CHARACTERIZATION OF SELECTED *BACILLUS* ISOLATES EXHIBITING BROAD SPECTRUM ANTIFUNGAL ACTIVITY

by

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Submitted in the fulfilment of academic requirements for the degree of
Master of Science in Microbiology

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October 2004

ABSTRACT

The genus *Bacillus* is comprised of Gram-positive, rod-shaped, spore-forming bacteria which are well known for their ability to produce a diverse array of antimicrobial compounds. Of particular interest is the ability of certain strains to produce antifungal compounds. Such organisms have the potential for application in agriculture where they can be used as biocontrol agents against selected plant pathogenic fungi. A study was undertaken to further characterize selected *Bacillus* isolates that exhibit broad spectrum antifungal activity.

Dual culture bioassays were used to screen seven selected *Bacillus* isolates for activity against four plant pathogenic fungi *in vitro*. All isolates were able to inhibit the pathogens to varying degrees. Two isolates, R29 and B81, were selected for further testing and characterization. Further bioassays were performed on five complex nutrient media which were adjusted to pH 5.5 and 7, and both incubated at 25^0C and 30^0C, respectively. It was found that pH and media composition showed significant influences on the antifungal activities of the isolates tested, but that a 5^0C temperature difference in incubation temperature did not. Tryptone soy agar was found to give rise to the largest inhibition zones.

Both isolates were tentatively identified using standard biochemical and morphological tests. Based on its phenotypic characteristics, R29 was identified as a strain of *B. subtilis*. B81 proved to be more difficult to assign to a specific group or species of *Bacillus,* though *B. subtilis* and *B. licheniformis* were considered to be the nearest candidates. Genomic DNA was extracted from both isolates and a portion of each of their 16s rDNA genes were amplified and sequenced for homology testing against the GeneBank database. Homology testing confirmed that both isolates were members of the genus *Bacillus* and most probably strains of *B. subtilis*. The DNA fragment used for sequencing proved to be too small to give conclusive identification of the isolates.

Isolate R29 was selected for further characterization of its antifungal compound/s. Growth curve studies using a defined synthetic medium showed that antifungal activity arose during the stationary phase and appeared to be closely linked to sporulation. The antifungal component of cell free culture supernatant was extracted using various methods including thin layer chromatography, acid precipitation, hydrophobic interaction chromatography and methanol extractions. High performance liquid chromatography (HPLC) analysis of extracts from acid

precipitation and hydrophobic interaction chromatography revealed two active peaks indicating that at least two antifungal compounds were produced. Methanol extracted samples produced the cleanest sample extract but only revealed one active peak from the HPLC fraction.

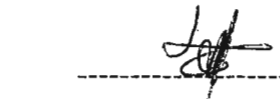
Nuclear magnetic resonance analysis of purified samples indicated that the antifungal compound/s have aromatic complex and peptide structures. The extracted antifungal compounds were Protease K resistant and found to be thermostable at temperatures ranging 80-121^{0}C, and, were active at pH ranges of 3-13. The antifungal compounds were found to exhibit similar properties to known antifungal lipopeptides i.e. iturin A and fengycin A and B.

Further characterization and identification of the active compounds is recommended using methods such as liquid chromatography mass spectrometer and matrix-assisted laser desorption ionisation time-of-flight.

The results presented in this dissertation provide a basis from which antifungal compounds produced by strains of *Bacillus* can be further characterized.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations and that it has not been submitted for a higher degree in any other university.



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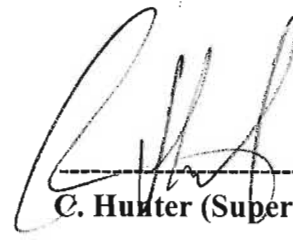
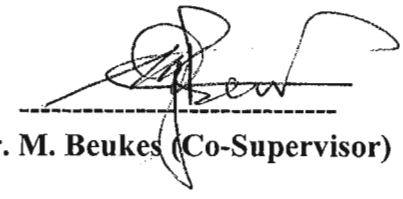


C. Hunter (Supervisor)



Dr. M. Beukes (Co-Supervisor)

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to:

My supervisor Mr. C. Hunter for his guidance, valuable advice, encouragement and assistance in reviewing and editing this thesis.

My co-supervisor Dr. M. Beukes for his guidance, valuable advice, his technical advice and assistance with some of the analyses.

Dr. R. Robinson and Mr. C. Grimmer for their assistance with NMR analysis and interpretation.

All staff and postgraduate students in the Disciplines of Microbiology and Plant Pathology, for their cooperation whenever needed.

I am also grateful to the Eritrean Human Resource Development (EHRD) for their sponsorship.

Special thanks go to my mother, brothers and sisters who always encouraged me.

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CHAPTER 1

LITRATURE REVIEW

1.1 THE GENUS *BACILLUS*

The family Bacillaceae comprises Gram-positive, rod-shaped bacteria, which are able to form endospores. Two main genera can be distinguished:

1. Anaerobic bacteria of the genus *Clostridium;* and,
2. Aerobic or facultatively anaerobic bacteria of the genus *Bacillus* (Priest, 1993a)

The genus *Bacillus* comprises a large heterogeneous group of bacteria, which are ubiquitous in nature. Most species are harmless and easy to isolate. Many species are economically important exhibiting useful attributes such as biocontrol and plant growth promotion activities, insecticidal activity, antibiotic and extracellular enzyme production (Claus and Fritze, 1989).

1.1.1 *Bacillus* Taxonomy

Although the generic name *Bacillus* was first established in 1872, by Ferdinand Cohn, the first comprehensive taxonomic study of the genus was published by Smith *et al.* (1952) and was based on a comparative study of 1134 strains representing over 150 species. From this study 19 type species were distinguished (Claus and Fritze, 1989; Priest, 1993a & b). Further taxonomic studies carried out by Gordon *et al.* (1973) resulted in 65 distinct species being described (Priest, 1993b).

The genus is catalase positive and usually motile by means of lateral or peritrichous flagella (Lancini and Lorenzetti, 1993; Prescott *et al.*, 1999). Cells of *Bacillus* occur singly or in chains. The rod-shaped cells have rounded or squared ends and range in size from (0.5 x 1.2μm) to (2.5x 10μm) (Claus and Berkeley, 1986; Prescott *et al.*, 1999).

Gibson and Gordon (1974) (cited by Claus and Fritze, 1989) promoted the concept of morphological grouping of *Bacillus* based on the shape (oval or spherical) and position of endospores in the sporangium, in order to place species into more manageable groups for easier identification. Based on numerical classification studies six large groups have been established by Gordon (1981) (Priest 1993a) (Table1.1). Numerical taxonomic studies have been successful for analysis of bacteria from soils and have contributed enormously to the understanding of the distribution of *Bacillus* species in various soils.

Taxonomic classification of bacteria within the genus *Bacillus* was originally based on their ability to sporulate and their biochemical, morphological, and physiological characteristics (Bron *et al.*, 1999). Modern classification is based on the comparative sequence analysis of the 16S rDNA base composition (Jonson, 1989).

Although classified within one genus, *Bacillus* species are more heterogeneous than other bacterial genera, as evidenced by the wide range of DNA base ratios (32 to 69 mol% G + C) between species, which is far wider than that usually considered reasonable for a genus (Claus and Berkeley, 1986; Priest, 1989a; Lancini and Lorenzetti, 1993). Species in a genus should vary by no more than 10-12 mol% G+C (Jonson, 1989). The heterogeneity of *Bacillus* became apparent with the introduction of modern taxonomic techniques such as numerical phenetics, DNA base composition determination and DNA reassociation, which allows DNA sequence homology between strains to be estimated (Claus and Berkeley, 1986; Priest, 1993a).

Recent studies using 16S rRNA gene sequence analysis have confirmed the high levels of phylogenetic heterogeneity within the genus (Garveba *et al.*, 2003). It has been proposed that the genus *Bacillus* be subdivided into eight genera, namely *Bacillus, Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus* and *Gracilibacillus* (Ash *et al.*, 1991; Heyndrickx *et al.*, 1996; Heyndrickx *et al.*, 1998; Nazina *et al.*, 2001; Nakamura *et al.*, 2002, cited by Garveba *et al.*, 2003). Garveba *et al.* (2003) developed polymerase chain reaction (PCR) and PCR-DGGE (denaturing gradient gel electrophoresis) methods selective for *Bacillus* and used them for assessing the diversity of the genus and related organisms directly from soil DNA. Using newly developed primer sets they found that the amplified fragments affiliated exclusively

with Gram-positive bacteria with up to 95% of sequences originating from *Bacillus* species. The remainder of sequences identified belonged to Gram-positive genera such as *Arthrobacter* and *Frankia* spp., which have high G+C% contents.

The 16S rDNA sequence alone, however, does not differentiate between isolates at the subspecies level. To reach these fine taxonomic levels a polyphasic approach based on a variety of techniques, both genotypic and phenotypic have been proposed to achieve a consensus classification (Gillis *et al.*, 1989).

Table 1.1 *Bacillus* spp. groups based on phenotypic similarities (Priest, 1993b)

Species[a]	Mol % G+C	RNA group[c]	Characteristic of groups
Group I			
B. alvei	46	3	All species are facultative anaerobes and grow strongly in the absence of oxygen. Acid is produced from a variety of sugars. Endospores are ellipsoidal and swell the mother cell.
B. amylolyticus	53	3	
"B. apiarius"	ND	ND	
B. azotofixans	52	3	
B. circulans	39	1	
B. glucanolyticus	48	ND	
B. larvae	38	3	
B. lautus	51	1	
B. lentimorbus	38	1	
B. macerans	52	3	
B. macquariensis	40	3	
B. pabuli	49	3	
B. polymyxa	44	3	
B. popilliae	41	1	
B. psychrosaccharolyticus	44	1	
B. pulvifaciens	44	3	
B. thiaminolyticus	53	ND	
B. validus	54	3	
Group II			All species produce acid from a variety of sugars including glucose. Most are able to grow at least weakly in absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell.
B. alcalophilus	37	UG	
B. amyloliquefaciens	43	1	
B. anthracis	33	1	
B. atrophaeus	42	1	

"B. carotarum"	ND	ND	
B. firmus	41	1	
B. flexus	38	ND	
B. laterosporus	40	5	
B. lentus	36	1	
B. licheniformis	45	1	
B. megaterium	37	1	
B. mycoides	34	1	
B. niacini	38	ND	
B. pantothenticus	37	1	
B. pumilus	41	1	
B. simplex	41	1	
B. subtilis	43	1	
B. thuringiensis	34	1	
Group III			
(*B. alginolyticus*)	48	ND	These strict aerobes do not produce acid from sugars; names in brackets are exceptions. They produce ellipsoidal spores that swell the mother cell.
"B. aneurinolyticus"	42	UG	
B. azotoformans	39	1	
B. badius	44	1	
B. brevis	47	4	
(*B. chondroitinus*)	47	ND	
"B. freudenreichii"	44	ND	
B. gordonae	55	3	
Group IV			
(*"B. aminovorans"*)	40	ND	All the species produce spherical spores that may swell the mother cell wall. All species are strictly aerobic, but some have limited ability to produce acid from sugars.
B. fusiformis	36	2	
B. globisporus	40	2	
B. insolitus	36	2	
B. marinus	39	ND	
B. Pasteurii	38	2	
B. psychrophilus	42	2	
B. sphaericus[d]	37	2	
Group V			
B. coagulans	44	1	These thermophilic species all grow optimally at >50^{0}C. Physiologically and morphologically, they are heterogeneous, but most produce oval spores that swell the mother cell.
"B. flavothermus"	61	ND	
B. kaustophilus	53	5	
B. pallidus	40	ND	
B. schegelii	64	ND	
B. smithii	39	1	

B. stearothermophilus	52	5	
B. thermocatenulatus	69	5	
B. thermocloacae	42	ND	
B. thermodenitrificans	52	ND	
B. thermoglucosidasius	45	5	
B. thermoleovorans	55	5	
B. thermoruber	57	ND	
B. tusciae	58	ND	
Group VI			
A. acidocaldarius	60	6	Thermophilic, acidophilic species with
A. acidoterrestis	52	6	memberaneous ω-alicyclic fatty acids
A. cycloheptanicus	56	6	
Unassigned species			
B. benzoevorans	41	1	
B. fastidiosus	35	1	
B. naganoensis	45	ND	

[a] Name in quotation marks refers to taxa that do not appear in the Approved List of Bacterial Names and therefore have not been validly published. Names in parenthesis refer to species that are atypical of the general description.
[b] Gives either as the value for the type strain or as the mean of a range for several strains.
[c] RNA groups, UG, ungrouped, ND, no data available.
[d] *B. sphaericus* includes at least five " species " of round-spored strains.
-(Group VI) The thermophilic bacilli are phylogenetically diverse and acidophilic thermophles have been allocated to the new genera *Alicyclocabacillus*.

1.1.2 *Bacillus* Ecology

The genus *Bacillus* is among the most widely distributed groups of microorganisms in nature (Bron *et al.*, 1999). Though the main reservoir of *Bacillus* species is considered to be soil, they have been isolated from food, water and even from eukaryotic organisms (Dahl, 1999). *Bacillus* strains have also been isolated from extreme environments such as deserts and even the Antarctic. Representatives of this genus show extraordinary metabolic diversity and include thermophilic, phychrophilic, alkalophilic and acidophilic members (Priest and Sharp, 1989; Bron, 1999). Typically, most strains grow rapidly on inexpensive complex media, and most of them, but not all, are non-pathogenic and regarded as safe (Priest and Sharp, 1989; Dahl, 1999).

The most important aspect of *Bacillus* ecology is the endospore (Priest, 1993b). Endospores of *Bacillus* spp., due to their resistance to adverse conditions, are ubiquitous and can be isolated from a wide variety of sources (Claus and Berkeley, 1986; Nicholson *et al.*, 2000). Population densities ranging from 10^7 - 10^8 CFU. g^{-1} soil in cultivated soil to 10^3CFU.g^{-1} soil in poor soil have been reported (Alexander, 1977). The recovery of *Bacillus* endospores from certain habitats may not necessarily indicate their exact habitat or imply their contribution to that habitat. This is because *Bacillus* endospores can originate from different habitats and are metabolically inactive. However, the presence of a large number of spores of a particular species in a sample can be a good indication of previous or continued growth (Priest, 1993a).

Soil is the primary habitat of most saprophytic bacilli where they play important roles in the biological cycling of carbon and nitrogen. Plant material and plant root exudates are important sources of organic nutrients. Saprophytic bacilli are able to produce a range of extracellular carbohydrases and proteases that permit hydrolysis of cellulose, starch and other glucans, pectin and proteins (Claus and Berkeley, 1986; Priest, 1993a).

Bacillus spp. have been isolated from marine environments, but are thought to originate from water runoff from soil (Priest, 1993a). However, some species are present in high numbers and are considered to be marine organisms (Bonde, 1981, cited by Priest, 1993a). Most bacilli in fresh water are also thought to be of soil origin. Endospore formers occur in sediments in greater numbers than in the water column and their spores often account for the bulk of the total flora at lower levels (Priest, 1993a).

Most *Bacillus* species are considered to be harmless to humans and animals, and only a few are pathogenic i.e., *B. anthrax* (Claus and Berkeley, 1986; Priest, 1993b; Bron *et al.*, 1999; Turnbull, 2002). Although anthrax is primarily associated with herbivores, humans can acquire it from infected animals or animal products (Turnbull, 2002). Additionally, various Group II bacilli are known to contaminate and spoil certain foodstuffs (Priest, 1993b). With the exception of *B. cereus,* these organisms are considered non-pathogenic. *Bacillus cereus* is regarded as an opportunistic infectious bacterial species, which causes emetic and/or diarrhoeal type symptoms (Claus and Berkeley, 1986; Turnbull, 2002).

Williams and Susan (1983) isolated and identified 36 *Bacillus* strains, including *B. licheniformis, B. circulans, B. coagulans, B. laterosporus* and *B. pumilis,* from the rumen contents of cattle fed on hay. Each of these strains were able to utilize hemicelluloses as a carbon source indicating that enzymes capable of releasing reducing sugars from hemicelluloses were active under anaerobic conditions.

Many *Bacillus* sp. are associated with insects either as commensals or as pathogens (Priest, 1993a). Some are facultative anaerobes, which can be isolated from the gut or feaces of insects such as honeybees and generally, are not regarded as pathogens (Bucher, 1981, cited by Priest, 1993b). Several species such as *B. thuringiensis, B. sphaericus* and *B. larvae* are obligate pathogens of insects and produce toxins which affect various insect larvae and are important in the biological control of insects (Claus and Berkeley, 1986).

A number of *Bacillus* spp. are closely associated with plants, particularly facultatively anaerobic strains which have complex growth requirements including various amino acids and vitamins (Priest, 1993b). Large populations are typically found in the root region, namely the rhizosphere and rhizoplane, and grow at the expense of root exudates (Priest, 1989a). Some members such as *B. azotofixans* and *B. polymyxa* are capable of fixing nitrogen which is made available to plants in return for nutrients from roots exudates (Priest, 1989a; Priest, 1993b).

1. 2 COMMERCIAL IMPORTANCE OF *BACILLUS*

Members of the *Bacillus* genus have a long association with the field of biotechnology. The Japanese are thought to be the first to use *Bacillus* spp. in the fermentation of traditional food such as natto, which is derived form fermented rice straw and soybeans (Harwood and Archibald, 1990; Priest, 1993a). Since most bacilli are non-pathogenic and good producers of proteins and extracellular metabolites, they are considered to be well suited to large-scale commercial bioprocesses (Dahl, 1999). For example Kim *et al.* (1997) reported that out of eight commercially registered biological control microorganisms in the United States, two are *Bacillus* strains, namely *Bacillus sp.* Gb03 and *Bacillus subtilis* MBI600.

The genus contains a large number of commercially important species, which are responsible for the production of a range of products including enzymes, fine biochemicals, antibiotics and insecticides (Tables 1.2 and 1.3) (Harwood and Archibald, 1990; Bron *et al.*, 1999). The primary industrial use of selected *Bacillus* strains is the production of enzymes. The varieties of extracellular proteins produced have a range of hydrolytic activities and are used extensively in detergent industries. Large amounts of amylases are also produced for converting starch to sugar in food and brewing industries (Priest and Sharp, 1989; Lancini and Lerenzetti, 1993). Many *Bacillus* spp. are capable of producing antibiotics of which bacitracin, polymyxin, tyrocidin, gramicidine are some examples (Madigan *et al.,* 1997; Garveba *et al.*, 2003). Most of the antibiotics produced are classified as peptide antibiotics and exhibit a range of spectra. Most are active against Gram-positive bacteria whereas some inhibit Gram-negative bacteria and others exhibit antifungal properties (Lancini and Lerenzetti, 1993). The potential application of bacilli, capable of producing antifungal compounds, for the biological control of fungal pathogens, has been researched extensively (Fiddaman and Rossall, 1993).

Obligate insect pathogens, such as *B. thuringiensis,* sporulate efficiently in larval hosts. Their growth and sporulation causes larval death, releasing spores into the environment. Such insect pathogens have found application as biological control agents. *Bacillus thuringiensis*, the most important and successful of these, produces a crystalline parasporal body, or delta-endotoxin, during sporulation which is toxic to various insect larvae (Claus and Berkeley, 1986; Priest, 1993a). The application of insect pathogens as biological control agents is considered feasible alternative to chemical insecticides, which have significant shortcomings (Bron *et al.*, 1999).

Various *Bacillus* strains have also found applications in the feed industry. In the 1950s, antibiotics were added sub-therapeutically to feed as a way of controlling disease and improving feed efficiency. This practice was subsequently banned due to the risks of pathogens developing resistance to antibiotics that they are continuously exposed to. More recently, feed industries have introduced beneficial bacteria, or probiotics, as feed additives to control or positively influence the microbial environment in the gastrointestinal tract. *Bacillus* strains which produce compounds that are antagonistic to disease-causing bacteria have been used for this purpose (Vi-Cor, 2001).

Table 1.2 Example of important extracellular enzymes produced by *Bacillus* (Priest, 1989b)

Enzymes	Species	Comments
Starch hydrolyzing enzymes		
□-Amylase	*B. amyloliquefaciens*	The tradition "industrial " amylase
	B. licheniformis	Thermostable industrial amylase
	B. acidocaldarius	pH optima of 2-4.5, dependent on strain
	B. alcalophilllus sub-species haloduans	pH optimum about 9.0
□-Amlyase	*B. cereus*	Optimal temperature about 50^{0}C
	B. megaterium	Optimal temperature about 50^{0}C
	B. polymyxa	Optimal temperature about 37^{0}C
□-Amlyase	*B. stearothermophilus*	Exo-attacking, producing □-maltose from starch
Isoamylase	*B. amyloliquefaciens*	Hydrolyzes amylopectin and glycogen but not pullulan
Pollulanase	*B. acdiopullulyticus*	Hydrolyzes the 1,6-□branches in amylopectin
	B. cereus	
	B. polymyxa	
Proteases		
Metalloprotease	*B. amyloliquefaciens*	The traditional industrial enzyme
	B. thermoproteolyticus	Thermostable industrial enzyme
Serine protease	*B. licheniformis*	Temperature-stable, high pH optimum; used in detergents
	Alkaliphilic bacilli	Improved alklalline stabilitlly for inclusion in detergents
Penicilinases		
□-Lactamase	*B. cereus*	Inactivates penicillins
	B. lichenifomis	
Penicillin amidase (acylase)	*B. megaterium*	Used for synthesis of semisynthetic penicillins
□-glucanase	*B. amyloliquefaciens* *B. subtilis*	Commercial preparations used in brewing and related industries
Glucose isomerase	*B. coagulans*	Intracellular enzymes used in immobilized form for fructose manufacture from glucose

Many *Bacillus* spp. are beneficial to plants. In nitrogen deficient soils some are able to fix nitrogen and provide a source of nitrogen to plants (Priest, 1993b). Some bacilli, particularly strains of *B. subtilis*, produce antifungal compounds which have been shown to promote plant growth by suppressing diseases (Podile and Dube, 1988, cited by Podile and Prakash, 1996). Several types of fungal diseases associated with field crops and vegetables, such as *Rhizoctonia* sp. and P*ythium* sp. have been controlled to varying degrees (Podile and Prakash, 1996; Priest, 1993b; Leifert *et al.*, 1995).

Representative *Bacillus* sp. strains have been described as plant growth promoting rhizobacteria (PGPR), which are able to colonise roots and exert beneficial effects on plants. Increases in plant growth arise from the indirect effect of PGPRs, whereby they change the microbial balance by inhibiting plant pathogens in the rhizosphere, thereby improving plant growth (Sailaja *et al.*, 1997). Some of the modes of actions for disease-suppression include competition for iron, niche exclusion, antibiosis, induction of systematic resistance and hydrogen cyanide production (Sailaja *et al.*, 1997; Podile and Laxmi, 1998).

Microorganisms that can colonize the rhizosphere are ideal for use as biological agents, since the rhizosphere provides the first line defence for roots against attack by pathogens. Various *Bacillus* spp. have been reported to consistently colonize roots and survive for long periods in the rhizosphere /rhizoplane of a plant, making them interesting candidates for use as biological control agents. Tolerance to heat and desiccation are useful characteristics of endospores, because after inoculation, they remain viable under a wide range of conditions until suitable growth conditions arise (Podile and Prakash, 1996).

1.2.1 Secondary Metabolites of *Bacillus*

Bacillus spp. that inhabit complex ecological niches that are subjected to conditions of nutritional stress, typically produce a diverse array of secondary metabolites (Katz and Demain, 1977). Some of these products have been found to play crucial roles in the survival of their producers in complex ecological systems (Zuber *et al.*, 1993). Secondary metabolite production may be restricted to certain species or even strains within a particular genus and are usually produced as families of closely related components. Secondary metabolites are not essential for cell metabolism but in some instances appear to have a survival function in nature (Martin and Demain, 1980; Lancini and Lerenzetti, 1993). They are usually formed at the end of the logarithmic growth phase with the on-set of a stationary phase (Martin and Demain, 1980). Sporulating cells typically produce secondary metabolites, during sporulation stages 0-II (Nicholson and Setlow, 1990). For the purpose of this literature review only secondary metabolites exhibiting antimicrobial properties are discussed.

1.3 ANTIBIOTICS

The term antibiotic is generally defined as a low molecular weight compound that originates as a microbial metabolite and which inhibits the growth of other microorganisms, temporally or permanently, at low concentrations (Lancini and Parenti, 1982; Fravel, 1988). This definition is not strictly accurate because some antibiotics are produced synthetically (Quesnel and Russel, 1983).

Many bacteria including *Bacillus* spp. produce short polypeptides, which are categorized as peptide antibiotics (Katz and Demain, 1977; Zuber *et al.*, 1993). Antibiotics produced by *Bacillus* spp. include aminoglycosides, lipopeptides, and small polypeptides (Lancini and Parenti, 1982; Zuber *et al.*, 1993).

Although there are assumptions that antibiotics are not toxic to the producing organisms, most antibiotics are more toxic to these organisms than their precursors (Katz and Demain, 1977). Martin and Demain (1980) reported that during growth antibiotic producing species are sensitive to the antibiotic they produce. There are many mechanisms whereby organisms can protect themselves from the antibiotics they produce. The following are some of the mechanisms that prevent self- annihilation:

1. Producing an antibiotic only after the growth phase;
2. Alteration of the antibiotic target in the producing cell;
3. Permeability changes where cell permeability to the antibiotic decreases after it is excreted;
4. Modification (and thus detoxification) of the antibiotic by enzymes formed by the producing strains; and,
5. Maintaining an inactive form of the antibiotic intracellularly (Katz and Demain, 1977; Martin and Demain, 1980).

In the late 1970s, more than 3,000 antibiotics had been described with approximately 50–100 new antibiotics being discovered each year (Fravel, 1988). More recently, Borders (1999) stated that this number had grown to more than 17,000 antibiotics and other biologically active microbial metabolites. Most of these antibiotics are produced by soil inhabiting microorganisms.

1.3.1 Peptide Antibiotics

Peptide antibiotics are secondary metabolites, which are composed, in part, of short polypeptides. Amino acid sub-units can be linked to each other by peptide bonds or through lactones and esters (Zuber *et al.*, 1993). Peptide antibiotics are known to contain non-protein amino acids, D-amino acids, hydroxyl acids and other unusual constituents (Kleinkauf and Döhren, 1990). They are the predominant class of secondary metabolites produced by *Bacillus* (Katz and Demain, 1977). Though peptide antibiotics are composed of amino acids, they have few similarities to gene-encoded polypeptides in terms of structure and their biosynthesis (Zuber *et al.*, 1993). Many of these compounds are not required for growth. This is based on the observation that strains kept in laboratory cultures often lose their antibiotic synthesis ability (Kleinkauf and Döhren, 1990).

1.3.1.1 Properties of Peptide Antibiotics

General properties and/or chemical characteristics of peptide antibiotics are outlined as follows (Perlman and Bodanszky, 1971; Katz and Demain, 1977; Yao *et al.*, 2003):

1. Peptide antibiotics are generally much smaller in size than proteins. Their molecular weights range from 270da (bacilycin) to about 4500da (licheniformin);
2. Microorganisms generally produce a family of closely related peptides rather than a single substance;
3. Some peptide antibiotics produced by *Bacillus* spp. are composed solely of amino acids while others contain amino acids plus other constituents such as fatty acids, pyrimidines, and amino sugars;
4. Normally, peptide antibiotics contain both L- and D-amino acids, which are unique and are not found in proteins. Two isomers of the same amino acids may be present in a molecule;

5. Most of the peptides are cyclic structures with no α-amino or carboxyl terminal; few are linear and may have unusual linkage or arrangements of the amino acids in the antibiotics;
6. Peptide antibiotics are generally resistant to hydrolysis by peptidases and proteins of animal and plant sources; and
7. Two mechanisms of peptide antibiotic synthesis, either ribosomal or nonribsomal, have been distinguished.

1.3.1.2 Function of Peptide Antibiotics to the Producing Organisms

With the exception of siderophores, which have a specific function in the transport of Fe^{3+}, it is difficult to assign a single function to the variety of secondary metabolites produced by *Bacillus* (Kleinkauf and Döhren, 1990). A number of functions have been proposed.

Hodgson (1970) (cited by Betina, 1983) proposed that peptide antibiotics play important roles in modification of membrane permeability, accumulation of dipicolinic acid and Ca^{2+} ions in the spore cortex, removal of water and contraction of prespores, and, formation of the spore coat.

Katz and Demain (1977) hypothesised that the synthesis of peptide antibiotics is a means of avoiding cell death when there is unbalanced growth due to nutrient deficiencies. They suggested that it is a mechanism of detoxification where antibiotics play a key role in the termination of vegetative growth and/or inhibit vegetative cell macromolecular synthesis but allow sporulation.

Another function, which has been proposed, is that peptide antibiotics provide a competitive advantage to the antibiotic producing species (Zuber *et al.*, 1993). This competitive effect may be aimed at competing bacteria, fungi and even predatory protozoa. Competition has also been shown to exist between different antibiotic producing strains of the same species (Katz and Demain, 1977). Antibiotic production is not necessary for microorganisms present in nutritionally rich niches, such as the intestine, since enough food is available for all species present (Martin and Demain, 1980).

In some instances, spores of *Bacillus* spp. contain antibiotics as well as a novel spore associated protein, TasA, which are thought to serve a protective function during spore germination (Katz and Demain, 1977; Ristow and Paulus, 1982; Stöver and Driks, 1999a). Competitors in the immediate environment of the germinating spore are inhibited or effectively eliminated.

Peptide antibiotics are also thought to have a regulatory role and act as signal molecules in the sporulation process (Modest *et al.*, 1984). When environmental conditions are unfavorable for germination, they inhibit germination (Katz and Demain, 1977). They are also thought to play an important role in cellular differentiation during the transition from vegetative cell to spore under nutrient deficient conditions (Katz and Demain, 1977). Ristow and Paulus (1982) found that when actively growing *B. brevis* ATCC 8185 was transferred from a nutrient rich medium to one devoid of a nitrogen source, no further increase in cell number was observed and subsequently, only a very small number of cells developed into spores. However, when the medium was supplemented with a mixture of tyrocidine and/or gramicidin peptide antibiotics, efficient sporulation was observed. Kleinkauf and Döhren (1990) also showed that tyrocidine functions specifically in the early sporulation process.

Modest *et al.* (1984) discovered two mutants of *B. brevis* ATCC 8185, S18 and S19 were unable to synthesise tyrocidine and gramicidin and did not form spores. When these mutants transferred during exponential growth to a nitrogen free medium and supplemented with tyrocidine, they formed spores similar to that of the wild type. It was also observed that tyrocidine induced gramicidin production, which is accompanied by RNA synthesis and other sporulation specific events.

Similarly, Marahiel and Döhren (1982) found that *B. brevis* cultured in a medium lacking a nitrogen source and supplemented with tyrocidine, induced a series of biochemical events related to sporulation and the formation of a high percentage of heat stable spores. There was no sporulation in the absence of biologically active antibiotics. They also found that in *B. brevis* ATCC 9999, gramicidin S-negative mutants produced abnormal spores, which were heat sensitive and had lower dipicolinic acid levels than the parental strains. The addition of

gramicidin to the medium of the growing mutant partly restored the dipicolinic acid levels, heat resistance and the out growth properties of spores.

Observations of Katz and Demain (1977) also support the hypothesis that antibiotic production plays an important role in sporulation. These are summarised as follows:

1. All sporulating bacteria produce antibiotics;
2. Antibiotics at low concentrations inhibit vegetative cells;
3. Peptide antibiotics produced by *Bacillus* spp. inhibit important cellular processes such as DNA and cell wall synthesis, membrane function and structure;
4. Peptide antibiotic production starts towards the end of the logarithmic growth phase and continues up to the sporulation stage in the *Bacillus* life cycle;
5. Both antibiotic production and sporulation are induced by depletion of some essential nutrients; and
6. There are genetic links between the synthesis of antibiotics and sporulation.

1.3.2 Peptide Antibiotics Produced by *Bacillus*

Many of the peptide antibiotics produced by *Bacillus* spp. are antibacterial, but because of their limited antibacterial activity and toxicity to the recipient, their clinical application is very limited (Katz and Demain, 1977; Lancini and Lorenzetti, 1993). Nevertheless, some, e.g. as polymyxin, tyrocidine and bacitracin are produced on a commercial scale. Certain strains of *Bacillus* also produce antifungal compounds that are important in controlling plant pathogens (Priest and Sharp, 1989). Lebaddi *et al. (*1994) found that *B. licheniformis* M4 produces fungicin M4 (3.4 kDa hydrophilic peptide) that has antifungal activity. Silo-Suh *et al.* (1994) purified two antifungal antibiotics (zwittermicin A and antibiotic B) from a culture filtrate of *B. cereus* UW85. Zwittermicin A was found to be an aminopolyol (cationic at pH 7.0) whereas antibiotic B was an aminoglycoside containing a disaccharide. Examples of peptide antibiotics produced by *Bacillus* spp. are presented in Table 1.3.

In some instances more than one type of antibiotic is produced. Leifert *et al.* (1995) detected three antifungal antibiotics from the fermentation broth of *B. subtilis* CL27. Two of the three antibiotics produced by CL27 were found to be peptide antibiotics. Antibiotic production was

found to be dependent on the composition of growth substrate. The activity of the antibiotics was also dependent on the pH and nutrient concentration of the medium.

Table 1.3 Peptide antibiotics produced by *Bacillus* species (Adapted from Zuber *et al.*, 1993)

Antibiotic	**Producing organism(s)**	**Structure**	**Properties**
Alboleutin	*Bacillus subtilis*		Antifungal, used in agriculture
Bacillomycin	*Bacillus subtilis*	Cyclic (8n)	Antifungal
Bacilycin	*Bacillus subtilis*	Dipeptide	Antibacterial, antifungal
Bacitracin	*Bacillus licheniformis*	Branched cyclic (12n)	Antibacterial, topical antibiotic, metal ion binding, membrane acting, cell wall synthesis inhibitor
Botrycidin	*Bacillus subtilis*	Polypeptide (62n)	Gene incoded, antifungal
Brevistin	*Bacillus brevis*	Acylcyclic peptidolactone (11n)	Antibacterial
Cerexins	*Bacillus cereus*	Acylpeptide (10n)	Antibacterial
Chlorotetain	*Bacillus subtilis*	Dipeptide	Antifungal
Edeines	*Bacillus brevis Vm4*	Modified (5n)	Antibacterial, antitumor, translation inhibitor, nucleic acid binding
EM 49	*Bacillus circulans*	Cyclic acylated (8n)	Antibacterial
Esperin	*Bacillus mesentericus*	Lacton (8n) antimycobacterial	Antimycobacterial
Fengycin	*Bacillus subtilis F-29-3*	Modified acylpeptide (10n)	Antifungal
Gramicidin S	*Bacillus brevis*	Cyclic (10n)	Antibacterial, surfactant, nucleotide binding
Iturin	*Bacillus subtilis*	Cyclic lipopeptide (7n)	Antifungal, clinically used
Mycosubtilin	*Bacillus subtilis, Bacillus subtilis* subsp.*niger*	Cyclic (9n)	Antifungal
Octapeptins	*Bacillus circulin ATCC 31805*	Branched cyclic acylpeptide (8n)	Antibacterial, antimycobacterial, antifungal, antiprotozoal
Polymycins	*Bacillus polymyxa*	Branched cyclic acylpeptide (10n)	Antibacterial, membrane acting
Polypeptins	*Bacillus circulans*	Peptidolactone (10n)	Antibacterial, broad spectrum proteinase inhibitor
Rhizoctincins	*Bacillus subtilis*	Dipeptide and tripeptide	Antifungal
Subtilin	*Bacillus subtilis*	Lantibiotic (32n)	Gene encoded, antibacterial, antitumor, used in agriculture
Surfactin	*Bacillus subtilis*	Acylated cyclic (7n)	Antimycobacterial, membrane acting
Tridecaptins	*Bacillus polymyxa*	Acylated (13n)	Antibacterial
Tyrocidine	*Bacillus brevis ATCC 8185*	Cyclic (10n)	Antibacterial, topical antibiotic, hemolytic

* n-indicates the number of amino acids in peptide molecule.

1.3.2.1 Classification and Nomenclature of Antibiotics

Based on their structure and activities antibiotics produced by *Bacillus* are grouped into four, classes (Shoji, 1978; Priest and Sharp, 1989). These are:

1. Cyclic oligopeptides, comprising antibiotics that inhibit cell wall synthesis, e.g., bacitracin produced by *B. licheniformis*;
2. Linear or cyclic oligopeptides, that interfere with membrane function, e.g., tyrocidines and linear gramicidins;
3. Basic peptides (e.g., edeine) which inhibit the formation of the initiation complex on small ribosomal subunits; and
4. Aminoglycoside antibiotics comprising antibiotics that affect ribosome function.

The best-characterized *Bacillus* antibiotics are bacitracin, gramicidin S and tryocidine which all show antibacterial activity (Lebbadi *et al.*, 1994). Antibiotics showing antifungal activity comprise lipopeptides such as iturin, fengycin (Phae *et al.*, 1990; Yao *et al.*, 2003) surfactins (Kluge *et al.*, 1988, cited by Lebbadi *et al.*, 1994), bacillomycins (Eshita and Roberto, 1995), and mycosubtilins (Zuber *et al.*, 1993). Most of these antibiotics are cyclic peptides containing D- and L-amino acids closed by a beta-amino acid carrying a long aliphatic chain. They all appear to be active against selected fungi and yeasts. The mode of action of these antibiotics is penetration into the cytoplasmic membrane by the hydrophobic tail followed by auto-aggregation to form a pore which causes cellular leakage (Maget-Dana *et al.*, 1985, cited by Volpon *et al.*, 1999). A second family comprises hydrophilic antifungal antibiotics, such as the dipepetide bacilysin which is an inhibitor of glucosamine synthetase (Kenig *et al.*, 1976) and the hydrophilic phosphono-oligopeptide rhizoctin (Lebbadi *et al.*, 1994).

1.3.2.2 Synthesis of Peptide Antibiotics

Based on their synthesis mechanisms, peptide antibiotics can be grouped into two classes namely; non-ribosomally synthesized peptide antibiotics (e.g., gramicidin, polymyxin, bacitracin) (Hancock and Chapple, 1999) and ribosomally synthesized antibiotics (e.g., nicin, subtilin,

subtilosin) (Fig1.1) (Zuber *et al.*, 1993). In general, peptide antibiotic synthesis requires the following amino acids of the peptide antibiotic in question, adenosine 5'-triphosphate, Mg^{+2} ions and a reducing agent (Katz and Demain, 1977). Ribosomally synthesized peptide antibiotics undergo post-translational processing and modifications before being exported out of the cell (Zuber *et al.*, 1993).

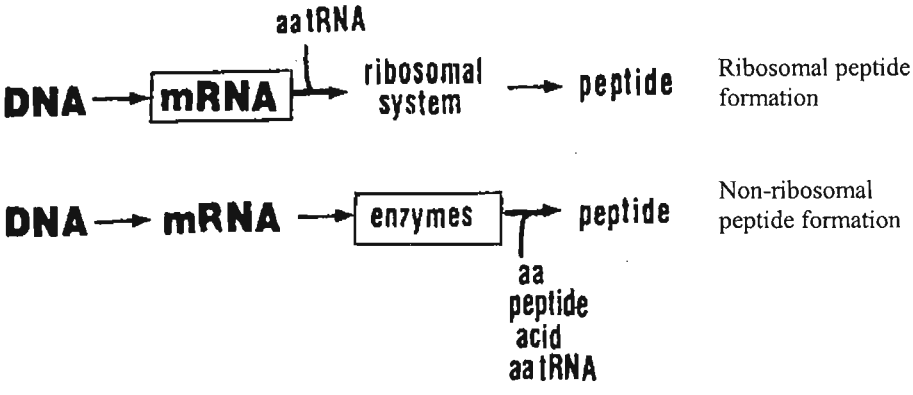

Figure 1.1 Flow of information and origin of templates in ribosomal and nonribosomal peptide formation (adapted from Kleinkauf and Döhren, 1982).

1.3.2.2.1 Non-ribosomal Synthesis

Non-ribosomal peptide synthesis is achieved by the thiotemplate function of large, modular enzyme complexes known collectively as peptide synthetases (Zuber *et al.*, 1993; Hancock and Chapple, 1999; Neilan *et al.*, 1999). tRNA, mRNA and ribosomes are not directly involved (Katz and Demain, 1977). Peptide synthetase complexes are composed of some of the largest polypeptides produced in all living cells ranging from 100,000 to >600,000da (Zuber *et al.*, 1993). The mechanism involved has more in common with fatty acid synthesis than with the normal ribosomal mechanism of peptide synthesis (Kleinkauf and Döhren, 1990; Zuber *et al.*, 1993). Non-ribosomal mechanisms give rise to peptide antibiotics which exhibit a broader specificity than that shown for ribosomally synthesized peptides (Katz and Demain, 1977).

Non-ribosomally synthesized peptide antibiotics are composed of between 2 and 20 amino acids and are organized in linear, cyclic and/or branched cyclic structures. The constituent amino acids often undergo extensive modifications including methylation, acetylation, glycocylation,

racemization L to D forms, and, covalent linkage to a variety of functional groups including nucleotides (Zuber *et al.*, 1993; Hancock and Chapple, 1999). Stein *et al.* (1996) divided the multi-enzyme thiotemplate model process into two steps and explained it as follows: The first step involves an aminoacyl adenylation similar to the amino acid activation process catalysed by tRNA ligases and the second an aminoacyl thioesterification at specific reactive thiol groups of the multienzyme.

The modular structures of peptide synthetases are responsible for the sequential, and amino acid-specific, elongation of peptide chains. The specific combination of modules and various functional domains within the peptide synthetase determines the structure and hence the activity of the peptide product (Neilan *et al.*, 1999).

1.3.3.2.2 Ribosomal Synthesis

Ribosomally synthesized peptide antibiotics are typically gene encoded and are produced by a number of prokaryotic and eukaryotic organisms as major components of defense system molecules (Hancock and Chapple, 1999).

For example, lantibiotics are ribosomally synthesized, antimicrobial amphiphilic peptides that are almost exclusively produced by Gram-positive bacteria. Lantibiotics are often active against closely related Gram-positive organisms (Stein *et al.,* 2002). In general, lantibiotics are characterized by the presence of unusual amino acids 2,3-dehydroalanine and/or 2,3-didehydrobutrine, which are formed by dehydration of serine and theonine residues, respectively (Guder *et al.*, 2000). Genes on plasmids and chromosomes encoded for precursors and these undergo extensive post-translational modifications including amino acid dehydration and thioether bridge formation (Stein *et al.,* 2002; Upton *et al.*, 2001). Thioether linkages provide a heterocyclic structure and thermal stability. Lantibiotics are so called because of the presence of uncommon amino acids, i.e. lanthionine and methyl lanthionine (Zuber *et al.*, 1993).

1.3.3 Mode of Action of Antibiotics

Though peptide antibiotics have a uniform biosynthetic origin, they present a variety of antimicrobial activities and mechanisms of action (Lancini and Lorenzetti, 1993). These include

inhibition of DNA synthesis and functioning, impairment of murein (peptidoglycan) biosynthesis and of cell wall formation, damaging and interfering with cytoplasmic membrane function and structure (Katz and Demain, 1977; Lancini and Lorenzetti, 1993).

1.3.4 Spectra of Antifungal Peptide Antibiotics

Most of the antibiotics of *Bacillus* are inhibitors of Gram-positive bacteria; some inhibit Gram-negative bacteria while others are antifungal agents (Lancini and Lorenzetti, 1993). *Bacillus* species produce antibiotics and other metabolites, which are responsible for biological control *in vitro* as well as *in vivo* (Leifert *et al.*, 1995). Pusey and Wilson (1984) and Gueldner *et al.* (1988) stated that *B. subtilis* stain B-3 was antagonistic against *Monilinia fructicola* on wounded stone fruit. Pusey and Wilson (1984) also reported that it was able to control brown rot of a number of fruit types. Similarly, Kim *et al.* (1997) reported that *Bacillus* sp. strain L324-92 exhibits broad-spectrum inhibitory biological activity against fungi that cause Rhizoctonia root rot; Pythium root rot and take all of wheat caused by *Rhizoctonia solani* AG8, *Pythium iiregulare* and *P. ultimum,* and *Gaeumannomyces graminis* var. *tritici* respectively.

Bacillus pumilus (MSH) produces a compound inhibitory to *Mucor* and *Aspergillus (*Bottone and Peluso, 2002). Baker and Stavely (1984) found that *B. subtilis*, Isolate PPl-3 showed an inhibitory effect on *Uromyces appendiculatus* that causes bean rust. A strain of *B. subtilis* was found to produce volatile antifungal compound(s) which inhibit *R. solani* and *P. ultimum* (Fiddaman and Rossall, 1993). A disease of alfalfa caused by *Phytophythora medicaginis* was also controlled by zwittermicin A and antibiotic B the products of *B. cereus* UW85 (Silo-Suh *et al.*, 1994).

1.3.5 Other *Bacillus* Compounds and Their Activities

The antagonistic nature of certain *Bacillus* spp. toward fungal pathogens is not restricted to antibiosis. Other mechanisms of antagonism include competition for nutrients or space, induced host resistance and the production of hydrolytic enzymes (Priest, 1993a; Maurhofer *et al.*, 1994; Silo-Suh *et al.*, 1994; Glick, 1995; Podile and Prakash, 1996). For example, hydrolytic enzymes such as chitinase degrade the cell wall of fungal pathogens causing cell lysis and death. Such microorganisms have been investigated for biocontrol purposes (Mitchell and Alexander, 1962).

Podile and Prakash (1996) reported that *B. subtilis* AF 1 induces host plant resistance and produces diffusible antibiotic-like substances that inhibit the growth of plant pathogenic fungi. Podile and Prakash (1996) reported that *B. subtilis* AF 1 adheres to the fungus *Aspergillus niger* and multiplies rapidly on the surface of the fungus. Extensive colonization and direct lytic activity of *Bacillus* resulted in the dissolution of the cell wall and disintegration of the mycelium. Continuous degradation of the mycelium and protoplasts led to the lysis of the fungus.

1.4 ENDOSPORE, SPORULATION AND GERMINATION

The ability to respond flexibly to a varying environment is essential for bacterial survival (Stöver and Driks, 1999). The bacterial endospore is a highly specialized structure capable of maintaining the bacterial genome in a protected, viable state for extended periods (Nicholson *et al.*, 2000). With reference to *B. subtilis* as a model, sporulation results from the integration of a wide range of environmental and physiological signals, culminating in the activation of a key transcriptional regulatory protein, Spo0A. These signals are triggered by nutrient depletion, cell density, the Krebs cycle, DNA synthesis, and DNA damage (Stragier and Losick, 1996). *Bacillus subtilis* responds to these signals by spore formation, the production of degradative enzymes and antibiotics (Stöver and Driks, 1999b).

1.4.1 Endospores

Bacillus endospores are encased within a complex multilayered protein structure known as the spore coat (Fig. 1.2) (Priest, 1993a; Serrano *et al.*, 1999). The coat has two major layers; the inner and the outer coat. The inner coat, which is composed of several layers, has a fine lamellar appearance and stains lightly. The outer coat is often thicker and stains darker than the inner coat and has a coarse layered appearance. The coat is composed largely of proteins, with minor amounts of carbohydrates and lipids. The coat proteins are especially rich in the amino acids tyrosine and cysteine (Driks, 1999).

The major function of the coat is to protect the spore. The outer coat protects the spore from bactericidal enzymes and chemicals, such as lysozyme and chloroform. The ability of the outer coat to withstand lysozyme is due to a sieving (filtering) function (Driks, 1999; Serrano *et al.*, 1999). The coat also influences the spore's ability to monitor its environment and to germinate

within min of exposure to the appropriate germinants (Serrano *et al.*, 1999). Driks (1999) stated that the coat is unlikely to play an active role in germination, since none of the germination machinery is coat associated. Its role in germination is regarded as being passive in that it merely permits or inhibits the passage of germinants to the interior of the spore, where the germinant receptors are likely to be found.

Endospores of *Bacillus* spp. especially *B. subtilis*, have served as experimental models for exploring the molecular mechanisms underlying spore longevity and resistance to environmental stresses (Nicholson *et al.*, 2000). In general terms they exhibit resistance to heat, irradiation and harsh chemicals (Priest, 1993a). Driks (1999) and Nicholson *et al.* (2000) stated that spore resistance mechanisms during dormancy depend on different physiological events that occur throughout the life cycle of the bacteria (i.e. growth, sporulation and germination). Spore resistance also depends on a number of other factors, such as the genetics of the sporulating species, the precise sporulating conditions specifically temperature, the relative impermeability of the spore core, the mineral content of the core, the concentration of acid-soluble spore proteins and the repair mechanisms that operate during germination and outgrowth (Nicholson *et al.*, 2000).

The resistance properties of endospores do not depend only on the spore coat. Heat resistance depends on the dehydration state of the spore core, which in turn, depends on the cortex for its maintenance. The cortex also provides resistance against organic solvents (Driks, 1999). Small acid soluble proteins, which are found in the core and bind to DNA, are responsible for UV radiation protection (Setlow, 1995; Slieman and Nicholson, 2001). Nicholson *et al.*, (2000) also stated that the spore coat layers, particularly the inner coat, play a protective role in the solar UV resistance of spores.

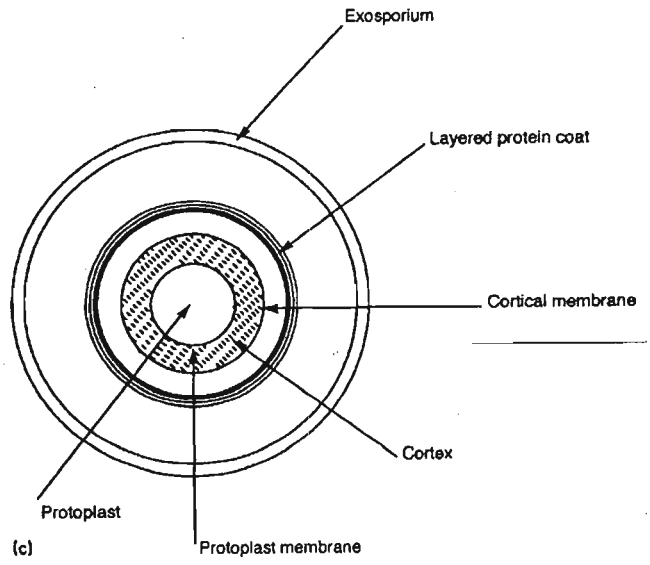

Figure 1.2 Structure of a typical *Bacillus* endospore (Adapted from Priest 1993a).

1.4.2 Sporulation

Bacillus subtilis is one the most extensively studied Gram-positive bacteria in terms of microbiological, biochemical and genetic studies (Dahl, 1999). The sporulation processes and role of the endospore coat described in this review refers primarily to findings derived from *B. subtilis*.

Sporulation is the ultimate response to complete deprivation of nutrients (Doi, 1989). The morphological process of sporulation is driven by a temporary and spatially controlled programme of gene expression, which is initiated at a certain stage of the cell cycle, and is activated by environmental stimulants. At this moment sporulation is triggered and the vegetative gene expression is largely replaced with a specialized programme of sporulation gene expression (Driks, 1999).

Sporulation is initiated by a number of environmental conditions, which include limitation or exhaustion of essential nutrients such as carbon, nitrogen and phosphorus (Nicholson and Setlow, 1990) and partial or transient deprivation of one or more amino acids (Ochi *et al.*, 1982).

When *Bacillus* lacks nutrients, several cellular responses are triggered to prevent starvation or prepare the cell for a long dormant stage. The responses can be categorized into three main parts. The first response involves the synthesis of flagella to increase motility of the cell. The second involves repression of a number of genes that code for intra-and extra-cellular enzymes, and synthesis of a new array of enzymes that can utilize secondary carbon sources. These extracellular enzymes hydrolyze biopolymers and substrates in their environment providing the cell with monomeric nutrients. If these responses fail to provide nutrients, the cell resorts to the third and last option, which is sporulation (Doi, 1989).

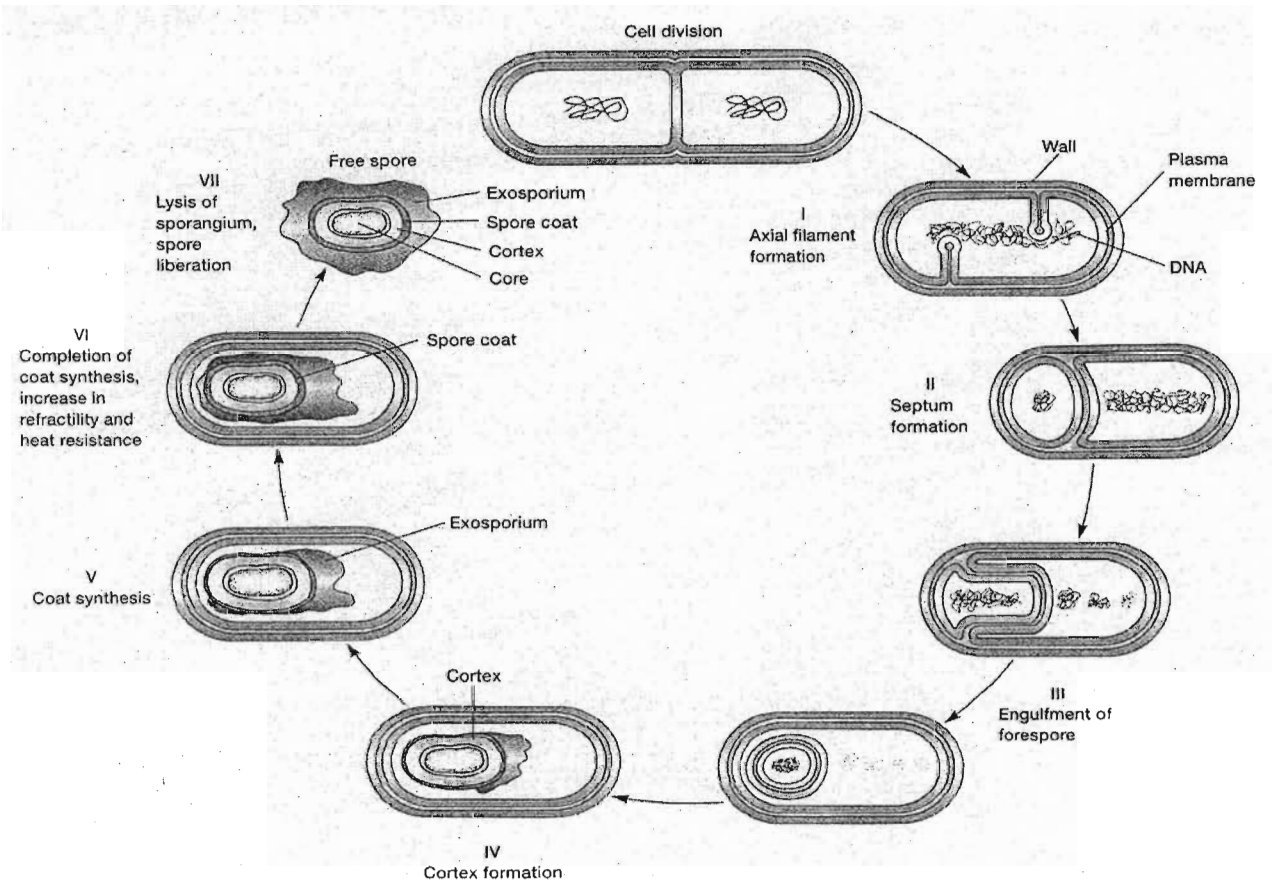

Figure 1.3 The seven stages of sporulation. (Adapted from Prescott *et al.*, 1999).

Based on the cytological changes that occur, sporulation has seven stages (Fig. 1.3) (Doi, 1989; Nicholson and Setlow, 1990; Priest, 1993a). An early morphological event in sporulation is the formation of an asymmetrically positioned septum that divides the sporulating cell into two cells of unequal size. The smaller chamber, the forespore, ultimately becomes the spore. The larger compartment, the mother cell, nurtures the developing spore until its development is completed (Driks, 1999; Stöver and Driks, 1999a).

A series of sequentially activated transcription factors ensures that sporulation genes are activated at the proper times and in the correct compartments with over 125 genes involved in this process (Stragier and Losick, 1996). Prior to the appearance of the sporulation septum, the transcription factors Spo0A and σ^H direct the expression of a large group of genes. Once the septum is formed, sigma factors σ^F and σ^E become active in the forespore and mother cell, respectively. After engulfment, the activity of σ^F in the forespore, is replaced by that of σ^G. Similarly, the activity of σ^E in the mother cell is replaced by that of σ^K (Stöver and Driks, 1999a).

The spore is built from the outside as well as from within (Driks, 1999). The edge of the septum migrates toward the forespore pole of the cell and engulfs the smaller forespore compartment, resulting in a protoplast with two membrane layers that is entirely surrounded by the mother cell. The space between the double layers of membranes becomes the site of assembly of two layers of specialized peptidoglycan, called the germ cell wall and the cortex. The last structure to be formed is a proteinacious shell, the coat, that encircles and protects the spore. The final step in sporulation involves the lysis of the mother cell, and the release of a mature spore into the environment (Nicholson and Setlow, 1990; Stöver and Driks, 1999a).

Endospores are the hardiest known form of life on earth and can remain dormant for long periods (Nicholson *et al.*, 2000). They are resistant to extremes of temperature and pH, to desiccation, UV irradiation, enzyme action, and organic chemicals (Gould, 1969, cited by Moir and Smith, 1990). Pyridine2-, 6-dicarboxylic acid plays a significant role in the resistance of *B. subtilis* spores exposed to wet heat and UV radiation (Slieman and Nicholson, 2001). Spores contain high levels of divalent ions, in particular Ca^{2+}, and are more resistant to wet heat than those spores which have monovalent ions (Nicholson *et al.*, 2000).

1.4.3 Spore Germination

Germination is generally triggered by the interaction of germinant molecules such as amino acids, sugars, and nucleotides with receptors on the spore. The triggered reaction commits the spores to a series of successive events, which leads to the loss of spore dormancy and resistance properties resulting in the resumption of vegetative growth (Moir and Smith, 1990; Gould, 1996; Paidhungat and Setlow, 1999; Paidhungat *et al.*, 2001).

The transformation from a dormant spore into an active vegetative cell has three stages (Prescott, 1999). The first is loss of the spore's dormancy and resistance; this is followed by reactivation of metabolism to form a vegetative cell, and finally the subsequent stage is the synthesis of RNA, proteins and DNA that leads to cell growth and division (Moir and Smith, 1990; Prescott, 1999).

Genetic analysis of spore germination of *B. subtilis* has shown that, *ger*A, *gerB*, *ger*D and *gerK*, are the principle spore germination genes (Moir and Smith, 1990). The products of genes required for the germination response to multiple types of germinants could represent proteins activated by the initial signal transduction mechanism.

The first measurable event in spore germination is the loss of heat resistance. Later events include the activation of spore lytic enzymes, selective cortex hydrolysis, and rehydration of the spore core followed by a rise in spore internal pH, a release of monovalent ions (H^+) (Moriyama *et al.*, 1996), and excretion of dipicolinic acid (DPA) and divalent cations (predominantly Ca^{2+}) (Paidhungat *et al.*, 2001). Spore germination proteins, which exhibit homology to a widely distributed group of putative cation transporters, are involved in the outward fluxes of Na^+, K^+, and H^+, as well as the subsequent uptake of K^+ (Southworth *et al.*, 2001).

1.5 SCREENING METHODS FOR SECONDARY METABOLITES

Various methods have been developed to isolate, screen, identify and analyse biologically active secondary metabolites, such as antibiotics (Cannell, 1998). Preparation of biological samples requires optimisation of the growth conditions for the synthesis of the secondary metabolites. Extraction procedures and related protocols for purification must also be optimised (Higgs *et al.*, 2001).

The quantitative expression of antimicrobial activity of many antibiotics may be markedly influenced by the composition of the culture medium used. In addition, general factors such as pH, incubation atmosphere, and protein concentration may also affect the susceptibility of a given test microorganism (Prescott *et al.*, 1999).

1.5.1 Isolation of Endospore Formers from Natural Sources

Bacillus spp. commonly found in soil, are isolated by incubating environmental samples at 80^0C for 10 min to destroy vegetative cells but not endospores. Successive aerobic incubation on a suitable medium at 30^oC eliminates anaerobic clostridia (Alexander, 1977; Priest, 1993a). Even though this procedure is valuable, it has several shortcomings. Firstly, it isolates only spores and not cells that are at their vegetative phase of growth. Secondly, heat sensitive spores of psychrophiles can be destroyed and, thirdly, the germination conditions may not be suitable for a spore to germinate. Due to these shortcomings, some other spore selection agents such as ethanol or other solvents, and UV light irradiation have been adopted as alternative selection procedures (Priest, 1989a).

1.5.2 Primary Test of Antibiotic Activity and Production

Strains, which may be isolated from the environment or obtained from culture collections, are tested for antibiotic activity in primary screens. The methods used usually rely on the observation of inhibition of microbial growth on an agar plate bioassay (Betina, 1983). Ideally these primary screens should be rapid, sensitive and inexpensive (Nakayama, 1981). Various approaches have been developed. For example when an appropriately diluted sample is made, separated colonies will grow on the surface of the medium. After a few days of incubation and growth, a layer of agar, containing an indicator organism, is poured on the growing colonies and the inhibition zones are observed after an additional incubation period.

A second approach involves agar plugs cut out from fungal cultures and placed on agar media seeded with an indicator organism. The test substance diffuses into the agar and inhibition zones are observed indicating a positive reaction (Nakayama, 1981; Betina, 1983). Soaking filter paper discs in filtrate containing an antibiotic, and placing them on agar that is seeded with the test

organism can also assay the activity of an antibiotic. The appearance and size of the inhibition zone indicates the activity of the antibiotic(s) (Lancini and Parenti, 1982).

Secondary screening allows for further evaluation of those microorganisms that exhibit potential as biological control agents or use in industrial processes. Identification and classification of newly isolated microorganisms allows for comparison with known organisms which, in turn, can provide information with regard to pathogenicity, growth characteristics and types of antibiotics that can be produced (Nakayama, 1981). The active organisms grown on several media and aqueous concentrate cultures are tested for activity. The aqueous concentrate cultures are also used to prepared pure samples for testing such as biochemical, biological, chemical, and pharmacological tests. The purity of the extract is one of the most important factors in these methods (Nakayama, 1981; Betina, 1983).

1.5.3 Extraction and Purification Methods

To characterize and define a biomolecule it has to be isolated and purified. Peptide antibiotics can be extracted, purified and characterized using a range of different techniques which include centrifugation, filtration and extraction, solvent extraction, thin layer chromatography (TLC), electrophoresis and high performance liquid chromatography (HPLC) (Boyer, 1993; Gibbons and Gray, 1998). Bioautography, a version of paper chromatography, is used to track activity through the isolation/purification process and is sufficiently sensitive to locate the appropriate fraction containing the active compound (Gibbons and Gray, 1998). This method can be applied by either direct bioautography or by a bioautography overlay assay (Krieg and Gerhardt, 1981; Gibbons and Gray, 1998). Determining the physical properties and overall concentrations of the antimicrobial compound in a fermentation broth are important factors for the characterisation of antimicrobial molecules (Borders, 1999).

Centrifuging liquid cultures at high speed can remove bacterial cells and particulate matter, resulting in a cell free supernatant (Boyer, 1993). Peptide antibiotics have been precipitated from the supernatant by adjusting the pH of the medium to pH 2.5 with acid. The precipitate, which contains the active fraction, can then be extracted with suitable solvents (McKeen *et al.*, 1986; Gueldner *et al.*, 1988; Lebbadi, 1994; Munimbazi and Bullerman, 1998). Alternatively,

antimicrobial substances in cell free filtrates can be adsorbed on cation exchange resin; washed and eluted with suitable solvents, before the active compounds are purified and isolated (Pinchuk *et al.*, 2001). The separation process technique depends on the shape, size and density of the molecules being purified. Biomolecules can also be separated and purified by chromatography. TLC, due to its simplicity and rapidity, is widely used to purify and check the purity of products (Boyer, 1993; Gibbons and Gray, 1998).

Electrophoresis, the transport of charged particles through a gel solvent by an electric field, is also an important method used to purify biomolecules (Dunbar, 1987). The sieving effect of cross-linked gel influences the movement of biomolecules and leads to separation due to their size and shape (Wood, 1981). For successful separation of the molecules, a suitable buffer must be chosen to maintain a constant pH within the system. The buffer also acts as an acceptable electrolyte conducting current across the electric field (Dunbar, 1987).

1.6 INDUSTRIAL FERMENTATION

Information with regard to laboratory scale fermentations of *Bacillus* is readily available in the literature. Unfortunately as a process become more commercially significant, the availability of published information decreases. The information available on large-scale industrial processes is more generalized and the more significant details often remain patent protected (Priest and Sharp, 1989; Sharp *et al.*, 1989).

Peptide antibiotic fermentation biotechnology uses pure cultures. Manufacturing of most bioactive peptides requires incorporating self-protective systems to protect organisms from their own products. Fortunately, *Bacillus* spp. cells are inherently immune to their own antibiotics (Kleinkauf and Döhren, 1997). Antibiotic producing microbes protect themselves from the antibiotics they produce by different mechanisms as mentioned in earlier (1.3) (Katz and Demain, 1977; Martin and Demain, 1980).

There are two main strategies for improving productivity of a fermentation processes. The first involves mutation and selection for an enhanced mutant strain (Priest and Sharp, 1989; Sharp *et al.*, 1989). The second revolves around the development and optimisation of suitable production media (Arbige *et al.*, 1993). A suitable production medium includes the provision of a

nutritionally and physically balanced growth environment (Priest, 1989b; Arbige *et al.*, 1993). A suitable culture medium should meet the organism's basic needs such as source of carbon, nitrogen, phosphorus, sulphur, potassium and trace elements. The physiochemical conditions that need to be monitored and controlled during a fermentation process include temperature, aeration rate, pressure, feed rate, agitation, foaming, volume, pH, CO_2, dissolved oxygen, power input, and draw (Arbige *et al.*, 1993).

1.6.1 Formation of Antibiotics in Relation to Growth

The synthesis of antibiotics is typically initiated after a rapid growth phase with the onset of a stationary phase. But, this is not always true, as in some instances antibiotic synthesis is controlled by carbon and nitrogen repression as well as growth rate. Modifying these conditions can shift the time of antibiotic synthesis (Katz and Demain, 1977). *Bacillus subtilis* was found to produce considerable amounts of antibiotics in a synthetic medium during active cell growth and stopped at the end of the logarithmic growth. However, when small amounts of casamino acid was supplemented, antibiotics were produced at the end of growth and in smaller amounts (Ochi and Ohsawa, 1984).

AIMS AND OBJECTIVES

The main aims and objectives of this project are:

1. To investigate the influence of pH, temperature and medium composition on the antifungal activity of selected *Bacillus* isolates, *in vitro*;
2. To identify selected *Bacillus* isolates exhibiting broad spectrum antifungal activity using phenotypic and genotypic methods; and,
3. To extract, purify and partially characterize antifungal compound/s produced by a selected *Bacillus* isolate, R29.

CHAPTER 2

MATERIALS AND METHODS

2.1 DUAL CULTURE *IN VITRO* ANTAGONISM BIOASSAY

2.1.1 Isolates and Test Organisms

Selected antibiotic producing *Bacillus* spp. strains, designated R29, B81, B81c, B77, B69, B69c, and R16 were used in the study and were sourced from a culture collection housed within the Discipline of Microbiology, School of Applied and Environmental Sciences, University of KwaZulu-Natal, RSA.

Master cultures were established by resuspending a spore suspension of each isolate in sterile 25% (v/v) glycerol and storing at -82^0C (Bron, 1990). Isolates were sub-cultured from each master culture onto tryptone soy agar (TSA) and incubated at 30^0C for 48hr before testing.

Representative soil borne plant pathogenic fungi, *Rhizoctonia solani*, *Pythium* sp., *Phytophthora* sp. and *Fusarium sp.* were used as test organisms to screen for antifungal activity. The fungal isolates used were isolated from diseased plant material, cultured and identified by members of the Discipline of Plant Pathology, School of Applied and Environmental Sciences, University of KwaZulu-Natal, RSA. The fungi obtained were grown on either vegetable juice (V8) or potato dextrose agar (PDA) media and incubated at 25^0C.

2.1.2 Media

Various complex media namely TSA (Merck), PDA (Merck), malt extract agar (MEA) (Merck) and nutrient agar (NA) (Merck) were used in bioassay testing and were made up according to the manufacturers instructions. Tryptone soy broth (TSB) (Merck) was used to culture the *Bacillus* isolates before inoculating the different agar media. Half strength PDA (HPDA) was also evaluated and was prepared by weighing out half of the recommended media and the supplementing it with 7.5g bacteriological agar so as to allow for normal agar solidification.

2.1.3 Dual Culture *In Vitro* Bioassay

In the initial round of antagonism bioassays, the seven *Bacillus* isolates were tested individually for antagonism against four plant pathogens on four agar media, namely PDA, HPDA, MEA and NA using a modified dual culture technique described by various authors (Utkhede, 1983; Utkhede and Rahe, 1983; Sadfi *et al.*, 2002; Anith *et al.*, 2003).

Overnight cultures of each bacterial isolate were established in 50ml of tryptone soy broth (TSB), incubated at 30^0C and shaken at 170 rpm in a controlled environment shaker incubator (CESI)(New Brunswick Scientific co. Inc). Bacteria were incubated until spectrophotometric readings of approximately 1.040 (625nm) were achieved (~10^8 CFU.ml^{-1}) (Milton Roy Spectronic 302). Aliquots (10μl) of bacterial suspension were then spotted on the periphery of duplicate agar plates and incubated for 24h at 25^0C and 30^0C. Mycelial plugs (1.5mm X 1.5mm) taken from fungi grown on either V8 or PDA agar plates were transferred using a sterile scalpel to the center of each agar plate. Since *Phytophthora* sp. is regarded as slow growing, it was inoculated at the same time as the *Bacillus* isolates, whereas the other three pathogens were inoculated 24h after the *Bacillus* isolates. The radius of each inhibition zone was measured from the center of the bacterial colony to the edge of the fungal mycelium. *Rhizoctonia solani* and *Pythium* sp. were measured after two days incubation, *Fusarium* sp. after three to four days and *Phytophthora* sp. after four to five days. Each dual culture experiment was repeated three times.

Two isolates exhibiting the greatest degree of inhibition against the four fungal pathogens were selected for further bioassay testing. The pH of the agar media was adjusted to pH 5.5 and 7, and the bioassay repeated as previously described.

Inhibition zone data was analyzed using Genstat^R Statistical Analysis Software (Genstat, 2002). Analysis of Variance (ANOVA) was used to select isolates showing the greatest degree of antagonism. For the second data set the influence of pH, temperature and media on fungal inhibition was also assessed using Genstat^R Statistical Analysis Software (Genstat, 2002), Analysis of Variance (ANOVA).

2.2 BIOCHEMICAL AND NUTRITIONAL CHARACTERIZATION OF *BACILLUS* ISOLATES R29 AND B81.

2.2.1 Catalase Test

Catalase tests were performed using the methods described by Smibert and Krieg (1981) and Ferrar and Reboli (1992). Bacterial isolates were streaked onto NA and TSA Petri dishes and slants and incubated at 30^0C for 48hr prior to testing.

According to the Slepecky and Hemphill (1992) method, 0.5ml of 10%(v/v) H_2O_2 solution was flooded onto colonized agar. For the Smibert and Krieg (1981) method a single drop of 3% (v/v) H_2O_2 solution was placed on a bacterial colony. Alternatively, a single colony was transferred to a glass slide to which one drop of 3% H_2O_2 was added. The catalase test was considered to be positive if vigorous bubbling of the H_2O_2 solution occurred upon contact with bacterial colonies.

2.2.2 Growth in Sodium Chloride

The growth of *Bacillus* isolates R29 and B81 at different sodium chloride (NaCl) concentrations was assessed using methods described by Ferrar and Reboli (1992). A basal medium was prepared by dissolving the following in distilled water and making up to 1 litre: peptone, 5.0g; yeast extract, 2.0g; beef extract, 1.0g. Medium containing NaCl at concentrations of 0.0%, 4%, 7% and 10%(w/v) were then made up. The pH of each medium was adjusted to 7 with 5N NaOH. Each medium was then dispensed into test tubes (10ml) and 250ml Erlenmeyer flasks (75ml). In addition to this, nutrient broth (NB) (Merck) was made up according to the manufacturers instructions. All were autoclaved for 15 min at 121^0C.

Test tubes were inoculated with a loop-full of bacterial culture and then incubated at 30^0C and checked for turbidity every day. Flasks were inoculated with 0.1ml of an overnight broth culture and incubated in a CESI at 30^0C and 170 rpm for seven days. Spectrophotometer readings (625nm) were made daily to assess bacterial growth.

2.2.3 Nitrogen Fixation Assay Using Hino-Wilson Nitrogen-Free Medium for *Bacillus*

The Hino-Wilson Nitrogen-Free Medium (Ferrar and Reboli, 1992) comprises two solutions A and B which were prepared by dissolving the following compounds:

Solution A

Sucrose, 20g; $MgSO_4.H_2O$, 0.5g; NaCl, 0.01g; $FeSO_4.7H_2O$, 0.015g; $NaMoO_4.2H_2O$, 0.005g; and $CaCO_3$, 10g dissolved in 500ml distilled water.

Solution B

p-Aminobenzoic acid, 10μg and Biotin, 5μg dissolved in 500ml 0.1M K_2HPO_4-KH_2PO_4 buffer, at pH 7.7.

Both solution A and B were autoclaved separately for 15min. at 121^{0}C (15psi). Upon cooling these solutions were combined aseptically in equal volumes.

75ml of the Hino-Wilson medium were decanted aseptically into autoclaved 250ml flasks. Flasks were inoculated with 0.5ml of either R29 or B81 spores suspended in quarter strength Ringer solution ($1.53x10^7$ and $5.6x10^6$ CFU.ml^{-1}, respectively). The spores were taken from sporulated agar medium and heat treated for 10min. at 80^{0}C in quarter strength Ringer solution. This medium was then incubated at 30^{0}C at 170 rpm in a CESI for seven days. Samples (1ml) were taken daily over this time and assessed for growth by preparing serial dilutions and plating onto TSA.

Serial dilutions were made to give dilutions of 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} (Figure 2.1) by transferring 1ml broth culture to 9ml Ringer solution. Using the spread plate method, 0.1ml aliquots were transferred onto TSA in triplicate for CFU.ml^{-1} counts. Total CFU counts were calculated by multiplying the dilution factor (DF) of the solution being transferred by the volume transferred to the plate (Leboffe and Pierce, 1996). The DF of the plates then became 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}, respectively.

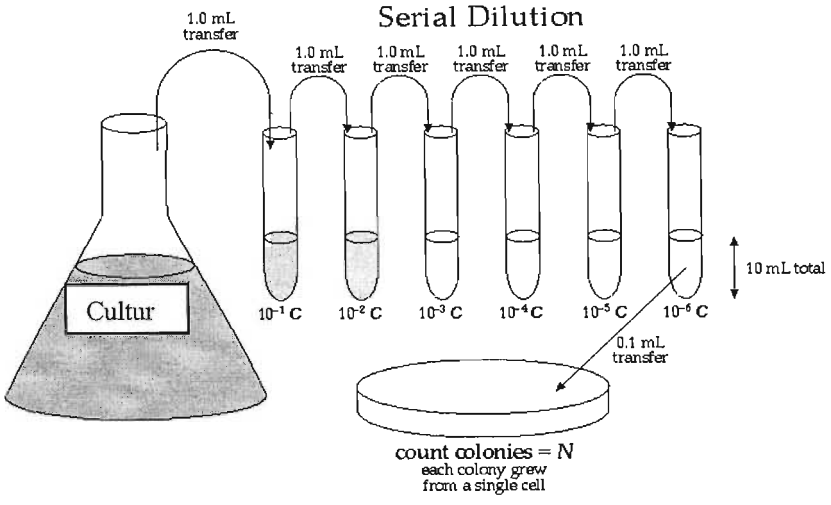

Figure 2.1 Serial dilutions used for colony forming unit counts.

2.2.4 Starch Hydrolysis Assay

Starch hydrolysis media (Ferrar and Reboli, 1992; Atlas, 1995) were prepared by adding 0.2% (w/v) soluble starch to both NA and TSA before autoclaving at 115^0C for 10min.

The ability of R29 and B81 to hydrolyse starch was tested by inoculating the above-mentioned media and incubating for 48h at 30^0C. Starch hydrolysis was assayed by flooding the agar plates with iodine solution. Zones of clearing around bacterial colonies indicates that starch hydrolysis occurred.

2.2.5 Anaerobic Growth Assay

Anaerobic agar medium for anaerobic cultivation of *Bacillus* spp. was prepared by dissolving the following components in distilled water and diluting to 1 litre: Casein, 20.0g; bacteriological agar, 15.0g; NaCl, 5.0g; sodium thioglycollate, 2.0g and sodium formaldehyde sulfoxylate, 1.0g (Atlas, 1995).

The medium was heated gently to dissolve ingredients and the pH was adjusted to 7.2 using 5N NaOH, and then dispensed into test tubes (10ml) and Erlenmeyer flasks (500ml) before autoclaving for 20min. at 121^{0}C (15-psi). When cool the medium was poured in to Petri dishes.

Test tubes were inoculated with overnight cultures of *Bacillus* isolates grown on TSA using the stabbing inoculation technique. Test tubes were then overlaid with sterilised mineral oil (±3ml) and incubated at 30^{0}C for seven days. Each test tube was assessed for bacterial growth on a daily basis.

Agar plates were inoculated using the streak method and inserted into an anaerobic jar (GasPak). Methylene blue indicator strips were moistened with distilled water and inserted into the anaerobic jar. A gas-generating unit (Merck) was then activated with 35ml water, inserted into the anaerobic jar, which was then closed, sealed (Instruction booklet (Merck); Costilow, 1981; Leboffe and Pierce, 1996) and incubated at 30^{0}C for seven days.

2.2.6 Casein Utilization Test

Two milk agar media (Smibert and Krieg, 1981; Ferrar and Reboli, 1992) were prepared by dissolving bacteriological agar 5g and either 2.5g or 25g of skimmed milk powder in distilled water and diluting to 250ml. Each medium was autoclaved for 10min. at 115^{0}C (15psi) and when cool, poured into Petri dishes.

Isolates R29 and B81 were streaked onto both milk agar media and incubated at 30^{0}C. The agar plates were examined at 7 and 14 days for zones of clearing which is indicative of casein degradation.

2.2.7 Nitrate Reduction and Denitrification Test

The nitrate reduction test medium (Ferrar and Reboli, 1992) was prepared by dissolving the following components in distilled water and diluting to 1 litre: peptone, 5g; meat extract, 3g; and potassium nitrate, 1g.

Nitrite Test Reagents (Ferrar and Reboli, 1992) were prepared in two separate solutions as follows.

Solution-A

N-(-1-Naphthyl)-ethlendiamine dihydrochloride,0.02g was dissolved in 100ml 1.5N HCl.

Solution-B

Sulfanilic acid, 1.0g was dissolved in 100ml 1.5N HCl.

Solutions A and B were mixed in equal volumes shortly before assaying for nitrate.

To detect the ability of organisms to reduce nitrate to nitrite, 0.1ml of bacterial suspension cultured in the nitrate reduction test medium was mixed with 2ml of test reagent. Water was added to make the final volume 4ml and left for 15min. to allow for colour development. The formation of a red colour is indicative of the presence of nitrite. The ability of an isolate to reduce nitrite further, i.e. denitrification, can be assessed by measuring the intensity of the red colour over time using a spectrophotometer to monitor absorbance at 540nm (Smibert and Krieg, 1981). If complete denitrification occurs NO_2 is reduced to N_2 and hence colouration should decrease or disappear.

2.2.8 Voges-Proskauer Reaction Test

The Voges-Proskauer reaction test medium (Smibert and Krieg, 1981, Ferrar and Reboli, 1992) was prepared by dissolving the following components in distilled water and diluting to 1 litre: bacteriological peptone, 7g; NaCl, 5g and glucose, 5g. pH was adjusted to 6.5 with 1N NaOH and 1N HCl and autoclaved for 10min. at 121^0C (15psi).

A 5% (w/v) α-naphthol stock solution was prepared by dissolving 0.5g α-naphthol in 10ml absolute ethanol. 40% (w/v) KOH solution was prepared by dissolving 4g KOH in 10ml water (Smibert and Krieg, 1981).

The medium was inoculated with isolates R29 and B81 and incubated at 30^0C for 48hrs. After 48hrs., 1ml of the culture was transferred into another test tube and 0.6ml of 5% (w/v) α-naphthol solution added and mixed thoroughly. Then 0.2ml of 40% KOH solution was added and left for 15min. to allow for colour development. A red colour is indicative of a positive reaction while a yellow colour is considered to be a negative reaction.

2.2.9 Growth at Different Temperatures

A defined growth medium (McKeen *et al.*, 1986) was prepared to assess *Bacillus* spp. growth over a range of temperatures. The following compounds were dissolved in distilled water and made up to 1 litre: D-glucose, 15g; DL-glutamic acid, 5g; $MgSO_4.7H_2O$, 1.02g; K_2HPO_4, 1g; KCl, 0.5g; and 1ml trace element solution. The trace element stock solution was made up as follows: to 100ml dH_2O add $MnSO_4.H_2O$, 0.5g; $CuSO_4.5H_2O$, 0.16g; $FeSO_4.7H_2O$, 0.015g and then adjust pH to 6.0- 6.2 with 5N NaOH or 1N HCl. Autoclaved for 15min. at 121^0C (15psi).

Erlenmeyer flasks (250ml) containing 75ml defined medium were inoculated with 0.1ml of overnight cultures of R29 and B81 and incubated in water baths set at 45^0C, 50^0C and 65^0C. The growth of each culture was monitored spectrophotometrically (625nm) for a period of 7 days.

2.2.10 Bromocresol Purple Broth-Acid Production Test

Bromocresol purple broth (Atlas, 1995) was prepared by dissolving the following compounds in distilled water and diluting to 1 litre: peptone, 10g; NaCl, 5g; beef extract, 3g; bromocresol purple, 0.04g and 5% (w/v) glucose solution, 10ml. The pH was adjusted to 7.0$\pm$ 0.2 with 5N NaOH or 1N HCl.

The medium was gently heated, brought to the boil and then dispensed into test tubes. In some of the test tubes, inverted Durham tubes were added to act as gas traps. The test tubes were then autoclaved for 15min. at 121^0C (15psi). Each test tube was inoculated with a loop full of the bacterial colony and some were then overlaid with sterile mineral oil to establish anaerobic conditions. The test tubes were incubated for 7 days at 30^0C and were checked daily of the colour change and gas production.

2.2.11 Hugh-Leifson's Glucose Broth

Hugh-Leifson's Glucose Broth (Atlas, 1995) is used to determine whether a bacterium is able to use glucose oxidatively or fermentatively and was prepared by dissolving the following components in distilled water and diluting to 1 litre: NaCl, 30g; glucose, 10g; bacteriological agar 3g; peptone, 2g; yeast extract, 0.5g and bromocresol purple, 0.015g. In addition, another medium, but with out glucose was also prepared to compare the colour changes brought about by oxidation-fermentation reactions. The pH was adjusted to 7.4 with 1N NaOH.

Each medium was heated gently, brought to the boil and then dispensed into test tubes, before autoclaving for 15min. at 121^{0}C (15psi). Test tubes containing semi-solid media were immersed in boiling water for a few minutes to remove oxygen and then quickly cooled in cold water. These were then inoculated with R29 or B81 by stabbing with an inoculation needle. Some of the media were overlaid with mineral oil and others not.

All cultures were incubated at 30^{0}C for 7 days checked for color changes and compared to the uninoculated controls.

2.2.12 CAS Siderophore Detection Assay

Modified chrome azurol s (CAS) siderophore detection agar medium (Barghouthi *et al.*, 1989) was prepared by dissolving 5.3g of NaOH and 30g of piperazine-N,N'-bis (2-ethanesulfonic acid) in 750ml distilled water. To this 20g agar and 100ml inorganic stock solution were added.

An inorganic stock salt solution was prepared by dissolving 0.3g of KH_2PO_4, 0.5g NaCl and 1.g NH_4Cl in 100ml distilled water.
The agar was dissolved by heating the solution; sterilized by autoclaving for 15min. at 121^{0}C (15psi) and kept at 50^{0}C in a heated water bath.

Filter sterilized solutions of the following components were then added: 30ml casamino acid (10%)(w/v), 10ml glucose (20%)(w/v), 10ml thiamine (200μg/ml), 10ml nicotinic acid (200μg/ml), 1ml $MgCl_2$ (1M) and 1ml $CaCl_2$ (0.1M).

A 100ml sterile solution containing a complex of chrome azurol S, iron and hexadecyltrimethylammonium bromide, (its preparation is described below), was gently mixed. Finally the medium was poured into Petri dishes.

The CAS-iron-hexadecyltrimethylammonium bromide solution was prepared by dissolving 60.5mg of CAS in 50ml of water and adding 10ml of 1mM $FeCl_3.6H_2O$ in 10mM HCl. This solution was then slowly added to a solution containing 72.9mg of hexadecyltrimethylammonium bromide in 40ml water. The resulting solution was then autoclaved separately for 15min. at 121^{0}C (15psi).

Petri dishes were separately inoculated with cultures using the streak method as well as with filter paper disks soaked in Ringer solution containing spores of either R29 or B81 isolates. These cultures were then incubated at 30^0C and checked for 10 days for zones of yellow-orange around the bacterial colonies, which indicates siderophore production.

2.2.13 Chitinase Assay

Colloidal chitin (Hsu and Lockwood, 1975; Chin-Min *et al.*, 2002) was prepared as follows: 13.33g of chitin powder (practical grade derived from crab shells) was dissolved in 133ml concentrated HCl by stirring for 50min. The chitin solution was then precipitated as a colloidal suspension by adding it to 667ml H_2O. The colloidal suspension was collected by filtration onto Whatman filter paper using a vacuum pump and then washed in 1.7 litre water and re-filtered. This process was repeated until the pH of the suspension was neutral. The colloidal chitin was then dried at 80^0C for 12hrs.

Colloidal chitin agar (Hsu and Lockwood, 1975) was prepared by dissolving the following compounds in distilled water and diluting to 1 litre: 4g dry weight of colloidal chitin, K_2HPO_4, 0.7g; $K_2H_2PO_4$, 0.3g; $MgSO_4.5H_2O$, 0.5g; $FeSO_4.7H_2O$, 0.01g; $ZnSO_4$, 1mg; $MnCl_2$, 1mg and 20g agar. The pH was adjusted to 7.10 with 5N NaOH.

The medium was autoclaved for 15min. at 121^0C (15psi), and poured into Perti dishes when cool ($\pm$ 47^0C).

The medium was inoculated using the single streak method as well as with filter paper discs soaked in quarter strength Ringer solution, containing spores of either R29 or B81, and then incubated at 30^0C and 37^0C. The plates were checked for a period of 14 days for growth of the bacterial isolates and zones clearing around the bacterial colonies, which is indicative of chitin utilization.

2.2.14 Cellulase Assay

CMC Agar Medium

Carboxymethyl Cellulose (CMC) agar (Spun, 2003) was prepared by dissolving the following compounds in distilled water and diluting to 500ml: CMC (Sigma), 2.5g; $NaNO_3$, 0.5g; K_2HPO_4,

0.5g; KCl, 0.5g; $MgSO_4$, 0.25g; yeast extract, 0.25g; glucose, 0.5g and bacteriological agar, 8.5g. The pH was adjusted to 7.00 with 5N NaOH or 1N HCl and autoclaved for 15min. at 121^0C (15psi).

Filter Paper Cellulose Medium

Filter paper cellulose medium was prepared in the same way as CMC agar with the exception that CMC was substituted with dried and ground Whatman filter paper. The filter paper was immersed in liquid nitrogen and then ground in a mortar and pestle.

0.1% (w/v) congo red solution was prepared by dissolving 1g congo red powder in 100ml distilled water.

Isolates R29 and B81 were assayed for their ability to hydrolyse CMC and filter paper cellulose by inoculating each culture onto the above-mentioned media in wells and filter paper discs and incubating for 48hrs for 7 days at 30^0C. Carboxymetyl cellulose and filter paper cellulose hydrolysis was assayed after flooding Petri dishes with 0.1% Congo red solution and de-staining with 1M sodium chloride solution. Unstained areas around the bacterial colonies indicate that cellulose hydrolysis has occurred.

2.2.15 Cell Size Measurements

Cell suspension samples were taken from actively growing TSB broth cultures of R29 and B81, and were added drop-wise to coat grids for viewing under transmission electron microscope (TEM). Excess liquid was drawn off using sharp edged filter paper. The grids were then stained with a drop of 2% (v/v) phosphotungstic acid (PTA) for 30sec. and excess liquid was drawn off as before. Discs were then viewed using TEM (Philips CM_120 BioTWIN) at 80Kv. The images were captured using a Meg-view III digital camera and length and width of cells were measured.

2.3 DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

2.3.1 Luria-Bertani Medium

Luria-Bertani (LB) medium was used for the cultivation of *Bacillus* isolates R29 and B81 prior to DNA extraction and was prepared by dissolving the following components in distilled water and

diluting to 1 litre: tryptone, 10.0g, yeast extract, 5.0g, NaCl, 10.0g and 1ml, 1M NaOH. The medium was then autoclaved for 30min. at 121^{0}C (15psi) (Harwood and Cutting, 1990).

2.3.2 DNA Extraction

Genomic DNA of *Bacillus* Isolates R29 and B81 was extracted using a NucleoSpin tissue DNA extraction Kit (Nage Machery) following the protocol recommended for Gram-positive bacteria. R29 and B81 were cultured in LB medium at 30^{0}C and agitated at 170rpm for 42hrs. in a CESI.

1ml from each culture was centrifuged (Hermle Z 160M) at 8,000xg for 5min. Each of the pellets was re-suspended in 180µl solution of 20mM Tris-HCl (pH 8.0), 2mM EDTA, 1%Triton X-100, and 25µl 20mg/ml lysozyme and incubated at 37^{0}C for 30min. Proteinase K (25µl) was added to each suspension and mixed by vortexing and then incubated at 56^{0}C for 30min. in a shaking water bath until the samples were lysed completely. This step was followed by the addition of 200µl buffer B3 to each of the samples, which were then vortexed and incubated at 70^{0}C for 10min. Ethanol 210µl of 96-100%was added to each of the sample and then vortexed.

NucleoSpin silica membrane columns were placed into 2ml collection tubes. Samples were added into the column and centrifuged at 11,000xg for 1min. 500µl BW buffer was added into the column, centrifuged at 11,000xg for 1min. and the flowthrough was discarded. 500µl B5 buffer was added to each spin column and centrifuged at 11,000xg for 2min. The flowthough was discarded and the process was repeated. The Nucleospin column was reinserted into the collection tube and centrifuged at 11,000xg for 1min. to remove any traces of buffer B5 from the membrane filter. The NucleoSpin column was placed in a clean 1.5 microcentrifuge tube and the DNA of each *Bacillus* isolate was eluted with 100µl BE buffer which was warmed at 70^{0}C. The preheated buffer was incubated in the spin columns at 60^{0}C-70^{0}C for 1min. This was centrifuged at 11, 000xg for 1min. to elute and collect DNA, which was then divided into to two 1.5ml minifuge tubes and stored at -20 and -80^{0}C until required.

2.3.3 Measuring Purity and Molecular Weight of Extracted DNA

Absorbance measurements determined at A_{260} and A_{280} were used to calculate the A_{260}/A_{280} purity ratio of the DNA sample and estimate the amount of DNA extracted (Linacero *et al.*, 1998) with a GeneQuant pro RNA/DNA calculator (Biochrom Ltd).

2.3.4 Detection and Analysis of Extracted DNA by Agarose Gel Electrophoresis

1X tris acetic acid + ethylenediaminetetra acetic acid (TAE) buffer (500ml) was prepared (pH 8.5) by adding 10ml of (50x) TAE stock solution and mixing with 490ml distilled water.

Agarose gel 0.8% (w/v) was prepared by adding 0.4g of electrophoresis grade agarose (Whitehead Scientific Ltd) to 50ml of (1x) TAE. The mixture was dissolved by heating using a magnetic stirrer hot plate (FE Freed Electric) until it cleared. When the solution had cooled to $\pm$ 55^0C, 2.5µl of ethidium bromide (50µg/ml) was added. The molten agarose was then poured into an electrophoresis unit (C.B.S Scientific Co. Model MGU-202T), a comb was inserted at one end to form sample wells and left to set for 30min. at room temperature. The comb was then gently removed and the gel electrophoresis unit was filled with 1X TAE until the buffer covered the surface of the gel by approximately 1mm.

The DNA samples (5µl) were amended with 3µl of (6x) loading dye (Promega) and then loaded into separate wells. A molecular weight marker was prepared by mixing 5µl 1kbp DNA ladder (Promega) and 3µl loading dye. Electrophoresis was initially carried out at 100volts for 5min. and then adjusted to 90volts for 75min. using a Bio-Rad power pack. The presence of DNA was verified by viewing the gel using UV transilluminator and the image of the gel was captured as a tiff file image using Image Store 5000 Annotator software.

2.3.5 Polymerase Chain Reaction (PCR)

Two PCR reaction mixes, with and without bovine serum albumin (BSA), were made up for each isolate (Table 2.1). All the components except the DNA template and Taq polymerase were added to a master mix, which was then dispensed (46µl) into separate PCR reaction tubes. DNA template (3µl) and Taq polymerase (1µl) were then separately added to each PCR reaction tube.

A negative control consisting of all the components of the mixture except the DNA template was also prepared.

The sequence of the Forward (P_f) and Reverse (P_r) primers were as follows:

P_f: 5'- CGC CCG CCG CGC CGC GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'

P_r: 5'- ATT ACC GCG GCT GCT GG-3'

These primers are from the variable region (V3) of the 16s rDNA, which corresponds to position 341 and 534 in *E. coli.*

The PCR was performed in an automated thermal cycler (Perkin-Elmer Applied Biosystem GeneAmp PCR system 2400, Nowalk, USA).

The PCR amplification sequence used included the following steps: (i) initial denaturation at 95^0C (5min.) (ii) 35 cycles of denaturation at 92^0C (1min.), annealing at 55^0C (1min.), elongation at 72^0C (1min.) (iii) final elongation at 72^0C (10min.) and (iv) cooling and storage at 4^0C.

Table 2.1 Reagents used for PCR amplification of DNA from isolates R29 and B81

Reagent	**Volume (µl) (with BSA)**	**Volume (µl) (without BSA)**
Millipore water	38	39
10X Taq buffer	5	5
BSA (20mg/ml)	1	0
dNTPs mix(10mM ea)	1	1
0.01M Primer (forward, 5µM)	0.5	0.5
0.01M Primer (reverse, 5µM)	0.5	0.5
DNA template	3	3
Taq polymerase	1	1
Total	50	50

The amplified PCR products were checked by agarose gel electrophoresis followed by direct viewing of the DNA using a UV transilluminator. A 100bp marker (Promega) was also run to confirm the approximate size of the amplified DNA.

2.3.6 Purification of Single DNA Band of Interest from Agarose Gel

To prepare purified single DNA band samples for sequencing, PCR amplified DNA samples (20µl) were mixed with 3µl loading dye, and then loaded and electrophoresed in 0.8% (w/v) (90volts for 80min.). Duplicates were loaded for each DNA sample. At the end of the run, the bands of interest were excised with a sterile scalpel blade by viewing the gel under a UV transilluminator and collected in Eppendorf tubes (according to GenElute^{TM} agarose Spin Column protocol). The excised gel samples were grounded with a sterile pestle in the Eppendorf tubes and then transferred into pre-washed spin columns.

Spin columns were pre-washed by adding 100µl of 1X TE buffer and centrifuging at 14000xg for 10sec. The excised (and ground) agarose gel, containing the DNA of interest, was placed into a spin column, and spun at 14000xg for 10min. Soluble molecules, including DNA, passed through the filter and were collected in a microcentrifuge tube (GenElute^{TM} agarose Spin column Protocol, technical bulletin code MB-725, Sigma).

The concentration and purity of the eluted DNA was determined by measuring UV absorbance measured values (A_{260} and A_{280}) in a Genequant spectrophotometer. DNA samples (5ul) were added to 95ul 1X TE DNA before absorbance readings were made. DNA concentration (µg/µl) was calculated using the following formula: A_{260} x 50 x 20 fold dilution.

2.3.7 DNA Sequencing and Homology Test

Purified and eluted DNA bands were sent to the University of KwaZulu-Natal's Molecular Biology Unit for sequencing.

The purified and eluted DNA bands were first cleaned up according to a modified BigDye Terminator v3.1 DNA sequencing reactions protocol (Applied Biosystem manual). The following components were added to sterile 1.5ml microcentrifuge tubes: 50µl of 100% ethanol, 2µl of 125mM EDTA and 2µl of 3M NaAc (sodium acetate, pH 4.6-5.2). 20µl of the DNA was added to each tube and vortexed gently for a few seconds and then placed in a fridge for 20min. to allow sequencing reaction extension products to precipitate. The tubes were then spun for 20min. at maximum speed with the cap hinges facing outward. All the supernatant was removed by tilting

the tube forwards (with the cap hinge uppermost) and inserting a pipette tip into the supernatant to carefully withdraw the liquid. Tubes were washed twice with 120μl of 100% ethanol and vortexed briefly. Placing the tubes in the same orientation as previously described tubes were centrifuged for 15min. at maximum speed and all supernatant removed. Finally, samples were dried by leaving opened tubes in the laminar flow hood (for 20min.).

Reaction mixes for PCR were prepared (Table 2.2), and were amplified in a thermo-cycler. The PCR amplification sequence used included 35 cycles with the following steps: (i) initial denaturation at 96^{0}C for 10sec. (ii) annealing (at 55^{0}C for 30sec.), elongation (at 60^{0}C for 4min., and (iii) cooling and storage at 4^{0}C. The primers used for this assay/trial were the same as those used in the first PCR).

Table 2.2 Reagents used for PCR amplification of the cleaned DNA of both isolates (µl)

Sample	**1**	**1**	**2**	**2**	**1**	**1**	**2**	**2**
Tube	1	2	3	4	7	8	9	10
Reagents								
RRM	2	2	2	2	2	2	2	2
2.5X CSRB	6	6	6	6	6	6	6	6
DNA template	0.4	0.8	0.4	0.8	0.4	0.8	0.4	0.8
P1	3.3	3.3	3.3	3.3				
P2					7.44	7.44	7.44	7.44
ddH_2O	8.3	7.9	8.3	7.9	4.16	3.76	4.16	3.76
Total	20	20	20	20	20	20	20	20

RRM-ready reaction mix; CSRB-cycle sequence reaction buffer; P1-forward primer, P2-reverse primer

Sequencing of cleaned up samples were performed on an Applied Biosystem ABI Prism® 3100 Gene Analyzer and the results were analyzed using the Staden sequence analysis package. Consensus sequences of the aligned Forward and Reverse chromatograms were produced, for both R29 and B81. These consensus sequences were then submitted to BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi) for homology testing against the existing GenBank database.

2.4 PRODUCTION, EXTRACTION, ISOLATION AND CHARACTERIZATION OF ACTIVE COMPOUNDS

2.4.1 Test Organism

Isolate R29 was selected for further antifungal metabolite characterization.

2.4.2 Antibiotic Production Medium

An antibiotic production broth medium (McKeen *et al.,* 1986) was prepared by dissolving the following compounds and diluting to 1 litre distilled water: D-glucose, 15g; DL-glutamic acid, 5g; $MgSO_4.7H_2O$, 1.02g; K_2HPO_4, 1.0g; KCl, 0.5g and 1ml of trace elements solution. The trace elements solution was prepared by dissolving $MnSO_4.H_2O$, 0.5g; $CuSO_4.5H_2O$, 0.16g and $FeSO_4.7H_2O$, 0.015g in 100ml of water. The pH of the medium was adjusted to 6.0-6.2 with 5N NaOH. 75ml aliquots were dispensed into 250ml Erlenmeyer flasks and autoclaved for 15min. at 121^0C (15psi).

Pure cultures of isolate R29 were established by taking a loop-full of inoculum from a master culture (stored at -80^0C) and streaking onto TSA plates and incubating for 24hrs. at 30^0C. A single colony of R29 was inoculated into 75ml of the antibiotic production medium to establish starter cultures, which were incubated at 30^0C for 18hrs. in a CESI set at 170rpm.

The overnight starter culture was inoculated (0.5ml) into 250ml Erlenmeyer flasks containing 75ml antibiotic production medium and incubated at 30^0C and shaken at 170rpm. Bacterial growth and pH changes of both cultures were monitored every 8-12hrs. for 7 days by using a spectrophotometer (625nm) (Milton Roy Spectronic 301) and pH meter (Micro pH 2000 Crison) respectively.

2.4.3 Activity Assay of the Cell Free Filtrates (Extracts)

Antimicrobial activity was assessed daily by taking samples (2ml) from the broth cultures. The samples were centrifuged at 10,000xg (Hermle Z 160M) for 10min. and the resulting supernatant filtered through either a cellulose acetate filter membrane (0.22µm pore size) (Cameo 25AS) or a cellulose nitrate filter membrane (0.2µm pore size) to obtain a cell-free culture supernatant. In

some instances cell-free supernatant samples were concentrated by freeze-drying (Edwards High Vacuum) and re-suspended in sodium phosphate buffer to achieve a hundred fold concentration.

To test for activity of the filter sterilized supernatants, 6mm diameter wells were made on the periphery of V8 and HPDA plates using a sterilized glass Pasteur pipette. A mycelial plug (5x5mm) from an actively growing *Rhizoctonia solani* culture was inoculated at the center of each plate. Aliquots (60µl) of the filter-sterilized supernatant were pipetted into each well and incubated for 2-3days at 25^0C. Concentrated samples were also spotted (10µl) onto the surface of the agar media and incubated with *R. solani* in the same way as previously described.

After testing for the production of the antifungal compounds from the synthetic medium, new cultures (1 litre) were established using the same medium and growth conditions. These cultures were incubated until the stationary phase was reached and were then centrifuged (Beckman J2-HS centrifuge) at 9,000xg (for 20min.) to remove bacterial cells. The cell free supernatant was freeze-dried at -40^0C, and then resuspended in 0.1M sodium phosphate buffer (pH 7.4) (8-10ml) and stored at -20^0C.

2.4.4 Separation and Extraction of the Antifungal Compound/s

2.4.4.1 Detection and Assaying of the Active Compounds by Thin Layer Chromatography (TLC).

For successful detection and separation of antifungal metabolites, different solvents were tested using TLC (aluminium sheet Silica gel 60 F_{254}, Merck). The solvents evaluated were:

1. acetone: methanol (1:1,v/v)
2. butanone: ethanol: water (35:35:30,v/v)
3. ethylacetate: acetone: water: acetic acid (50:25:15:10,v/v)
4. methanol: chloroform: water (45:40:15,v/v)
5. butanone: acetic acid water (40:10 50, v/v)
6. n-propanol: water(70:30 v/v)
7. hexane: methanol: water (40: 30: 30,v/v)
8. ethyl acetate
9. butane-1-ol

10. acetone
11. acetic acid: water (7:3 v/v)
12. butane-1-ol: acetic acid: water (70:15:15, v/v)
13. butane-1-ol: acetic acid: water (60:20:20, v/v)
14. butane-1-ol acetic acid: water (70:15:15, v/v)

The solvents listed above were prepared just before an experimental run. TLC was performed in sealed glass chambers. Solvents were added into each chamber to a depth of about 1cm. A piece of filter paper was placed at the center of the chamber so that the solvent was absorbed thereby helping to saturate the atmosphere of the chamber. The chamber was tightly closed and left for 30min. to saturate the atmosphere of the chamber before adding a TLC plate.

TLC (aluminium sheet Silica gel 60 F_{254}, Merck) plates were cut to an appropriate size (10cm x 14cm) and 10µl samples were spotted on a line 2cm from the bottom edge. The TLC plate was then immersed in the closed chamber and left till the solvent almost reached the opposite side of the plate. The developed TLC plates were viewed under UV light and bands were outlined with a pencil. The distances of the bands and the solvent were measured to calculate the relative mobility (R_f).

Active bands can be located and detected from a developed TLC by direct bioautography and bioautography overlay assays (Krieg and Gerhardt, 1981; Gibbons and Alexander, 1998). Gibbons and Alexander (1998) also stated that active compounds can be recovered from a developed TLC by scrapping off the relevant band/s from the plate, dissolving in a suitable solvent and then eluting them from the solvent. These three methods were used in this particular study to locate, detect and recover antifungal compounds on/from TLC plates.

Once an active band was detected, more TLC plates were developed and to remove solvent from the plate, they were incubated in a drying oven until completely dried. Active bands were then scraped from the silica gel plate onto foil. These were then transferred into Eppendorf tubes to which 80% ethanol solvent was added. This solution was mixed by vortexing and left for about 30min. to facilitate leaching of the antifungal metabolites into the solvent. The liquid fraction was separated from silica gel by centrifugation. This process was repeated three times to ensure a good recovery of the antifungal metabolites. The solvent was removed from samples containing

the antifungal metabolites by rotary vacuum evaporation (Jouan RCT60). The dried residue was re-suspended in (0.1M) sodium phosphate buffer and stored at -4^0C until it could be assayed for antifungal activity and HPLC analysis.

2.4.4.2 Separating the Antifungal Compound Using Two-Dimensional TLC

In order to confirm whether the active band on the TLC is a single compound or not, two-dimensional TLC was run with n-propanol-water (30:70 v/v). Plates were dried, and then turned through 90^0 and run a second time with the following solvents; ethanol-water (67:33 v/v), methanol-chloroform-water (45:40:15 v/v), acetone-water (70:30 v/v), acetic acid-water (70:30 v/v) and butanon1-ol (100%). All the TLC plates were dried and then viewed under UV light.

In order to purify extracts containing antifungal metabolites, three purification methods were evaluated. These were precipitation of the antifungal metabolites by adjusting the pH to 2.5 (McKeen *et al.,* 1986; Gueldner *et al.*, 1988; Chitarra *et al.*, 2003), hydrophobic interaction chromatography using Amberlite XAD-4 resin (Pichuk *et al.*, 2001) and solvent chemical extraction (methanol) (Bernal *et al.*, 2002).

2.4.4.3 Precipitation at 2.5 pH

Antifungal metabolites were precipitated by adjusting the pH of the culture broth to 2.5 with concentrated HCl followed by centrifugation for 10min. at 16,500xg (McKeen *et al.*, 1986; Gueldner *et al.*, 1988; Lebbadi, 1994; Munimbazi and Bullerman, 1998). Both the precipitate and the supernatant were then tested for antimicrobial activity. The pH of the supernatant was adjusted to 7.4 with 5N NaOH before testing.

The precipitate was dissolved in 80% (v/v) ethanol with vigorous stirring and then centrifuged for 10min. at 9,000xg to remove the insoluble fraction. Both the precipitate and the supernatant fractions were tested for antimicrobial activity. Ethanol was removed from the supernatant by evaporation using a rotary vacuum evaporator (VV1 Heidolph) at 60^0C. The solid residue was resuspended in (0.1M) sodium phosphate buffer (8-10ml) and stored at 4^0C. Samples were developed on TLC and eluted as described in Section 2.4.4.1 and analyzed in HPLC Section 2.4.4.6.

2.4.4.4 Hydrophobic Interaction Chromatography: Amberlite XAD-4 Resin

The hydrophobic nature of the active compound/s was first tested by adding 1ml of cell free supernatant to 0.5ml of Amberlite XAD-4 resin. The suspension was vortexed and allowed to stand for 15min. before centrifuging at 9,000xg for 5min. The supernatant was removed and then 1ml of methanol was added to eluted compounds, which had absorbed to the resin. The methanol extract was decanted and then subjected to a rotary vacuum evaporator to remove the methanol. The remaining residue was resuspended in 10µl of 0.1M sodium phosphate buffer and tested for antimicrobial activity.

Subsequently, a hydrophobic interaction chromatography column was assembled to extract active compounds from large volumes of cell free supernatant. 400ml Amberlite XAD-4 resin (Sigma) was washed with distilled water in a beaker to remove sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) salts imbedded in the resin. The resin was then packed into a glass column (14cm x 8cm) (Figure 2.2) and activated by passing through methanol solutions (20%, 40%, 60%, 80%, 95% (v/v) methanol:water and lastly distilled water through the column.

The column was then filled with 1 litre of cell free supernatant and allowed to stand for 15min., before being slowly drained from the bottom of the column. The eluent was collected and evaluated for antimicrobial activity. After washing the resin with double volumes of methanol: water (20:80 (v/v)), the resin column was sequentially eluted with solutions of 40%, 60%, 80%, 95% (v/v) methanol-water, respectively. All eluents were collected separately and concentrated by vacuum and by freeze-drying. The dried residues were then resuspended in (0.1M) sodium phosphate buffer (3-5ml) and tested for activity to select the best methanol-water percentage for eluting.

Eluted samples obtained using methanol-water (95:5 v/v) were then subjected to absorbance spectrophotometry over the ranged 200nm-800nm to determine optimum absorbance wavelength. For subsequent HPLC analysis the Perkins Elmer UV/VIS detector (LC 295) was adjusted to 285nm for detection of the compounds of interest. Samples were then analyzed in HPLC Section 2.4.4.6.

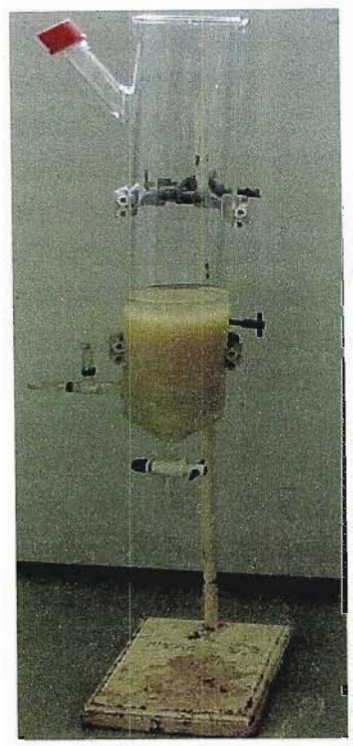

Figure 2.2 Hydrophobic interaction chromatography column.

2.4.4.5 Extraction of the Antifungal Metabolites with Methanol

Antifungal metabolites were extracted from the crude cell free supernatant using the methanol solvent extraction method described by Bernal *et al.* (2002). Equal volumes of methanol and supernatant were mixed and left at room temperature for at least 12hrs. and then centrifuged at 10,000xg for 15min. to remove insoluble residues. Both the soluble and insoluble fractions were individually collected. The methanol (boiling point 65^0C) was evaporated from the solvent supernatant mixture using rotary vacuum evaporation (Heidolph) at 76^0C until no more evaporation and condensation was observed in the condenser column. The remaining supernatant was freeze-dried at -40^0C. The resultant solid residue was then resuspended and dissolved in methanol to separate the methanol soluble and insoluble fractions. The dark insoluble methanol fraction was separated by centrifugation at 10,000xg for 15min. and was found to regain solubility when resuspended in (0.1M) sodium phosphate buffer. Each fraction was then separately tested for antifungal activity. The methanol soluble fraction (3ml) was separated from

the methanol using rotary evaporation and resuspended in (0.1M) sodium phosphate buffer before testing for antifungal activity. The rest of the sample arising from the methanol extraction was run in HPLC (Section 2.4.4.6).

2.4.4.6 Purification of Antifungal Compounds Using HPLC

Samples, which tested positive for activity against *R. solani* were analyzed with a Perkins Elmer UV/VIS detector LC 295, Series 200 IC Pump, and a PE NELSON model 1022 controller, HPLC.

Samples (50µl) recovered from TLC were loaded onto a C_{18} analytical RP-HPLC column, equilibrated at a flow rate of 1ml/min. Samples were eluted with a two-step linear gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) (0-90%)(solvent A) over 40min. followed 40-45min. with distilled water (10-100%)(solvent B) containing 0.1% (v/v) TFA.

Similarly, samples (250-500µl) from each of the extraction/purification steps in the amberlite resin extraction method (Section 2.4.7) were loaded in the HPLC and analysed in the same manner as described above. Peaks were collected manually and individually and taken to dryness with a rotary vacuum evaporator. Each of the dry residues from the collected peaks were resuspended in 0.1M sodium phosphate buffer and tested for activity.

Samples arising from the methanol extraction were also run in the HPLC using the same setting as previously described but differing in that 100% methanol (solvent A) and distilled water (solvent B) were used instead of acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) (solvent A) and 0.1% (v/v) TFA distilled water (solvent B). Fractions were collected at intervals corresponding to peaks arising in the UV detector (285nm). These samples were also assayed for antifungal activity.

Purified samples arising from the three different extraction methods were run in the HPLC; fractions corresponding to detectable peaks were collected individually and screened for activity against *R. solani*. Each fraction was dehydrated in a rotary evaporator and the residue resuspended in either (0.1M) sodium phosphate buffer, sterilized distilled water or methanol. These individual samples were re-run in the HPLC and the fraction collected. Samples reflecting

the activity of a single peak were dehydrated in a rotary evaporator and then subjected to nuclear magnetic resonance (NMR) analysis.

2.4.5 Partial Characterization of Antifungal Metabolites

2.4.5.1 Nuclear Magnetic Resonance (NMR)

NMR analysis was carried out on Varian, Unity Inova 500 using VNMR 6.1C software operated and housed within the Discipline of Chemistry, University of KwaZulu-Natal, RSA. Samples were analyzed in ^{13}C proton noise decoupled (PND) at 125MHz and ^{1}H at 500MHz by dissolving in chloroform ($CDCl_3$) and a few drops of methanol (CD_3OD) in 5mm glass tubes at 25 ± 0.2^0C.

2.4.5.2 Thermostability of Antifungal Compound/s

Thermostability of the antifungal metabolites was tested using methods previously described by Munimbazi and Bullerman (1998), Bernal (2002) and Nair *et al.* (2002). Samples (250μl) taken from the crude extract derived from precipitation at pH 2.5 and resuspended in 0.1M sodium phosphate buffer, were dispensed in sterile micro-fuge tubes. Two replicate tubes were exposed to the following temperatures: room temperature, heating in a water bath to 80^0C and 100^0C, and autoclaving at 110^0C and 121^0C for 15min., respectively. Upon cooling, each sample (60μl) was tested for antifungal activity against mycelial growth of *R. solani* on HPDA.

2.4.5.3 Determination of the Inhibitory Activity Unit (IAU)

Inhibitory activity units (IAU) were determined using a modification of the method described by Bernal *et al.* (2002). The pH of 1 litre of R29 supernatant was adjusted to 2.5 and the precipitate, containing the active compounds, was separated by centrifugation and extracted with 80% ethanol. The solid residue was discarded and the 80% ethanol solution dehydrated using a rotary vacuum evaporator. The solid residue was resuspended in 10ml (0.1M) sodium phosphate buffer to achieve 100-fold concentration. The concentrated sample was sequentially diluted with sodium phosphate buffer and each dilution then tested for antifungal activity. In order to determine activity units, the dilution factor was calculated as the reciprocal of the sample with the lowest dilution showing antifungal activity multiplied by the volume used for the bioassay and then by 10. The total final activity unit was multiplied by 100 as it was concentrated 100-fold (Table 2.3).

Table 2.3 Sequential dilution series used to determine Inhibitory Activity Unit's of the extracted antifungal compounds

	Crude extract sample (µl)	Sodium phosphate buffer (µl)	Dilution factors (1000 /volume of crude extract sample)
1	1000	0	1.0
2	875	125	1.1
3	750	250	1.3
4	625	375	1.6
5	500	500	2.0
6	375	625	2.7
7	250	750	4.0
8	125	875	8.0
9	100	900	10.0
10	75	925	13.3
11	50	950	20.0
12	25	975	40.0

Wells (6mm) were made on the periphery of HPDA plates and the media inoculated with plugs of actively growing mycelia of either *R. solani, Pythium* sp. or *Fusarium* sp. Each well was filled with 100µl of diluted sample and the plates incubated at 25^{0}C. Inhibition zones were measured in order to determine the minimal inhibitory concentration. This test was repeated twice.

2.4.5.4 pH Stability

The pH stability was tested according to the methods described by Pinchuk *et al.* (2001) and Yu *et al.* (2002). The crude extract was diluted three fold with (0.1M) sodium phosphate buffer and adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 using (1N) NaOH or (1N) HCl, in separate test tubes and incubated for 24hrs. at 25^{0}C. Each of these samples was then readjusted to pH 7 and tested for activity against *R. solani* as previously described (Section 2.4.5.2).

2.4.5.5 Protease Test

Sensitivity to Protease K enzyme was tested using the method described by Pinchuk *et al.* (2001). Purified extracts from the TLC and resin purification step were incubated for 1hr at 56^{0}C. The following mixtures were prepared for this experiment:

1. 10µl sample from TLC extract + 5µl protease K,
2. 10µl sample from TLC extract + 5µl buffer,
3. 10µl buffer + 5µl protease K and
4. 10µl sample from resin extract + 5µl protease K.

Each of these sample (15µl) were spotted on the periphery of HPDA on which *R. solani* was growing and incubated at 25^0C for two days and checked for the formation of inhibition zones.

CHAPTER 3

IN VITRO BIOASSAYS FOR SCREENING ANTIFUNGAL ACTIVITY OF SELECTED *BACILLUS* SPP. ISOLATES

RESULTS

All of the seven *Bacillus* isolates screened grew well on TSA with colonies appearing after 24hrs. incubation at 30^0C. Colonies of *Bacillus* isolates B81, B81c, B77 and B69c were similar forming small, creamy, circular and flat matt colonies, whereas *Bacillus* R29 and R16 colonies were mucoid, creamy, circular and convex in shape. *Bacillus* isolate B69 colonies also exhibited a red pigment especially when cultured in TSB where the bacterial cells flocculated as a red gelatinous mass.

Both *R. solani* and *Pythium* sp. fungal isolates grew well on PDA at 25 and 30^0C covering an agar plate in two days. The mycelial colour of *R. solani* was faded whereas *Pythium* sp. grew as a bright white with dense mycelia when cultured on PDA, HPDA and MEA. When cultured on NA and TSA the mycelium colour of the *Pythium* sp. isolate was faded. The *Fusarium* sp. isolate grew slower than other fungi covering the whole plate within three to four days when incubated at 25 and 30^0C. The colour of its mycelium was white and was furry having the appearance of cotton wool. *Phytophthora* sp. grew very slowly and mycelial growth was characterised by a scattered growth of a large number of colonies.

3.1 ANTIBIOTIC ACTIVITY OF SEVEN *BACILLUS* ISOLATES AGAINST FOUR FUNGAL PLANT PATHOGENS

All the isolates screened, inhibited growth of each of the fungal pathogens tested, to varying degrees. Mean inhibition zones between the *Bacillus* isolates and pathogenic fungi are presented in Table 3.1. Analysis of variance revealed that the seven *Bacillus* isolates showed significant differences ($P < 0.001$) on the basis of their mean inhibition zones on the four different complex media.

Isolate R29 and B69c consistently produced zones of inhibition ≥ 9.95mm for all the fungi tested indicating that both were the most antagonistic isolates (Table 3.1 and Figure 3.1). In general terms inhibition of *R. solani* and *Pythium* sp. was not as pronounced as that of *Phytophthora* sp. and *Fusarium* sp. for B69c, B81, B81c R16 and R29. Isolates B69 and B77 exhibited the lowest extent of fungal inhibition.

Based on these preliminary findings as well as on prior *in vitro* screenings of B81, which exhibited potential as a good biocontrol agent (Khubeka, 2003), R29 and B81 were selected for further testing and characterisation.

Table 3.1 Antibiotic activities of seven *Bacillus* isolates against four pathogens on four different complex media in terms of mean inhibition zones (in mm)

	Mean zones of inhibition (mm)			
***Bacillus* Isolates**	***R. solani***	***Pythium* sp.**	***Phytophthora* sp.**	***Fusarium* sp.**
B69	8.58d*	7.33d	4.59g	5.55f
B69c	9.95b	11.09a	10.39c	13.94a
B77	6.64g	6.17d	5.19f	5.88e
B81	7.63f	8.16c	7.73e	11.44c
B81c	7.98e	8.17c	11.53a	10.81d
R16	9.36c	8.31c	10.72b	12.66b
R29	11.05a	10.13b	10.08d	12.47b
LSD	**0.359**	**0.4327**	**0.3071**	**0.3537**
CV%	**5.8**	**7.2**	**5.0**	**4.8**

*Mean values within a column followed by common letter do not differ significantly according to Fischer's protected least significant difference ($P<0.001$).

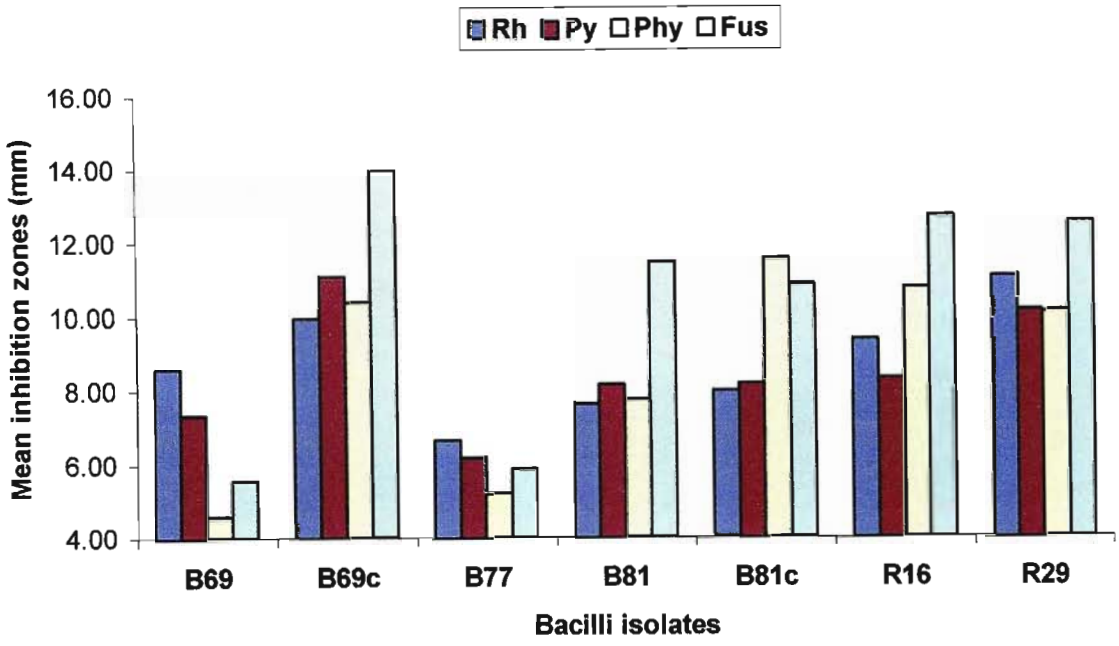

Figure 3.1 Antibiotic activities of the seven *Bacillus* isolates against four pathogens on four complex media in terms of mean inhibition zones (mm). ***Rh**: *Rhizoctonia solani*, **Py**: *Pythium* sp., **Phy**: *Phytophthora* sp. and **Fus** *Fusarium* sp.

3.2 INFLUENCE OF pH, TEMPERATURE AND MEDIUM COMPOSITION ON THE ANTIFUNGAL ACTIVITY OF *BACILLUS* ISOLATES, R29 AND B81

Influence of pH, incubation temperature and medium composition on the antifungal activity of isolates R29 and B81 are shown in Tables 3.2 and 3.3, respectively. Antifungal activity was apparent for each parameter\variable tested.

3.2.1 *Bacillus* Isolate R29

For *R. solani*: each of the parameters tested showed significant differences ($P < 0.001$) (Tables 3.2 and 3.3). The general trend showed that pH influenced antifungal activity of R29 more than a 5^0C difference in temperature. Generally, R29 showed higher antifungal activity against *R. solani* at pH 7. Of all agar media evaluated, TSA gave rise to the largest inhibition zones.

For *Pythium* sp., all the parameters tested showed significant differences ($P < 0.001$) and in general both pH and temperature influenced antifungal activity of R29. Media composition also

appeared to influence antifungal activity of R29. The highest activity was found on TSA and the lowest activity was found on NA.

For *Phytophthora* sp., both pH and temperature showed insignificant differences (P = 0.936 and 0.014 respectively) on the antifungal activity of R29. Media composition influenced antifungal activity of R29 with the highest level of activity being associated with growth on TSA.

For *Fusarium* sp., pH and media composition showed significant differences (P <0.001) on the antifungal activity of R29 whereas temperature did not. In general the highest antifungal activities were found on TSA.

Table 3.2 Antibiotic activity of R29 against four plant pathogens in terms of mean inhibition zones on five complex media adjusted to pH 5.5 and 7.0, which were incubated at temperatures 25 and 30^{0}C

				Mean inhibition (mm)			
	Temp	**pH**	**Media**	***R. solani***	***Pythium* sp.**	***Phytophthora* sp.**	***Fusarium* sp.**
1	25	5.5	PDA	12.92g	12.96ed	18.92bc	16.67d
2	25	5.5	HPDA	13.33g	13.17d	15.75cd	16.25de
3	25	5.5	MEA	13.08g	12ef	17.75bc	14.5f
4	25	5.5	NA	13.5g	5.96j	18.5bc	16.67d
5	25	5.5	TSA	18.08b	10.58g	18.33bc	19.08b
6	25	7	PDA	15.5e	12.75ed	19.08b	17.08cd
7	25	7	HPDA	15.5e	14.08c	17.25c	15.42ef
8	25	7	MEA	16.33d	11.58f	16.5cd	15.5e
9	25	7	NA	17.08c	8.83i	13e	17.5cd
10	25	7	TSA	18b	14.5bc	21.58a	19.58b
11	30	5.5	PDA	13.29g	12.5e	17.29c	15ef
12	30	5.5	HPDA	13.25g	13ed	18.08bc	14.92ef
13	30	5.5	MEA	11.5h	14.5bc	15.83cd	13.83f
14	30	5.5	NA	12.92g	6.04j	15.08d	17.17cd
15	30	5.5	TSA	17.33c	14.67b	18bc	20.92a
16	30	7	PDA	15ef	13.67cd	15.83cd	16.5d
17	30	7	HPDA	15.42e	12.92ed	17.17c	17cd
18	30	7	MEA	14.5f	11.96ef	15.17d	14.42f
19	30	7	NA	16.25d	9.17h	17.33c	17.83c
20	30	7	TSA	20.42a	17.46a	20.42ab	21.17a
LSD				**0.617**	**0.551**	**1.619**	**0.9312**
CV%				**3.6**	**4.0**	**8.1**	**4.8**

*Mean values within a column followed by the same letter do not differ significantly ($P<0.001$) according to Fischer's protected least significant difference

Table 3.3 ANOVA results of P values of *Bacillus* isolate R29 against four plant pathogens on five complex media

	R. solani	***Pythium* sp.**	***Phytophthora* sp.**	***Fusarium* sp.**
Source of variation	F pr.	F pr.	F pr.	F pr.
TEMP	<.001	<.001	0.014	0.737
PH	<.001	<.001	0.936	<.001
TEMP.PH	0.071	0.004	0.183	0.035
MEDIA	<.001	<.001	<.001	<.001
TEMP.MEDIA	<.001	<.001	<.001	<.001
PH.MEDIA	<.001	<.001	<.001	0.788
TEMP.PH.MEDIA	<.001	<.001	<.001	0.002
LSD	**0.617**	**0.551**	**1.619**	**0.9312**
CV%	**3.6**	**4.0**	**8.1**	**4.8**

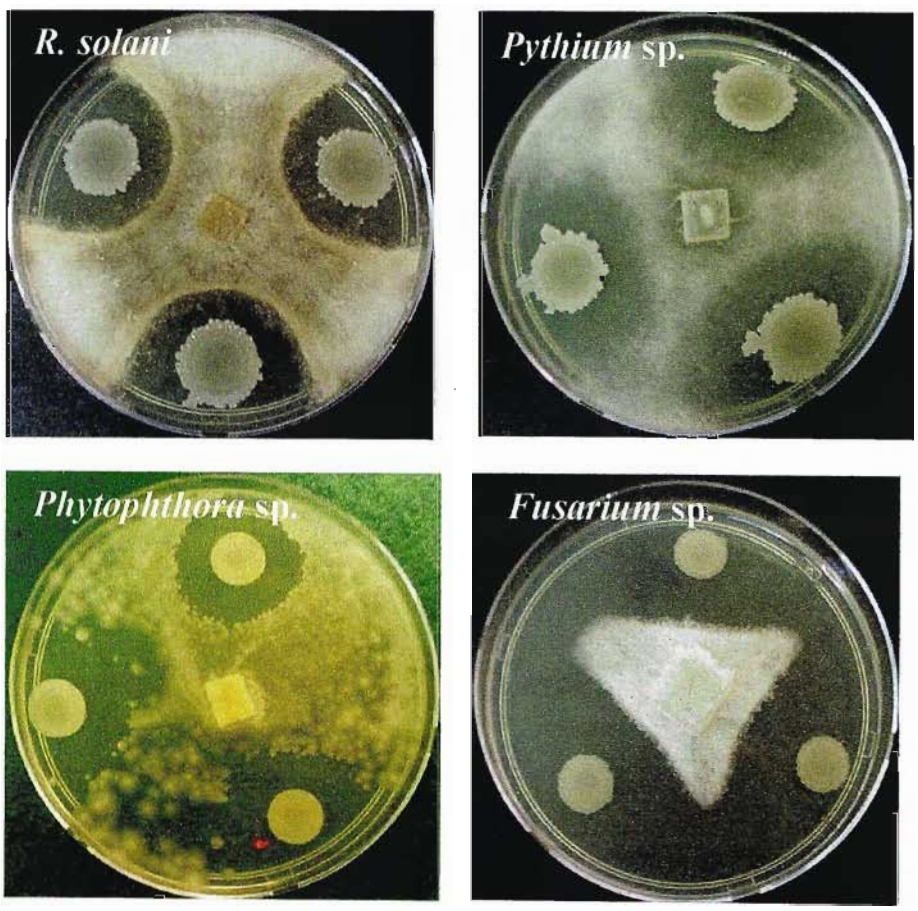

Figure 3.2 Antifungal activity of isolate R29 against *Rhizoctonia solani Pythium* sp., *Phytophthora* sp. and *Fusarium* sp. on PDA, PDA, NA and HPDA agar, respectively.

3.2.2 *Bacillus* Isolate B81

For *R. solani,* B81 showed significant differences in antifungal activity ($P < 0.001$) for all parameters tested except temperature ($P = 0.1000$) (Tables 3.4 and 3.5). In general, pH influenced antifungal activities of B81 more than did a 5^0C difference in temperature. Media composition also showed significant differences and TSA was found to give rise to the largest zones of inhibition of B81 against *R. solani.*

For *Pythium* sp., B81 showed significant differences in antifungal activity ($P < 0.001$) for all the parameters tested. Higher antifungal activities were recorded at pH 5.5 than at pH 7 on NA and TSA media.

For *Phytophthora* sp., B81 showed significant differences in antifungal activity on media composition ($P < 0.001$) but other of the parameters showed insignificant differences against *Phytophthora* sp. Highest antifungal activities were also found on TSA and MEA.

For *Fusarium* sp., B81 showed significant differences in antifungal activity ($P < 0.001$) for all parameters tested. In general the highest antifungal activities were recorded on TSA and NA.

For both isolates R29 and B81, it was observed that in dual cultures of *R. solani* and *Fusarium* sp. the mycelia and adjacent agar media turned a dark brown colour over time. Discoloration started in the region adjacent to the inhibition zones and slowly progressed toward the center of the agar plate eventually covering the entire plate.

Table 3.4 Antibiotic activities of B81 against four plant pathogens in terms of mean inhibition zones on five complex media adjusted to pH 5.5 and 7.0, which were incubated at temperatures 25 and 30^{0}C

				Mean inhibition (mm)			
	Temp	**pH**	**Media**	***R. solani***	***Pythium* sp.**	***Phytophthora* sp.**	***Fusarium* sp.**
1	25	5.5	PDA	11.75cd	11.75de	14.29bc	13.42de
2	25	5.5	HPDA	10.67de	10.38e	13bc	11.33f
3	25	5.5	MEA	11.75cd	13c	13.38bc	11.54ef
4	25	5.5	NA	10.17e	15.33a	13.75bc	17.08b
5	25	5.5	TSA	12.42c	13.54bc	18.08a	14.42d
6	25	7	PDA	11.83cd	10.92e	15.75ab	11.67ef
7	25	7	HPDA	11.25d	13.25bc	16ab	11f
8	25	7	MEA	13.58b	8.75f	17.5a	11.29f
9	25	7	NA	13.13bc	8.63f	6d	14.17d
10	25	7	TSA	15.17a	12.04d	17.42a	16.92bc
11	30	5.5	PDA	11.75cd	10.42e	11.83c	15.83c
12	30	5.5	HPDA	11.75cd	5.17g	12.08c	13.75de
13	30	5.5	MEA	13bc	13.92b	17.58a	14.17d
14	30	5.5	NA	10.42e	13.08bc	16.08ab	17.42ab
15	30	5.5	TSA	13.42b	13.25bc	17.17ab	17.75ab
16	30	7	PDA	12.25c	11.42de	16ab	12.67e
17	30	7	HPDA	12.25c	11e	14.08bc	12.33ef
18	30	7	MEA	10.25e	10.58e	17.42a	11.88ef
19	30	7	NA	13.54b	8.71f	6d	14.25d
20	30	7	TSA	15.08a	13.42bc	15.08b	18.42a
L.S.D				**0.756**	**0.889**	**2.337**	**1.136**
CV%				**5.4**	**6.8**	**14.1**	**7.1**

*Mean values within a column followed by the same letter do not differ significantly (P<0.001) according to Fischer's protected least significant difference

Table 3.5 ANOVA results of P values of *Bacillus* isolate B81 against four pathogens on five complex media

	R. solani	***Pythium* sp.**	***Phytophthora* sp.**	***Fusarium* sp.**
Source of variation	F pr.	F pr.	F pr.	F pr.
TEMP	0.100	<.001	0.624	<.001
PH	<.001	<.001	0.110	<.001
MEDIA	<.001	<.001	<.001	<.001
TEMP.PH	<.001	<.001	0.092	<.001
TEMP.MEDIA	<.001	<.001	0.004	0.004
PH.MEDIA	<.001	<.001	<.001	<.001
TEMP.PH.MEDIA	<.001	0.224	0.058	0.554
LSD	**0.756**	**0.889**	**2.337**	**1.136**
CV%	**5.4**	**6.8**	**14.1**	**7.1**

DISCUSSION

The appearance of *Bacillus* sp. colonies cultured on agar media can vary greatly depending on factors such as medium composition, incubation temperature and humidity. With the exception of certain strains, which consistently form only small colonies, colony diameter is largely dependent on the number of colonies developing and the concentration and type of nutrient available (Clause and Berkeley, 1986).

Development of an *in vitro* screening system that provides reliable and repeatable results is an important initial step for isolation of efficient bacterial antagonists for biological control of plant diseases (Anith *et al.*, 2003). In the present study, seven *Bacillus* isolates were subjected to *in vitro* dual culture assays to select the most active, stable and broad spectrum antagonistic isolates.

All four plant pathogens screened were inhibited by the seven *Bacillus* isolates tested. The inhibitory (antibiotic) activities of the *Bacillus* isolates were visible from day two for *R. solani* and *Pythium* sp. and day three for *Fusarium* sp. and *Phytophthora* sp. Antifungal activity was attributed to the bacterial ability to excrete inhibitory compound(s) most probably peptide antibiotics and/or lipopeptides (Katz and Demain, 1977; Vanittanakom *et al.*, 1986).

Antibiotics are able to kill or inhibit the growth of other organisms in nature thereby providing a competitive advantage to the producer (Katz and Demain, 1977; Zuber *et al.*, 1993). The antagonistic nature of certain *Bacillus* spp. toward fungal pathogens, however, is not restricted to antibiosis. A range of metabolites including chitinase, biosurfactants, fungal cell wall degrading enzymes (Leifert *et al.*, 1995) hydrolytic enzymes (Stöver and Driks, 1999a) and siderophores (Grossman *et al.*, 1993) may also be produced. Podile and Prakash (1996), for example, found that *B. subtilis* AF 1 produces extracellular enzymes(s) that lyse the cell wall of *Aspergillus niger*.

The sizes of inhibition zones formed between the different *Bacillus* isolates and the pathogens tested were found to differ (Table 3.1). Cook and Baker (1983) reported that *R. solani* is more sensitive to antifungal antagonism than most soil-born pathogenic fungi. In this study, *R. solani* inhibition was found to be fungicidal whereas *Pythium* sp. inhibition was fungistatic.
Inhibition of *Pythium* sp. was found to be temporary and when left for a sufficient incubation period it colonised the inhibition zone. This indicates that the inhibition of *Pythium* sp. was

fungistatic. Possible explanations for the fungistatic response include resistance to the antibiotics produced, reduced permeability of the antibiotics, a fungistatic concentration of the antibiotics being produced or that *Bacillus* isolates may not be able to continuously produce the antibiotics. Fiddaman and Rossall (1993) reported that *B. subtilis* (NCIMB 12376) produced a volatile antifungal compound, and that its activity was reduced over time. In addition to this it was found that *P. ultimum* was far less sensitive to the volatile compound than two *R. solani* isolates. Though the volatile nature of the inhibitory compounds produced by the *Bacillus* isolates is not known in this particular study, the increasing resistance of *Pythium* sp. over time agrees with this finding.

Although the sensitivity of the pathogens varied considerably, all the *Bacillus* isolates screened were able to inhibit all the pathogens indicating that the inhibitory compound(s) present had broad activity spectra. Similar findings were reported by Kim *et al.* (1997) in which *Bacillus* sp. strain L324-92 exhibited broad-spectrum inhibitory activity against *R. solani* AG8; *P. irreguare, P. ultimum* and *G. graminis* var *tritici.* Yoshida *et al.* (2001) and Yao *et al.* (2003) also found antifungal compounds produced by *B. amyloliquefaciens* and *B. subtilis,* respectively, which showed inhibitory activity against a broad range of plant pathogens.

Differences in the sensitivity of pathogens to inhibitory compound(s) produced could, in part, be attributed to differences in their cell wall composition. The cell wall of *R. solani* is composed of 1,3-β-glucan and chitin subunits (Bartnicki-Garcia, 1968; Papavizas, 1985). *Phytophthora* and *Pythium* both have cell walls comprising β-1,3- and β-1,6-glucan subunits covering fibrillar cellulose (Sietma *et al.*, 1969 cited by Thrane *et al.*, 1997; Bartnicki and Garcia, 1983). In addition, the cellulosic fraction of the cell wall of *Phytophthora* has β-1, 4-linked chains of cellulose, which is poorly crystalline (Bartnicki-Garcia, 1983). Schineider *et al.* (1977) reported that the cell wall of *Fusarium* contains glucosamine (derived from chitin), glucose, mannose, and galactose subunits. Chemical analysis on *Fusarium*'s cell walls has revealed the presence of hexosamine, hexose, pentose and lipids subunits (Marchant, 1984).

Extracellular enzymes can degrade specific cell wall components. For example, hydrolytic enzymes such as chitinase degrade the cell wall of fungal pathogens causing cell lysis and death (Mitchell and Alexander, 1962). Chet (1990) found that extracellular enzymes β-1, 3-glucanase

and chinitase degraded the cell wall of *R. solani* but that similar enzymes failed to degrade the cell wall of *Fusarium* sp. These differences in cell wall compositions, as well as differences in inhibitory compounds produced from different *Bacillus* isolates, could account for the varying antifungal antagonism.

Medium composition is a very important factor affecting the production of antibiotic compounds (Lancini and Parenti, 1882). Bernal *et al.* (2002) found that different culture media have direct bearing on the size of inhibition zones produced by *B. subtilis* A47. Similar observations were made in this study. Tryptone soy agar was found to produce the largest zones of inhibiton (Table 3.2 and 3.4). Yoshida *et al.* (2001) reported that media containing peptone, significantly increased production of antifungal compounds. By increasing the peptone concentration of a liquid culture, they were able to increase antifungal activity of the filtrate 50 by fold. They proposed that peptone (a mixture of several kinds of amino acids) is the key nutrient for the production of the antifungal compounds or that the production of the antifungal compounds require several amino acids. The fact that TSA contains soy peptone, seems to support this observation. However, NA which also contain peptone, did not produce favourable results suggesting that additional factors play an important role.

The pH was found to be a factor that caused antibiotic activity differences in media and similar findings have been reported by Leifert *et al.* (1995). In this study it was found that there was a significant difference ($P < 0.001$) in the antibiotic activities of the isolates on media, which differed in pH. Antibiotics often have maximum activity at their isoelectric point and pH of test media tends to select substances with an isoelectric point near the pH of the medium. The pH of a medium can affect the degree of ionisation of an antibiotic and hence its effectiveness by influencing its diffusion through agar and penetration of cell walls (Lancini and Parenti, 1982).

Temperature optimisation is an important consideration in the production of antibiotics (Arbige, 1993). Perez *et al.* (1992) found that production of antimicrobial substances by *B. subtilis* MIR 15 at 37^0C was 10 times higher than at 20^0C. Isolate R29 achieved slightly larger zones of inhibition at 30^0C whereas for B81, the temperatures tested (i.e. 25^0C an 30^0C) did not elicit marked differences in inhibition zones.

Dual culture bioassays with *R. solani* and *Fusarium* sp. both resulted in discolouration of the agar media. Discoloration started in the region adjacent to the inhibition zones and slowly progressed toward the center of the agar plate eventually covering the entire plate. Similar observations were made by Montealegre *et al.*, (2003) who determined that media discoloration was associated with cytoplasmic leakage, which resulted in hyphal deformation and slimming of the apex up to 1/10 of its original size.

The screening methods used in this study were found to be reproducible and were used to select two isolates for further characterization.

CHAPTER 4

PHYSIOLOGICAL AND NUTRITIONAL CHARACTERISATION AND TENTATIVE IDENTIFICATION OF *BACILLUS* ISOALTES R29 AND B81

RESULTS

The isolates used for this study were identified as members of the genus *Bacillus* based on the Gram stain and spore staining. From these tests, it was confirmed that the isolates were rod shaped, Gram-positive, spore-forming bacteria. However, the genus *Bacillus* is a very heterogeneous group of unicellular bacteria and so it was decided to carry out further biochemical and physiological characterizations using the taxonomic key proposed by Slepecky and Hemphill (1992). The results of these taxonomic, biochemical and nutritional characteristics are detailed as follows.

4.1 CATALASE TEST

Immediately after flooding bacterial colonies, cultured in test tubes with 10% (v/v) H_2O_2, gas bubbles rapidly formed (Figure 4.1) indicating isolates R29 and B81 are both catalase positive.

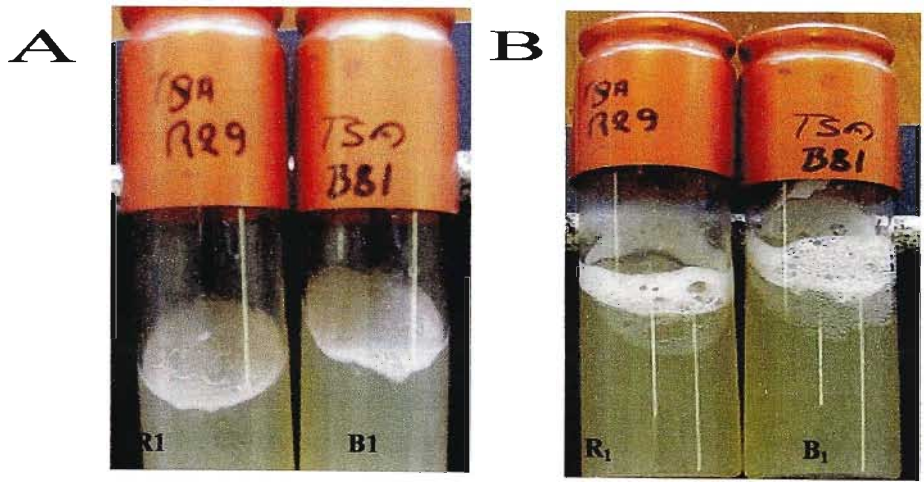

Figure 4.1 Catalase reaction of isolates R29 (R1) & B81 (B1) before (A) and after (B) the addition of H_2O_2.

4.2 VOGES- PROSKAUER REACTION

When test reagents were mixed with samples from both reaction cultures, solutions turned red (Figure 4.2) indicating that isolates R29 and B81 are both positive for the Voges-Proskauer reaction test. The indicator turned red at a pH < 4.4 whereas the reaction mix remained yellow at a pH > 6.0 (Leboffe and Pierce, 1996).

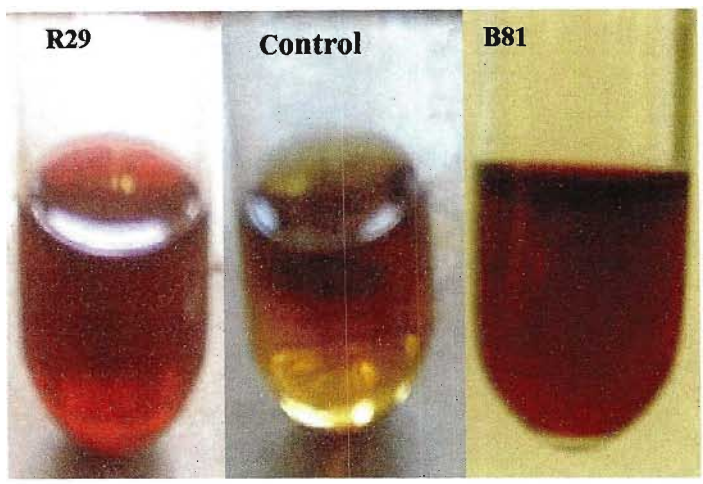

Figure 4.2 Voges-Proskauer reaction test showing positive reactions (redish colour) of isolates R29 and B81 compared to the negative control (yellow colour).

4.3 GROWTH ON ANAEROBIC AGAR

Isolate B81 exhibited minute colony growth throughout the anaerobic agar after incubation in an anaerobic jar whereas isolate R29 did not. In a second test involving agar in tubes overlaid with mineral oil there was no growth. Results showed that B81 was capable of anaerobic growth whereas R29 was not.

4.4 GROWTH AT DIFFERENT TEMPERATURES

Both isolates were able to grow at 45^0C in TSB but not at 50^0C or 60^0C.

4.5 BROMOCRESOL PURPLE BROTH

After two weeks incubation, both the aerobic cultures and the cultures overlaid with mineral oil turned from purple to a yellow color (Figure 4.3). These reactions are due to the accumulation of acid end products of metabolism or fermentation. Bromocresol purple pH indicator is purple at a

pH > 6.8 and yellow at pH < 5.2 (Leboffe and Pierce, 1996). Results indicate that both isolates are capable of aerobic and anaerobic metabolism. In both instances no gas was trapped in the Durham tubes.

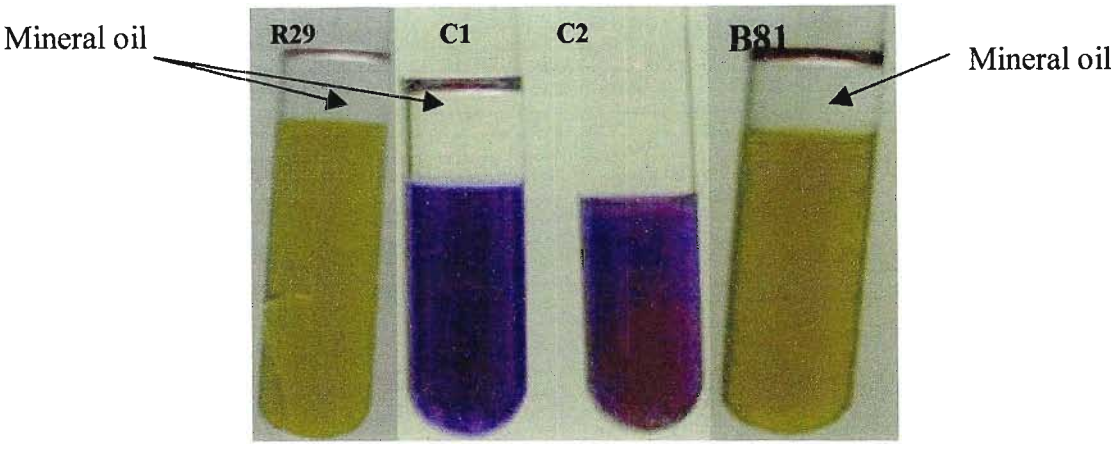

Figure 4.3 Bromocresol purple broth showing positive reactions of isolates R29 and B81. (Controls C1 and C2 with and without a mineral oil overlay, respectively).

4.6 HUGH-LEIFSON'S GLUCOSE BROTH

For both isolates the Hugh-Leifson's glucose broth changed from purple to yellow in colour (Figure 4.4). A positive reaction indicates that isolates can utilize glucose with the accumulation of acidic end products.

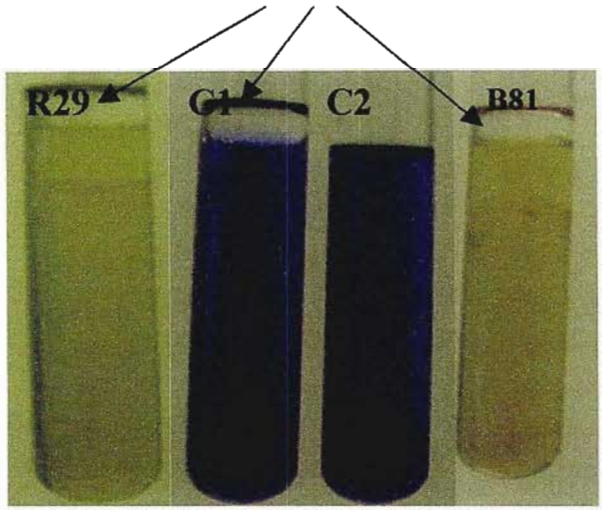

Figure 4.4 Hugh-Leifson's glucose broth showing positive reactions of isolates R29 and B81. (Control C1 and C2 with and without a mineral oil overlay, respectively).

4.7 GROWTH AT DIFFERENT NaCl CONCENTRATIONS

Figures 4.5 (a) and (b) show the growth of isolates R29 and B81 over a range of NaCl concentrations. Both isolates were able to grow at all concentrations of NaCl tested. At NaCl concentrations of 7 and 10% (w/v), B81 growth was markedly reduced and/or delayed over a 24-36hrs. period.

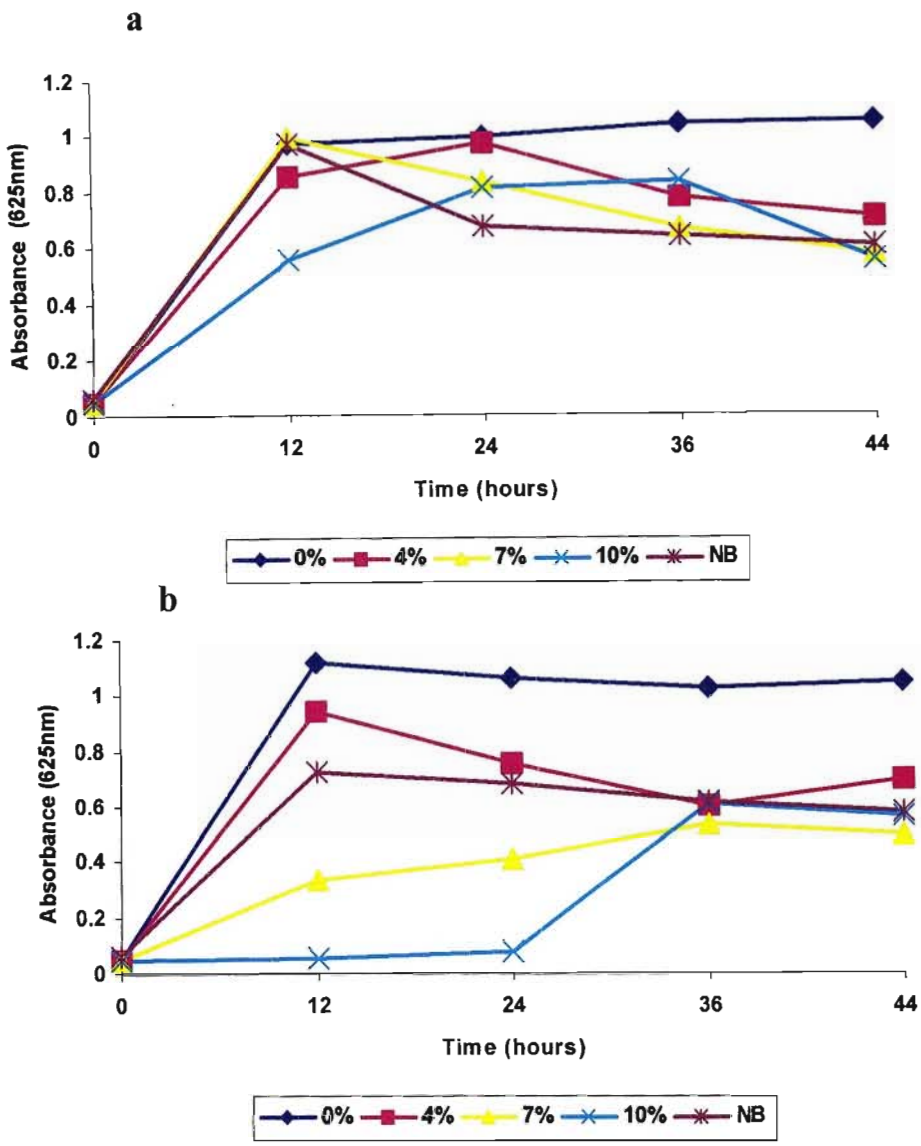

Figure 4.5 (a) and (b) Growth of isolates R29 (a) and B81 (b) over a range of different NaCl concentrations.

4.8 NITRATE REDUCTION AND DENITRIFICATION TEST

When the reagents and samples from the cultures were mixed, the solution turned a purple-red colour (Figure 4.6). This indicated that both R29 and B81 are able to reduce nitrate to nitrite. In addition, when the intensity of the purple-red colour reaction was measured at 540nm over a 6-day period, it was found that the intensity of the colour for isolate R29 peaked at 96hrs. and then decreased over the remainder of the experiment (Figure 4.7). For B81 the intensity of the colour

reaction increased or remained stable. This indicated that R29 was able to further reduce nitrite but that B81 was not.

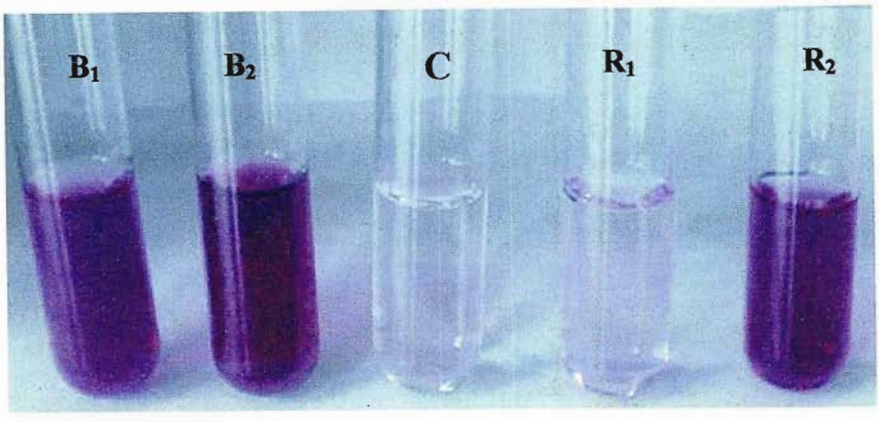

Figure 4.6 Nitrate reduction showing the positive reactions and intensity of color at different time intervals. Samples B_1 (B81) and R_1 (R29) are 24hrs older than samples B_2 (B81) and R_2 (R29), respectively. (C = control).

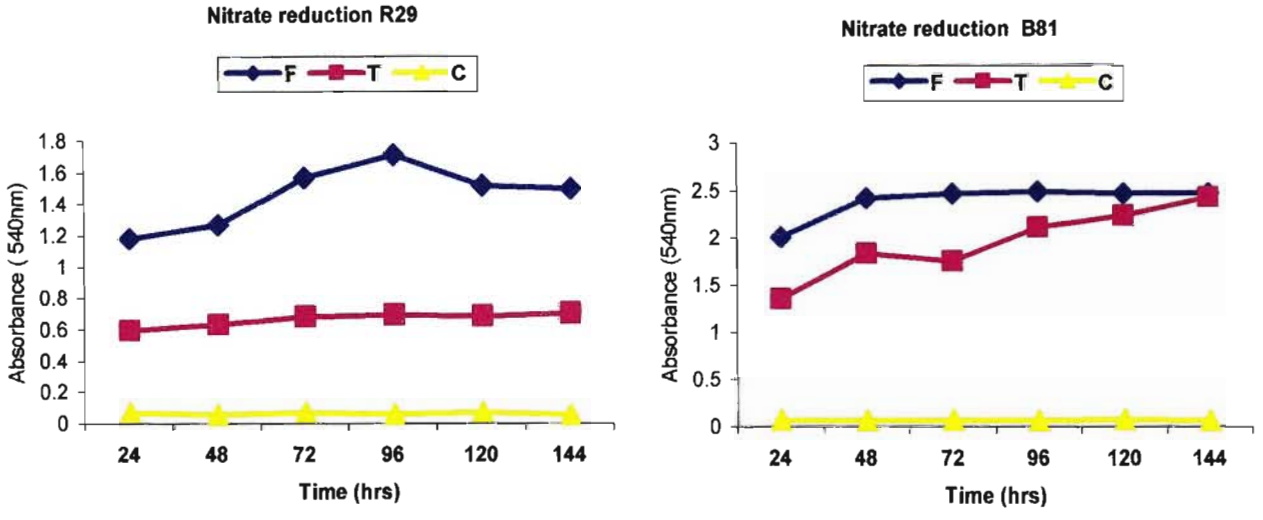

Figure 4.7 Colour intensity of nitrate reduction test over time (cultured in 100ml Erlenmeyer flasks (F) Test tube (T) control (C)).

4.9 STARCH HYDROLYSIS

After 48hrs incubation at 30^0C, the starch agar plates were flooded with iodine. Each plate turned a dark blue colour except in the region adjacent to the bacterial colonies of both R29 and B81

(Figure 4.8) indicating that they are positive for the production of amylase enzymes that hydrolyse starch.

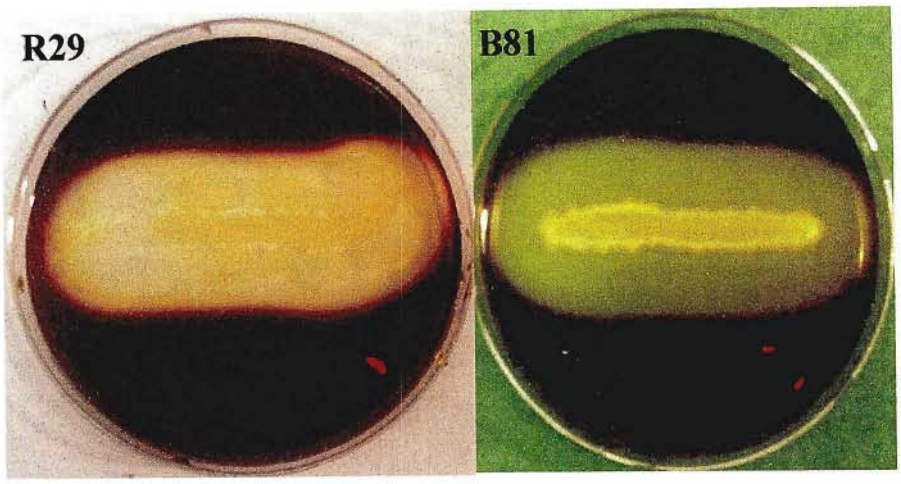

Figure 4.8 Iodine staining of nutrient agar-starch medium to detect starch hydrolysis by isolates R29 and B81.

4.10 CASEIN DECOMPOSITION TEST

Both R29 and B81 showed zones of clearing around their bacterial growth when grown on milk agar indicating a positive reaction for the decomposition of casein (Figure 4.9).

Figure 4.9 Milk agar showing zones of clearing indicating the hydrolysis of casein around the bacterial growth due to casease produced by isolates R29 and B81.

4.11 HINO-WILSON NITROGEN-FREE MEDIUM

Plate counts of R29 and B81 grown in Hino-Wilson nitrogen free medium showed that the CFU.ml^{-1} of the isolate B81 increased daily over the course of the experiment whereas R29 did not (Figure 4.10). This indicated that B81 was able to fix nitrogen but R29 was not.

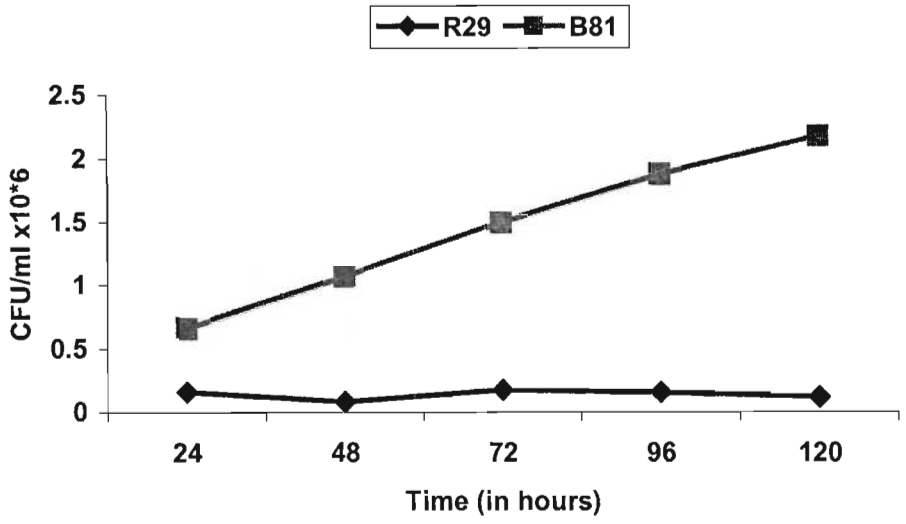

Figure 4.10 Growth of R29 and B81 in Hino-Wilson nitrogen free medium.

4.12 CAS SIDEROPHORE DETECTION AGAR MEDIUM

The CAS Siderophore detection agar media showed positive reactions for both isolates indicating the production of siderophores (Figure 4.11). The zones of clearing were more pronounced for R29 than B81. The size of these clear zone increased with time as bacterial growth increased.

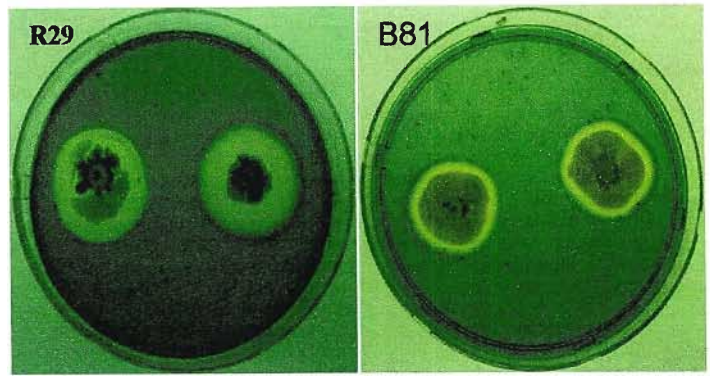

Figure 4.11 CAS agar cleared around the *Bacillus* isolates R29 and B81 indicating the production of siderophores.

4.13 CHITINASE ASSAY

Neither isolate exhibited an ability to grow on chitin agar indicating that they are unable to produce chitinase.

4.14 CELLULASE ASSAY

Both isolates exhibited an ability to degrade carboxymethyl cellulose (CMC) (Figure 4.12) but were unable to break down the Whatman No.1 filter paper (crystalline cellulose) agar.

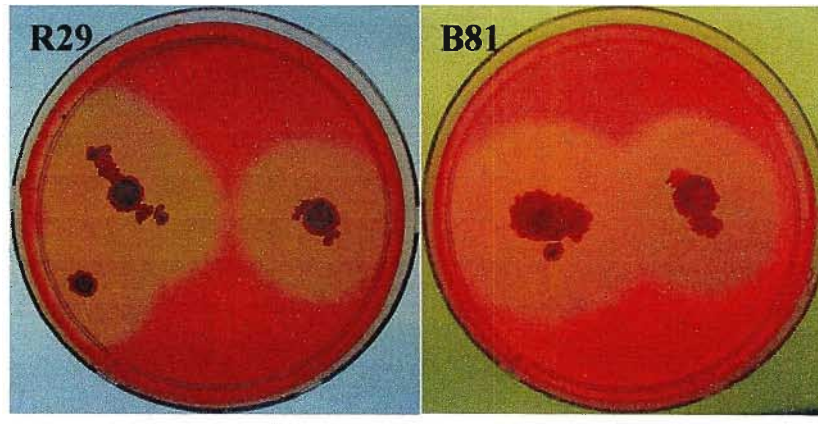

Figure 4.12 Zones of clearing indicating R29 and B81 ability to hydrolyse carboxymethy cellulose.

4.15 CELL DIMENSIONS OF R29 AND B81 CELLS

Using TEM to measure cell dimensions it was found that the *Bacillus* isolates R29 and B81 cells were 1.45-1.75μm and 1.28-1.9μm long and 0.61-0.78μm and 0.6-0.8μm wide, respectively. Phase contrast microscopy also showed that these *Bacillus* isolates both produced central ellipsoidal spores that do not swell the mother cell (Figure 4.13). The spore morphology and location within vegetative cells were characteristics of *Bacillus* species allotted to Group II as proposed by Priest (1993b).

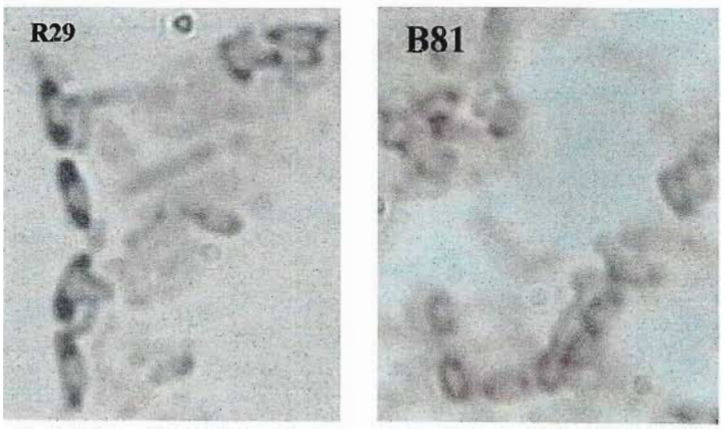

Figure 4.13 Spores of the *Bacillus* isolates R29 and B81 (1000x).

A summary of all the results is presented in Table 4.1.

Table 4.1 Results of the biochemical and nutritional tests of both *Bacillus* isolates R29 and B81

Tests	R29	B81
Gram stain	Positive	Positive
Spore stain	Positive	Positive
Spore shape	Ellipsoidal	Ellipsoidal
Spore position	Central	Central
Catalase test	Positive	Positive
Voges-Proskauer agar	Positive	Positive
Growth in Anaerobic agar	**Negative**	**Positive**
Growth at 45^0C	Positive	Positive
Growth at 50^0C	Negative	Negative
Growth at 60^0C	Negative	Negative
Growth in 0% NaCl	Positive	Positive
Growth in 4% NaCl	Positive	Positive
Growth in 7% NaCl	Positive	Positive
Growth in 10% NaCl	Positive	Positive
Acid and gas from glucose		
• Bromocresol purple broth		
-Acid production	Positive	Positive
-Gas production	–	–
• Hugh-Leifson's glucose broth		
- Acid production	Positive	Positive
- Gas production	–	–
Reduction of nitrate to nitrite	Positive	Positive
Reduction of Nitrite	**Positive**	**Negative**
Hydrolysis of starch	Positive	Positive
Cell dimensions: length rod	1.45-1.75μm	1.28-1.9μm
Cell dimension: width	0.61-0.78μm	0.6-0.8μm
Decomposition of casein	Positive	Positive
Siderophore production	Positive	Positive
Chitinase production	Negative	Negative
Cellulase test on CMC	Positive	Positive
Cellulase test on filter paper	Negative	Negative
Growth in nitrogen free medium	**Negative**	**Positive**

Tentative species identification of the two *Bacillus* isolates was carried out according to a simplified phenotypic key proposed by Slepecky and Hemphill (1992) (Appendix 4.1). Based on this, the results of the present study (Table 4.2) were used to identify the *Bacillus* isolates R29 and B81 as follows:

R29 showed positive reactions for catalase, the Voges-Proskauer test and starch hydrolysis but was not able to grow in anaerobic agar. Based on these R29 was identified as *B. subtilis* (Table 4.2).

Similarly, B81 showed positive catalase and Voges-Proskauer reactions and was able to grow in anaerobic agar and produce acid but not gas from glucose. B81 partially matched the phenotypic characteristics of *B. polymyxa*, *B. subtilis* and *B. licheniformis* according to the Key used (Appendix 4.1). However, *B. polymyxa* produces a terminal spore whereas *B. subtilis* and *B. licheniformis* produce a central ellipsoidal spore (Clause and Berkley, 1986). B81 was therefore tentatively identified as either a strain of *B. subtilis* or *B. licheniformis* (Table 4.2).

Table 4.2 Comparison of phenotypic characteristics of isolates R29 and B81 with *B. subtilis, B. polymyxa* and *B. licheniformis.*

		B. subtilis	**R29**	***B. polymyxa***	**B81**	***B. licheniformis***
1	Catalase	+	+	+	+	+
2	V-P reaction	+	+	+	+	+
3	Growth in anaerobic agar	-	-	+	+	+
4	Growth at 50^0C	+	-	-	-	+
5	Growth in 7% NaCl	+	+	-	+	+
6	Acid and gas in glucose	-	+	+	+	-
7	$N0_3$ reduced to NO_2	+	+	+	+	+
8	Starch hydrolysis	+	+	+	+	+
9	Growth at 65^0C	-	-	-	-	-
10	Rods1.um or wider	-	-	-	-	-
11	pH in V-P medium < 6.0	v	+	v	+	v
12	Acid from glucose	+	+	+	+	-
13	Hydrolysis of casein	+	+	+	+	+
	Character similarities		11/13		12/13	
	Spore shape	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
	Spore position	Central	Central	**Terminal**	Central	Central

* (+) means Positive reaction and (-) negative reaction, (v) variable characters.

DISCUSSION

The genus *Bacillus* comprises a heterogeneous group of bacteria with over 60 species being distinguished (Claus and Fritze, 1989; Priest, 1993b). They are widespread and can be recovered from almost every environment in the biosphere (Priest 1993a). Key features for identification of the genus include a Gram-positive stain, rod shaped cells an ability to grow aerobically and perhaps most significantly, an ability to produce resistant endospores. Isolate R29 and B81 both exhibited all these traits indicating that they indeed were members of the genus *Bacillus*.

Endospore shape, size and location within a bacterial cell have been used as criteria to further group *Bacillus*. *Bacillus* endospores are usually cylindrical, ellipsoidal, oval or round. Spores may be located at central, paracentral, subterminal, terminal or lateral positions within the sporangium (Claus and Berkley, 1986). During sporulation a mother cell may swell or retain its usual shape. In both instances R29 and B81 produced central ellipsoidal spores that do not swell the parental cell, suggesting that both isolates belong to Group II as proposed by Priest (1993b).

Phenotypic testing of both isolates showed that virtually all the test responses were similar with the exception that B81 exhibited an ability to grow anaerobically and in Hino-Wilson nitrogen free medium. Although both isolates were able to reduce nitrate, R29 exhibited an ablility to reduce nitrate completely, whereas B81 did not.

Matching the results with Slepecky and Hemphill's (1992) key resulted in a tentative identification of R29 as *B. subtilis*. This finding was further supported by morphological and phenotypic trait reports in Bergey's Manual of Systemic Bacteriology. B81 was initially identified as *B. polymxa* according to Slepecky and Hemphill (1992) but when checked with Bergey's Manual of Systemic Bacteriology it was found that *B. polymxa* could be excluded based on spore size and location and its inability to grow at different NaCl concentrations. Subsequently, B81 was tentatively identified as either a strain of *B. subtilis* or *B. licheniformis*.

Bacillus subtilis is a common soil resident. Although *B. subtilis* is generally regarded as aerobic, it can grow slowly and sporulate under strictly anaerobic conditions in complex media particularly if nitrate is present (Priest, 1993b). It is a chemoorganotrophy having the ability to oxidise a wide variety of organic compounds and has simple growth requirements. It can grow on

simple media containing glucose, or other simple sugars as carbon and energy source and inorganic nitrogen, provided there is an adequate supply of oxygen (Harwood and Archibald, 1990). Vegetative cells range in size from 2-3μm long and 0.7-0.8μm wide (Claus and Berkley, 1986). *Bacillus subtilis* spores are ellipsoidal and located in the center of a cell and do not swell the parental cell (Priest 1993b).

Bacillus licheniformis is prevalent in soil particularly in low nutrient soil (Priest, 1993b). *Bacillus licheniformis* is a facultative anaerobic bacterium. In the absence of oxygen, it can derive the energy needed for growth from fermentation of glucose, respiration of nitrate, or degradation of L-arginine (Broman *et al.*, 1978). It is a known producer of □-amylase (Ferrari *et al.*, 1993) and the peptide antibiotic bacitracin (Priest, 1998b). *Bacillus licheniformis* is 1.5-3μm long and 0.6-0.8μm wide. A *B. licheniformis* spore is central, ellipsoidal that does not swell the parental cell (Claus and Berkley, 1986).

Catalase activity is a taxonomic property of microorganisms that are capable of producing the enzyme catalase, which detoxifies hydrogen peroxide. Aerobic and facultative anaerobic bacteria produce hydrogen peroxide as a byproduct of respiratory metabolism. Hydrogen peroxide is a powerful and potentially harmful oxidizing agent. Catalase promotes the conversion of hydrogen peroxide to water and molecular oxygen and prevents the cell from harming itself (Alef and Nannipieri, 1995; Leboffe and Pierce, 1996). Usually the genus *Bacillus* is catalase positive (Holt *et al.*, 1994).

The bromocresol purple broth and Hugh-Leifson' glucose broth tests indicated that both *Bacillus* isolates are capable of fermenting glucose under aerobic and anaerobic conditions. These results correlate with those of Nakano and Zuber (1998) who showed that *B. subtilis* is capable of growing anaerobically using either nitrate or nitrite as a terminal electron acceptor or by fermentation. Although *B. subtilis* is regarded as a strict aerobe it grows weakly in the absence of oxygen with the production of acetoin and 2,3-butanediol from pyruvate via acetolactate (Priest, 1993a). Buchanan and Gibbons (1974) stated that *B. subtilis* ferments glucose without gas production whereas *B. polymyxa* ferments glucose with the production of gas.

The Voges-Proskauer test is used to identify bacteria that are able to ferment glucose to form stable acid end products. This test detects the precursor of 2,3-butanediol namely, acetoin; both substances always being found together. The reagents react with acetoin and oxidise it to diacetyl, which in turn reacts with the guanidine component of peptone to form a red color (Leboffe and Pierce, 1996). These three tests confirmed that both the isolates are capable of fermenting glucose, which is a characteristic feature of the genus *Bacillus*.

Though representatives of the genus show extraordinary metabolic diversity and include thermophilic, phychrophilic, alkalophilic and acidophilic members, the *Bacillus* isolates tested were not able to grow at 50^0C or above (Priest and Sharp, 1989; Bron, 1990). The isolates grew well at temperatures 25- 37^0C on nutrient agar indicating that they were mesophiles. Both isolates were also able to grow at NaCl concentrations as high as 10% (w/v). However, B81 growth was not as vigorous as R29 at the higher NaCl concentrations tested. Priest (1993a) reported that several species assigned to the *B. subtilis* group by rRNA sequence comparisons are halotolerant, but are associated with marine and estuarine habitats.

The ability of B81 to grow in the Hino-Wilson nitrogen free medium suggested that it was able to fix atmospheric nitrogen. Several *Bacillus* species such as *B. polymyxa*, *B. macerans*, and *B. azotofixans* fix nitrogen under anaerobic conditions (Slepecky and Hemphill, 1992; Priest 1993a and b). With the exception of *B. polymyxa* none of these showed phenotypic characteristics similar to B81. *Bacillus subtilis* has not been previously reported as being able to fix nitrogen and therefore this result should be viewed with caution and requires further confirmation.

Both isolates were able to utilize starch as a carbon source indicating that they were able to produce extracellular amylases. Production of extracellular enzymes such as amylase is a characteristic feature of the genus *Bacillus,* particularly members of Group II.

Both isolates were able to grow on soluble carboxymethyl cellulose (CMC) but not on crystalline cellulose. Ferrarri *et al.* (1993) found that strains of *B. subtilis* produced endoglucanases, which enable them to hydrolyse CMC but not crystalline cellulose. Górska *et al.* (2001) isolated a *B. polymyxa*, strain that was able to degrade both CMC and crystalline celluloses.

A number of reports in the literature indicate that some *Bacillus* spp. produces chitinase, which degrades chitin, a major structural component of fungal cell walls (Podile and Prakash, 1996). However, isolates R29 and B81 were not able to degrade and utilize colloidal chitin.

Both isolates exhibited siderophore producing abilities. Grossman *et al.* (1993) reported that in response to iron deprivation, *B. subtilis* secretes a catecholic siderophore, 2,3-dihydroxybenzoyl glycine. Schwyn and Neilands (1987) also stated that bacteria express a high affinity uptake system only up to a level that satisfies their requirements for the metal. In addition, the production of siderophores and the proteins required for their uptake are tightly regulated in order to avoid energy wastage and accumulation of iron that can be toxic to the cells (Braun, (1997) cited Cornelis and Matthijs, (2002)).

Phenotypic identification of the *Bacillus* isolates is not clear-cut. In many instances it is difficult to distinguish, closely related species. In the case of *B. subtilis* there appears to be numerous strains exhibiting varying traits which complicate matters further (Nakamura *et al.*, 1999). Our findings suggested that R29 is a strain of *B. subtilis* whereas the identification of B81 is more difficult to assign to a specific group or species of *Bacillus*. Possible candidates include a *B. subtilis* and *B. licheniformis*.

Further confirmation of the findings can be achieved using methods such as sequencing of the 16s rDNA, which will be addressed in chapter 5.

CHAPTER 5

DNA EXTRACTION, PCR AMPLIFICATION, SEQUENCING AND IDENTIFCATION OF *BACILLUS* ISOLATES R29 AND B81

RESULTS

Genomic DNA from isolates R29 and B81 was successfully extracted using NucleoSpin DNA Extraction Kits. UV illumination of the ethidium bromide stained agarose gel (0.8%) (w/v) revealed that single bands were obtained from each sample (Figure 5.1). Spectrophotometer readings of each DNA sample at 260nm and 280nm, were made to determine sample purity and estimate DNA concentrations (Table 5.1). In a pure DNA sample the A_{260}/A_{280} ratio should be a value close to 2. If the ratio is below 1.6 it indicates that protein or other UV absorbing material is present (Bron, 1990; Linacero *et al.*, 1998). Based on this, the purity of the DNA sample of isolate R29 was satisfactory whereas that of B81 was not.

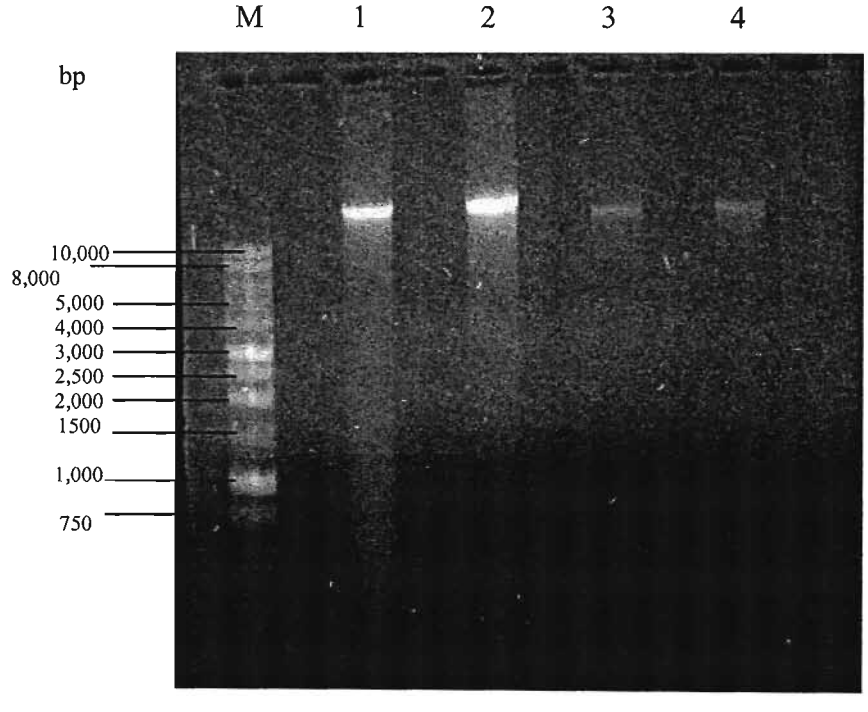

Figure 5.1 Agarose gel electrophoresis showing single DNA bands extracted from isolates R29 (lanes 3 and 4) and B81 (lanes 1and 2) (M = DNA molecular weight marker 1kb DNA ladder).

Table 5.1 Spectrophotometer readings at 260nm and 280nm used to determine purity and concentration of DNA samples extracted from isolate R29 and B81

	A_{260}	A_{280}	Purity of DNA	Concentration of DNA μg/μl
Blank	0	0		
R29	0.258	0.135	1.911	0.258
B81	0.570	0.433	1.316	0.57

The results of PCR amplification using 16s rDNA universal primers, is shown in Figure 5.2. Both isolates produced an intensely stained band approximately 200bp in size. This correlated well to the amplicon size anticipated for the primer set used in this study. In addition, both samples also exhibited a faint band that was slightly larger than the 400bp marker (Figure 5.2). This band was considered to be an anomaly originating during PCR and was attributed to annealing times that were too short. As a result of this anomalous band, the band corresponding to the PCR amplicon was excised and eluted directly from the agarose gel before being sent for sequencing. The purity and concentration of the excised DNA is shown in Table 5.2 and Figure 5.3.

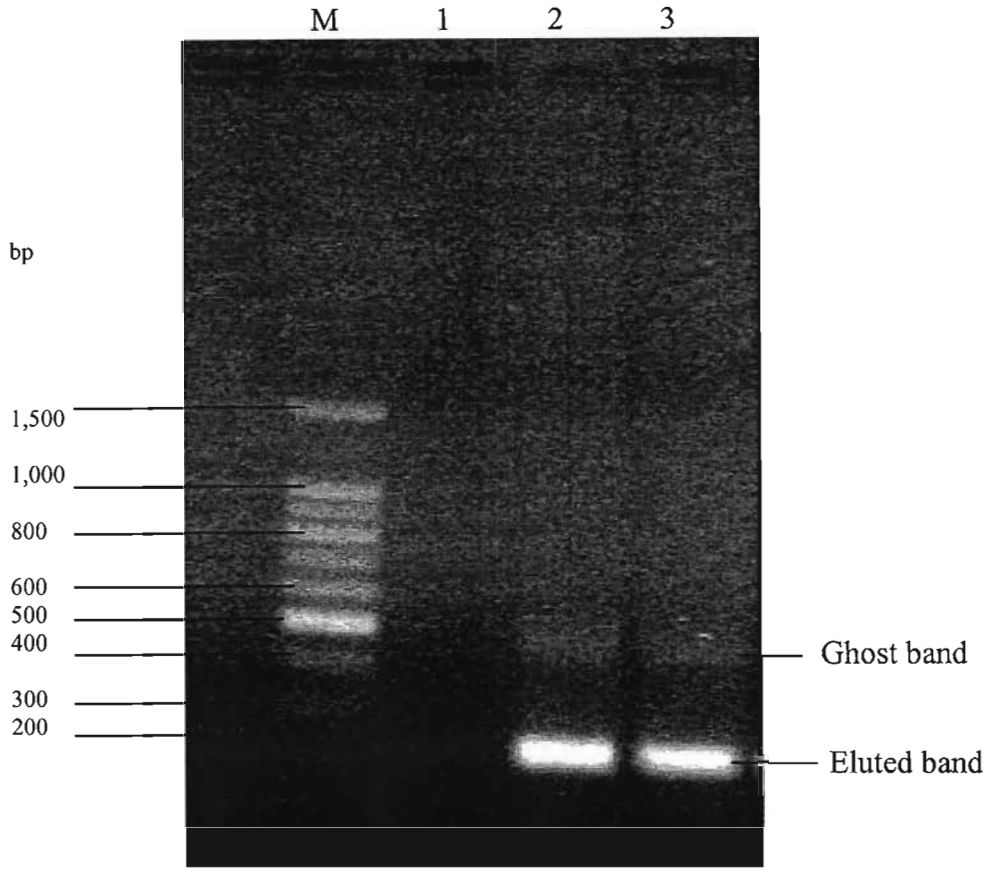

Figure 5.2 Agarose gel electrophoresis showing the PCR amplified DNA of B81 (lane 2) and R29 (lane 3). M=DNA molecular weight marker 100bp, lane 1:negative control.

Table 5.2 Spectrophotometer readings at 260nm and 280nm used to determine purity and concentration of the eluted DNA samples

	A_{260}	A_{280}	Purity of DNA	Concentration of DNA μg/μl
Blank	0	0		
R29	0.264	0.14	1.886	0.264
B81	0.263	0.138	1.906	0.263

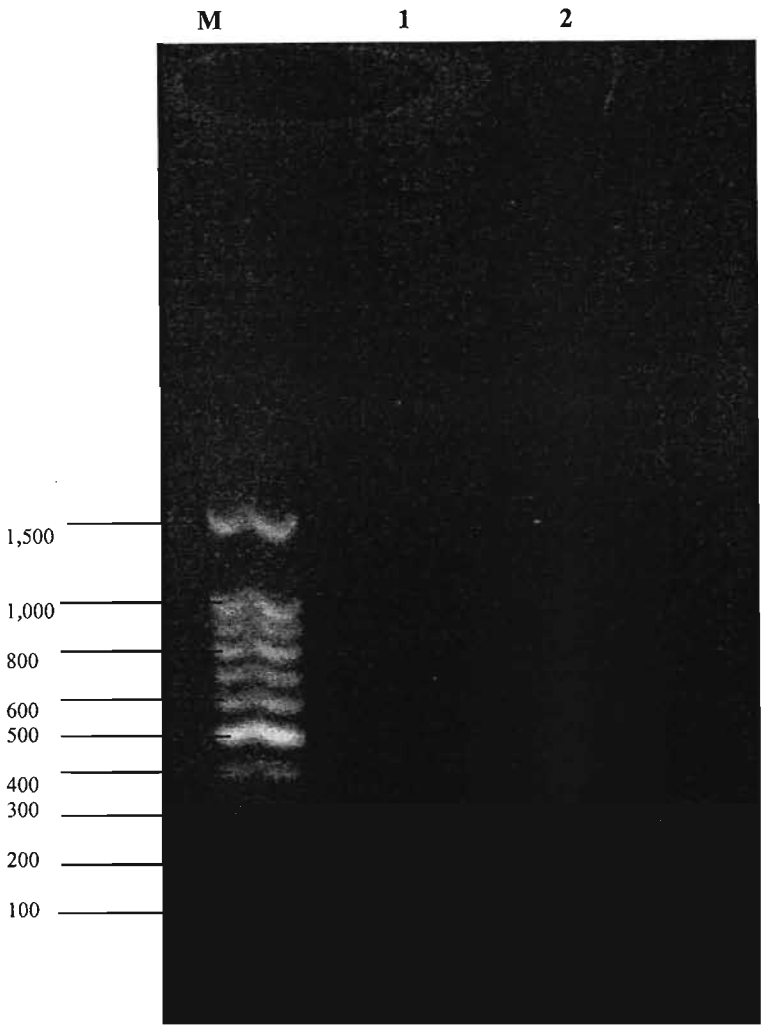

Figure 5.3 Agarose gel electrophoresis showing single DNA bands from PCR amplified and gel eluted products. M= DNA molecular weight marker (100bp), DNA samples of R29 (lane 1) and B81 (lane 2).

Analysis of the PCR amplification revealed sequences 176 nucleotides long for each isolate. Both had high levels of homology to one another, exhibiting only a three nucleotide difference (Figure 5.4).

R29

TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GT**G**CCGTTC**A**AATAGGGCGG**C**ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
CTACGTG

B81

TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GT**A**CCGTTC**G**AATAGGGCGG**T**ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
CTACGTG

Figure 5.4 Nucleotide sequences of 176 basal pair amplicon derived from isolates R29 and B81. Nucleotides, which are underlined and in bold, indicate the differences between the two sequences.

Homology testing of the consensus sequences against the GenBank database were submitted through BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi). Isolate R29, revealed 100% sequence homologies with 100 bacteria. Of these 93% belonged to the genus *Bacillus.* Nineteen were *B. subtilis,* followed by *B. amyloliquefaciens* which had 11 hits, *B. velesensis* 3 hits and *B. atrophaeus* 2 hits. *Bacillus licheniformis, B. vallismortis,* and *B. vietnamensis* each had 1 hit each. The remainders were unassigned *Bacillus* spp. (Table 5.3). Similarly out of 110 bacteria which revealed 100% sequence homologies with isolate B81, 109 (99%) are *Bacillus,* of which 85 (78%) are *B. subtilis,* followed by *B. mojavensis* (4 hits), *B. licheniformis* (2 hits) and one hit each for the remainder (Table 5.4).

Table 5.3 Taxonomy Report of BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi) for isolate R29 (accessed on July 9, 2004)

Bacteria	100 hits	66 orgs	[root; cellular organisms]
. Bacillales	95 hits	61 orgs	[Firmicutes; Bacilli]
. . **Bacillus**	**93 hits**	**59 orgs**	**[Bacillaceae]**
. . . **Bacillus subtilis**	**18 hits**	**2 orgs**	
. . . . **Bacillus subtilis subsp. endophyticus**	**1 hits**	**1 orgs**	
. . . **Bacillus amyloliquefaciens**	**11 hits**	**1 orgs**	
. . . Bacillus sp. B1(XC)	1 hits	1 orgs	
. . . Bacillus sp. CGTase	1 hits	1 orgs	
. . . **Bacillus velesensis**	**3 hits**	**1 orgs**	
. . . Bacillus sp. WL-3	1 hits	1 orgs	
. . . Bacillus sp. YNPRH8P-2	1 hits	1 orgs	
. . . Bacillus sp. Ni57	1 hits	1 orgs	
. . . Bacillus sp. Ni56	1 hits	1 orgs	
. . . Bacillus sp. Ni54	1 hits	1 orgs	
. . . Bacillus sp. Ni53	1 hits	1 orgs	
. . . Bacillus sp. Ni52	1 hits	1 orgs	
. . . Bacillus sp. Ni51	1 hits	1 orgs	
. . . Bacillus sp. Ni50	1 hits	1 orgs	
. . . Bacillus sp. Ni49	1 hits	1 orgs	
. . . Bacillus sp. Ni48	1 hits	1 orgs	
. . . Bacillus sp. Ni45	1 hits	1 orgs	
. . . Bacillus sp. Ni41	1 hits	1 orgs	
. . . Bacillus sp. Ni34	1 hits	1 orgs	
. . . Bacillus sp. Ni31	1 hits	1 orgs	
. . . Bacillus sp. Ni26	1 hits	1 orgs	
. . . Bacillus sp. Ni25	1 hits	1 orgs	
. . . Bacillus sp. Ni23	1 hits	1 orgs	
. . . Bacillus sp. Ni20	1 hits	1 orgs	
. . . Bacillus sp. Ni19	1 hits	1 orgs	
. . . Bacillus sp. Ni18	1 hits	1 orgs	
. . . Bacillus sp. Ni17	1 hits	1 orgs	
. . . Bacillus sp. Ni16	1 hits	1 orgs	
. . . Bacillus sp. Ni15	1 hits	1 orgs	
. . . Bacillus sp. Ni14	1 hits	1 orgs	
. . . Bacillus sp. Ni13	1 hits	1 orgs	
. . . Bacillus sp. Ni11	1 hits	1 orgs	
. . . Bacillus sp. Ni10	1 hits	1 orgs	
. . . Bacillus sp. Ni9	1 hits	1 orgs	
. . . Bacillus sp. Ni7	1 hits	1 orgs	
. . . Bacillus sp. Ni4	1 hits	1 orgs	
. . . Bacillus sp. Ni2	1 hits	1 orgs	
. . . Bacillus sp. Ni1	1 hits	1 orgs	
. . . Bacillus sp. SG-1	1 hits	1 orgs	
. . . Bacillus sp. SD-18	1 hits	1 orgs	
. . . Bacillus sp. MB-12	1 hits	1 orgs	
. . . Bacillus sp. EBI3	1 hits	1 orgs	
. . . Bacillus sp. BBK-1	1 hits	1 orgs	
. . . Bacillus sp. Bch1	1 hits	1 orgs	
. . . Bacillus sp. 5/117	1 hits	1 orgs	
. . . Bacillus sp. 2/117	1 hits	1 orgs	
. . . Bacillus sp. BAC2	1 hits	1 orgs	
. . . **Bacillus licheniformis**	**1 hits**	**1 orgs**	
. . . Bacillus sp. 'Mali 14'	1 hits	1 orgs	
. . . Bacillus sp. AH-E-1	1 hits	1 orgs	
. . . Bacillus sp. PP19-H3	1 hits	1 orgs	
. . . Bacillus sp.	6 hits	1 orgs	
. . . **Bacillus vallismortis**	**1 hits**	**1 orgs**	
. . . **Bacillus atrophaeus**	**2 hits**	**1 orgs**	
. . . Bacillus sp. SXQ-2004	1 hits	1 orgs	
. . . Bacillus sp. Ni43	1 hits	1 orgs	
. . . Bacillus sp. Ni42	1 hits	1 orgs	
. . . **Bacillus vietnamensis**	1 hits	1 orgs	
. . Paenibacillus popilliae	1 hits	1 orgs	[Paenibacillaceae; Paenibacillus]
. . marine bacterium SIMO-1598	1 hits	1 orgs	[unclassified Bacillales; unclassified Bacillales (miscellaneous)]
. unclassified Bacteria (miscellaneous)	5 hits	5 orgs	[unclassified Bacteria]

. . soil bacterium is02	1 hits	1 orgs
. . bacterium Te90R	1 hits	1 orgs
. . bacterium Te70R	1 hits	1 orgs
. . bacterium Te41R	1 hits	1 orgs
. . bacterium Te22R	1 hits	1 orgs

Table 5.4 Taxonomy Report of BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi) for isolate B81 (accessed on July 9, 2004)

Bacteria	110 hits	34 orgs	[root; cellular organisms]
. Bacillus	**109 hits**	**33 orgs**	**[Firmicutes; Bacilli; Bacillales; Bacillaceae]**
. . Bacillus subtilis	**75 hits**	**3 orgs**	
. . . Bacillus subtilis subsp. subtilis str. 168	**9 hits**	**1 orgs**	**[Bacillus subtilis subsp. subtilis]**
. . . Bacillus subtilis subsp. spizizenii	**1 hits**	**1 orgs**	
. . Bacillus mojavensis	**4 hits**	**1 orgs**	
. . Bacillus sp. ROO2A	1 hits	1 orgs	
. . Bacillus sp. 63	1 hits	1 orgs	
. . Bacillus aquimaris	1 hits	1 orgs	
. . Bacillus sp. CJ11043	1 hits	1 orgs	
. . Bacillus axarquiensis	1 hits	1 orgs	
. . Bacillus malacitensis	1 hits	1 orgs	
. . unclassified Bacillus	**3 hits**	**3 orgs**	
. . . marine bacterium SIMO IS-S74-237	1 hits	1 orgs	
. . . marine bacterium SIMO IS-574-241	1 hits	1 orgs	
. . . PAH-contaminated sludge bacterium PB-21	1 hits	1 orgs	
. . Bacillus sp. RZ-256	1 hits	1 orgs	
. . Bacillus sp. RZ-254	1 hits	1 orgs	
. . Bacillus sp. MB23	1 hits	1 orgs	
. . Bacillus licheniformis	**2 hits**	**1 orgs**	
. . Bacillus sp. Ni35	1 hits	1 orgs	
. . Bacillus sp. Ni29	1 hits	1 orgs	
. . Bacillus sp. Ni21	1 hits	1 orgs	
. . Bacillus sp. A4-7-1-2	1 hits	1 orgs	
. . Bacillus sp. A4-7-1-1	1 hits	1 orgs	
. . Bacillus sp. TUT1010	1 hits	1 orgs	
. . Bacillus sp. VAN14	1 hits	1 orgs	
. . Bacillus sp. CH10-1	1 hits	1 orgs	
. . Bacillus sp. CH7-1	1 hits	1 orgs	
. . Bacillus sp. CH20-1	1 hits	1 orgs	
. . Bacillus sp. CH19-3	1 hits	1 orgs	
. . Bacillus sp. CH15-2	1 hits	1 orgs	
. . Bacillus sp. CH4-5	1 hits	1 orgs	
. . Bacillus sp. CH4-4	1 hits	1 orgs	
. . Bacillus carboniphilus	**1 hits**	**1 orgs**	
. . Bacillus sp.	1 hits	1 orgs	
. Brevibacterium halotolerans	1 hits	1 orgs	[Actinobacteria]

DISCUSSION

Classification systems for microorganisms have historically been based on the existence of observable characteristics. However, because of the increasing numbers of species being identified and the limitations in the discriminatory power of these characteristics, classification and identification of microorganisms has more recently involved comparison of genetic traits (Holt *et al.*, 1994; Harmsen *et al.*, 2001). Modern classification and identification of microorganisms is based on comparative sequence analysis of the 16s rDNA base composition (Jonson, 1989). To achieve final and consensus classification, both the phenotypic and genotypic techniques are ideally combined (Gillis *et al.*, 1989).

Ribosomal DNA (rDNA), which codes for the RNA components of ribosomes, is particularly suited for identification purposes since it is ubiquitous to all living organisms. Ribosomal DNA occurs as multicopy genes, making its detection relatively easy. Additionally, rDNA is composed of conserved, variable, and highly variable regions so that probe or primer sets may be designed to meet a desired level of specificity (Schlötterer, 2001; Harmsen *et al.*, 2001). The ubiquity and conservation of rDNA sequences has made these DNA regions important tools for reconstructing phylogenetic relationships (Schlötterer, 2001). PCR can be employed to amplify the genes encoding 16s rRNA from genomic DNA, which is then used for sequencing and homology testing (Madigan *et al.*, 2003).

Although sequencing of the 16s rRNA gene is widely used for modern bacterial classification, it has some shortcomings in that it shows limited variation among closely related taxa (Fox *et al.*, 1992). Protein-coding genes, on the other hand, exhibit much higher genetic variation and potentially can be used for the classification and identification of closely related taxa (Yamamoto *et al.*, 1999). Chun and Bae (2000) recommended that amplification and sequencing of partial gyrA genes are useful for the rapid identification of *B. subtilis* and allied taxa, especially organisms in these taxa which cannot be differentiated by using conventional phenotypic tests and 16s rRNA analysis.

$A_{260/280}$ ratio indicated that the purity of DNA extracted from R29 was satisfactory, whereas B81 appeared to be contaminated with impurities such as protein (Boyer, 1993). Subsequent elution of

PCR amplicons directly from agarose gel resulted in DNA samples from both isolates which were of a high purity and suitable for sequencing.

Sequencing of the 176 bp amplicon of isolate R29 revealed 100% sequence homologies with 100 bacterial isolates. Of these 93% belonged to the genus *Bacillus.* Nineteen isolates were identified as *B. subtilis,* followed by *B. amyloliquefaciens* with 11 hits, *B. velesensis* with 3 hits, *B. atrophaeus* with 2 hits and *B. vallismortis*, *B. licheniformis* and *B. vietnamensis* with only one hit each. The remaining isolates were unidentified *Bacillus* spp.

Based on the phenotypic identification in Chapter 4, it was suggested that isolate R29 is a strain of *B. subtilis*. The results from DNA sequence homology testing supports this finding but was not conclusive because several species were also represented. *Bacillus amyloliquefaciens, B. vallismortis* and *B. atrophaeus* are considered to be closely related to *B. subtilis* (Prescott *et al.,* 1999; Roberts *et al.,* 1996). Phenotypically they are similar to *B. subtilis* and it is not always possible to separate these organisms solely on the basis of classical testing. *Bacillus licheniformis* could be excluded based on phenotypic differences, primarily, that isolates R29 did not exhibit an ability to grow under anaerobic conditions.

DNA from strains of *B. amyloliquefaciens* have consistently been found to share less than 25% homology with DNA from strains of *B. subtilis*. Within a species, strains should share at least 50 to 60% DNA homology. The 25% DNA homology between *B. subtilis* and *B. amyloliquefaciens* is too low for these two organisms to be considered a single species (Priest *et al.*, 1987). *B. amyloliquefaciens* strains can be distinguished from *B. subtilis* strains by the inability of most strains to hydrolyze DNA and pectin, failure of the organism to produce acid from inulin and the formation of long chains of cells in most *B. amyloliquefaciens* strains (Logan and Berkeley, 1984). *B. atrophaeus* can be distinguished phenotypically from *B. subtilis* by its pigmentation (Nakamura, 1989). Roberts *et al.* (1994, 1996) introduced two new species similar to *B. subtilis* namely *B. mojavensis* and *B. vallismortis.* These species can only be differentiated from the *B. subtilis* type strain using fatty acid composition analysis, restriction digest analysis, genetic transformation and DNA-DNA hybridization data and not by phenotypic differences (Roberts *et al.*, 1996; Chun and Bae, 2000).

Phenotypic identification of isolate B81 in the previous chapter was more difficult to assign to a specific group or species of *Bacillus*. Possible candidates were *B. subtilis* and *B. licheniformis*. The DNA sequence homology test for isolate B81 showed 100% homology with 110 bacterial isolates, of which 109 (99%) belonged to the genus *Bacillus* and of these 78% were *B. subtilis* isolates. *Bacillus mojavensis* had 4 hits and *B. licheniformis* had 2 hits and the remaining isolates were unidentified *Bacillus* spp. Whilst inconclusive, the results for B81 obtained from sequencing analysis validated the tentative phenotypic identification. Although *B. subtilis* and *B. licheniformis* are distantly related, they can be distinguished by only five phenotypic traits (Logan and Berkeley, 1984).

It is known that closely related taxa show limited variation on their 16s rRNA gene sequence making it difficult for their identification. The fact that both isolates showed high levels of homology (98.3%) for each 176bp DNA fragment indicates that the region of the 16s rDNA amplified was not sufficiently varied or that the DNA fragment was too small to provide definitive information with regard to identifying the isolates.

To make a more reliable identification of the isolates the following potions are recommended; optimization of PCR amplification using different primers that can amplify larger DNA fragments for sequencing and homology testing through BLAST. In addition, partial gyrA nucleotide sequencing, translated amino acid sequences tests, DNA-DNA hybridization are modern identification methods, which can be applied (Chun and Bae, 2000). Fatty acid composition and DNA hydrolysis tests are some of the phenotypic tests that can also be recommended.

CHAPTER 6

EXTRACTION AND PARTIAL CHARACTERISATIONS OF THE ANTIFUNGAL METABOLITES

RESULTS

Bacillus isolate R29 was selected for further characterization of the antifungal metabolite produced when it was cultured in a defined synthetic medium. Findings with regard to production, extraction and characterisation of the antifungal metabolite are presented as follows.

6.1 ANTIFUNGAL METABOLITE PRODUCTION ASSAY FROM CELL FREE SYNTHETIC CULTURE

Activity tests of the filter-sterilised, cell-free supernatants revealed evidence of antifungal activity although zones of inhibition were small. Confirmation of antifungal activity was achieved by concentrating samples by freeze-drying, followed by re-suspension in a small volume of sterilized distilled water before testing.

Subsequently, growth curve studies (Figure 6.1a) were performed to find out when the antifungal compounds were produced in the culture medium. Samples were taken intermittently during the growth cycle and assessed for antifungal activity. Samples taken from day four onwards, inhibited *Rhizoctonia solani* in subsequent assays but samples taken from day 1-3 did not. It was also observed that the colour of the culture medium changed from an opaque white to a dark coca cola colour during the stationary phase. Based on these results all further cultures were incubated for at least 4-5 days to ensure production of antifungal metabolites before extraction and purification of the active compound/s was attempted.

pH measurements showed that pH increased to maximum of 7.5 in the R29 culture after $\pm$ 60h and then dropped to pH 6.0 by the end of the experiment (Figure 6.1b).

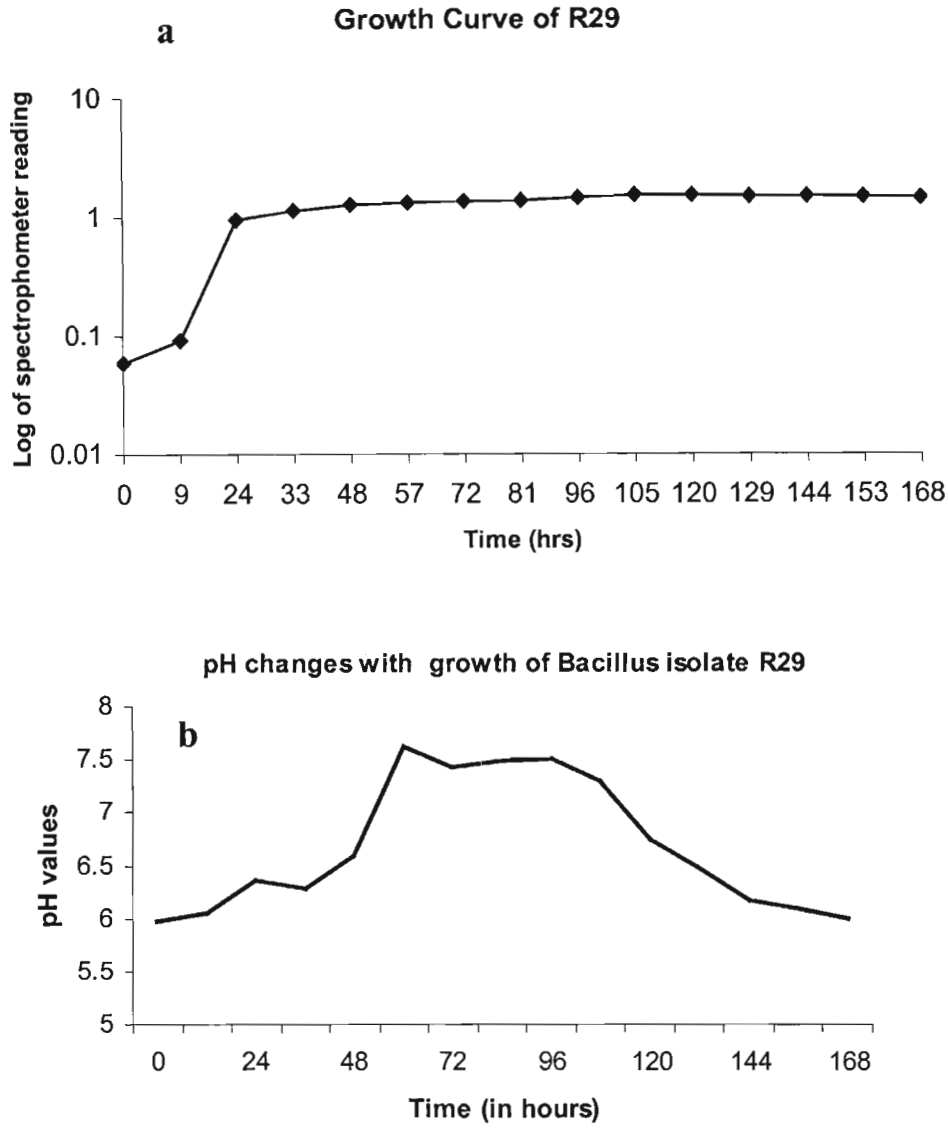

Figure 6.1 (a) Growth curve of *Bacillus* sp. R29 cultured in a defined synthetic medium (**b**) pH changes during growth.

6.2 ISOLATION OF ANTIFUNGAL METABOLITE ON THIN LAYER CHROMATOGRAPHY (TLC)

Of all the solvents tested for use in TLC, n-propanol:water (70:30, v/v) was found to give the best separation and resolution of component bands of the concentrated supernatant. Two bands were resolved with Rf values of 0.636 and 0.736, respectively which were visualized clearly under UV light.

6.2.1 Bioautography Assay

Attempts to use a bioautography assay to assess antifungal activity proved to be unsuccessful. The test organism *R. solani* completely overgrew the agar plate without any evidence of inhibition.

6.2.2 Bioautography Overlay Assay

When the TLC plate was overlaid with agar and screened for antifungal activity, the region corresponding to the band with a Rf value of 0.636 inhibited growth of *R. solani.*

6.2.3 Elution and Assay of the Active Compounds from the TLC

Zones on the developed TLC plate corresponding to resolved bands and adjacent regions were scraped from the TLC plate, eluted with 80% ethanol and bioassayed for antifungal activity. Only the band with a Rf value of 0.636 showed antifungal activity. The active compound was eluted three times from the silica and the extraction efficiently assessed by means of a plate bioassay. The majority of the active compound was extracted after two elutions (Figure 6.2).

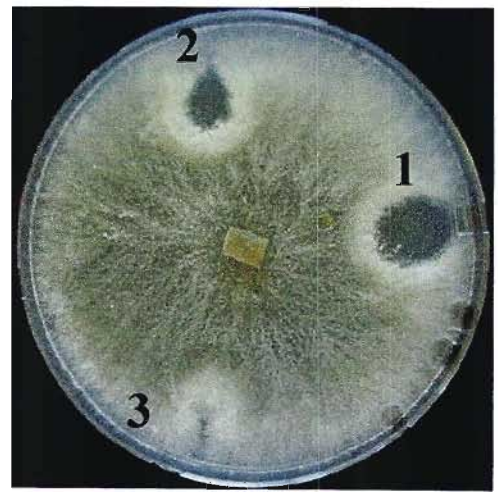

Figure 6.2 Plate bioassay showing antifungal activity of TLC resolved band (Rf 0.636) after elution with 80% ethanol (**1**: first elution, **2**: second elution and **3**: third elution).

In order to confirm whether the active band on the TLC is a single compound or not, the eluted active metabolite was subjected to two-dimensional TLC using different solvents. No further separation of the active band into two or more bands (compounds) was achieved.

The separation and extraction of antifungal metabolites by TLC proved to be costly, time consuming and impractical for extraction of the active compound from large volumes of supernatant. To avoid the shortcomings of TLC, other extraction methods were evaluated.

6.3 ACID PRECIPITATION AND EXTRACTION OF ANTIFUNGAL COMPOUND/S BY RE-SUSPENDING IN 80% ETHANOL

When the cell free supernatant was adjusted to pH 2.5 an insoluble fraction resulted which was removed from the supernatant by centrifugation. The precipitate was then resuspended in 80% ethanol but only partial dissolution was achieved. A further centrifugation step was introduced to remove the insoluble fraction from the 80% ethanol. This precipitate was successfully resuspended in sodium phosphate buffer.

When the ethanol-dissolved fraction was evaporated with a rotary vacuum evaporator, two fractions were discerned, i.e. a white-yellow residue, which accumulated on the walls of the evaporating bottle and a dark liquid concentrate, which accumulated at the bottom of the bottle. The liquid concentrate was collected separately and then divided into two containers. One part was re-adjusted to pH 7.4 and the other left as is. The residue was resuspended in sodium phosphate buffer which cause of this solution to turn yellow. Activity tests were made on all four fractions mentioned above as well as on the original supernatant the extracted supernatant, after re-adjusting the pH to 7.4. The results of the antifungal bioassay are presented in Table 6.1 and Figure 6.3.

Table 6.1 Relative antifungal activities of the different components arising from the pH adjusted culture supernatant

	Test growth agar media inhibition zones (mm)	
Sample	V8	1/2PDA
White-yellow residue	10	10
Dark liquid concentrate	8	8
Dark liquid concentrate after adjusting pH to 7.4	8	8
80% ethanol insoluble precipitate	-	-
Extracted supernatant (pH 7.4)	-	-
0.1M sodium phosphate buffer (pH 7.4)	-	-
80% ethanol	-	-
Un-extracted supernatant	6	7

These results revealed that the precipitated fraction which subsequently dissolved in 80% ethanol contained the active compound whereas the 80% ethanol insoluble fraction did not. The extracted supernatant lost its antifungal properties compared to the untreated cell-free supernatant. In addition, the sodium phosphate buffer and 80% ethanol did not affect the growth of the test organism *R. solani*.

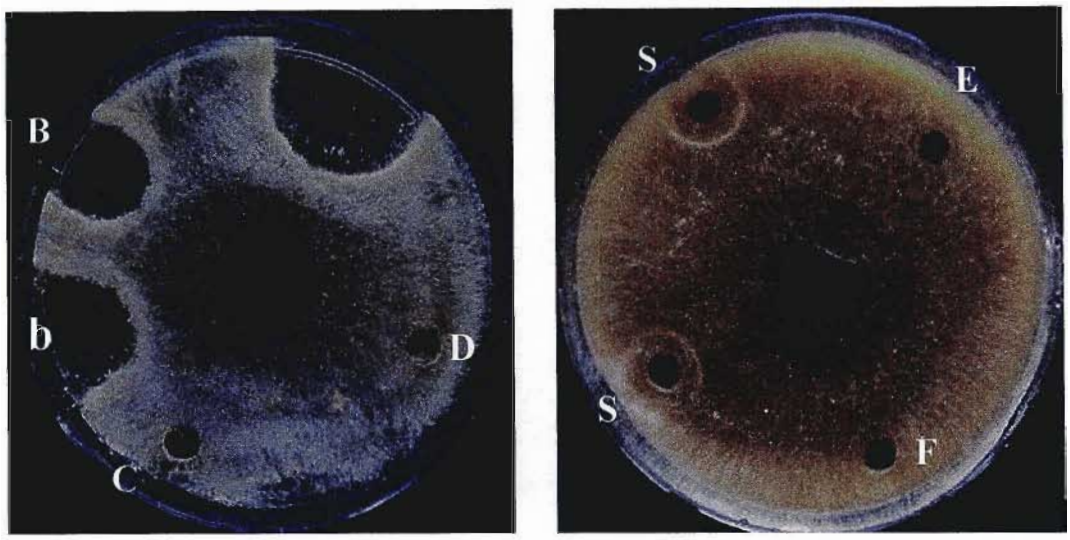

Figure 6.3 Antifungal bioassay showing activity of the different components of pH-80% ethanol extracted samples. **A**: the white-yellow residue part extracted from the 80% ethanol supernatant after evaporation, **B**: the dark liquid concentrate extracted from the 80% ethanol supernatant after evaporation, **b**: the dark liquid concentrate after adjusting pH, **C**: 80% ethanol insoluble precipitate, **D**: the extracted supernatant after adjusting pH, **E**: sodium phosphate buffer, **F**: 80% ethanol and **S**: un-extracted supernatant.

6.4 EXTRACTION USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY: AMBERLITE XAD-4 RESIN

Once the supernatant was passed through an Amberlite XAD-4 resin column, the column was eluted with a series of methanol solutions of increasing purity. Elution with 20% and 40% methanol resulted in pigmented compounds being washed from the column. Upon testing, these fractions did not show any antifungal activity. Methanol eluents, 60%, 80% and 95%, were collected separately and tested for activity after evaporating off the methanol. Of these, only the supernatant, the 80% and 95% eluted samples showed low levels of activity against *R. solani,* suggesting that the active compound/s was not fully extracted. Subsequently, the column was eluted with 95% methanol and this fraction was successfully tested for activity against *R. solani* after evaporating off the methanol (Figure 6.4).

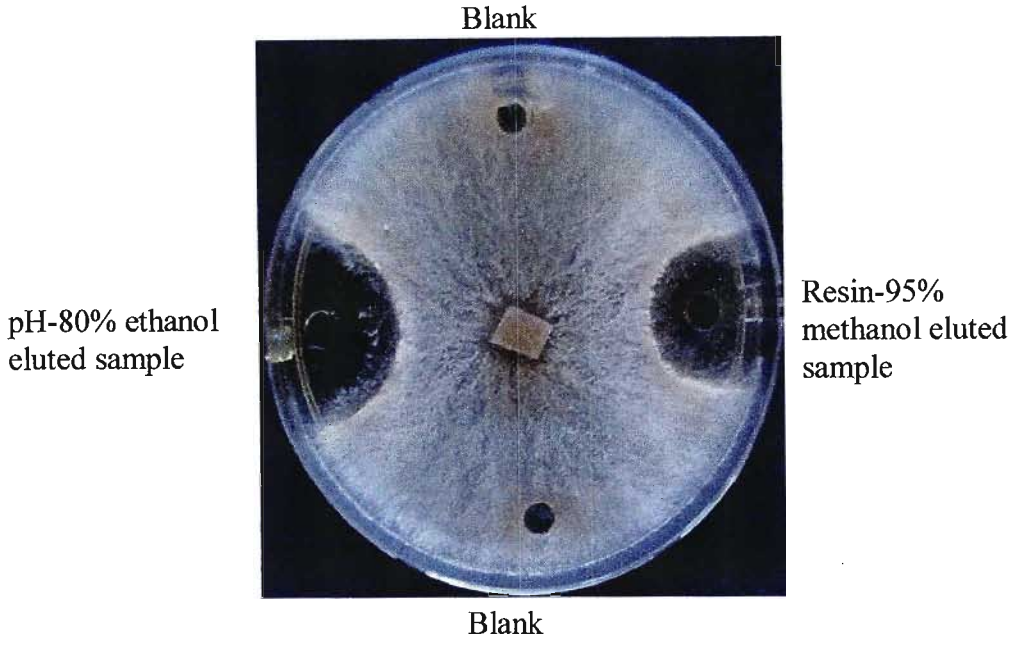

Figure 6.4 Comparison of antifungal activities against *R. solani* arising from the pH-80% ethanol and Amberlite resin extracts.

6.5 EXTRACTION OF ANTIFUNGAL METABOLITES USING METHANOL

When the supernatant and methanol (50:50, v/v) were mixed and left overnight, a precipitate was formed which was separated out by centrifugation. After evaporating off the methanol, the remaining solution was freeze dried and then resuspended in methanol; again, an insoluble precipitate formed which was removed by centrifugation. This fraction was soluble in sodium phosphate buffer and proved to be inactive against *R. solani* in a subsequent bioassay.

The methanol soluble fraction was evaporated and then resuspended in sodium phosphate buffer, and it turned to a yellow colour. It was found to be active against the test organism.

6.6 COMPARATIVE HPLC ANALYSIS OF THE EXTRACTED ANTIFUNGAL METABOLITES

Maximum UV absorbance of a purified sample was achieved at 285nm. Based on this, wavelength detection of the HPLC was calibrated to 285nm. Similarly, the optimum UV absorbance of un-inoculated medium was measured. No correlation between the two samples was found.

Samples (250μl) eluted from amberlite resin were run on TLC and the active band was collected and extracted for HPLC analysis. Eluent fractions corresponding to resolved peaks were collected and then concentrated by evaporating off the solvent. Fractions were re-suspended in sodium phosphate buffer and tested for antifungal activity, without success. To find out why none of these fractions showed activity the following ideas were postulated:

1. The samples could be too dilute to inhibit the test organism because the 250μl sample was effectively diluted with 40ml of solvent during HPLC analysis;
2. The antifungal compound or its activity could have been affected by the HPLC solvent; or,
3. That heat could have denatured the active compounds during evaporating processes used in purification steps.

By preparing different mixtures of the samples and solvents (Table 6.2) antifungal activities were again tested in order to try and address some of these questions.

Table 6.2 Samples and solvent mixtures used to assess effects on active compounds

	Mixtures
1	20μl pH-80% ethanol extracted sample + 20μl acetonitrile
2	20μl Amberlite resin extracted sample + 20μl acetonitrile
3	20μl TLC extracted sample + 20μl acetonitrile
4	20μl pH-80% ethanol extracted sample + 20μl 0.1% TFA
5	20μl Amberlite resin extracted sample + 20μl 0.1% TFA
6	20μl TLC extracted sample + 20μl 0.1% TFA
7	The following samples were also tested at the same time as controls; 20μl pH-80% ethanol extracted sample 20μl Amberlite resin extract sample 10μl TLC extract from the pH-80% ethanol extracted sample 10μl TLC extract from the Amberlite resin extracted sample

It was found that all the above listed mixtures showed activity against *R. solani*. These tests confirmed that neither the solvent nor heat had affected activity. Therefore, it was concluded that

the loss of antifungal activity was due to low concentrations of the active compounds being eluted during HPLC analysis. In order to resolve this problem sample volumes injected into the HPLC were increased to 1ml.

After confirming this, a 1ml sample from the pH-80% (v/v) ethanol extract was run in the HPLC. Four major peaks were resolved (Figure 6.5). Two peaks, P-2 and P-3, were found to be active against *R. solani* while the other peaks were devoid of antifungal activities. Similarly, the sample eluted from the amberlite resin showed more or less similar peaks (Figure 6.6). Again peaks, P-2 and P-3 exhibited antifungal activity. The methanol-extracted sample showed only two peaks (Figure 6.7) and only one peak, P-2, was active against *R. solani.* This sample appeared to be much cleaner than the other two samples although one of the active peaks had disappeared.

By running replicate samples in the HPLC, individual active peak fractions were collected and pooled. These pooled samples were checked for purity by re-running samples in the HPLC (e.g. from the methanol extraction-Figure 6.8). It was found that a single peak was resolved. Active peak fractions were collected from all samples extracted using the three methods.

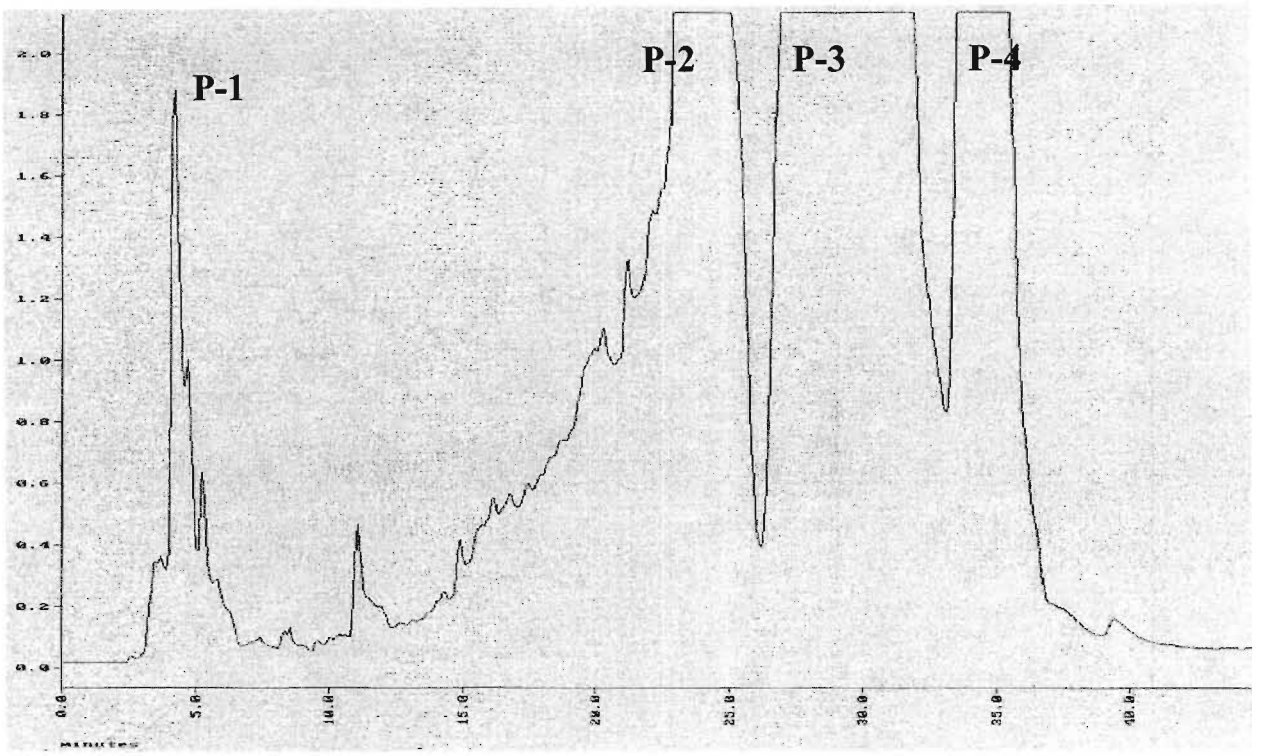

Figure 6.5 HPLC analysis of the pH-80% ethanol extracted, and TLC eluted sample(P = peak).

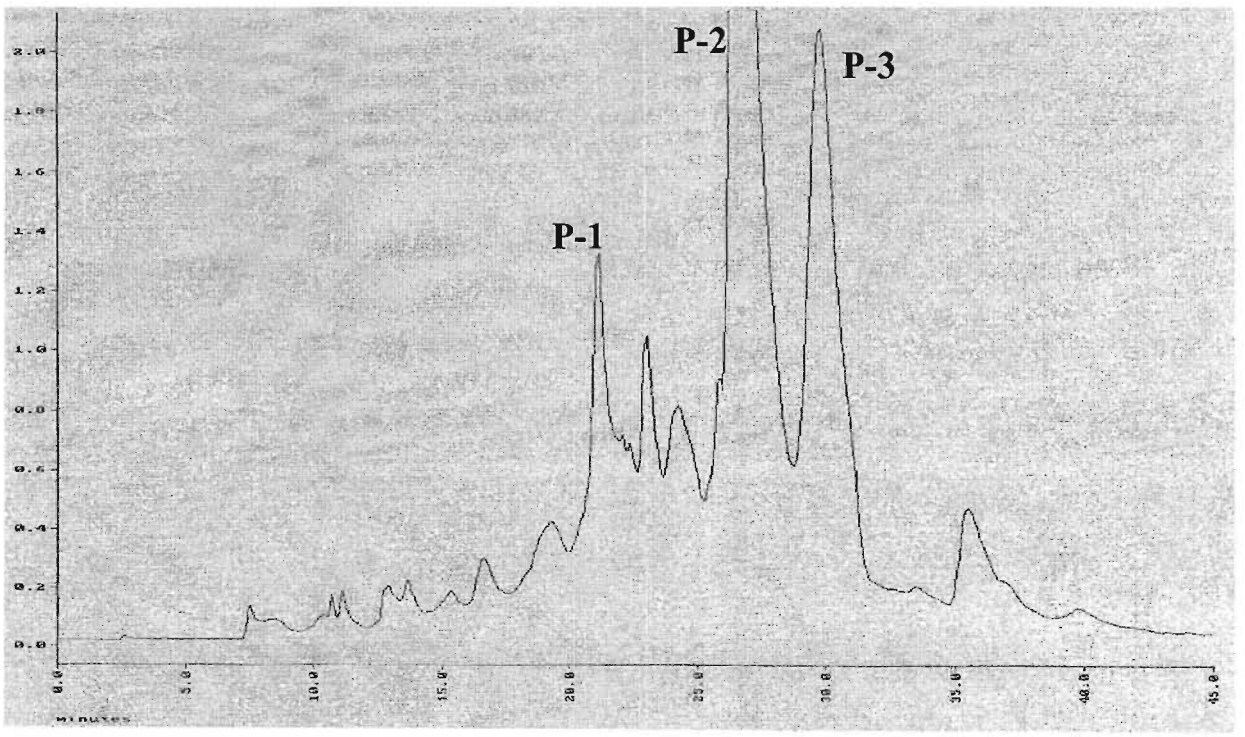

Figure 6.6 HPLC analysis of the amberlite resin extracted sample eluted with 95% methanol. (P = peak).

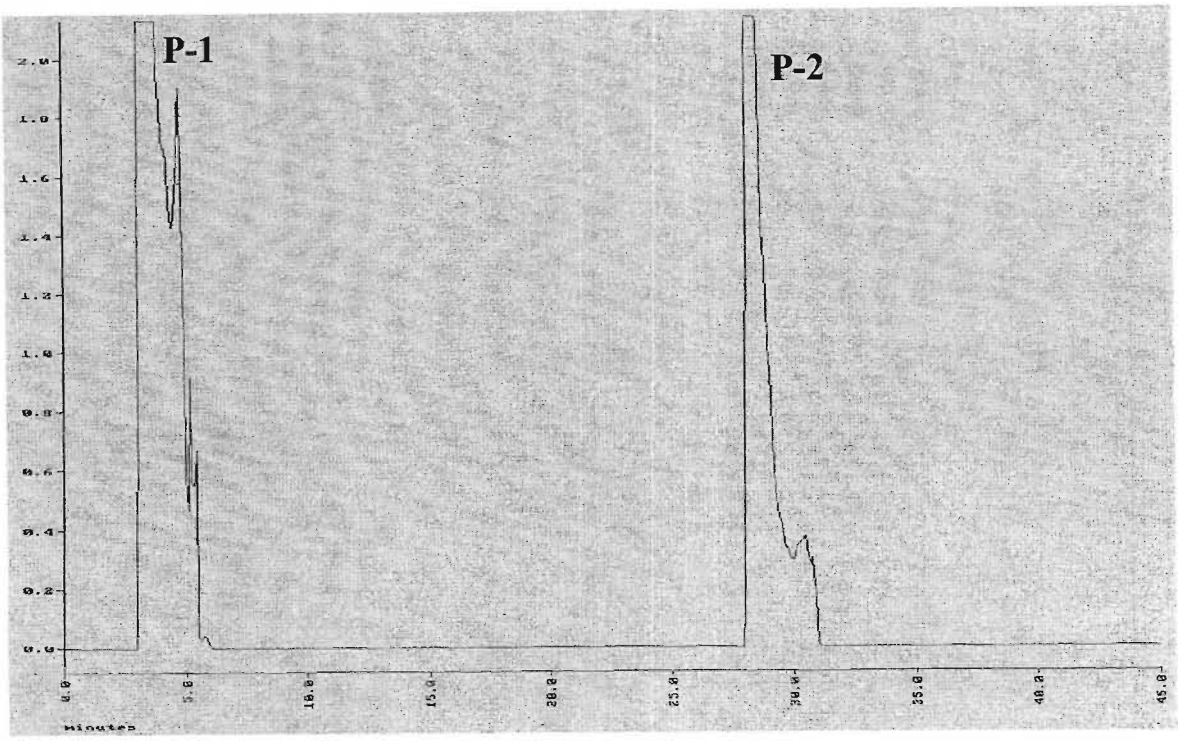

Figure 6.7 HPLC analysis of the methanol extracted sample, (P = peak).

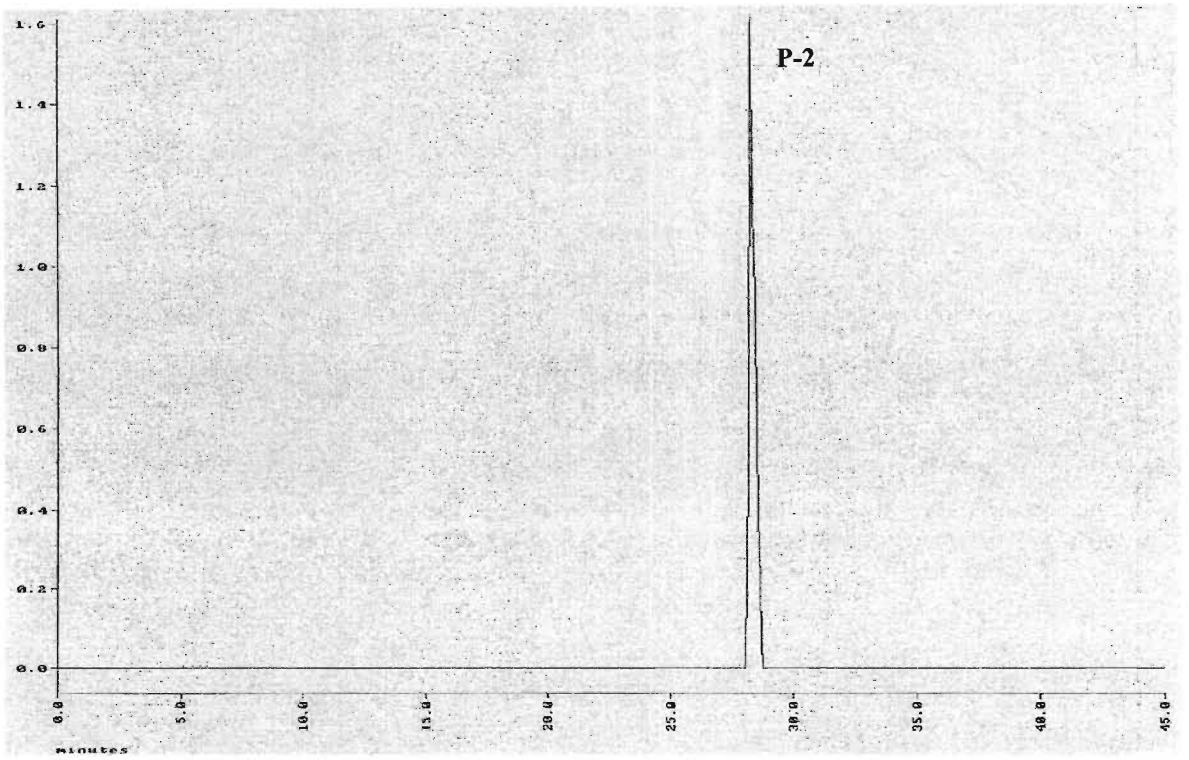

Figure 6.8 HPLC analysis of the pooled sample collected from a single active peak fraction (P=Peak).

6.7 PARTIAL CHARACTERIZATION OF ANTIFUNGAL METABOLITES

6.7.1 Thermostability of Crude Extracts Exhibiting Antifungal Activity

Temperatures ranging from 80^0C to 121^0C did not effect the antifungal activity of the crude extract after a 15min heat treatment period (Table 6.3). No differences in the size of the inhibition zones were observed between the heat-treated and control samples.

Table 6.3 Influence of temperature on activity of antifungal compounds present in R29 cell free supernatant

	R29 Inhibition (mm)	
	I	II
Room Temp (Control)	11	
80^0C	11	10.5
100^0C	10	10
110^0C	10	10
121^0C	10	10

6.7.2 Determination of Crude Extract Inhibitory Activity Units (IAU)

From serial dilution of crude extract in sodium phosphate buffer, it was found that the minimum inhibitory activity against *R. solani* was 2 x 103 IAU/ml while for *Pythium* sp. and *Fusarium* sp. it was 4 x 102 IAU/ml (Table 6.4).

Table 6.4 Serial dilution of crude extract of R29 in sodium phosphate buffer and mean inhibition zones formed from 100μl of the different dilutions

Crude extract per 1000μl)	Dilution factor (DF)	Mean size of inhibition zones (mm)		
		R. solani.	***Pythium* sp.**	***Fusarium* sp.**
1000	1.0	12	8.5	10.5
875	1.1	12	8	10
750	1.3	11.5	7	9.5
625	1.6	11.25	7	9
500	2.0	11	6	9
375	2.7	10.25	5.25	7.5
250	4.0	9.5	3.5	6.5
125	8.0	9	0	0
100	10.0	8.5		
75	13.3	7		
50	20.0	5		
25	40.0	0		

Inhibitory activity units were calculated as follows,

IAU = DF x volume assayed x 10 x 100fold

R. solani IAU = 20 x 0.1ml x 10 x 100fold = 2 x 10^3/ml

Pythium sp. and *Fusarium* sp. IAU = 4 x 0.1ml x 10 x 100fold = 4 x 10^2/ml

6.7.3 pH Stability of the Antifungal Activity of the Crude Extract

Antifungal activity was observed over the pH range (3-13) tested (Figure 6.9). Antifungal activity was not significantly effected over the range pH 5-9, but activity decreased upon exposure to acidic or basic extremes. In addition to this, precipitates formed at pH 3.

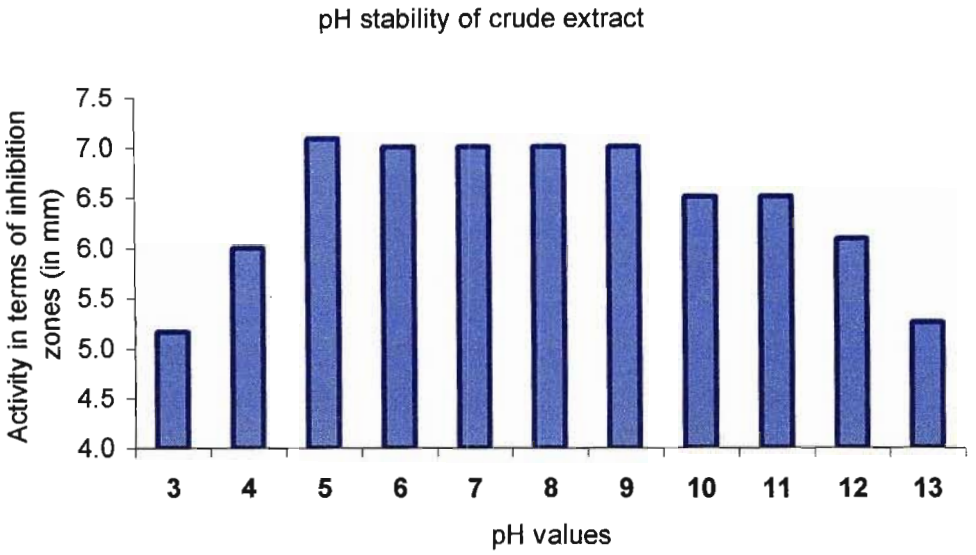

Figure 6.9 pH effect on the activity of a cell free crude extract from R29cultured on defined synthetic medium.

6.4.4 Protease Test of the Eluted Antifungal Active Metabolite

Protease K did not effect the antifungal activity of the pure extract (Figure 6.10).

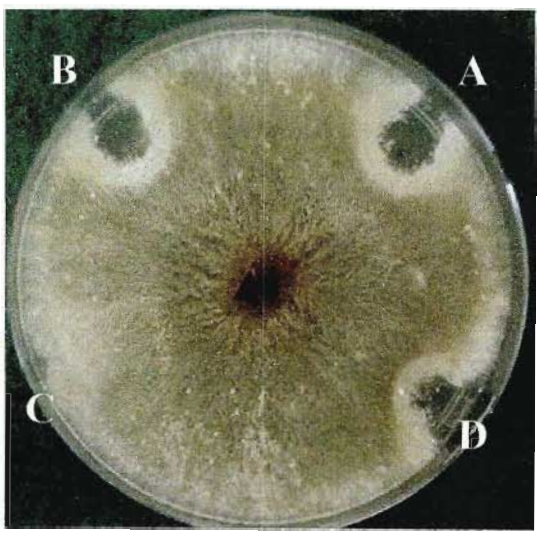

Figure 6.10 Protease K test showing the activity of 10µl of protease K treated antifungal metabolite placed on agar surface. **A**: 10µl sample from TLC extract + 5µl protease K, **B**:10µl sample from TLC extract + 5µl buffer; **C**:10µl buffer + 5µl protease K and **D**:10µl sample from resin extract + 5µl protease K.

6.8 NMR ANALYSIS

Assistance in interpreting NMR results was obtained from Dr. Ross Robinson (Discipline of Chemistry, University of KwaZulu Natal, Pietermaritzburg, RSA). The ^{13}C-NMR spectrum between 120-130ppm indicated that the active antifungal compound contains an aromatic complex (Figure 6.11). Unfortunately, the sample concentration was too low to detect quaternary carbons. In addition the ^{1}H -NMR spectrum between 6.4-7.2ppm also indicated the presence of aromatic complex in the active antifungal compound (Figure 6.12). The presence of peptides was also indicated in the ^{1}H-NMR spectrum between 1-4ppm. Similar results were obtained for each of the samples arising from different extraction methods. However, there were some impurities in the samples extracted by acid precipitation. These analyses revealed that all the active peak fractions from the three methods contained the aromatic and peptide components.

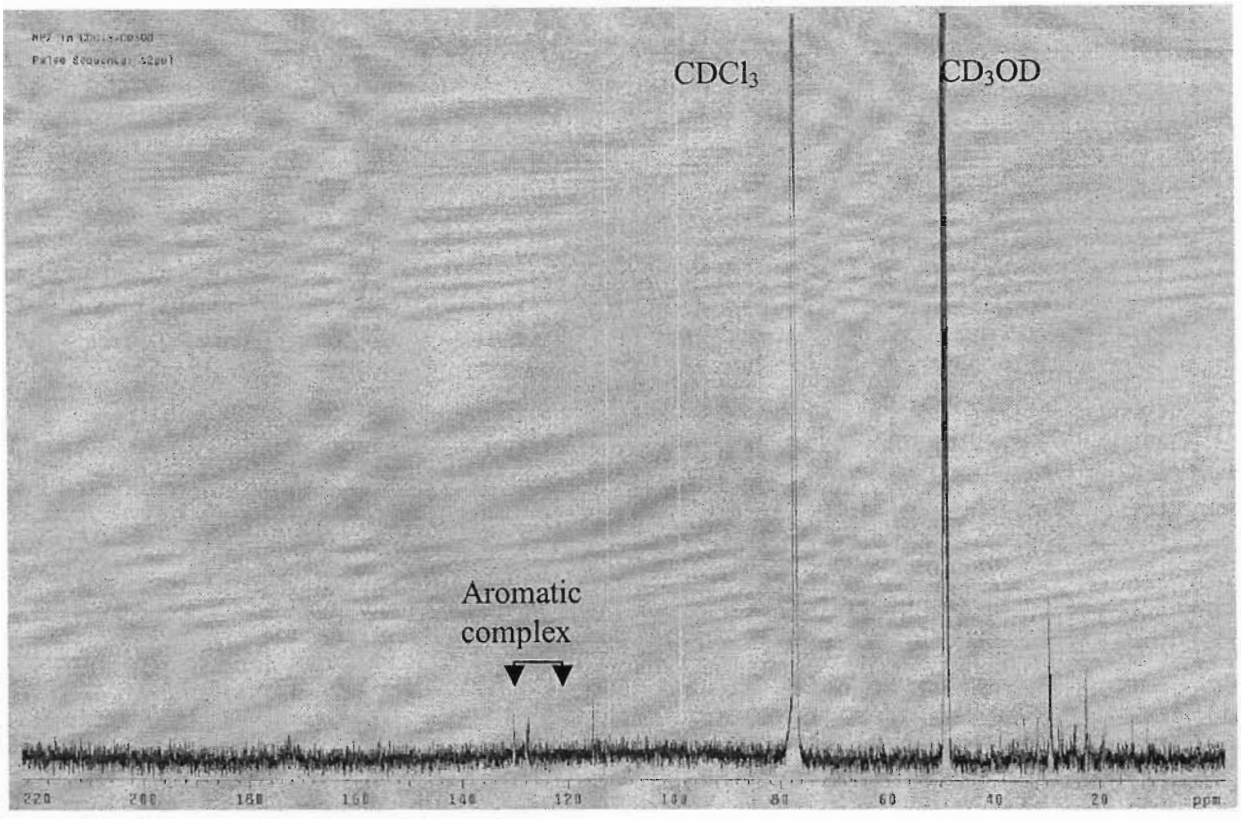

Figure 6.11 ^{13}C- NMR analysis of the methanol extracted sample. Chloroform ($CDCl_3$) and methanol (CD_3OD) solvents.

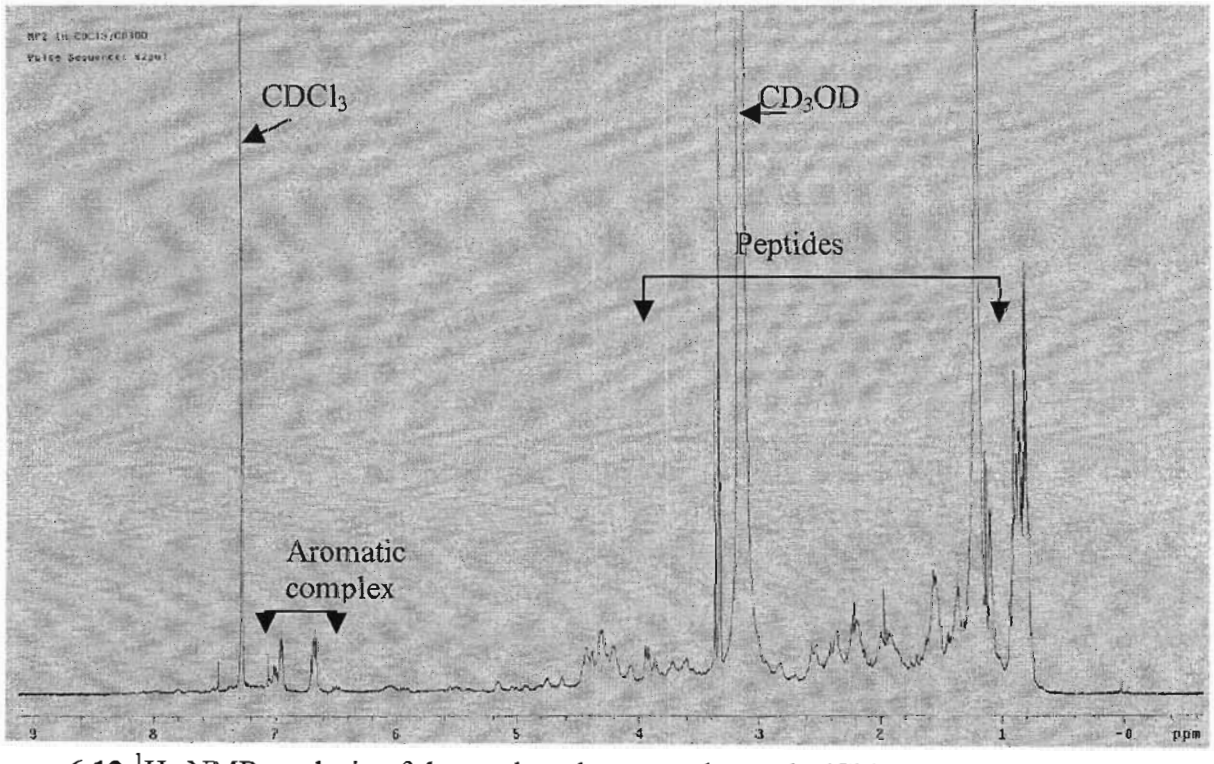

Figure 6.12 ^{1}H- NMR analysis of the methanol extracted sample (Chloroform-$CDCl_3$, metahnol-CD_3OD.

DISCUSSION

Most *Bacillus* spp. are capable of producing antibiotics (Garveba *et al.*, 2003) and many of these antibiotics are peptide antibiotics which exhibit a range of antimicrobial spectra (Lancini and Lerenzetti, 1993). Peptide antibiotics are typically secondary metabolites, which are produced during the late logarithmic or early stationary phase in batch cultures (Martin and Demain, 1980; Slepecky and Hemphill, 1992). This is not always the case, as in some instances peptide antibiotics are produced during the growth phase (Katz and Demain, 1977). Ochi and Ohsawa (1984) reported a *B. subtilis* strain that produced considerable amounts of antibiotics in a synthetic medium during active cell growth which stopped at the end of the logarithmic growth. When small amounts of casamino acid were supplemented, antibiotic was produced only at the end of the growth but in smaller amounts.

Bacillus isolate R29 was found to produce extractable secondary metabolites which exhibited antifungal activity during the stationary phase (but not in the logarithmic phase) in a defined synthetic medium. Spore staining revealed that sporulation during the stationary phase correlated well with the onset of antifungal activity. Bernal *et al.* (2002) reported that *Bacillus* strain M40 exhibited a prolonged growth phase and that maximum CFU counts were achieved after 114hrs. of cultivation. The growth for *Bacillus* isolate R29 indicated that the transition from the logarithmic to stationary phase occurred after 24hrs (Figure 6.1a).

When the cell free supernatant was run on TLC, two bands were distinguished and separated from the remaining impurities. Although Krieg and Gerhardt (1981) reported that bioautography is a sensitive method to locate the position of an active compound on TLC, it was not successfully used in this study. This was attributed to low concentrations of antifungal metabolites being present, or alternatively, that the active compound was not able to diffuse into the agar medium. In contrast to this, the bioautography overlay assay successfully showed inhibition of the test organism corresponding to a single band (Rf value 0.636) on the TLC plate. The activity of this band was also confirmed by scraping and eluting the antifungal metabolite from the TLC plate. The antifungal metabolites were eluted completely using 80% (v/v) ethanol solvent and the silica was easily separated by centrifugation.

Each extraction method tested proved to be successful in separating the antifungal compound/s from the cell free culture. Each method had its advantages and drawbacks. Acid precipitation was fast and simple to perform but unwanted impurities were also precipitated along with the antifungal compound which affected subsequent HPLC analysis. Hydrophobic interaction chromatography was time consuming and requires a large volume of solvent. In addition, the amount of sample collected was small relative to the acid precipitation method. Amberlite resin adsorbs hydrophobic compound, which can then be eluted with water miscible solvents e.g. methanol (Roham and Haas, 2003). This indicates that the active compound/s have a hydrophobic nature. Methanol extraction was easy to perform but only a single peak was distinguished using HPLC analysis. In general, the three methods were useful in soalting and extracting the active compound from the cell free cultures.

Proteins are amphoteric molecules that carry positive, negative or no net charge depending on the pH of the local environment. The overall charge of a protein is determined by the ionizable acidic and basic side chains of its constituent amino acids and prosthetic groups (Garfin, 1990). In globular and fibrous proteins, the interior of the structure is shielded from the aqueous medium by a concentration of hydrophobic amino acid residues (Englard and Seifter, 1990). In acidic environments the structure of water creates hydrophobic interactions with hydrophobic compounds causing them to precipitate out. Although not strictly proteins, peptide antibiotics appear to behave in a similar fashion. The hydrophobic nature and presence of peptides in the antifungal compound/s extracted were confirmed by the success of amberlite resin extraction and NMR analysis.

The number of peaks, which were resolved by HPLC analysis, differed for the three extraction methods, indicating differences in their purification efficiency. As mentioned earlier, the dark melanin like pigment was co-extracted with the active compounds and affected the purity of the sample analysis. Gibson *et al.* (1996) and Espinasse *et al.* (2002) stated that the presence of a dark brown color in bacterial cultures is characteristic of the production of melanin or a melanin-type polymer that can be collected from culture broth by acid precipitation. The methanol extraction produced the purest extract with fewer peaks. Collection of the active peak was relatively simple because the peaks were clearly resolved from each other. However, when the

numbers of active peaks were compared, the second active peak was lost presumably during the extraction process.

As indicated by TLC the antifungal activity produced in the synthetic medium resulted from a single antifungal band (peptide antibiotic or lipopetide), which had an Rf value of 0.636. Iturin A, an antifungal cyclic polypeptide produced by several strains of *B. subtilis*, produces a single band when it is chromatographed on TLC (Besson *et al.*, 1976 cited by McKeen *et al.*, 1986). This agrees with the result of the present study where the crude extract produced a single active band.

HPLC analysis of the samples from the first two extraction methods revealed that two active peaks (compounds) were extracted. These findings are supported in the literature. Leifert *et al.* (1995) detected three antifungal antibiotics from the fermentation broth of *B. subtilis* CL27. Two of the antibiotics produced by CL27 were found to be peptide antibiotics whereas the third antibiotic was not. Similarly, the HPLC analysis in the present study showed two active peaks implying two antifungal compounds were produced.

The purified antifungal compound/s were soluble in distilled water, methanol, 80% (v/v) ethanol and sodium phosphate buffer. These are polar solvents. Polar compounds dissolve in polar compounds and non-polar dissolve in non-polar compounds (Cannel, 1998). Results therefore indicate that the active compound/s are most probably polar. In addition to these, it was observed that all extracts from the three different methods used, when suspended in these solvents, the solution turned yellow. This indicates that similar compound/s were collected in all the methods used. Similarly, Gillespie *et al.* (2002) extracted an orange coloured antimicrobial compound, turbomycin A, from a metagenomic library of soil microbial DNA.

The thermostability of the extracted compound/s is also supported by findings in the literature. Munimbazi and Bullerman (1998) and Bernal *et al.* (2002) both reported that strains of *Bacillus* spp. produced thermostable metabolites active against phytopathogens. In both instances active compounds were soluble in methanol and were protease resistant. Iturin and fengycin antibiotics have both been reported to exhibit thermostability (Vanittanakom *et al.*, 1986; Bernal *et al.* 2002). In addition, Vanittanakom *et al.* (1986) reported that fengycin produced by *B. subtilis* strain F-29-3 exhibits a high range of thermostability and only denatures at 177^0C.

It is also reported in the literature that many antifungal polypeptides produced by different *Bacillus* spp. are resistant to hydrolysis by proteases, peptidases and various other enzymes (Perlman and Bodanszky, 1971; Katz and Demain, 1977; Munimbazi and Bullerman, 1998). For example Lebbadi *et al.* (1994) reported that fungicin M-4 is resistant to protease K, trypsin, carboxypeptidase A and lipase. Our findings support these observations and further supporte the hypothesis that the active compound was indeed a lipopolypeptide.

Antibiotics showing antifungal activity comprise lipopeptides such as iturin, fengycin, (Phae *et al.*, 1990; Yao *et al.*, 2003), surfactins (Kluge *et al.*, 1988, cited by Lebbadi *et al.*, 1994), bacillomycins (Eshita and Roberto, 1995), and mycosubtilins (Zuber *et al.*, 1993). Most of these antibiotics are cyclic peptides containing D- and L-amino acids closed by a beta-amino acid carrying a long aliphatic chain. A second family comprises hydrophilic antifungal antibiotics, such as the dipeptide bacitracin and the hydrophilic phospho-oligopeptide rhizoctin (Lebbadi *et al.*, 1994). Since the extracted compound/s exhibit antifungal activity and showed a number of properties similar to known *Bacillus* antibiotics, it is possible that it belongs be one of the above-mentioned antibiotic classes/groups.

The inhibitory activity unit assay method revealed that *R. solani* was the most sensitive. This observation supports the findings of Cook and Baker (1983) that *R. solani* is more sensitive to microbial antagonists than most other soil-born pathogenic fungi. The crude extracts bioassayed against *R. solani*, *Pythium* sp. and *Fusarium* sp. produced clear inhibition zones. Both *R. solani* and *Fusarium* sp. never overgrew the inhibition zones when left for a longer time whereas *Pythium* sp. did. From the plate bioassay it was observed that inhibition of *R. solani* and *Fusarium* sp. was fungicidal whereas for *Pythium* sp. it was fungistatic.

NMR analysis revealed the presence of aromatic ring and peptide structures in the antifungal compound. These findings support the hypothesis that the compound belongs to the lipopolypeptide group of compounds produced by members of the genus *Bacillus*. Iturin A, fengycin A and B are all examples of lipopeptide antibiotics that contain amino acids characterised by having an aromatic ring structure (Freifelder, 1983). Iturin A has a sequence of α amino acids (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser-) (Huang *et al.*, 1993; Yu *et al.*,

2002) and fengycin A has (D-Ala, L-Ile,L-Pro,D-allo-Thr,L-glx, D-Tyr, L-Tyr D-Orn-) whereas in fengycin B the D-Ala is replaced by D-Val (Vanittanakom *et al.*, 1986).

Further characterization of the active compound was restricted by instrument limitations and unavailability of analytical apparatus such as HPLC-MS and matrix-assisted laser desorption ionisation time-of-flight MALDI-TOF. Further research is needed to establish the biochemical nature of the antifungal compounds and confirm their identification as lipopolypeptide compounds.

CHAPTER 7

CONCLUDING REMARKS

The results of this study showed that all *Bacillus* isolates screened are antagonistic to the four plant pathogenic fungi tested *in vitro*, to varying degrees. The dual culture bioassay screening method showed reproducible results and were useful for selecting isolates exhibiting high levels of antifungal activity. Additional antagonistic assays of other plant pathogenic fungi and bacteria could be carried out to further define the actual spectrum of the isolates and their antifungal compounds.

It was relatively simple to identify the test isolates to the genus level based on phenotypic traits such as Gram reaction, cell morphology, ability to grow aerobically and the production of resistant endospores. However, phenotypic characterization based on biochemical and nutritional testing proved more difficult to ascribe isolates to a specific species of *Bacillus*. R29 was identified as a strain of *B. subtilis*, whereas B81 was tentatively identified as either *B. subtilis* or *B. licheniformis*. Since closely related *Bacillus* species show similar phenotypic profiles, identification of the isolates to the species level was not conclusive. Possible phenotypic tests which could have improved isolate identification include fatty acid composition analysis and DNA hydrolysis tests.

DNA sequence homology tests of both isolates further confirmed the phenotypic results. It showed that both isolates are definitely members of the genus *Bacillus*. Although both isolates were identified as strains of *B. subtilis* or its close relatives, the DNA fragment used was too small to provide definitive information for the final and conclusive identification of the isolates. For a more reliable identification of each isolate, further optimization of PCR amplification is required. Additionally, the use of different primers that can amplify larger DNA fragments for sequencing and homology test though BLAST could be used. In addition, partial gyrA nucleotide sequencing, translated amino acid sequence tests, restriction digest analysis, genetic transformation and DNA-DNA hybridization can be applied for conclusive identification of the isolates.

Isolate R29 was found to produce extractable antifungal compound/s from defined synthetic medium during the stationary phase. Antifungal compound/s were successfully extracted using three different extraction methods. Even though the acid precipitation extraction method was useful and easy, some impurities were also precipitated along with the antifungal compounds. These were not easily removed and interfered with the HPLC analysis. Antifungal compounds extracted with methanol resulted in a purer extract relative to other extraction methods. However, a major shortfall was that only one active peak was resolved during HPLC analysis. The importance of assessing a range of extraction methods is therefore highlighted. In addition, investigation into the clean-up or purification of the acid precipitation extract is warranted.

HPLC analysis revealed that two active peaks were resolved from both the acid precipitation and hydrophobic interaction extracts. This implies that two active antifungal compounds were produced by R29 from the defined synthetic medium. Other defined synthetic media as well as complex media also need evaluation to determine the effect of medium composition on the quantity and quality of the antifungal compounds produced. From the agar media used in the preliminary bioassays, TSA showed the largest inhibition zones. Therefore, TSB could possibly be used as a suitable medium for the large scale production of antifungal compounds. The purity of extracts arising from a complex medium would have to be assessed.

Physical characterization and NMR analysis of the extracted antifungal compound/s showed that they possessed similarities to antifungal lipopeptides such as iturin. Further characterization of the purified compounds is required to confirm this. Determination of parameters such as molecular weight and structure, using instrumentation such as liquid chromatography–mass spectrometer (LC-MS) and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) would be useful in this regard. Further research is needed to optimize antibiotic production and to make a detailed analysis to establish the biochemical nature of the antifungal compounds.

The results presented in this dissertation provide a basis from which the diversity of antifungal compounds produced by a *Bacillus* strains, exhibiting biocontrol activity, can be assessed.

REFERENCES

Alef, K. and Nannipieri, P. 1995. Catalase activity. In: Methods in Applied Soil Microbiology and Biochemistry. Eds. Alef, K. and Nannipieri, P. Academic Press Limited. London. Pp. 345-349.

Alexander, M. 1977. Bacteria. In: Introduction to Soil Microbiology. John Wiley & Sons, Inc. USA. Pp. 19-44.

Anith, K.L, Randhakrishnan, N.V. and Manomohandas, T.P. 2003. Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (*Piper nigrum*). Microbial Research **158**, 1-7.

Arbige, M.V., Bulthuis, B.A., Schultz, J. and Crabb, D. 1993. Fermentation of *Bacillus*. In: *Bacillus subtilis* and Other Gram-Positive Bacteria. Eds. Sonenshain, A.L., Hoch, J.A. and Losick, R. ASM Press. Washington, D.C. Pp. 871-895.

Ash, C., Farrow, J.A.E., Wallbanks, S. and Collins, M.D. 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit ribosomal RNA sequences. Letters in Applied Microbiology **13**, 202- 206.

Atlas, R.M. 1995. Handbook of Microbiological Media for the Examination of Food. CRC Press. USA. Pp. 1-310.

Baker, J.C. and Stavely, J.R. 1984. Biocontrol of bean rust by *Bacillus subtilis* under field conditions. Plant Diseases **69**, 770-772.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annual Review of Microbiology **22**, 87-108.

Bartnicki-garcia, S. and Wang, M.C. 1983. Biochemical aspects of morphogenesis in *Phytophthora*. In: *Phytophthora* its Biology, Taxonomy, Ecology and Pathology. Eds. Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. The American Phytopathological Society. USA. Pp. 121-137.

Bernal, G., Illanes, A. and Ciampi, L. 2002. Isolation and partial purification of a metabolite from a mutant strain of *Bacillus* sp. with antibiotic activity against plant pathogenic agents. Electronic Journal of Biotechnology ISSN:0717-3558.V 5.n 1.

Besson, F., Peypoux, F., Michel, G. and Delcambe, L. 1976. Characterizations of iturin A in antibiotics from various strains of *Bacillus subtilis*. Journal of Antibiotics **29**, 1043-1049.

Betina, V. 1983. Methods of screening, identification and determination of antibiotics. In: The Chemistry and Biology of Antibiotics. Elsevier Scientific Publishing Company. Czechoslovakia. Pp. 57-114.

Betina, V. 1983. Regulation of antibiotic biosynthesis. In: The Chemistry and Biology of Antibiotics. Elsevier Scientific Publishing Company. Czechoslovakia. Pp. 158-190.

Bonde, G.J. 1981. *Bacillus* from marine habitats: allocation to phena estimation by numerical techniques. In: The Aerobic Endospore-forming Bacteria, Classification and Identification. Eds. Berkeley, R.C.W. and Goodfellow, M. Academic Press. London. Pp. 181-216.

Borders, D.B. 1999. Isolation and identification of small molecules. In: Manual of Industrial Microbiology and Biotechnology. 2nd Edition. Eds. Demain, A.L and Davies, J.E. ASM Press. Washington, D.C. Pp. 392-416.

Bottone, J.E. and Peluso, W.R. 2002. Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against *Mucoraceae* and *Aspergillus* species: Preliminary report. Journal of Medical Microbiology **52,** 67-74.

Boyer, R.F. 1993. Separation and purification of biomolecules by chromatography. In: Modern Experimental Biochemistry. 2nd Edition. Cumming Publishing Company, Inc. California. Pp. 1-532.

Braun, P. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. Biological Chemistry **378**, 779-786.

Broman, K., N. Stalon, L.V. and Wiame, J.M. 1978. Oxygen and nitrate in utilization by *Bacillus licheniformis* of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their synthesis. Journal of Bacteriology **135,** 920-927.

Bron, S. 1990. Plasmids. In: Molecular Biological Methods of *Bacillus*. Eds. Harwood, C.R. and Cutting, S.M. John Wiley & Sons Ltd. England. Pp. 75-173.

Bron, S., Meima, R., Dijl, J.M.V., Wipat, A. and Harwood, C.R. 1999. Molecular biology and genetics of *Bacillus* spp. In: Manual of Industrial Microbiology and Biotechnology 2nd Edition. Eds. Demain, A.L. and Davies, J.E. ASM Press. Washington, D.C. Pp. 392-416.

Buchanan, R.E. and Gibbons, N.E. 1974. Endospores-forming rods and cocci. In: Bergey's Manual of Determinative Bacteriology. 8th Edition. Williams and Wilkins Company. Baltimore. Pp. 529-551.

Bucher, G.E. 1981. Identification of bacteria found in insects. In: Microbial Control of Pests and Plant Diseases. Ed. Burgs, R.C. 1970-1980. Academic Press. London. Pp. 7-33.

Cannell, R.J.P. 1998. How to approach the isolation of natural products. In: Natural Products Isolation. Ed. Cannell, R.J.P. Human Press. New Jersey. Pp.1-51.

Chet, I. 1990. Mycoparasitism- recognition, physiology and ecology. In: New Direction in Biological Control Alternative for Suppressing Agricultural Pests and Diseases. Eds. Baker, R.R. and Dunn, P.E. Alan R. Liss, Inc. USA. Pp. 725-733.

Chin-Min, W., Chien-Sheng, T., Chih-Yu, C. and Yaw-Kuen, L. 2002. Purification, characterization and cloning of a chitinase from *Bacillus* sp. NUTU2. Biotechnology and Applied Biochemistry **35**, 213-219.

Chitarra, G.S., Breeuwer, P., Nout, M.J.R., van Aelst, A.C., Rombouts, F.M. and Abee, T. 2003. An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Pencillium roqueforti* conidiospores. Journal of Applied Microbiology **94**, 159-166.

Chun, J. and Bae, K.S. 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial gyrA gene sequences. Antonie van Leeuwenhoek **78**, 123-127.

Claus, D. and Berkeley, R.C.W. 1986. Genus *Bacillus*. In: Bergey's Manual of Systematic Bacteriology. Volume 2. Eds. Sneath, P.H.A. Williams and Wilkins. USA. Pp. 1105-1139.

Claus, D. and Fritze, D. 1989. Taxonomy of *Bacillus*. In: Biotechnology Handbook, *Bacillus*. 1st Edition. Ed. Harwood, C.R. Plenum Press. New York. Pp. 5-26.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control agents against soil-born plant pathogens. ASP Press. Minnesota. USA.

Cornelis, P. and Matthijs, S. 2002. Diversity of siderophore-mediated iron uptake system in fluorescent pseudomonads: not only pyoverdines. Environmental Microbiology **4**, 787-802.

Costilow, R.N. 1981. Biophysical factors in growth. In: Manual of Methods for General Bacteriology. Eds. Gerhardt. P., Murray R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. ASM Press. Washington. Pp. 66-78.

Dahl, M. K. 1999. *Bacillus*. In: Encyclopedia of Food Microbiology. Eds. Robison, E.K., Batt, C.A. and Patel, P.D. Volume 1. Academic Press. UK. Pp. 113-158.

Doi, R.H. 1989. Sporulation and Germination. In: Biotechnology Handbooks *Bacillus*. 1st Edition. Ed. Harwood, C.R. Plenum Press. New York. USA. Pp. 168-204.

Driks, A. 1999. *Bacillus subtilis* spore coat. Microbiology and Molecular Biology Reviews **63**, 1-20.

Dunbar, B.S. 1987. Basic theories and principles of electrophoresis. In: Two-Dimensional Electrophoresis and Immunological Techniques. Plenum Press. New York, USA. Pp. 1-21.

Englard, S. and Seifter, S. 1990. Precipitation techniques. In: Methods in Enzymology. Volume 182. Ed. Deutscher, M.P. Academic Press, Inc. USA. Pp. 285-300.

Eshita, S.M. and Roberto, N.K. 1995. Bacillomycin L_c, a new antibiotic of the iturin group: isolation, structures, and antifungal activity of the congeners. The Journal of Antibiotics **48**, 1240-1247.

Espinasse, S., Gohar, M., Didier Lereclus, D. and Sanchis, V. 2002. An ABC Transporter from *Bacillus thuringiensis* is essential for ß-exotoxin I production. Journal of Bacteriology **184**, 5848-5854.

Farrar, W.E. and Reboli, A.C. 1992. The genus *Bacillus*-Medical. In: The Prokaryotes 2nd Edition. A Handbook on the Biology of Bacteria: Eds. Balow, A.S., Trüper, H.G., Dworkin. M., Harder, W. and Schileifer, K. Springer-Verlag Inc. New York. USA. Pp. 1747-1768.

Ferrari, E., Jarnagin, A.S. and Schidt, B.R. 1993. Commercial production of extracellular enzymes. In: *Bacillus subtilis* and Other Gram-Positive Bacteria. Eds. Sonenshain, A.L., Hoch, J.A and Losick, R. ASM Press. Washington, D.C. USA. Pp. 917-937.

Fiddaman, P.J. and Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology **74**, 119-126.

Fox, G.E., Wisotzkey, J.D. and Jurtshuk, P.J. 1992. How close 16s rRNA sequence identity may not be sufficient to guarantee species identity. International Journal of Systematic Bacteriology **42**, 166-170.

Fravel, D.R. 1988. Role of antibiotics in the biocontrol of plant diseases. Annual Review of Phytophatology **81**, 283-287.

Freifelder, D. 1983. Genetic analysis in molecular biology. In: Molecular Biology. 2nd Edition. Jones and Bartkett Publisher, Inc. Boston. USA. Pp. 35-55.

Garfin,V.E. 1990. Isoelectric focusing. In: Methods in Enzymology. Volume 182. Ed. Deutscher, M.P. Academic Press, Inc. USA. Pp. 459-477.

Garveba, P. Van Veen, J.A. and Van Elsas, J.D. 2003. Predominant *Bacillus* spp. In: Agricultural soil under different management regimes via PCR-DGGE. Microbial Ecology **45**, 302- 316.

Gibbons, S. and Alexander, I.G. 1998. Isolation by planar chromatography. In: Natural Products Isolation. Ed. Cannel, R.J.P. Humana Press. New Jersey. USA. Pp. 209-245.

Gibbons, S. and Gray, A.I. 1998. Isolation by planar chromatography. In: Natural Products Isolation. Ed. Cannell. R.J.P. Humana Press. USA. Pp. 209-243.

Gibson, L. F., Ritz, K. and Glover, L. A. 1996. Melanin one and novel precursors from *Aeromonas* media. FEMS Microbiological Letters **169**, 261-268.

Gibson, T. and Gordon, R.E. 1974. *Bacillu.* In: Bergey's Manual of Determinative Bacteriology. Eds. Buchanan, R.E. and Gibbons, N.E. Williams and Wilkins. Baltimore. USA. Pp. 529-550.

Gillespie, D. E., Brady, S. F., Bettermann, A. D., Cianciotto, N. P., Liles, M. R., Rondon, M. R., Clardy, J., Goodman, R. M. and Handelsman, J. 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Applied and Environmental Microbiology **68**, 4301-4306.

Gillis, M., De Vos, P. and Vancanneyt, M. 1989. Taxonomy and identification of *Bacillus* (Eubacteria) and relatives. http://betula.br.fgov.be/cgi-bin/BIODIV/ research.pl?res id= 196 . 20/2/2003.

Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology **41**, 109-117.

Gordon, R.E. 1981. One hundred and seven years, of the genus *Bacillus*, In: The Aerobic Endospore Forming Bacteria: Classification and Identification. Eds. Berkeley, R.C. and Goodfellow, W. M. Academic Press. London. UK. Pp. 1-15.

Gordon, R.E., Haynes, W.C. and Pang, C.H.N. 1973. The Genus *Bacillus*. United States Department of Agriculture. Washington, D.C..USA.

Górska, E., Tudek, B. and Russel, S. 2001. Degradation of cellulose by nitrogen-fixing strains of *Bacillus polymyxa*. Acta Microbiologica Polonica **50**, 129-137.

Gould, G. W. 1996. Stimulation of L-alanine-induced germination of *Bacillus cereus* spores by D-cycloserine and O-carbamyl-D-serine. Journal of Bacteriology **92,** 1261-1262.

Gould, G.W. 1969. Germination. In: The Bacterial Spore. Eds. Gould, G.W. and Hurts, A. Academic Press. London. UK. Pp. 397-444.

Grossman, T.H., Tuckman, M., Ellestad, S. and Osburne, M.S. 1993. Isolation and characterization of *Bacillus subtilis* genes involved in siderophore biosynthesis: relationship between *B. subtilis* sfpO and *Escherichia coli* endD genes. Journal of Bacteriology **175**, 6203-6211.

Guder, A., Wiedemann, I. and Sahl, H. G. 2000. Posttranslationally modified bacteriocins - the lantibiotics. Biopolymers **55**, 62-73.

Gueldner, C.R., Reilly, C.C., Pusey, L.P., Costello, E.C., Arreendale, F.R., Cox, H.R., Himmelsbach, S.D., Crumley, G.F. and Ulter, G.H. 1988. Isolation and identification of iturin as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry **36**, 366-370.

Hancock, R.E.W. and Chapple, D.S. 1999. Minireview, peptide antibiotics. Antimicrobial Agents and Chemotherapy **43**, 1317-1323.

Harmsen, D., Singer, C., Rothgänger, J., Tonjum, T., Hoog, G.S., Shah, H., Albert, J. and Frosch, M. 2001. Diagnostic of *Neisseriaceae* and *Moraxellanceae* by ribosomal DNA sequencing: ribosomal differentiation of medical microorganisms. Journal of Clinical Microbiology **39**, 936-942.

Harwood, C.R. and Archibald, A.R. 1990. Growth, maintenance and general techniques. In: Molecular Biological Methods for *Bacillus*. Eds. Harwood, C.R. and Cutting, S.M. John Wiley & Sons Ltd. England. Pp. 1-23.

Harwood, C.R. and Cutting, S.M. 1990. Molecular Biological Methods for *Bacillus*. Eds. Harwood, C.R. and Cutting, S.M. John Wiley & Sons Ltd. England. Pp. 546.

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. and Logan, N.A. 1998. *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight, 1950). Emended description of *virgibacillus pantothenticus*. International Journal of Systematic Bacteriology **48**, 99-106.

Heyndrickx, M., Vandemeulebroecke, K., Schaldeman, P., Kersters, K., De Vos, P., Logan, N.A, Aziz, A.M., Ali, N. and Berkeley, R.C. 1996. A phylophasic reassessment of the genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura, 1984) as a *Paenibacilllus lautus* combs. Nov. and *Bacillus peoriae* (Montifusko *et al.*, 1993) as a *Paeenibacilllus lautus* and *P. peoriaae*. International Journal of Systematic Bacteriology **46**, 988-1003.

Higgs, R.E., Zahn, J.A., Gygi, J.D. and Hilton, M.D. 2001. Rapid methods to estimate the presence of secondary metabolites in microbial extracts. Applied and Environmental Microbiology **67**, 371-376.

Hodgson, B. 1970. Possible roles for antibiotics and other biologically active peptides at specific stages during sporulation of *Bacillaceae*. Journal of Theoretical Biology **30**, 111-119.

Holt, J.G., Krieg, N.R., Staley, J.T. and Williams, S.T. 1994. Endospore Forming Gram-positive Rods and Cocci. In: Bergey's Manual of Determinative Bacteriology. 9th Edition. Williams and Wilkins Company. Baltimore. Pp. 559-563.

Hsu, S.C. and Lockwood, J.L. 1975. Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. Applied Microbiology **29**, 422-426.

Huang, C., Ano, T. and Shoda, M. 1993. Nucleotide sequence and characteristics of the gene, lpa-14 responsible for biosynthesis of the lipopeptide antibiotics iturin A and surfactin from *Bacillus subtilis* RB14. Journal of Fermentation and Bioengineering **76**, 445-450.

Jonson, J.L. 1989. Nucleic acid in bacterial identification. In: Bergey's Manual of Systemic Bacteriology, Volume 4. Eds. Williams, S.T., Sharpe, M.E. and Holt, J.G. Williams and Wilkins Press. Baltimore. USA. Pp. 206-209.

Katz, E. and Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. Bacteriological Review **41**, 447-474.

Kenig, M., Vandamme, E. and Abraham, E.P. 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. Journal of General Microbiology **94**, 46-54.

Khubeka, B.P. 2003. *In vitro* and *in vivo* screening of *Bacillus* spp. for biological control of *Rhizoctonia solani*. MSc Thesis, University of KwaZulu-Natal, South Africa.

Kim, D.S., Cook, R.J and Weller. D.M. 1997. *Bacillus* Sp.L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87**, 551-558.

Kleinkauf, H. and Döhren, H. 1982. A survey of enzymatic peptide formation. In: Peptide Antibiotics. Eds. Kleinkauf, H. and Döhren, H. Walter de Gruyter. Berlin. Pp. 1-45.

Kleinkauf, H. and Döhren, H.V. 1990. Non-ribosomal biosynthesis of peptide antibiotics. European Journal of Biochemistry **192**, 1-15.

Kleinkauf, H. and Döhren, H.V. 1997. Peptide antibiotics. In: Encyclopedia of Biotechnology. Volume 7. Eds. Reed, G. in cooperation with Puhler, A. and Stadler, P. VCH a Wiley company. Germany. Pp. 278-316.

Kluge, E., Vater, J., Salnikow, J. and Eckart, K. 1988. Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC21332. FEBS Letters **231**, 107.

Krieg, N.R. and Gerhardt, P. 1981. Solid culture. In: Manual of Methods for General Bacteriology. Eds. Gerhardt, P., Murray, R.G.E., Costilow, R., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. ASM Press. Washington, D.C. USA. Pp. 143-150.

Lancini, G. and Lorenzetti, R. 1993. Biotechnology of antibiotic-producing microorganisms. In: Biotechnology of Antibiotics and Other Bioactive Microbial Metabolites. Plenum Press. New York. USA. Pp. 19-72.

Lancini, G. and Parenti, F. 1982. The Antibiotics. In: Antibiotics an Integrated View. Springer-Verlag. New York, USA. Inc. Pp. 1-241.

Lebbadi, M., Galvez, M., Maqueda, M., Bueno, M. and Valdivia, E. 1994. Fungicin M4: a narrow spectrum peptide antibiotic from *Bacillus licheniformis* M4. Journal of Applied Bacteriology **77,** 49-53.

Leboffe, M. J. and Pierce, B.E. 1996. Quantitative techniques-viable count. In: A Photographic Atlas for the Microbiology Laboratory. Morton Publishing Company. Colorado. USA. Pp. 81-87.

Leifert, C., Li, H., Chidburee, S., Hampson, S. Workman, S., Sigee, D., Epton, H.A.S. and Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* C27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology **78**, 97-108.

Linacero, R., Rueda, J. and Vázquez, A.M. 1998. Quantification of DNA. In: Molecular Tools for Screening Biodiversity. Kluwer Academic Publishers. Netherlands. Pp. 18-24.

Logan, N.A. and Berkeley, R.C.W. 1984. Identification of *Bacillus* strains using the API system. Journal of General Microbiology **130**, 1871-2882.

Madigan, M.T., Martinko, J.M. and Parker, J. 1997. Brock Biology of Microorganisms. 8th Edition. Prentice Hall International Inc. USA. Pp. 1-987.

Madigan, M.T., Martinko, J.M. and Parker, J. 2003. Microbial evolution and systematics. In: Brock Biology of Microorganisms. 10th Edition. Prentice Hall. USA. Pp. 322-350.

Maget-Dana, R., Ptak, M., Peypoux, F. and Michel, G. 1985. Pore-forming properties of iturin A, a lipopeptide antibiotic. Biochimim et Biophysica Acta **815**, 405-409.

Marahiel, M.A. and Döhren, H. 1982. A survey of possible functions of peptide antibiotics in the producer organism. In: Peptide Antibiotics. Eds. Kleinkauf, H. and Döhren, H. Walter de Gruyter. Berlin. Germany. Pp. 375-380.

Marchant, R. 1984. The ultrastructure and physiology of sporulation in *Fusarium*. In: The Applied Mycology of *Fusarium*. Eds. Moss, M.O. and Smith. J. E. Pritman Press. Bath. UK. Pp. 15-37.

Martin, J.F. and Demain, A.L. 1980. Control of antibiotic biosynthesis. Microbiological Reviews **44**, 230-251.

Maurhofer, M., Hase, C., Meuwly, P., Metraux, J.P., and Defego, G. 1994. Induction of systemic resistance of tobacco to necrosis virus by the root-colonizing *Pseudomonas florescences* strain CHAO: influence of the gacA gene and of pyoverdine production. Phytopathology **84**, 139-146.

McKeen, C.D., Reilly, C.C. and Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicol* from *Bacillus subtilis*. Phytopathology **76**, 136-139.

Mitchell, R. and Alexander, M. 1962. Lysis of soil fungi by bacteria. Canadian Journal of Microbiology **15**, 689-696.

Modest, B., Marahiel, M., Pschorn, W. and Ristow, H. 1984. Peptide antibiotics and sporulation: induction of sporulation in asporogenous and peptide-negative mutants of *Bacillus brevis*. Journal of General Microbiology **130**, 747-755.

Moir, A. and Smith, D. A. 1990. The genetics of bacterial spore germination. Annual Review Microbiology **44**, 531-553.

Montealegre, J.R. Reyes, R., Pérez, M.L. and Herrera, R. 2003. Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato. Electronic Journal of Biotechnology ISSN: 0717-3458. V.6 n.2.

Moriyama, R., Kudoh, S., Miyata, S., Nonobe, S., Hattori, A. and Makino. S. 1996. A germination-specific spore cortex-lytic enzyme from *Bacillus cereus* spores: cloning and sequencing of the gene and molecular characterisation of the enzyme. Journal of Bacteriology **178**, 5330-5332.

Munimbazi, C. and Bullerman, L.B. 1998. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. Journal of Applied Microbiology **84**, 959-968.

Nair, J.R. Singh, G. and Sekar, V. 2002. Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. Journal of Applied Microbiology **93**, 772-780.

Nakamura, L.K. 1989. Taxonomy relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. International Journal of Systematic Bacteriology **39**, 295-300.

Nakamura, L.K., Roberts, M. and Cohan, F.M. 1999. relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. subtilis subsp. nov. and *Bacilllus subtilis* subsp. spizizenii subsp. nov. International Journal of Systematic Bacteriology **49**, 1211-1215.

Nakamura, L.K., Shida, O., Takagi, H. and Komagata, K. 2002. *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., round-spored bacteria from soil. International Journal of Systematic Bacteriology **52**, 501-505.

Nakano, M.M. and Zuber, P. 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). Annual Review of Microbiology **52**, 165-190.

Nakayama, K. 1981. Sources of industrial microorganisms. In: Biotechnology-Microbial Fundamentals. Volume 1. Eds. Rehm, H.J. and Reed, G. Verlag Chemie. Berlin. Germany. Pp. 355-410.

Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, S.S., Balyaev, S.S. Ivanov, M.V. 2001. Taxonomic study of aerobic thermophilic bacilli: description of *Gebacillus subterraneus* gen. Nov. sp. Nov. and *Geobacillus uzenesis* sp. nov. from petroleum reservoirs and transfer and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus , G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucasidasius and G. thermodenitrificans*. International Journal of Systematic Bacteriology **51**, 433-446.

Neilan, B.A., Dittmann, E., Rouhiainen, L., Bass, R.A., Schaub, V., Sivonen, K. and Borner, T. 1999. Non-ribosomal peptide synthesis and toxigenicity of cyanobacteria. Journal of Bacteriology **181**, 4089-4097.

Nicholson, W.T and Setlow, P. 1990. Sporulation, germination and outgrowth. In: Molecular Biological Methods for *Bacillus*. Eds. Harwood, C.R and Cutting, S.M. John Wiley & Sons Ltd. UK. Pp. 391-429.

Nicholson, W.T., Munakata, N., Hhorneck, G., Melosh, H.J. and Setlow, P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Review **64**, 548-572.

Ochi, K. and Ohsawa, S. 1984. Initiation of antibiotic production by the stringent response of *Bacillus subtilis* marbarg. Journal of Microbiology **130**, 2473-2482.

Ochi, K., Kandala, J. and Freese, E. 1982. Evidence that *Bacillus subtilis* sporulation induced by the stringent response is caused by the decrease in GTP or GDP. Journal of Bacteriology **151**, 1062-1065.

Paidhungat, M. and Setlow, P. 1999. Isolation and characterization of mutation in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. Journal of Bacteriology **181**, 3341-3350.

Paidhungat, M., Ragkousi, K. and Setlow, P. 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+}-Dipicolinate. Journal of Bacteriology **183**, 4886-4893.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annual Review of Phytopathology **23**, 23-54.

Perez, C. Suarez, C. and Castro, G.R. 1992. Production of antimicrobials by *Bacillus subtilis* MIR 15. Journal of Biotechnology **26**, 331-336.

Perlman, D. and Bodanszky, M. 1971. Biosynthesis of peptide antibiotics. Annual Review of Biochemistry **40**, 449-464.

Phae, C.G., Shoda, M. and Kubota, H. 1990. Suppressive effect of *Bacillus subtilis* and its products on phytophatogenic microorganisms. Journal of Fermentation and Bioengineering **69**, 1-7.

Pinchuk, I.V., Bressollier, P., Verneul, B., Fenet, B., Sorokulova, I.B., Megraud, F. and Urdaci, M.U. 2001. *In vitro* anti-helicobacter pylori activity of the probiotic strain *Bacillus sutillis* 3 is due to secretion of antibiotics. Antimicrobial Agents and Chemotherapy **45**, 3156-3161.

Podile, A.R. and Dube, H.C. 1988. Plant growth promoting activity of *Bacillus subtilis* AF1. Current Science **57**, 183-186.

Podile, A.R. and Laxmi, V.D.V. 1998. Seed bacterization with *Bacillus Subtilis* AF 1 increases phenylalanine ammonia-lyase and reduces the incidence of fusarial wilt in pigeonpea. Journal of Phytopatholgy **146**, 255-259.

Podile, A.R. and Prakash, A.P. 1996. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF1. Canadian Journal of Microbiology **42**, 533-538.

Prescott, L. M., Harley, L. M. and Klein, J. P. 1999. Microbiology. 4th Edition. WCB McgGraw Hill. New York. USA. Pp. 1-931.

Priest, F.G. 1989a. Isolation and identification of aerobic endospore-forming bacteria. In: Biotechnology Handbooks *Bacillus*. Volume 2. Ed. Harwood, C.R. Plenum Press. New York. USA. Pp. 27-51.

Priest, F.G. 1989b. Products and applications. In: Biotechnology Handbooks *Bacillus*. Volume 2. Ed. Harwood, C.R. Ed. Plenum Press. New York. USA. Pp. 293-320.

Priest, F.G. 1993a. *Bacillus*. In: Encyclopaedia of Biotechnology. Volume 1. Eds. Reed, G. in cooperation with Puhler, A. and Stadler, P., VCH Publisher Inc. New York. Pp. 368-400.

Priest, F.G. 1993b. Systematics and ecology of *Bacillus*. In: *Bacillus subtilis* and Other Gram-Positive Bacteria. Eds. Sonenshain, A.L., Hoch, J.A and Losick, R. ASM Press. Washington, D.C. USA. Pp. 3-16.

Priest, F.G. and Sharp, R.J. 1989. Fermentation of *Bacillus*. In: Fermentation Process Development of Industrial Organisms. Ed. Neway, J.O. Marcel Dekker, Inc. New York.USA. Pp. 73-132.

Priest, F.G., Goodfellow, M., Shute, L.A. and Berkeley, R.C.W. 1987. *Bacillus amyloliquefaciens* sp. nov., nom. rev. International Journal of Systematic Bacteriology **37**, 69-71.

Pusey, P.L. and Wilson, C.L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Disease **68**, 753-756.

Quesnel, L.B. and Russel, A.D. 1983. Introduction. In: Antibiotics: Assessment of Antimicrobial Activity and Resistance. Ed. Quesnel, L. B. and Russel, A.D. Academic Press. New York. USA. Pp. 1-17.

Ristow, H. and Paulus, H. 1982. Induction of sporulation in *Bacillus brevis*. Biochemical events and modulation of RNA synthesis during induction by tyrocidine. European Journal of Biochemistry **129,** 395-401.

Roberts, M.S. Nakamura, L.K. and Cohan, F.M. 1994. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. International Journal of Systematic Bacteriology **44**, 256-264.

Roberts, M.S., Nakamura, L.K. and Cohan, F.M. 1996. *Bacillus vallimortis* sp. nov., a close relative of *Bacillus subtilis*, isolation from soil in death valley, California. International Journal of Systematic Bacteriology **46**, 470-475.

Roham and Haas Company. 2003. Product data sheet.

http://www.rohmhaas.com/ionexchange/Pharmaceuticals/Bioprocessing_doc/us_english/xad4.pdf12/6/2003.

Sadfi, N., Cherif, M., Hajlaoui, M.R. and Boudabbous, A. 2002. Biological control of the potato tuber dry rot caused by *Fusarium roseum* var. *Sambucium* under greenhouse, field and storage conditions using *Bacillus* spp. isolates. Journal of Phytopathlogy **150**, 640- 648.

Sailaja, P.R., Podile, A.R. and Reddanna, P. 1997. Biocontrol strain of *Bacillus subtilis* AF 1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. European Journal of Pant Pathology **104**, 125-132.

Schineider, E.F., Barran, L.R., Wood, P.J. and Siddiqui, I.R. 1977. Cell wall of *Fusarium suphpureum*. II. Chemical composition of the conidial and chlamydospore wall. Canadian Journal of Microbiology **23**, 763-769.

Schlötterer, C. 2001. Ribosomal DNA probes and primers. In: Molecular Tools for Screening Biodiversity. Eds. Karp, A., Isaac, P.G. and Ingram, D.S. Kluwer Academic Publishers. Dordrecht. Netherlands. Pp. 267-276.

Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection determination of siderophores. Analytical Biochemistry **160**, 47-56.

Serrano, M., Zilhão, R., Ricca, E., Ozin, A.Z., Morgan, C.P. and Henriques, A.O. 1999. A *Bacillus subtilis* secreted protein with a role in endospores coat assembly and function. Journal of Bacteriology **181**, 3632-3643.

Setlow, P. 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annual Review of Microbiology **49**, 25-54.

Sharp, R.J., Scawen, M.D. and Atkinson, T. 1989. Fermentation and downstream processing of *Bacillus*. In: Biotechnology Handbooks *Bacillus*. Volume 2. Ed. Harwood, C.R. Plenum Press. New York. USA. Pp. 255-292.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology **24**, 187-214.

Sietma, J.H., Eveleigh D.E. and Haskins, R.H. 1969. Cell wall composition and protoplast formation of some oomycete species. Biochimica et Biophysica Acta **184**, 306-317.

Silo-Suh, L.A., Lethbridge, B.J., Raffel, S.J., He, H., Clardy, J. and Handelsman, J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Applied and Environmental Microbiology **60**, 2023-2030.

Slepecky, R. and Hemphill, E. 1992. The genus *Bacillus*-Nonmedical. In: The Prokaryotes. 2nd Edition. Eds. Balow, A.S., Trüper, H.G., Dworkin. M., Harder, W. and Schileifer, K. Volume 2. Springer-Verlag, New York Inc. USA. Pp. 1663-1696.

Slieman, T.A. and Nicholson, W.L. 2001. Role of dipicolinic acid in survival of *Bacillus subtillis* spore exposed to artificial and solar UV radiation. Applied and Environmental Microbiology **67**, 1274-1279.

Smibert, R.M. and Krieg, N.R. 1981. General characterization. In: Manual of Methods for General Bacteriology. Eds. Gerhardt. P., Murray R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. ASM Press. Washington. USA. Pp. 409-449.

Smith, N.R., Gordon, R.E. and Clark, F.E. 1952. Aerobic spore-forming bacteria. U.S. Dept. Agr. Monogr. No16. Washington, D.C. USA.

Southworth, T.W., Guffanti, A.A., Moir, A. and Krulwich, T.A. 2001. GerN, an endospore germination protein of *Bacillus cereus*, is a Na^+/H^+-K^+ antiporter. Journal of Bacteriology **183,** 5896-5903.

Spun, K. 2003. Cellulase production. http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/PRACBIOTECH/PDF/ cellprod.pdf 22/10/2003.

Stein, T., Borchert, S., Conrad, B., Feesche, J., Hofemeister, B., Hofemeister, J. and Entian, K.D. 2002. Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. Journal of Bacteriology **184,** 1703-1711.

Stein, T., Vater, J., Kruft, V., Otto, A., Witmann-Liebold, B., Franke, P., Panico, M., McDowell, R. and Morris, H.R. 1996. The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic template. The Journal of Biological Chemistry **271**,15428-15435.

Stöver, G.A. and Driks, A. 1999a. Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein. Journal of Bacteriology **181**, 1664-1672.

Stöver, A.G. and Driks, A. 1999b. Control of synthesis and secretion of the *Bacillus subtilis* protein Yqxm. Journal of Bacteriology **181**, 7065-7069.

Stragier, P. and Losick, R. 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annual Review of Genetics **30**, 297-341.

Thrane, C., Tronsmo, A., and Jensen, D.F. 1997. Endo-1, 3- β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of, and biological activity against plant pathogenic *Pythium* spp. European Journal of Plant Pathology **103,** 331-344.

Turnbull, C.B.P. *Bacillus.* http://gsbs.utmb.edu/microbook/ch015.htm 12/12/2002.

Upton, M., Tagg, J. R., Wescombe, P. and Jenkinson, H. F. 2001. Intra- and interspecies signalling between *Streptococcus salivarius* and *Streptococcus pyogenes* Mediated by SalA and SalA1 lantibiotic peptides. Journal of Bacteriology **183,** 3931-3938.

Utkhede, S.R. 1983. Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. Canadian Journal of Botany **62**, 1032-1035.

Utkhede, R.S. and Rahe, J.E. 1983. Interactions of antagonist and pathogen in biological control of onion white rot. Phytopathology **73**, 890-893.

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. 1986. Fengycin-A novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. The Journal of Antibiotics **39**, 888-901.

Vi-Cor, 2001. Introduction. http://www.vi-cor.com/html/microserin/mic-about.html 12/01/2003.

Volpon, L., Besson, F. and Lancellin, J. 1999. NMR structure of active and inactive forms of the sterol-dependent antifungal antibiotic bacillomycin L. European Journal of Biochemistry **264**, 200-210.

Williams, A.G. and Susan, W.E. 1983. *Bacillus* spp. in the rumen ecosystem. Hemicellulose depolymerase and glycoside hydrolase of *Bacillus* spp. and rumen isolates grown under anaerobic conditions. Journal of Applied Bacteriology **55**, 283-292.

Wood, W.A. 1981. Physical methods. In: Manual of Methods for General Bacteriology. Eds. Gerhardt, P., Murray, R.G.E., Costilow, R., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. ASM Press. Washington, D.C. USA. Pp. 286-327.

Yamamoto, S., Buvet, P.J. and Harayama, S. 1999. Phylogenetic structure of the genus *Acinetobacter* based on gyrB sequences: comparison with the grouping by DNA-DNA hybridization. International Journal of Systematic Bacteriology **49**, 87-95.

Yao, S., Gao, X., Fuchsbauer, N., Hillen, W., Vater, J. and Wang, J. 2003. Cloning sequencing, and characterization of the genetic region relevant to biosynthesis of the lipopeptide iturin A and surfactin in *Bacillus subtilis*. Current Microbiology **47**, 272-277.

Yoshida, S., Hiradate, S., Tsukamoto, T., Hatakeda, K. and Shirata, A. 2001. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. The American Phytopathological Society **91**, 181-187.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry **34**, 955-963.

Zuber, P., Nakano, M. M. and Marahiel, M. A. 1993. Peptide antibiotics. In: *Bacillus subtilis* and Other Gram-Positive Bacteria. Eds. Sonenshein, A. L., Hoch, J. A. and. Losick R. ASM Press. Washington, D.C. USA. Pp. 897–916.

APPENDICES

Appendix 3.1 Statistical analysis of the data of the seven isolates were analysed in Genstat® statistical analysis software (Genstat 2002) as in the following combination.

General Analysis of Variance.
BLOCK "No Blocking"
TREATMENTS TEMP*MEDIA*ISOLATE

COVARIATE "NO COVARIATE"

ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes; PSE=diff,lsd; LSDLEVEL=5]

Appendix 4.1 Simplified key for the tentative identification of typical strains of *Bacillus* species (Slepecky and Hemphill, 1992).

1.	Catalase: positive ...2	
	a. negative...17	
2.	Voges-Proskauer:positive ...3	
	i. negative...10	
3.	Growth in anaerobic agar: positive ...4	
	1. negative...9	
4.	Growth at 50^0C: positive ...5	
	i. negative...6	
5.	Growth in 7% NaCl: positive	*B. licheniformis*
	1. negative.....	*B. coagulans*
6.	Acid and gas from glucose (inogranic N): positive	*B. polymyxa*
	i. .....negative...7	
7.	Reduction of NO_3 to NO_2: positive ...8	
	1. negative.....	*B. alvei*
8.	Parasporal body in sporangium: positive	*B. thuringiensis*
	a. negative.....	*B. cereus*
9.	Hydrolysis of starch: positive	*B. subtilis*
	i. negative.....	*B. pumilus*
10.	Growth at 65^0C: positive	*B .stearothermophilus*
	i. negative...11.	
11.	Hydrolysis of starch: positive ...12	
	i. negative... 15	
12.	Acid and gas from glucose (inorganic N): positive	*B .macerans*
	i. negative...13	
13.	Width of rod 1.0μ or greater: positive	*B. megaterium*
	a. negative...14	
14.	pH in V-P broth < 6.0: positive	*B. circulans*
	1. negative.....	*B. fimus*
15.	Growth in anaerobic agar: positive	*B. laterosporus*
	1. negative...16	
16.	Acid from glucose (inorganic N): positive	*B. brevis*
	a. negative.....	*B. sphaericus*
17.	Growth at 65^0C: positive	*B. stearothermophilus*
	i. negative...18	
18.	Decomposition of casein: positive	*B. larvae*
	1. negative...19	
19.	Parasporal body in sporangium: positive	*B. popilliae*
	a. negative.....	*B. lentimorbus*

Numbers on the right indicate the number (on the left) test to be applied until the right-hand number is replaced by a species name.