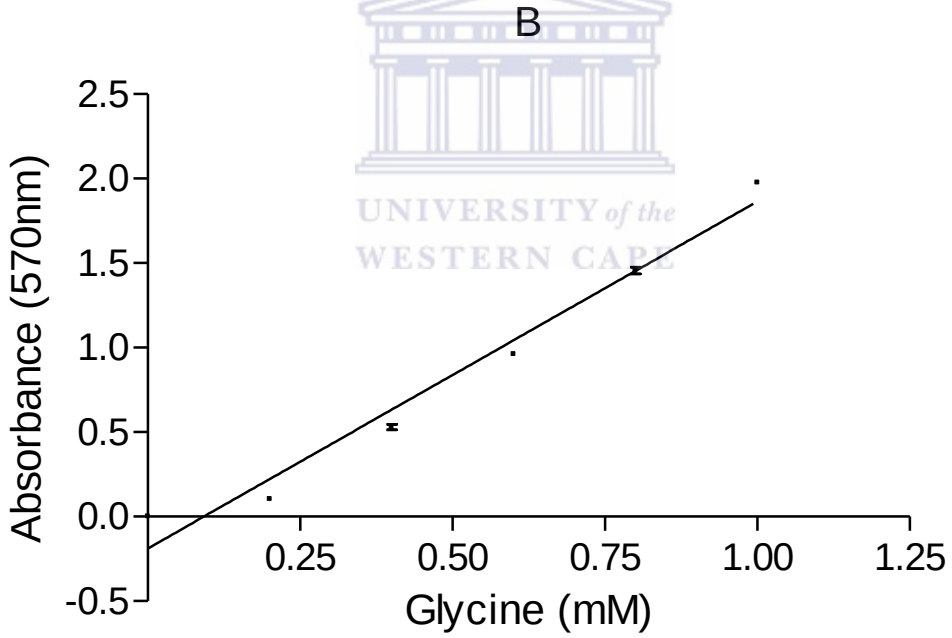
y= 0.011 x – 0.0311, R^2 = 0.98

Figure A (iii) Typical standard curves for N-carbamoyl b alanine (A) and N-carbamoyl glycine (B) with Ehrlichs reagent.

Appendix (iv)



$y = 0.8844 x – 0.04272$, $R^2 = 0.98$



$y = 2.054 x – 0.189$, $R^2 = 0.97$

Figure A (iv): Typical standard curves for alanine (A) and glycine (B) with Ninhydrin reagent

Appendix (v)

Ammonium sulphate precipitation table. Initial saturation percentages are highlighted in the red box and the final saturation percentages are highlighted in green. The intercept indicates the amount of ammonium sulphate (in grams) required to achieve the desired saturation in one litre.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

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