The development of a putative microbial product for use in crop production.

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Abstract

The challenges faced by the agricultural sector especially around improving production yields using environmentally friendly solutions have received market attention. Biological intervention can range from application of biological products to enhance the nutritional value of crops or to control plant pathogens. Biostart®, a biological product that demonstrated growth enhancement when applied in lettuce crops is currently in the market. The product is comprised of a consortium of bacterial isolates (*Bacillus licheniformis*, *Brevibacillus laterosporus* and *Bacillus laterosporus*) but the contribution of the individual isolates to growth enhancement had not been elucidated. Green house experiments on lettuce seedlings with individual and mixed treatments were commissioned to determine such contribution. There was either no or marginal growth enhancement observed in the experiments. The results showed that the product was effective as a consortium and not as individual isolates. Further isolation and screening for potential *Bacilli* with antifungal properties was undertaken. An isolate identified as *Bacillus subtilis* that demonstrated inhibition against a wide spectrum of fungi, and especially the phytopathogenic *Verticillium dahliae* and *Fusarium oxysporum*, was successfully identified. The isolate was cryo-preserved and cultivated to significant levels at bench scale. A characterized comparison of different putative products with known systematic fungicide showed potential application even of heat treated products. The product showed control *V. dahliae* when tested in green houses with potatoes and tomatoes as test crops. This isolate has been targeted for further development as a biological control product.

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Abbreviations

ARC……Agricultural Research Council
BCA……Biological control agent
CCC ……..…….Chlorcholinchlorid
CFU…….……...Colony forming units
CSIR……..Council of Scientific and Industrial Research
DRB……Deleterious rhizobacteria
HTH……...Calcium hypochlorite
IPM……Integrated pest management
IPA…… Isopropyl alcohol
NA……..Nutrient agar
OD……..Optical density
PCA……Plate count agar
PCNB……..Pentachloronitrobenzene
PDA……....Potato dextrose agar
PGPR……..Plant growth promoting rhizobacteria
TSB……Tryptone soy broth
WCB……...Work cell bank

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CHAPTER 1

LITERATURE REVIEW

1 INTRODUCTION

Agricultural farming is regarded as one of the most important pillars of every nation. This has been the case ever since mankind embarked on farming as a source of food production. There are however many challenges that are continuously faced by the agricultural sector with regards to maintaining high production outputs and high quality of the desired cultivars. These challenges include infestation of plantations by weeds, destruction of crops by undesired insects and also contamination of cropland by undesirable micro-organisms resulting in competition for nutrients (Gerhardson, 2002) and proliferation of disease (Winding *et al*., 2004).

Table 1.1.: Estimated annual crop losses as caused by insect pests, plants diseases and weeds in the world (Deacon, 1983).

Commodity	Value ($1000 M)*		Losses (%) DUE TO			Total loss (%)
	Actual	Potential	Insects	Diseases	Weeds	
Wheat	18.5	24.3	5.0	9.1	9.8	23.9
Rice	19.6	36.4	26.7	8.9	10.8	46.4
Maize	11.4	17.5	12.4	9.4	13.0	34.8
Other cereals	11.4	19.8	6.6	8.6	12.1	27.3
Potatoes	10.6	15.6	6.5	21.8	4.0	32.3
Sugar	7.6	13.9	16.5	16.5	12.2	45.3
Vegetables	16.7	23.1	8.7	10.1	8.9	27.7
Fruits	14.3	20.1	5.8	16.4	5.8	28.8
Oil crops	10.6	15.7	11.5	10.2	10.8	32.5
Fibres/rubber	8.6	12.7	14.2	11.8	6.3	32.3

* year 1965

Huge economic losses are therefore experienced annually (Table 1.1) due to crop losses caused by diseases, insects and weeds (Deacon, 1983) and this is of great concern to the agricultural community as it challenges the economic viability and social benefit of agricultural practice. Such crop losses also exacerbate the global challenge of food shortage for the ever-growing population, especially in Africa. There is therefore a need to develop suitable solutions to address these challenges.

Chemical pesticides have been developed and widely utilised to combat plant diseases. However, there is growing reluctance in the agricultural community to persist in utilizing chemically derived compounds for the control of plant ailments, as this practice has a detrimental effect on the environment, animal and human life (Shoda, 2000). Accumulation of such chemicals in the soil can contaminate the water table and compromise continual safe water supply. Continuous exposure of chemicals to farm personnel could also result in health complications and immuno-compromised individuals could be at greater risk. The possibility of a target pest population developing resistance to pesticides is of great concern as it would result in increased resistance and virulent pathogenic strains (Cook and Bakker 1983; Gerhardson, 2002). Some pesticides have a narrow spectrum of application e.g. pentachloronitrobenzene (PCNB) and this can result in a significant increase in diseases caused by pathogens insensitive to this chemical (Lockwood, 1990). The poor biodegradability of most commonly utilised chemical pesticides is of further concern regarding their usage. The cost associated with the development and production of pesticides also limits accessibility to subsistence farmers. Chet (1990) had estimated the cost of developing a pesticide to be over $30 million and this cost is expected to have significantly increased over time. For these reasons, there is an urgent need to develop control measures and treatments and new alternatives to conventional chemical treatments.

Micro-organisms such as bacteria and fungi inhabit virtually all environments and most have been demonstrated to have a beneficial influence on the environment. It is well known that certain bacteria when applied to seed, soil or planting material can suppress soil-borne diseases and promote the health of crop plants (Thomashow, 1996). This practice has been in operation from ancient times (Winding *et al*., 2004) but without recognition that disease suppressing organisms were being introduced even by such traditional practices as the application of manure. These beneficial organisms have been referred to by several names, including biological control agents (BCA), plant growth promoting rhizobacteria (PGPR), or biopesticides. Although BCA are regarded as a safer alternative to chemical pesticides, they also have greater benefits than just disease suppression. Rhizobacteria produce a wide array of secondary metabolites and phytohormones (Ryder *et al*., 1999; Joo *et al*., 2004) that can impact on plant growth

positively. These secondary metabolites may enhance the availability of nutrients to the plant, improve nitrogen fixation ability, and decrease susceptibility to frost damage, induce systemic plant resistance and facilitate plant growth and development (Sturz and Christie, 2003).

1.1 The impact of rhizobacteria on growth and plant health

A limited understanding of the interaction between soil, plants and soil microbial populations has resulted in slow scientific development and limited large scale commercial usage of biological control agents (Gerhardson, 2002). Understanding the functionality of BCA and subsequent interactions in the rhizosphere improves the potential for successful exploitation of these products in the field (Raupach and Kloepper, 1998; Collins and Jacobsen, 2003).

The rhizosphere is referred to as the zone of soil adjacent to and influenced by the roots (Nehl *et al.*, 1996) and most interactions between plants and soil micro-organisms occur in this region. It is usually inhabited by both deleterious rhizobacteria (DRB), that may inhibit plant growth, and plant growth promoting rhizobacteria (PGPR), that may enhance plant growth. Through the selective release of root exudates, plants can influence which rhizobacterial community will be dominant in the rhizosphere (Sturz and Christie, 2003). Organisms which assimilate the exudates will proliferate and those which cannot will be nutrient limited. The effects of an individual isolate of the rhizobacterial population associated with a plant can however fluctuate from growth inhibition to growth stimulation (Nehl *et al.*, 1996). Such effects could be influenced by environmental conditions such as pH, temperature, host genotype and plant developmental stage. As plants excrete different types of exudates during different developmental stages, this may impact on the physiological state of the soil and proliferation of a micro-organism in the rhizosphere. A change in the biochemical state of the presiding organisms is a direct consequence of the change in substrate availability (Upadhyay and Rai, 1986, Cook and Baker, 1983). Exudates excreted by plant roots provide the plant with great physical and chemical benefits (Nehl *et al.*, 1996). The extent of friction between root tips and the soil is related

to root mucilage secretion, which assists in reducing root desiccation, improving root and soil contact and contributes to soil structural stability (Cook and Baker, 1983). For effective development of biological agents, extensive research should be undertaken to enhance and improve the understanding of rhizosphere interactions.

Bacteria that stimulate plant growth have enormous potential in agriculture and these PGPR are generally responsible for positive contributions towards plant development. These bacteria become abundant through the selective excretion of essential exudates by the host plants. Through microbial metabolism of these substrates by the PGPR, arrays of secondary metabolites are released. Lucas García *et al*., (2004) reported on the ability of *Bacillus licheniformis* to promote plant growth of tomato and pepper seedlings. This effect was attributed to the production of phytohormones such as auxins that improved plant growth and health (Cook and Baker, 1983; Sturz and Christie, 2003; Vessey, 2003; Lucas García *et al*., 2004). Siderophores that are produced by certain organisms such as *Pseudomonas fluorescence* are beneficial as they solubilize and sequester iron from the soil (Défago *et al*., 1990; Shoda, 2000) and they have also been reported to enhance nitrogen fixation. Siderophores [2,3-Dihydroxybenzoyl-glycine (2,3 DHBG)] are also produced by gram positive *Bacillus subtilis* (Shoda, 2000) and their effects could be referred to as indirect plant growth stimulation.

Intensive research has been conducted to establish the effect of phytohormones on plant development. A number of phytohormones have been reported to enhance plant growth and these include auxins, gibberellins (Joo *et al*., 2004), cytokinins (Ferreira and Kieber, 2005) and ethylene (Cook and Baker 1983). Auxins (indole -3-acetic acid) have been reported to increase the rate of transcription and the control of activity of some enzymes but the total benefit is demonstrated by a significant increase in plant development (Cook and Baker, 1983). Gibberellins are di-terpenoids which are steroid based compounds and are responsible for stem elongation of the plant (Joo *et al*., 2004). Cytokinins have been reported as being responsible for multiple functions during plant development and these include seed germination and cell multiplication (Ferreira and Kieber, 2005), while ethylene has been shown to suppress certain pathogenic fungi and promote maturation of

fruits (Cook and Baker, 1983). The introduction of organisms that can produce these phytoproducts to the soil may have extensive benefits to the plant production process.

The rhizosphere possesses both growth promoting and deleterious rhizobacteria. Sturz and Christie (2003) referred to DRB as being rhizosphere micro-organisms that adversely affected plant growth and development without destroying plant tissue, but through the production of secondary metabolites, meaning that they are not directly pathogenic. Nehl *et al.,* (1996) also defined DRB as minor plant pathogens that occupy the rhizosphere, rhizoplane and root cortex. Such definitions may be misleading as the influence of rhizobacteria has been shown to fluctuate with environmental conditions, host species, plant developmental age, and accompanying mycorrhizal status (Sturz and Christie, 2003).

Consideration should also be given to deleterious rhizobacteria that have the ability to inhibit root and shoot growth without causing any other visually obvious symptoms (Nehl *et al*., 1996). This is sometimes confused with nutrient deficiencies. Although the DRB are of serious concern to the agricultural sector, very few, if any, negative effects have been reported as a direct consequence of their action (Nehl *et al.*, 1996), apart from growth and yield depression both of which can be a consequence of negative agricultural practice such as continuous monoculture.

A number of mechanisms have been outlined for growth inhibition by DRB such as the production and excretion of chlorcholinchlorid (CCC) which causes plant inhibition. This compound has been directly implicated in the prevention of plant elongation even at small dosages (Gans *et al*., 2000). Bakker and Schippers (1987) suggested that the production of a volatile compound of cyanide origin, by rhizobacteria was a consequence of fields being continuously cropped with potato. A *Pseudomonas* sp. was also found to produce labile phytotoxic metabolites of a non-volatile nature that were active over a wide pH range (Nehl *et al.*, 1996) and were shown to inhibit elongation of wheat roots. It is therefore apparent that rhizosphere organisms also have the potential of exerting a negative impact on the general plant health and survival of crops due to the ability of some of these organisms to produce phytotoxins.

1.2 Crop pathogen- target for BCA

The need to improve agricultural outputs to supply enough food for the world has been driven by an ever increasing population without a concomitant increase in agricultural resources (Cook and Bakker, 1983) and therefore there is a need to increase agricultural productivity. Plant pathogens have however been an additional challenge to the realisation of increased productivity, as such infections result in a decrease in crop yield and infected crops that are harvested may not be fit for consumption due to the presence of undesirable products such as mycotoxins that have detrimental effects to the consumer. A fungus, *Claviceps purpurea* is a rice parasite that causes ergot (Prescott *et al*., 1996) which killed about 40 000 people in France in the earlier centuries and therefore controlling the proliferation of this fungus continues to be of great interest.

Tomatoes are one of the major crops cultivated in South Africa. Abstracts of Agricultural Statistics (2002) reported an estimated tonnage of 256 600 in 2002 realising a price of R2 098/ton. Unfortunately tomato growers are faced with challenges of infections by different plant pathogens. *Verticillium dahliae* has been reported to have caused excessive losses of up to 30% in susceptible cultivars and therefore decreases the actual crop productivity (Goud and Termorshuizen, 2003). This crop is also susceptible to other infections such as *Rhizoctonia solani* which causes both stem canker and black scurf of potato (Larkin and Brewer, 2005).

Verticillium dahliae has also been reported to cause vascular wilt in potato crops (Goud and Termorshuizen, 2003). Potatoes represent about 44% of the total vegetable production and this crop is a significant staple food in South Africa. Losses due to infections therefore cause great concern to the industry. Infections by *V. dahliae* are further exacerbated by the ability of the organism to form dormant structures that can survive for extended periods under adverse conditions of desiccation and heat (Collins and Jacobsen, 2003) and therefore are unlikely to be eliminated even with application of chemical based products. *Phytium ultimum*, *Meloidogyne incognita* (nematode), and *Rhizoctonia solani* have all

been reported to contribute to significant crop losses (Roberts *et al*., 2005). *Fusarium* wilt of banana is another disease of great concern and is caused by *Fusarium oxysporum* which blocks the vascular system resulting in wilting (Cao *et al*., 2005). These are only a few examples of the many pathogens that challenge agricultural productivity but serve to illustrate the great need to develop products that are effective against plant pathogens.

1.3 Methods of disease control in agriculture

The application of chemically derived pesticides has yielded benefits in the past and a number of chemicals are currently available for use against fungal pathogens. Koeller *et al*., (1982) demonstrated that Benomyl, a broad spectrum fungicide registered for use against many fungal pathogens, exerted an irreversible inhibition of cutinase, consequently such a phenomenon prevents any potential penetration of plants by fungi. As this chemical has a broad spectrum of application, its negative impact on beneficial mycorrhizal fungi, is of great concern. Mycorrhizal fungi are beneficial symbiotic organisms which ensure the availability of essential nutrients and they have been reported as being effective in the solubilization of some nutrients (Altomare *et al*., 1999). Interference with these beneficial fungi may therefore have negative outcomes that were not intended through use of this fungicide. Methyl bromide is another chemical which is in the process of being withdrawn because of its negative effects on the environment (Asaka and Shoda, 1996; San-Lang *et al*., 2002). Methyl bromide application was planned for termination by 2005 in developed countries due to its negative impact on the ozone layer (Webster *et al*., 2002) even though it is an effective soil fumigant. As South Africa is an article 5 country according to the Montreal Protocol, phasing out of methyl bromide is targeted for year 2015.

Solarization, which is a method of physical disinfection of the soil, has also been used to reduce the number of pathogenic organisms infesting the soil (Cook, 1990). Solarization is predominantly used in hot countries by covering the soil with transparent film thus elevating the soil temperature (Spadaro and Gullino, 2004). This method is regarded as a potential alternative to chemical control of soil pathogens (Katan 1980) and great successes have been reported using this method, not only in controlling soil pathogens, but also in catalysing the release of and the cycling of soil nutrients for the enhancement of

beneficial rhizobacteria (Patrício *et al*., 2005). The importance of making nutrients readily available cannot be over emphasised as the general deficiency of essential minerals in humans can be attributed to a lack of those minerals in edible plants (White and Broadley, 2005). Attempts to address such challenges have been through bio-fortification of products. Any technique that enhances the bioavailability of nutrients in soil advances the goal of minimizing the deficiency in plants.

Seed treatment is another method used to protect crops from pathogen infection. Mehrotra *et al*., (1986) reported that treatment of seed with a bacterial antagonist demonstrated some control over fungal pathogens but such treatments have only showed limited success as the level of control was not extensive. Crop rotation has also been reported to have a positive impact on the control of pathogens, but due to economic pressures and demand for certain crops, farming communities are sometimes forced to grow a single crop to respond to economic demands which is to the detriment of proper soil management (Deacon, 1983).

Although use of BCA's may seem the best alternative to the farmer and the consumer, to ensure success it cannot be applied in isolation. The application of chemical derived pesticides are envisaged to continue to play an integral part of an Integrated Pest Management (IPM) strategy which utilises a mixture of suitable control strategies integrated with relevant production techniques. The ultimate goal is to significantly reduce disease proliferation to levels that would allow a net economic gain to the farmer (Gan-Mor and Matthews, 2003). The approach of IPM is an accepted trend as it is apparent that single symptom treatment strategies are not necessarily the ultimate solution.

1.4 **Biological control agents**

Biological control was defined by Nigam and Mukerji (1986) as a direct or indirect manipulation of soil microbial flora by man to increase their efficacy against plant pathogens. This manipulation should result in reduced levels of pathogens as described by Garrett (cited by Mehrotra *et al*., 1986). An essential feature of a successful BCA is an ability to reduce the activity of undesired plant pathogens. An understanding of the

behaviour of a BCA in the environment can lead to developments that can improve the performance of the BCA.

Several mechanisms are involved in the actions of BCA's against plant pathogens (Shoda, 2000) such as parasitism, antibiosis, competition, induced systematic resistance and protection from infection (Shoda, 2000; Gerhardson, 2002; Sturz and Christie, 2003). Parasitism is the dependence of a species on the host species for survival, with detrimental effects on the host. Saxena and Mukerji (1986) reported *Bacillus penetrans* as a parasite of nematodes. The entire life cycle of *B. penetrans* is well adapted to parasitism of certain plant nematodes (Saxena and Mukerji, 1986). The relationship results in death to the host nematode or it may reach maturity without producing eggs. A similar phenomenon is observed in fungal pathogen control whereby the BCA may have the ability to produce β-(1, 3) -gluconase and/or chitinase, causing the death of the fungal pathogen by enzymatic degradation (Shoda, 2000). *Trichoderma* spp. have broad spectrum modes of action against plant pathogens such as the productions of gluconase and chitinase enzymes, competition for nutrients and the stimulation of the defence mechanisms of the crop (Szekeres *et al*., 2004). The ability of *Bacillus thurengiensis* to produce BT toxin has also given rise to development of products to control certain plant pests, such as caterpillars through ingestion of the lethal toxin (Gan–Mor and Matthews, 2002). The efficacy of this toxin may however be limited due to the selective nature of the toxin as different strains of *B. thurengiensis* will produce BT toxins effective against specific pests without a general efficacy being exerted for a wide range of pests. This selectivity can be exploited by applying the organism producing the desired toxin therefore limiting the spectrum of activity to non-target organisms. Organisms such as *Brevibacillus laterosporus* have the ability to produce toxic inclusions during cell growth which are released during lysis of the cell (de Oliveira *et al*., 2004). Because of these toxic compounds, the organism can be used in biological control with toxicity having been observed in some beetles and nematodes.

Some micro-organisms have the ability to synthesise antibiotics or other antimicrobial compounds and can be used as BCA's. *Bacillus subtilis* is effective against a range of fungal pathogens such as *Verticilium dahliae* and *Cercospora beticola* (Shoda, 2000; Collins and Jacobsen, 2002). The ability to produce a variety of antibiotics such as subtilin

is considered the key mode of action for disease suppression by these organisms (Mercer, 1986). The quality and quantity of the antibiotic is determined by the type of substrate and growth conditions, thus efficacy will differ depending on environmental conditions and soil type (Singh and Faull, 1986). Strains of *B. licheniformis*, *B. pumilus*, *Brevibacillus laterosporus* and *Paenibacillus polymyxa* were demonstrated to control pathogens in wheat (Alippi *et al*., 2000). The ability of BCA's to compete in the soil environments may contribute greatly to their ability to control plant pathogens.

Competition occurs when there is a demand by two or more micro-organisms for the same resource that is limited in the immediate environment (Kaye and Hart, 1999). As iron is a fundamental element for the respiration of aerobic and facultative anaerobic micro-organisms, its availability in the soil is essential (Montealegre *et al*., 2003). Siderophores are low molecular weight compounds with an affinity for iron that are produced under limiting concentrations of iron. Organisms producing siderophores have been found to suppress plant pathogens such as *Fusarium* spp. that have a nutritional requirement for iron in iron-limited soils (Sturz and Christie, 2003). Furthermore, a mutant strain defective in siderophore synthesis (siderophore-minus) was less effective in suppressing disease than its wild-type counterpart (Bakker 1990). Rhizobacteria can also create partial sinks for nutrients and therefore reduce the amount of nutrients available for plant pathogens, as a result limiting the proliferation of pathogens (Stack *et al*., 1986). However, concerns have been raised with regards to competing pathogenic micro-organisms that can make use of siderophores produced by the biological agent for their own benefit (Bakker, 1990) and therefore growth rate coupled to ability to compete for nutrients are both important features of a BCA in regard to competitive exclusion of pathogenic organisms (Spadaro and Gullino, 2004)

The ability of a BCA to induce resistance to disease has also been suggested as a mechanism of controlling plant pathogens. Application of some bacterial strains has been reported to promote growth and elicit induced resistance to plants against pathogens when applied as seed treatments or soil drenches (Raupach and Kloepper, 1998). This phenomenon can however not be entirely divorced from growth enhancement as the

applied organisms also could be exerting other forms of indirect disease suppression such as phytohormone production.

Induced systematic resistance is the ability of certain rhizobacteria to elicit some disease resistance in crop species. This phenomenon is visible in pathogen-suppressing micro-organisms that show little or no direct antagonism against the target organism yet they exert some biocontrol activity (Gerhardson, 2002). Two types of systemic resistance have been reported namely; systematic acquired resistance (SAR) that is typically induced by pathogens (Gerhardson, 2002) and induced systemic resistance (ISR) when root colonization by certain non-pathogenic rhizobacteria stimulates defence-related genes in plants (Sturz and Christie, 2003). Although systemic resistance can result in broad-spectrum resistance (Thomashow, 1996), it is however mostly not a single effort, but rather a result of multiple allelophatic events with synergistic output that will realise an effective control of plant pathogens (Sturz and Christie, 2003).

The ability of some organisms of the genus *Bacillus* to produce spores under nutrient limiting conditions and the ability to control plant pathogens has made them ideal candidates for development as BCA's. Upudhyay and Rai (1986) reported that for effective biological control of plant pathogens in a field environment, the desired agent has to be produced ideally in a spore form and from inexpensive media. Such a final product will ensure viability over an extended period and if lower cost materials are used, the cost of production can be reduced. The general classification of *B. subtilis* to a GRAS (generally recognised as safe) status further improved interest in this organism as a candidate biological control agent (Wester *et al*., 2004).

Extensive research has been undertaken in recent years to elucidate the ability of *B. subtilis* to control plant pathogens (Wulff *et al*., 2003; Spadaro and Gullino, 2004; Brewere and Larkin, 2005). A number of products derived from different isolates of this organism demonstrated a variety of applications including growth enhancement of certain crops such as tomatoes (Asaka and Shoda, 1996; Kim *et al*., 1997). The ability of *B. subtilis* to produce a wide array of pathogen growth inhibiting substances has resulted in increasing interest in the development of some *B. subtilis* strains as potential BCA's (Asaka and

Shoda, 1996). Furthermore, products of *B. subtilis* origin are currently commercially available and registered, but there is a need for ongoing research and development in this area to provide isolates with higher levels of efficacy and appropriate production methods for *B. subtilis* based BCA's.

1.5 The role of biological agents in integrated pest management.

The application of an individual BCA can have limitations with regards to the consistency of the product and efficacy in different environments (Robert *et al*., 2005). This phenomenon emphasises the importance of an integrated approach to controlling plant pathogens. Integrated pest management (IPM) is a strategy combining several complex control measures to realise synergies in the control of plant pathogens. IPM strategies can be successful when characteristics of the environment, pesticide and antagonist are integrated to act against disease (Cook and Baker, 1983). The aims of IPM are to suppress pathogen proliferation, reduce the negative impact of pathogens on crops, reduce environmental pollution and to limit human exposure to chemical pesticides (Uphunday and Rai, 1986). An IPM theory was reported by Chet (1990) regarding *Trichoderma* application in conjunction with chemicals, for the control of *Fusarium oxysporum*. A strain of *Trichoderma* resistant to a selected chemical can be utilized as a BCA by co-application with a chemical, whereby the chemical would eradicate the soil bacteria including pathogens, but the *Trichoderma* would survive. Another approach would be to introduce *Trichoderma* after fumigation as this would prolong and enhance the effects of the treatment through synergistic effects. The application of *Trichoderma* in the control of *Rhizoctonia solani* after fumigation with methyl bromide showed better control than when the chemical was applied by itself (Chet, 1990).

To ensure the sustainability of the agricultural sector, integrated approaches have to be designed and implemented. IPM can result in positive economic implications such as reduced chemical usage, environmental impact and human exposure. This can result in improvements in crop yield through improved control of pathogens, plant growth

stimulation (Raupach and Kloepper, 1998; Siddiqui and Shaukat, 2003; Roberts *et al*., 2005) and an improvement in the environment.

1.6 Considerations for development of biological control agents as useful products

As the use of chemical pesticides has several disadvantages, alternatives to such products have to be developed. Certain bacteria can suppress soil-borne diseases and promote health and productivity of crop plants, when applied to seed, soil or planting material. For such reasons and by virtue of their physiological adaptability and metabolic versatility, bacteria in plant root zones have been identified as potential agents for biological control (Sturz and Christie, 2003). Because of this, micro-organisms can be isolated and selected based on screens for desired effects and further developed into products for use as biological control agents.

For effective isolation of biological agents, soil from different sources can be used because soils contain a diverse array of useful micro-organisms. Ideally soil sources will be from the fields of target cultivars as these soils should have some level of natural ability to suppress diseases (Ryder, 1999). Soils showing disease suppression especially in the event of a particular outbreak are likely to harbour organisms that posses antimicrobial properties against the pathogen. Soil sources should therefore be selected to maximize antagonist isolation. For effective isolation of *Bacillus* spp., their ability to withstand elevated temperatures and dehydration may be incorporated into an isolation procedure (Földes *et al*., 2000).

Screening is a critical step in the development of a BCA as the suitability of a screening procedure to identify an appropriate candidate with antagonistic potential against pathogens is a key requirement of the procedure (Cook, 1993). The most common method of screening for antibiosis as the mode of action is by monitoring for inhibition of pathogenic organisms due to the activity of the candidate biological control agent in plate inhibition assays (McSpadden Gardener and Fravel, 2002). Although this is an effective

screening technique, it is limited because it does not identify biocontrol agents with other modes of action such as parasitism, induced plant resistance, or some form of competition. Suitable methods should therefore be developed that assess a broader spectrum of useful features of candidate BCA's. Screening methods for parasitism may include burying and retrieving propagules of the pathogen to isolate parasites. Methods to assess competition may incorporate looking for organisms that quickly colonize sterilized soil and have the ability to eliminate other organisms that attempt to invade this space, and micro-organisms that aggressively colonize the infection court (Cook and Baker, 1983)

The selection process for a putative BCA is one of the most important steps required. Firstly the organism of choice should be compatible to the target crop. Vurro *et al.,* (2006) reported on a bacterium that was capable of over producing an amino acid, isoleucine, while growing in tobacco roots. As a consequence, such a phenomenon resulted in the inhibition of acetolactate thus shutting down the synthesis of valine and leucine in the plant. This organism could have demonstrated antagonistic properties *in vitro*, but the application would have been limited. It is therefore a challenge for scientists engaged in development of BCA's to select appropriate organisms while being aware of any undesirable impacts on the environment or on plant growth (Mills, 2005).

The strategy to developing a useful biological agent is driven by an understanding of target ailments and the economic value of affected crops (Mills, 2005). Such an understanding is essential to elucidate the economic viability of developing a production process for any biological product (San-Lang *et al*., 2002). Inoculum effectiveness and production costs are two key factors that require consideration when developing a BCA production method (Yan and Riley, 2003). A dependable and consistent source of the desired culture is required and it is therefore essential to ensure a standard supply of high quality isolates of the desired organisms. This can be achieved by developing appropriate microbial preservation (Meza *et al*., 2003) and culture maintenance methods. Such methods must prevent microbial death, exclude contaminating agents and prevent changes in the biochemical, morphological, and genetic characteristics of the preserved culture (Bargabus *et al*., 2003). The development of appropriate cell storage and inoculum protocols is therefore vital for the success of a production process to manufacture BCA based products.

The performance and efficacy of a biological agent is dependent on appropriate production, product formulation, packaging and application knowledge. The development of these process steps are more challenging than for chemical pesticides (Gerhardson, 2002). Several reports have detailed the tendency of BCAs to lose their capability and ecological competence during large-scale production processes (Sturz and Christie, 2003) and some BCA have lost their efficacy due to changes in the type of substrate used in the fermentation process (Papavisas and Lewis cited in Upuday and Rai, 1988). Understanding such dynamics is therefore vitally important when developing BCAs.

Biological control agents are mainly cultivated in submerged fermentation. The major objective of the fermentation process is to maximize the volumetric productivity (Riesenberg and Gurthke, 1999). As such, the fermentation process is used to maximize biomass production while reducing the fermentation time as this will impact on the cost of production of the BCA (Feng *et al*., 2003). Stringent process development and process optimisation strategies have to be implemented to ensure production processes that are cost effective and efficient (Feng *et al*., 2003).

It is therefore important that suitable operating parameters are established. These include aeration, temperature, dissolved oxygen and agitation. Submerged fermentation processes for the production of BCAs have resulted in viable cell concentrations of $1X10^{10}$ $CFU.mL^{-1}$ (Garrett, 1986), through the development of appropriate media composition and operating conditions.

The stability of the final product is essential, especially in the South African context as a product that is stable for extended periods at non-refrigerated temperature will ensure availability of an active product that is not limited to commercial farming communities. In South Africa, there is a continuous emphasis on the quality of life of citizens and this entails accessibility of agricultural interventions that will realise better agricultural outputs. For this reason, emerging, small and medium enterprise (SMME) farmers must also have access to such products that should have extended shelf lives without a requirement for refrigeration. The ability of some potential biological agents to form endospores is

therefore an added advantage. The ideal BCA product must therefore be stable for extended periods, under relatively high temperatures, during periods of low nutrient levels and show resistance to toxic compounds (Knudsen and Spurr, Jr, 1986; Wolken *et al*., 2003).

Product formulation and application also plays a significant role in determining the effectiveness of BCAs. The ability of *Bacillus* spp. to form spores eases the complexity of product formulation and the application process. A spore can remain dormant for extended periods of time and endure almost all known stresses. Once nutrients are abundantly available in the environment, spores can germinate into viable vegetative cells (Driks, 2002). Different product formulations are available for spores, such as wettable powders, dusts, granules, and liquid products including aqueous cell suspensions, oils and emulsions. The spores can also be microencapsulated or adhered to seeds. The choice of formulation is determined by many factors such as the type of production processes, the mode of action of the strain, the durability of the life-stage of the BCA, the physical, chemical and biological characteristics of the desired application and the equipment used for field application (Jackson *et al*., 1996).

The final product packaging can impact on the efficacy of the product. Warriner *et al*., (1999) reported that application of hydrogen peroxide (H_2O_2) during the sterilization of packaging material resulted in reduced activity of the bioactive compound produced by BCAs due to the carry over of H_2O_2. It is therefore imperative that all factors that can influence the efficacy of the BCA be properly investigated.

Understanding the behaviour of a BCA in the condition that it is expected to be applied in, is of greatest importance (Jacobsen and Collins, 2002) as this will determine the effectiveness and the limitations of the desired BCA. It is essential for the BCA to be applied through an appropriate dosage regime to effect control (Garrett, 1986) and therefore an understanding of the life cycle of target pathogens is also of importance. It has been proposed that beneficial micro-organisms should be introduced at the earliest possible stage of crop development (Sturz and Christie, 2003) to effectively realise the desired

outcomes. Gan-Mor and Matthews (2002) further emphasised the importance of understanding population density and distribution of both the target pathogen and the antagonist as this population dynamic can influence the dose and frequency of application of the BCA. The timing for application of a spore suspension is thus important to ensure the desired success (Yan and Riley, 2003). It is apparent that application of BCAs is never recommended to compromise the introduction of IPM, and all systems aimed at controlling pathogen levels should be purposely utilised in combinations (Gan-Mor and Matthews, 2003). The product should thus be compatible with other existing products. Such an approach is vital as the majority of biocontrol agents applied on their own often have limited success (Roberts *et al*., 2004). Spore suspension formulations are also advantageous as they are compatible with IPM as the spores are generally more resistant to the other chemical compounds that could be applied in the programme (Wolken *et al*., 2003).

1.7 **Motivation for the research.**

The use of chemically derived pesticides has many disadvantages, such as pollution of the water and environment. This is compounded by the overuse of chemical pesticides, particularly in an indiscriminately preventative manner, which has resulted in soil and water pollution and has had harmful effects on human beings (Shoda, 2000). In conjunction with such societal concerns, the reality that there are mostly no resistant cultivars that meet specified standards had also contributed to the requirement for the development of BCAs (Kirk *et al*., 2005). It is therefore essential that alternative control strategies are developed to prevent increasing shortfalls in annual productivity within the agricultural sector, which consequently threatens global food security. Bacteria have been reported to suppress soil-borne plant pathogens and to promote health and productivity of crops when applied to seed, soil or planting material. The fact that they are resident organisms of the soil renders their application ideal as they can easily adapt to the new environment. Products based on *Bacillus* spp. further offer benefits due to the ability of these organisms to form spores which can be viable for extended periods, even under unfavourable conditions, thus extending the shelf-life of products. The development of technologies for mass production of these products will further ensure extended

availability at a reasonable price. This is crucial towards the introduction of integrated strategies in the emerging and commercial agricultural sector and will furthermore minimize the negative impact of excessive application of chemical based products.

1.8 Project objectives and aims

The increasing resistance faced by the agricultural sector in treatment of infectious plant pathogens with chemical pesticides has resulted in a need for alternatives to be developed. It is the ultimate objective of this research to develop a bacterial product or products which can be directed for use in crop production. Such a product should enhance crop growth, crop yield and/or promote biological control of plant pathogens relevant to economically important plant diseases. Such a product should effectively and consistently act against target pathogens. However, the agricultural sector is perceived to be extremely conservative and resistant to change and it is therefore a requirement that the development of the biological agents should offer the end user an improved benefit to cost ratio.

In order to achieve the objectives, the following three aims will be examined and investigated

1.8.1 Bench scale development of a biological agent showing growth enhancement activity. This will be achieved by:

- Successful cryo-preservation of isolates to be investigated;
- Demonstration of the bench scale production process; and
- Stabilization of a model organism and testing the efficacy of isolates as growth promoters on selected crops.

1.8.2 Isolation, screening and selection of potential isolates showing plant pathogen inhibition. This will be achieved by undertaking:

- Isolation and *in vitro* screening of existing and new isolates (CSIR culture collection and new soil samples) for antifungal properties; and
- Identification of isolates showing greatest biocontrol potential.

1.8.3 Bench scale process development of a selected isolate showing antifungal activity. This will be achieved by:

- Cryo-preservation of the selected isolate to be investigated;
- Demonstration of the bench scale production process;
- Stabilization of the product and testing the efficacy of the product through *in vitro* and *in vivo* trials;
- Determining the effect of high temperature on product activity; and
- Comparing the efficacy of the product with a known agrichemical.

CHAPTER 2

EVALUATION OF THE POTENTIAL OF BIOSTART®

AS A GROWTH ENHANCER

2 INTRODUCTION

There is an increasing effort towards the improvement and sustainability of crop production by enhancing plant growth promotion and control or suppression of phytopathogens. Rhizobacteria, such as *Bacillus* species, have been shown to increase plant growth, yield and productivity of a number of important crops (Reddy *et al*., 1999; Siddiqui and Shaukat, 2003). There is thus an interest in developing such micro-organisms towards potential commercial products in agriculture (Gerhardson, 2002) due to their ability to address multiple agricultural challenges and improve crop productivity. Plant growth promoting Rhizobacteria (PGPR) have been shown to solubilise insoluble or sparingly soluble minerals such as iron and other minerals *in vitro* (Altomare, 1999) and can thereby increase the availability of these minerals for assimilation by plants. The capability of some PGPR to produce phytohormones that enhance plant growth and development (Khalid *et al*., 2005) also demonstrates the potential to exploit these micro-organisms in agriculture. Lucas García *et al*., (2004) reported on a *B. licheniformis* strain with ability to promote plant growth of tomato and pepper seedlings. The effect could be attributed to the ability of the organism to produce phytohormones thus resulting in growth enhancement. Some PGPR have the ability to control the proliferation of phytopathogens thus resulting in improved growth and the prevention of crop losses (Kavitha *et al*., 2005; Bernal *et al*., 2002). Isolates of *B. licheniformis* together with *B. pumilus*, *Brevi. laterosporus* and *Paenibacillus polymyxa* were also reported by Alippi *et al*., (2000) to have the ability of controlling a virulent wheat pathogen. Consequently, an improved harvest could be realized due to less competition for nutrients. A need to develop biological agents demonstrating growth enhancement would therefore yield significant outcomes for the agricultural community.

The first step in the development of a biological product is the isolation and selection of potential isolates while taking into account the application requirement of the isolates (Montealegre *et al*., 2003). Once a suitable isolate has been identified, a validated production process has to be developed using competitive raw materials (Upadhyay and Rai, 1986). The final product must also demonstrate efficacy and be stable. The continuous availability and maintenance of an inoculum source is an integral part of every successful

fermentation process. This is because the source and quality of cultures used for inoculation impacts on the final product quality and the validity of *in vivo* efficacy tests. Maintenance of a standardized supply of high quality culture for process development and further commercial production is essential. This can be achieved by applying suitable microbial preservation techniques (Meza *et al*., 2003) and effective culture maintenance methods. The objective of microbial preservation is to restrain microbial death, exclude the introduction of contaminating agents and prevent changes in the biochemical and morphological characteristics, nucleotide sequence and plasmid stability of the conserved culture (Bargabus *et al*., 2003). It is thus important to prepare an appropriately preserved culture of putative biological control agents for subsequent evaluation and process development purposes.

The key advantages of *Bacillus* based biological products are their ability to sporulate. Endospores produced by *Bacillus* spp. are resilient structures capable of surviving desiccation, heat, oxidizing agents, UV and γ radiation (Jacobsen and Douglas, 2002; Wuytack *et al*., 1999). This ability improves the prospect of developing a highly stable product that will survive extreme temperature and adverse storage conditions while retaining viability (Knudsen and Spurr, Jr, 1986; Wolken *et al*., 2003).

Biostart® is a commercially available product that is composed of three isolates, namely *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus*. The basis for the product formulation was the ability of the isolates to enhance growth (Lucas García *et al*., 2004; Alippi *et al*., 2000). The growth enhancement ability of the product has been demonstrated in green-house trials on lettuce seedlings by Relihan[1] (Personal communication) but these experiments did not evaluate the contribution of each isolate towards the observed growth enhancement. Determining the growth enhancement efficacy of individual isolates was therefore imperative as potential elimination of just one isolate could have great economic benefits by lowering production cost by one third.

([1] June 2004, project report)

The objectives of this study were therefore to:

1 Prepare cryo-preserved cell banks of the isolates under investigation and validate the cell banks.

2 To cultivate and stabilise *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* at bench scale.

3 To test each individual isolate for plant growth promoting efficacy on lettuce seedlings.

2.1 Materials and Methodology

2.1.1 Cryo-preservation and culture maintenance

Cultures of *B. licheniformis, B. laterosporus* and *Brevi. laterosporus* were obtained from Microbial Solution (PTY) LTD[2]. All cultures had been cryo-preserved and kept in 1.8mL Nalgene cryo-vials (Nalgene Nunc Int, Rochester, NY) at -70ºC in an ultra-freezer. A semi-defined cultivation/sporulation media containing 0.8%m.v^{-1} Yeast extract, 0.5% m.v^{-1} Peptone, 0.3% m.v^{-1} Meat extract, 0.005% m.v^{-1} $MnSO_4.4H_2O$, 0.01% m.v^{-1} $CaCl_2.2H_2O$ and 0.03% m.v^{-1} $MgSO_4.7H_2O$ was prepared and the pH adjusted to 6.8 with 25% m.v^{-1} NH_4OH and 30% m.v^{-1} H_2SO_4. A total volume of 700mL was sterilized in a 1.8 L Fernbach flask for 20min at 121ºC and 100 kPa pressure using an autoclave (Anzy JSD400, Getinge Inc, Rochester, NY). Three flasks were prepared in this manner and after the flasks had been cooled to room temperature, each flask was inoculated with the contents of one cryo-vial of each isolate per flask and incubated at 32ºC at an agitation of 200rpm on an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ). After 24hrs incubation flasks were aseptically sampled (10mL) for microscopic examination to determine the purity of each culture and the sporulation efficiency at 1000X magnification using a microscope and image analysis software (Olympus BX40, Olympus Optical CO. LTD, Japan and Optimas Software version 6.2, Optimas Corporation, USA).

[2] Microbial Solutions (PTY) LTD, P.O Box 103, Kya Sands, Randburg, 2163.

The following equation was used to determine percentage sporulation efficiency:

$$\%Sporulation\ Efficiency = Mean\ Count_{(spore)} / [mean\ count_{(veg)} + mean\ count_{(spore)}] * 100 \quad (Eq: 1)$$

When approximately 90% sporulation efficiency was achieved, the culture was cryo-preserved. Equal aliquots of the medium were aseptically mixed with cold 50% $v.v^{-1}$ sterile glycerol solution (Baudot and Odagescu, 2004; Woelders, 2004, Wharton *et al*., 2004). After proper mixing, aliquots (1.5mL) of the mixture were aseptically dispensed into 1.8mL Nalgen cryo-vials (Nalgene Nunc Int, Rochester, NY) using an auto dispenser (Eppendorf AG, Humburg, Germany). The cryo-vials were then placed into Nalgen Mr Frosty cryo-containers, containing approximately 250 mL cold Isopropyl Alcohol (IPA). The IPA is used to control the freezing rate of the cryo-vials when placed in the ultra-freezer. The Mr Frosty containers were stored in an ultrafreezer at –70ºC for 48 hours (Meza *et al*., 2003). Five percent of the total number of preserved cryo-vials was randomly selected to check the purity of the preserved culture by streaking onto Plate Count Agar (PCA) plates (Biolab, 1024507, Merck, RSA) and incubated for 24-48 hrs at 32ºC. The viable spore count in each of the cryo-vials was also assessed by transferring 1mL from each cryo-vial into 9mL of sterile peptone water resulting in 10^{-1} dilution, followed by serial dilution up to 10^{-9}. From selected dilutions, 0.1mL was spread on PCA plates which were incubated at 32ºC for 24hrs and the resultant colonies were counted (Perez-Zohar *et al*., 2002) to enumerate the viable cell concentration of each cell bank.

To assess the growth rate and transfer time of each working cell bank (WCB), three cryo-vials were randomly selected from each cryo-preserved culture and the contents inoculated into individual flasks containing sterile 3% $m.v^{-1}$ Tryptone Soy Broth (TSB) (Biolab, 102506, Merck, RSA), that was used as a modified inoculum stage medium (Feng *et al*., 2003). The flasks were aseptically sampled two hourly and the optical density (OD) was measured at 660 nm using a spectrophotometer (Spectronic® 20 Genesis®, Spectronic instruments, USA) according to Prescott *et al*., (1996). Purity of the culture was assessed by microscopic examination and by streak plating on PCA as previously described.

The maximum growth rate was determined (John Pirt, 1975) from OD measurements using the following equation for data points conforming to high linearity ($r^2 > 0.9$) of a plot of ln OD against time:

$$\mu = d \ln (OD_{660}).dt^{-1} \qquad (Eq: 2)$$

Where, μ is maximum growth rate;

The doubling time of the organism was calculated using the following equation (John Pirt, 1975):

$$Doubling\ time = \ln (2).\mu^{-1} \qquad (Eq: 3)$$

During late exponential growth phase, samples were taken to determine the viable cell number as outlined earlier in this section.

2.1.2 Growth of isolates for biomass production

The cryo-preserved cultures of *B. licheniformis, B. laterosporus* and *Brevi. laterosporus* were inoculated into pre-sterilized TSB (100mL), contained in a 1L Erlenmeyer flasks (unbaffled), resulting in a 10% media to volume ratio (Feng *et al.*, 2003). After sterilization at a temperature of 121°C for 20 minutes at 100 kPa pressure in an autoclave (Anzy JSD400, Getinge USA, Inc, Rochester, NY), the media was cooled to ambient temperature and each flask was inoculated with the contents of one cryo-vial per organism and incubated at 32ºC on an orbital shaker at 200rpm (Innova 2300, New Brunswick Scientific, Edison, NJ). Each experiment was done in triplicate. After 12hrs incubation, the flasks were sampled and checked for purity as described previously in section 2.1.1. One flask of each isolate was used for the inoculation of individual fermenters.

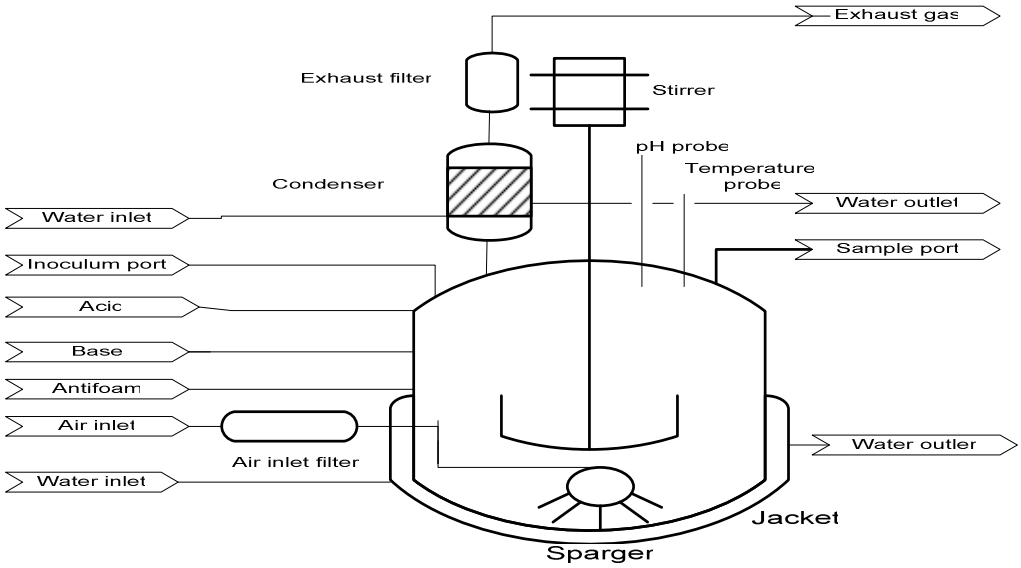


Figure 2.1.2.1.: Schematic representation of Braun B fermenter

The fermentation media was prepared (Appendix 1) and transferred into three 2L fermenters (Biostat B, B. Braun Biotech, International, Germany). A schematic representation of a Biostat B Fermenter is shown in figure 2.1.2.1. The fermenters were sterilized for 30 minutes at 121°C and 100 kPa pressure in an autoclave (Anzy JSD400, Getinge USA, Inc, Rochester, NY). Once the fermenters were cooled to a temperature of 32°C, the pH was adjusted to 6.8 with 25% $m.v^{-1}$ NH_4OH and 30% $m.v^{-1}$ H_2SO_4. Carbon source solutions were prepared by dissolving 15g glucose monohydrate in 10mL of water contained in 50mL flasks followed by sterilization for 20 minutes in an autoclave as previously described. After cooling to room temperature the carbon source solutions, were aseptically added to each fermenter, Vitamin solutions were prepared (Appendix 2) by dissolution of the compounds in water (10mL) and the solutions were thereafter filter sterilized through a 0.22µm sterile filter, into the inoculum flasks prior to inoculation. Samples were taken prior to inoculation of the fermenters to confirm sterility by microscopic observation of the starting media.

The contents of the inoculum flasks were aseptically transferred to each of the designated fermenters. The working volume of each fermenter was 1200mL. The pH was controlled at

6.8 with 25% m.v^{-1} NH_4OH and 30% m.v^{-1} H_2SO_4, aeration at 1v.v^{-1}.m^{-1} and agitation at 500 rpm. The fermenters were sampled (20mL) at four hourly intervals and assayed for glucose by a modified Accutrend method (Appendix 3) using an Accutrend instrument (Roche Diagnostics Gmbh, Germany), Cell biomass was determined by centrifugation of 2 mL of broth sample in a pre-weighed eppendorf at ~ 10 000 x *g*. The supernatant was discarded and the pellet was re-suspended and rinsed in 1mL of 0.1 N HCl. The contents were centrifuged and the supernatant discarded. The pellets were further rinsed by re-suspending in 1 mL de-ionised water before centrifugation. The supernatants were discarded and the eppendorf tubes containing the pellets were dried in a convection oven at 105ºC (Labcon, C.C Imelmann (PTY) LTD, Jhb) for 24 hours. The eppendorf tubes were cooled and weighed and the differential masses were used to calculate biomass content (Brenton 1999).

$$Dry\ biomass = [(FW - EE)/2]*1000 \qquad (Eq: 4)$$

With :

FW= final weight of eppendorf tube with the pellet

EE = empty eppendorf tube weight

Microscopic examination was conducted at 1000X magnification to confirm the monoculture status of the growing cultures. Final samples were taken after termination of the process and analysed for viable cell count, streak plate for purity and sporulation efficiency. The fermentation was terminated when the sporulation efficiency reached ~90%.

2.1.3 Stabilization and formulation of final broths

The final sporulated broths from each fermentation were stabilized. The stabilization procedure involved the addition of pre-sterilized stabilizing agents, constituted by glacial acetic acid, HCl and potassium sorbate, to the final product contained in a 2L flask with continuous stirring by a magnetic stirrer bar. The broth temperature was adjusted to 4ºC by placing it in a cold room with continuous agitation for approximately one hour. The pH

was adjusted to 4.25 by drop-wise addition of glacial acetic acid while the pH was monitored using a pH meter (Jenway 3310 pH meter, Barloworld Scientific, England). Proper mixing for three minutes was allowed after the target pH had been reached. Potassium sorbate was added to each of the broths to a final concentration of 0.15% $m.v^{-1}$, followed by the addition of 4N HCl drop wise to a pH of 3.9. After proper mixing, a sample was taken to determine the cell concentration after stabilization by performing viable cell count assays of the final sample. The final product was kept in a cold room at 4ºC. Samples were taken over six months to determine the viability of the preserved spores. A student T-test was done to compare results and P values greater than 0.05 were regarded as non significant.

2.1.4 Product activation

To eliminate the effects of complex activator application in growth enhancement of lettuce seedlings, a mixture of 75% Corn Steep Liquor (CSL) and 25% dextrose with a total concentration of $1g.L^{-1}$, was applied to all seedlings. The activator solution was prepared by dissolving 0.75g of CSL in 750mL of water. The pH was adjusted to 7.2 using 4N HCl and 32% $v.v^{-1}$ NH_4OH and the final volume was made to 800mL by addition of distilled water. Dextrose (0.25g) was weighed in a separate flask and dissolved in 80mL distilled water with the final volume being made up to 100mL. The flasks were sterilized at 121ºC for 20 minutes at 100 kPa pressure using an autoclave (Anzy JSD400, Getinge USA, Inc, Rochester, NY). The solutions were combined after cooling with a total volume of 900mL. The extra 100mL to realize a concentration of $1g.L^{-1}$ was compensated with the addition of designated isolate suspensions just prior to application. The recommended product application rate in the field is $5L.ha^{-1}$ of 1×10^9 $CFU.mL^{-1}$. To achieve the same dosage in the greenhouse the product was diluted to a concentration of 8.3×10^5 $CFU.mL^{-1}$ with an application volume of 200mL. $tray^{-1}$. The final application solution contained bacterial concentrations of 8×10^5 $CFU.mL^{-1}$ with $0.75g.L^{-1}$ CSL and $0.25g.L^{-1}$ dextrose. This application regime was also adopted even for applications with mixed isolates. The target concentration remained 8×10^5 $CFU.mL^{-1}$ but this was achieved by aseptic mixing of isolates in appropriate volumes to result in the target concentration. The same methodology was used for the preparation of either vegetative cells or spores which were

applied as separate treatments to the seedlings to determine the impact of a pre-activation step of the biological agents.

The flasks were incubated at 32°C on an orbital shaker at 180rpm for 8h. Microscopic slides were prepared after the incubation time had elapsed to confirm the purity status of the solution and to determine germination efficiencies. The following equation was used for germination efficiency.

$$\%GE = Mean\ count_{(veg)} / [\ mean\ count_{(veg)} + mean\ count_{(spores)}] * 100 \qquad (Eq: 5)$$

where a minimum of six fields at 1000x magnification were examined (Prescott *et al*., 1996).

2.1.5 Testing of product efficacy in vivo

Commercial nursery test

The *B. licheniformis, B. laterosporus* and *Brevi. laterosporus* organisms that were cultivated and stabilized, were tested for product efficacy. Individual isolates were grown as outlined in Section 2.1.2 and stabilized as per Section 2.1.3. The stabilized broths were kept in the cold room at 4ºC until they were required. Efficacy trials were planned by Halalisani Gumede and subcontracted for execution to Plant Aid Services[3] at Sunshine Seedling's[4] green houses in Pietermaritzburg. The products to be tested were designated as follows, *B. licheniformis* (B1) at 1.2 x 10^9 CFU.mL^{-1}, *B. laterosporus* (B2) at 1.1 x 10^8 CFU.mL^{-1} and *Brevi. laterosporus* (B3) at 1.5 x 10^9 CFU.mL^{-1}.

Lettuce seedlings (*Lactuca sativa*) cv Frosty, were prepared in Unigro 128 trays at Plant Aid Services. Ten Unigro 128 trays with removable inserts were surface sterilized with 1% HTH (calcium hypochlorite) solution and rinsed with autoclaved water. Composted pine bark seedling mix that had been sterilized in a pressure cooker for 20 minutes and cooled to ambient temperature was then dispensed into individual inserts. Lettuce seeds that had been surface sterilized for 3 minutes with 1% HTH and rinsed at least twice in autoclaved water were sowed into trays at a rate of one seed per cavity. Trays were incubated in the dark with watering to saturation using autoclaved tap water until seedling emergence. Once proper emergence of seedlings was observed the Unigro inserts were sorted such that each of the eight trays to be used had fairly uniform seedling heights. Each tray was subdivided into sixteen treatments as outlined in the tray layout (Appendix 4) and selected seedlings were inoculated with the relevant treatments.

Each designated seedling was inoculated with the aid of a sterile syringe and needle with 1.56mL of the respective formulation based on a dosage of 200mL.tray^{-1}. Once the trays were inoculated, they were placed in a demarcated area in a commercial nursery and irrigated by a boom sprinkler.

[3] Plant Aid Services, 4 Temple Street, P.O Box 101007, Scottville, 3209.
[4] Sunshine Seedlings, Springbank Farm, P.O. Box 81, Rynie, 4182.

A second inoculation was applied to half of the treatments after seven days in the nursery to evaluate the effect of a booster dose of the biological agent. A similar procedure was employed for the preparation and inoculation of both the pre-incubated and non-incubated application.

At completion of the nursery trial, the seedling development was assessed by visual inspection. Final seedling weight was determined by cutting the seedling top off with scissors, allowing them to drain excess water and the fresh weight of each seedling was measured. Potting soil from selected seedling pots was sampled for the determination of viable cell counts of the *Bacillus* spp. added.

Agronomy tunnel test

A second experiment on lettuce seedlings (*Lactuca sativa*) cv Frosty, was done to provide more clarity on product efficacy, and to demonstrate the growth enhancement effect of all test isolates. Trials were conducted in an agronomy tunnel in Pietermaritzburg using an improved experimental design. Experiments were conducted as outlined in Section 2.1.4. Eight trays were used for this trial and only one product application was investigated and not the double product application as had been previously done. Two extra trays that had pine bark seedling media mixed with top soil were included to evaluate differences in seedling development by measuring seedling top fresh weights. Different combinations of the isolates were applied as outlined in the experimental designs (Table 2.1.4.1).

Table 2.1.5.1.: Experimental design for the agronomy trials on lettuce seedlings.[5]

01	01		02	02		03	03
01	01		02	02		03	03
04	04		05	05		06	06
04	04		05	05		06	06
07	07		08	08		09	09
07	07		08	08		09	09
10	10		11	11		12	12
10	10		11	11		12	12
13	13		14	14		15	15
13	13		14	14		15	15
		16	16	16	16		

Table 2.1.4.1 illustrates the different treatments for the modified experimental design. Means and totals of the seedling weights were calculated and the data analyzed using analysis of variance applying Rayner (1969) formulas.

[5] Where each cluster of same numbers represents a block of seedlings with a similar test application. 1- No product application, 2- application of activator only, 3- *B. licheniformi*s and activator, 4- *B. laterosporus* and activator, 5- *Brevi. laterosporus* and activator, 6- (*B. licheniformis* + *B. laterosporus*) and activator, 7- (*B. licheniformis* + *Brevi. laterosporus*) and activator, 8- (B. *laterosporus* + *Brevi. laterosporus*) + activator, 9- (*B. licheniformis* + *B. laterosporus*+ *Brevi. laterosporus*) and activator. Treatments 10-16 were replicates of 3-9 but these treatment were pre-incubated prior to application.

2.2 Results

2.2.1 Cryo-preservation and culture maintenance

Working cell bank's (WCB) were prepared for *B. licheniformis, B. laterosporus* and *Brevi. laterosporus*. Microscopic examination of the flasks inoculated from cryo-vials and streak plates on Nutrient Agar indicated that the cryo-vials were monoseptic. Growth of each of the 3 isolates was evaluated in shake flasks by optical density and is presented in Figure 2.2.1.1.

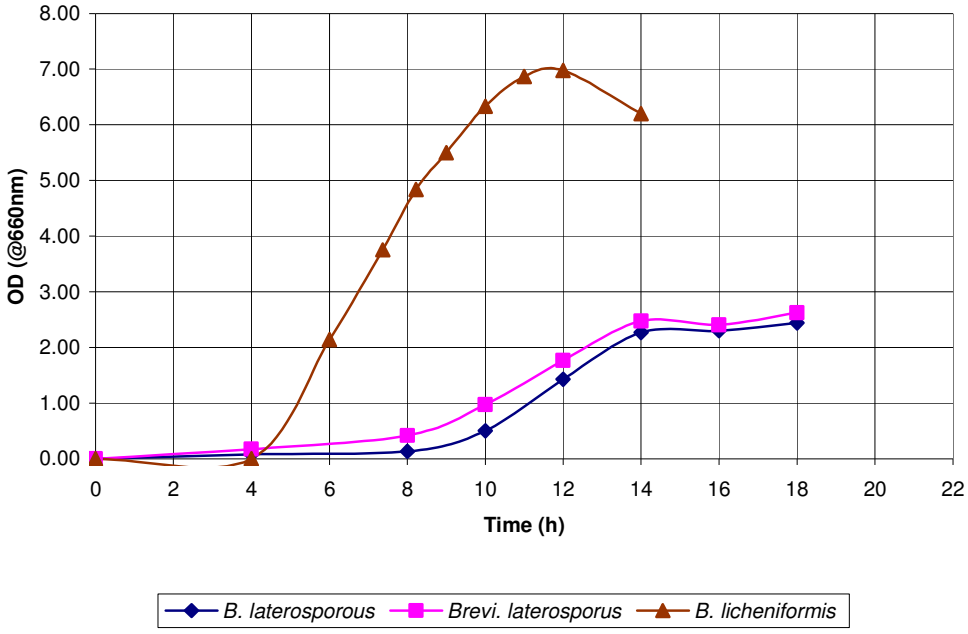


Figure 2.2. 1.1.: Growth curves of *B. licheniformis, B. laterosporus* and *Brevi. laterosporus* based on optical density.

B. licheniformis had a four hour lag phase compared to *B. laterosporus* and *Brevi. laterosporus*, with the later two organisms only demonstrating exponential growth after an eight hour lag phase. The stationary growth phase of *B. licheniformis* was reached after eleven hours in contrast to fourteen hours for *B. laterosporus* and *Brevi. laterosporus.* The latter two organisms displayed similar growth patterns. The differences in growth rate and lag phase cannot be attributed to the inoculum size as the viable spore counts indicated that

B. licheniformis and *Brevi. laterosporus* had the same viable cell counts after cryo-preservation. The maximum optical density achieved by *B. licheniformis* was 2.8 fold higher than that of the other two isolates.

The viable spore counts were determined for each WCB by plate-count assay of the contents of the cryo-vials.

Table 2.2.1.1.: Average viable cell counts for the cryo-preserved *Bacillus* spp.

Organism	**Mean viable count ($CFU.mL^{-1}$)**	**SD**
B. licheniformis	1.2×10^9	1.00×10^8
B. laterosporus	5.3×10^7	4.17×10^7
Brevi. laterosporus	1.2×10^9	2.00×10^8

The viable cell count assays of the cryo-preserved cultures showed that *B. licheniformis* and *Brevi. laterosporus* had average viable cell counts of 1.2×10^9 $CFU.mL^{-1}$ while *B. laterosporus* had viable cell counts of 5.3×10^7 $CFU.mL^{-1}$.

Table 2. 2.1.2.: Mean growth rates and doubling times for *B. licheniformis, B. laterosporus* and *Brevi. laterosporus.*

	B. licheniformis	***B. laterosporus***	***Brevi. laterosporus***
Doubling time (Dt) (h)	0.6	1.58	1.00
SD	0.16	0.10	0.05
Growth rate (μ) (h)	1.21	0.44	0.68
SD[6]	0.30	0.03	0.04
R^2	0.992	0.998	0.995

Growth of the cryo-preserved *B. licheniformis* culture resulted in an average doubling time of 0.6 hours and an averaged maximum growth rate of 1.21 hours. The growth rate plots of

[6] SD :- Standard deviation

B. laterosporus demonstrated a doubling time of 1.58 hours with maximum growth rate of 0.44 hours. *Brevi. laterosporus* had a maximum growth rate of 0.68 hours with a doubling time of 1.00 hours.

2.2.2 Growth of isolates for biomass production

The production of *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* was demonstrated in batch fermentation at 2L scale. The final broth was to be used for product testing in green house experiments. The summary of the fermentation performance for each of the organisms is tabulated in Table 2.2.2.1.

Table 2.2.2.1.: Summary of the fermentation performance for each of the *Bacillus* spp.

Organism designation		***B. licherniformis***	***B. laterosporus***	***Brevi. laterosporus***
Age	h	20	26	28
Total TSAI[7] added	g	14.3	15	14.8
Final Cell Count	$CFU.mL^{-1}$	5.0×10^{9}	3.4×10^{9}	5.6×10^{9}
Total Cell Count	CFU	6.0×10^{12}	4.1×10^{12}	6.7×10^{12}
CFU yield on TSAI	$CFU.g^{-1}$	4.2×10^{11}	2.7×10^{11}	4.5×10^{11}
Cell Productivity	$CFU.\ L^{-1}.h^{-1}$	2.5×10^{8}	1.3×10^{8}	2.0×10^{8}
Culture Purity	-	Monoculture	Monoculture	Monoculture
Sporulation Efficiency	%	98	92	96

The batch fermentation of *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* at bench scale was successfully executed. The fermentation termination signal was sporulation of above 90% after 20 hour fermentation bracket. A total viable cell concentration of 6.0×10^{12} CFU was achieved for *B. licheniformis* with cell productivity of

[7] TSAI- Total sugars as inverts

2.5 x 10^8 CFU. $L^{-1}.h^{-1}$ after 20 hours of fermentation. *B. laterosporus* was cultivated to a viable cell concentration of 4.1 x 10^{12} CFU and a cell productivity of 1.3 x 10^8 CFU.$L^{-1}.h^{-1}$ after 26 hours of fermentation. The cultivation of *Brevi. laterosporus* at bench scale yielded a total concentration 6.7 x 10^{12} CFU with cell productivity of 2.0 x 10^8 CFU.$L^{-1}.h^{-1}$ after 28 hours of fermentation. The determination of viable cell yields with sugar as a variable showed that *Brevi. laterosporus* resulted in a higher yield on consumed sugar of 4.5 x 10^{11} CFU.g^{-1} with *B. licheniformis* and *B. laterosporus* having yields of 4.2 x 10^{11} CFU.g^{-1} and 2.7 x 10^{11} CFU.g^{-1} respectively. Even though *Brevi. laterosporus* had resulted in a higher yield, it was also fermented for longer at 28 hours in contrast to *B. licheniformis* that was fermented for 20 hours. The fermentations for *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* were confirmed to be pure by microscopic examination and uniform colonies observed on streaked PCA plates.

2.2.3 Stabilization and formulation of final broths

The full broth at the end of the fermentation was harvested and stabilised as per the method outlined in Section 2.1.3. The viable spore count of the stabilized product was done immediately after stabilization and after six months of storage at 4°C. The viable spore-counts are captured in Table 2.2.3.1.

Table 2.2.3.1: Stabilization data for *Bacillus* spp. observed over six months storage in a cold room (4ºC).

	B. licherniformis		***B. laterosporus***		***Brevi. laterosporus***	
	after Stabilization	Six months	after Stabilization	Six months	after Stabilization	Six months
Average counts (CFU.mL^{-1})	1.27 x10^9	1.13 x10^9	1.02 x10^8	1.03 x10^8	1.07 x10^9	1.02 x10^9
SD	1.00 x10^8	5.77 x10^7	8.19 x10^6	5.77 x10^7	5.51 x10^8	6.81 x10^7
TTEST[8]	0.17		0.14		0.27	

[8] TTEST : Student test comparing two sets of quantitative data

Stabilization of all the isolates was demonstrated to have been successful as there was no significant difference in viable cell number after six months of product storage at 4°C. The statistical comparison of viable cell count data obtained for the stabilized broths resulted in P-values showing non-significant difference in the data with values of 0.17, 0.13 and 0.27 for *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* respectively.

2.2.4 Testing of product efficacy in vivo

Commercial nursery test

The efficacy of the stabilised product was evaluated on lettuce seedlings in a commercial nursery. The outcome from the trial is tabulated in Table 2.2.4.1. The data was statistically evaluated and the results reported in Table 2.2.4.2.

Table 2.2.4.1: Shoot fresh weight averages for lettuce seedlings at a commercial nursery after 20 days growth.[9]

Treatments	Mean (g)	Treatments	Mean (g)	Treatments	Mean (g)	Treatments	Mean (g)
NC	2.69	B1+ B2 + B3	2.77	NC	2.75	B1+ B2 + B3	2.59
BC	2.63	A1	2.77	BC	2.48	A1	2.51
B1	2.78	A2	2.81	B1	2.71	A2	2.71
B2	2.77	A3	2.69	B2	2.51	A3	2.74
B3	2.65	A1 + A2	2.85	B3	3.82	A1 + A2	2.81
B1 + B2	2.68	A1 + A3	2.34	B1 + B2	2.70	A1 + A3	2.69
B1 + B3	2.62	A2 + A3	2.81	B1 + B3	2.73	A2 + A3	2.83
B2 + B3	2.64	A1+ A2 + A3	2.71	B2 + B3	2.70	A1+ A2 + A3	2.71

[9] With NC (no treatement), BC (activator only), A1, A2, A3 being activated treatments of *B. licheniformins*, *B. laterosporus* and *Brevi. laterosporus* respectively and B1, B2 and B3 being non activated treatments of *B. licheniformins*, *B. laterosporus* and *Brevi. laterosporus* respectively.

Table 2.2.4.2: Statistical analysis of shoot weight results obtained for lettuce seedlings at a commercial nursery after 20 days growth.

Analysis of variance of lettuce top weights (g.plant^{-1})# Trial 1					
Source	d.f.	s.s.	m.s.	F-ratio	F-Prob.
Blocks	7	10.45	1.49	17.53	<0.001
Treatments	31	3.13	0.101	1.18	NS
L1: No Bacteria vs. Bacteria	{1}	{0.12}	{0.12}	1.41	NS
Deviations	{30}	{3.01}			
Error	217	18.47	0.085		
Total	255	32.05			
L1: No bacteria vs. bacteria = 0.064					
l.s.d. of treatment means differences = 0.286					
# Calculation done with calculator according to Rayner (1969)					
Abbreviations: d.f. = degrees of freedom; s.s. = sums of squares; m.s. mean squares; F-ratio = m.s./inter-plot error m.s.; F-prob. = probability of the F-ratio; l.s.d. = least significant difference comparing two means.					

The analysis of variance table (Table 2.2.4.2) indicates that there was no significant difference amongst any of the different treatments of the lettuce seedlings with bacterial application when compared to the controls (P = 0.064).

Agronomy tunnel test

The efficacy evaluation was redone on lettuce seedlings planted in an agronomy tunnel as it was felt that better control of the experiment could be given at this location compared to the nursery location. The results and statistical analysis are tabulated in Table 2.2.4.3 and Table 2.2.4.4 respectively

Table 2.2.4.3: Average shoot fresh weight of lettuce seedlings obtained at an agronomy tunnel after 18 days growth.[10]

Treatments	Mean (g)	Treatments	Mean (g)	Treatments	Mean (g)
NC	0.37	B1 + B3	0.40	A3	0.40
BC	0.37	B2 + B3	0.37	A1 + A2	0.37
B1	0.38	B1+ B2 + B3	0.42	A1 + A3	0.35
B2	0.37	A1	0.39	A2 + A3	0.38
B3	0.34	A2	0.36	A1+ A2 + A3	0.40
B1 + B2	0.41				

[10] With NC (no treatement), BC (activator only), A1, A2, A3 being activated treatments of *B. licheniformins*, *B. laterosporus* and *Brevi. laterosporus* respectively and B1, B2 and B3 being non activated treatments of *B. licheniformins*, *B. laterosporus* and *Brevi. laterosporus* respectively.

Table 2.2.4.4: Statistical analysis of shoot weight results obtained for lettuce seedlings at an agronomy tunnel after 18 days growth.

Analysis of variance of lettuce top weights (g.plant^{-1})# Trial 2					
Source	d.f.	s.s.	m.s.	F-ratio	F-Prob.
Blocks	9	0.0916	0.0216	9.33	<0.001
Treatments	15	3.13	0.045	1.35	NS
L1: No Bacteria vs Bacteria	{1}	{0.0026}	{0.0026}	0.77	NS
Deviations	{14}	{0.0655}			
Error	135	0.4541	0.00336		
Total	159	0.7138			
L1 : No bacteria vs. bacteria = 0.0123					
l.s.d. of treatment means differences = 0.0508					
# Calculation done with calculator according to Rayner (1969)					
Abbreviations: d.f. = degrees of freedom; s.s. = sums of squares; m.s. = mean squares; F-ratio = m.s./inter-plot error m.s.; F-prob. = probability of the F-ratio; l.s.d. = least significant difference comparing two means.					

The analysis of variance (Table 2.2.4.4) indicated that there was no significant difference amongst the different bacterial treatments with significant difference at 95% confidence level when comparing seedlings treated with bacteria to the un-inoculated controls (P= 0.0123).

2.3 Discussion

For commercial growth promoting bacteria to be successful in agriculture they have to be economically produced, be stable with adequate field persistence, be safe and easy to handle, mix, apply and provide consistently effective growth promotion on the targeted crop (Slininger *et al*., 2003). The requirements mentioned generally hold true for every microbial product that is to effectively penetrate the ever competitive market and for a consolidative approach towards integrative pest management strategies (Raupach and Kloepper, 1998).

A number of commercial biological products are currently available in the market with individual traits ranging from growth promotion through phytohormones to disease suppression (Gan–Mor and Matthews, 2002; Shoda, 2000). Biostart® is a commercial product marketed as a growth enhancer and it consists of three isolates. The possibility of locally manufacturing this product at a competitive price presented an opportunity for commercial exploitation. A competitive price advantage could be realized firstly by decreasing the number of isolates in the product and/or developing a cost effective fermentation process of the isolates to high cell number with a high sporulation percentage. The efficacy of the products as a growth enhancer had been demonstrated by Relihan (Personal communication) when applied to lettuce seedlings. However, the contribution by individual isolates towards the demonstrated efficacy had not been elucidated, hence the need to conduct research to this effect.

To ensure reliable supply of putative isolates during the research process (Meza *et al*., 2003), *B. licheniformis*, *B. laterosporus* and *Brevi. laterosporus* were cultivated and cryo-preserved with glycerol as had been reported by Baudot and Odagescu (2004). The cultures were cyro-preserved as endo-spores due to the ability of endo-spores to withstand unfavourable conditions and therefore to survive for prolonged periods (Knudsen and Spurr, Jr, 1986; Wolken *et al.*, 2003).

All three isolates were effectively cryo-preserved as demonstrated by viable cell counts (Table 2.2.1.1) and growth curves of flasks inoculated from the cryo-vials (Fig 2.2.1.1).

The different growth trends of the isolates observed were not as a result of the difference in inoculum size as *B. licheniformis*, and *B. laterosporus* cryo-vials had similar spore loading. *Bacillus licheniformis* grew at a growth rate of 1.21h which was a much faster growth rate than the other two isolates. *Bacillus. laterosporus* and *Brevi. laterosporus* strains grew at growth rates of 0.44h and 0.68h respectively. These differences in growth rates could be problematic when the three organisms are applied together in the soil as *B. licheniformis* could out-compete *B. laterosporus* and *Brevi. laterosporus* for nutrients (Park *et al*., 1992). As the ability of nutrient assimilation and utilization differs from isolate to isolate, this will impact on the levels of growth individual isolates may reach in the soil (Prescott *et al*., 1996).The lower final spore count of *B. laterosporus* was not perceived as being problematic as the counts were within the limits of between 10^6 and 10^7CFU.mL^{-1}as reported by Meza *et al*., (2003).

The major objective of fermentation in industrial research is to maximize the productivity of the final product (Reisenberg and Gurthke, 1999). The objective at bench scale was however not so much on optimum productivity but to demonstrate, that the isolates could be cultivated to the desired concentration of spores as determined by viable cell analysis. The bench scale fermentation results demonstrated that *B. licheniformis* could be effectively cultivated to a total cell count of 6.0 x 10^{12} CFU, and *B. laterosporus* and *Brevi. laterosporus* with total counts of 4.1 x 10^{12} CFU and 6.7 x 10^{12} CFU respectively. These results compare favourably with the maximum total counts of 3.9 x 10^{11} CFU achieved by Tonkova *et al*., (1993). A production process that can consistently produce high final cell counts will result in huge benefits especially for the end user.

The stability of the products, as presented in Table 2.2.3.1, was effective in maintaining the viability over a six month period at 4ºC. Microbial preservation systems in general must maintain viability and prevent changes in the biochemical and morphological characteristics (Meza *et al*., 2003). Although the viability of the organisms was confirmed, the retention of their biochemical characteristics and nucleotide sequence were not measured but the functionality of the isolates was assessed in the form of growth stimulation in this study. Further investigations to elucidate the impact of storing the product under non refrigerated conditions would be required, as some if not most of the

farmers, may not have facilities to keep products at low temperatures. If the developed product does not retain its activity at room temperatures, the extent of product availability may be restricted to the few farmers with the required infrastructure resulting in a smaller potential market.

The ability of rhizobacteria to promote growth has been known for some time and has been demonstrated in a number of agronomically important crops (Reddy *et al*., 1999). The growth enhancing effect of Biostart® was also demonstrated previously by Relihan[11] (Personal communication) using lettuce as a test crop. During follow up trials to elucidate the impact of individual isolates in promoting growth, either not or marginal benefit to seedling growth was observed with any of the individual isolates or combination treatments. As bacteria require specific substrates to be available in the soil for effective production of desired phytoproducts (Shoda 2000), the change in the activator as a substrate during the follow up trials could have resulted in target phytohormones essential for growth enhancement not being produced. During the initial evaluation of the product with three isolates, an activator consisting of a mix of crushed yeast and molasses was used. During the current evaluation an activator consisting of glucose and corn steep liquor was used as it was a semi-defined medium. Even though this recipe supported germination of *B. licheniformis*, its efficacy on field environments was not tested.

Microbial products applied as individual biological agents are likely not to perform consistently and it has been proposed that a consortium of biological agents with different individual traits should rather be applied (Roberts *et al.,* 2005, Raupach and Kloepper, 1998). Although this should be taken into consideration during development of a biological product, it does not fully elucidate the observations from the trials, especially as different combinations of all three organisms were also evaluated for growth enhancement with questionable results. For this reason the variation of the activator types during the different trials is assumed to have been a significant variable affecting the growth enhancement potential of the isolates.

([11] June 2004, project report)

Upadhayay and Rai (1986) reported that continuous sub-culturing of micro-organisms resulted in the loss of some distinct biochemical properties. This is an unsettling phenomenon especially during product and process development. In taking such potential changes into consideration and initiating steps to prevent this from happening, the cultures were subjected to cryo-preservation. This process is meant to prevent any microbial death of the culture while excluding any potential contaminant and preserving the biochemical, genetic and morphological characteristics of the culture (Bargabus *et al*., 2003; Meza *et al*., 2003). The cultures were successfully cryo-preserved and were therefore continuously available for the experimental process. The fact that spores were the final product and not an active compound (e.g. an enzyme) further introduced complications. The standard cell enumeration techniques determine the viability of the product providing only quantitative analysis and not a qualitative outcome and therefore such techniques are not capable of determining the efficacy of the final product (Prescott *et al*., 1996). Further investigations are therefore necessary to establish better assay methods that target the biochemical activity of the product especially if the active compound is a biochemical compound, e.g. phytohormones or enzymes. The ability of the rhizobacteria to solubilize minerals is known to promote crop growth (Altomare, 1999), and if this is the mode of action of the BCA, assay methods have to be devised to confirm and quantify such activity. It is also essential that the active compound is identified and its production profile determined as this could influence the timing and conditions under which the product should be applied for optimal efficacy.

The final products were targeted to be in a spore form and sporulation involves a number of complex genetic steps where retention of the target characteristics has not been proven (Stragier and Losick, 1996). The recipe was however successful in spore formation as sporulation efficiency above 90% was achieved on all isolates. This was imperative as such success would greatly limit production costs. Media used in the production process also has a great impact on the efficacy of the final product as different media will enable isolates to express certain different characteristics which may or may not be desirable (Lang-Sang *et al.,* 2002). It is therefore important that procedures to continuously measure the activity of the final product be developed, such procedures must ascertain the level of activity of the final product with regards to biochemical processes.

The current focus on the development and application of biological agents is on integrated pest management (IPM), which ensures an integrated approach to achieve the desired objective. As an individual biological agent is not likely to succeed especially when applied under unknown conditions, use of combinations of organisms with other growth promoting organisms' such as mycorrhizal fungi may be more effective (Raupach and Kloepper, 1998; Roberts *et al*., 2005). The challenge would be in formulating and applying the two different agents in a suitable multi-agent product.

CHAPTER 3

ISOLATION, SCREENING AND SELECTION OF *Bacillus* spp. WITH POTENTIAL APPLICATION AS BIOLOGICAL CONTROL AGENTS

3. INTRODUCTION

The use of micro-organisms for biological purposes has become an effective alternative for the control of plant pathogens (Bernal *et al.*, 2002), this is a direct response to the societal concerns regarding excessive usage of agrichemicals and genetically modified organisms as a means of managing crop diseases (Bargabus *et al.*, 2003).

The process of utilizing these micro-organisms commences at an isolation and screening stage. Cognisance should however be taken that if these processes are not properly executed, some potential isolates may be marginalized due to non-apparent mode of action. For example, some organisms may produce phytohormones that will enhance plant growth (Khalid *et al.*, 2004) while others may produce antimicrobial compounds that assist in curbing disease proliferation (Kavitha *et al.*, 2005; Bernal *et al.*, 2002). Furthermore, various modes of action may be present and act simultaneously to produce the desired effect therefore the isolation process should be aware of such variations (Shoda 2000; Gerhardson, 2002).

Screening of potential organisms with biological control properties is of integral importance for effective development of biological control products. In this study screening was conducted from isolates currently available in the CSIR culture collection as well as from soil samples. The selection criteria used were for potential isolates that demonstrated antifungal properties against a wide spectrum of known fungal pathogens. The screening technique applied targeted isolates displaying a mode of action that was essentially antagonism. Other potential isolates which could be of use in other applications i.e. growth enhancement (Khalid *et al.*, 2004; Medina *et al.*, 2002), nitrogen fixation (Bloemberg, 2001), solubilisation of minerals (Bonkowski *et al.*, 2000; Altomare *et al.*, 1999) and induced systematic resistance (Reddy *et al.*, 1999) were not the target of this investigation.

The selection process of the ideal biological control agent (BCA) should be cognisant of the physico-chemical condition the agent is going to operate under (Montealegre *et al.*, 2003) as this determines the effectiveness of the BCA in targeted field operations. Földes *et al.,* (2000) reported that bacterial endospores in natural environments had different heat sensitivities and heat activation requirements for optimal growth from those which have been cultured. It is therefore imperative that the conditions under which the BCA is to operate are fully understood so as to improve the product's effectiveness.

BCA generally do not perform convincingly under field conditions when compared with chemical fungicides (Dimbi *et al.*, 2004) and this could be attributed to a number of factors. It is therefore recommended that after the preliminary screening the efficacy of the selected isolate be assessed in natural non-sterile soil in pot trials aimed at identifying the key factors affecting the efficacy of the BCA (Kerry, 1993). Successful usage of existing BCA or construction of improved variants for control of plant disease can also be enhanced by understanding the mechanisms of the BCA at both the molecular and biochemical levels and by determining the basis for the variability in biological control in the ecosystem (Kavitha, 2005). Further understanding of the best timing for application of the BCA may further enhance its efficacy (Powell *et al.*, 1993).

The objectives of this study were therefore:

1. To screen *Bacillus* isolates from the CSIR culture collection and from soil samples for antagonist properties against a spectrum of pathogenic fungi by conventional plate well assay.
2. Preliminary identification of selected bacterial isolate.
3. Temperature stability studies of the biological control agent displaying activity.
4. Comparative activity study of the selected isolate against a known agrichemical.

3.1. Materials and Methodology

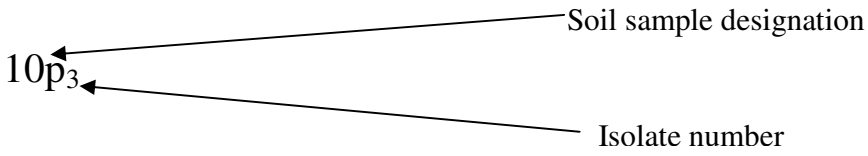
3.1.1. Bacterial sources and soil isolation protocol

A variety of bacterial isolates of *Bacillus* origin as listed in appendix 5 from CSIR Bioscience culture collection, were used to determine antifungal properties. Isolates (*Bacillus* spp.) to be tested had been cryo-preserved and were stored in 1.8mL Nalgene cryo-vials (Nalgen Nunc Int, Rochester, NY) at -70ºC in an ultra-freezer. One cryo-vial of each individual bacterial isolate was inoculated into a 3% $v.v^{-1}$ TSB solution, which is the inoculum stage medium. Flasks were incubated at 32°C for 12-18hrs with agitation of 200rpm in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ).

Isolation from soil samples was performed as follows. Soil samples were sourced at several locations in the Kwa-Zulu Natal Province, South Africa as indicated in Appendix 7 and kept at 4^{o}C until they were processed. From the soil samples, 4g of soil was placed in a McCartney bottle containing 9mL nutrient broth and incubated for 24 hours on a rotary shaker at 120rpm. Ten minutes prior to the actual isolation procedure, another 4g of the same soil was placed in a McCartney bottle containing 9ml peptone. Both samples were incubated at 45°C for 30 minutes. This step was necessary to activate proteins necessary for sporulation (Stragier and Losick, 1996). Samples were then dehydrated by adding a filter sterilised ethanol solution (50% $v.v^{-1}$) to aliquots of each sample (4ml) to obtain a final volume of 20 mL. The samples were incubated for one hour at ambient temperature which allowed dehydration of cells and the subsequent formation of endospores. This procedure was based on the assumption that all bacteria that are not capable of producing endospores would be killed (Prescott *et al.*, 1996). The resulting solution was centrifuged at 10 000 x g (Beckman J2-21M/E centrifuge, Beckman Instruments Inc., Palo Alto, California.) for 10 minutes. This supernatant (ethanol and water) was discarded and the resulting pellets were heat treated by incubation at 105°C for five minutes. This allowed residual ethanol to evaporate and any non-spore forming organisms that may have survived the ethanol treatment, to be destroyed. The cellular components were reconstituted in 20mL of sterilized distilled water and serially diluted to 10^{-4}, and 0.1mL aliquots of the respective dilutions were plated onto nutrient agar (NA)

plates as well as NA plates supplemented with polymyxin B, which allowed Gram positive bacteria resistant to polymyxin B to flourish (Prescott *et al.*, 1996). The plates were incubated at 32°C for 24h.

The colonies growing on these plates were sub-cultured by streaking three times to ensure purity and pure colonies were flooded with 3% hydrogen peroxide (H_2O_2) to determine the presence of the catalase reaction (Prescott *et al.*, 1996). A catalase positive result was confirmed by the generation of bubbles. Gram stains were also performed (Prescott *et al.*, 1996) and all gram positive and catalase positive organisms were considered to be potential *Bacilli* and were cryo-preserved as per section 2.1.1. The resulting colonies were labelled as follows:



The P was used to designate isolates that were cultivated in peptone. A bacillus isolate labelled as PWB was also included in the screening. This isolate had been previously isolated in soil and had shown some chitinolytic properties. The exercise was to determine if this isolate did demonstrate antifungal properties as well.

3.1.2. Fungal pathogens

A total of 11 fungal cultures known to be associated with plant diseases were obtained from Plant Aid Services[12] and are listed in Appendix 6a. The fungal isolate *Fusarium oxysporum* (Panama AVC 145) that was isolated from an infected banana plant and is known to cause Panama wilt was obtained from CSIR Biosciences, Plant Biotechnology

[12] Plant Aid Services , 4 Temple Street, P.O. Box 101007, Scottville, 3209.

Unit[13]. Two *Verticillium dahliae* racemates isolated from infected potato and tomato plants were obtained from ARC Roodeplaat[14].

All fungal isolates were grown on Potato Dextrose Agar (PDA) (Biolab, 1023789) and incubated at 25ºC until sufficient growth was observed. The fungal plates were kept at 4°C until they were used. The plates were sub-cultured on PDA every two weeks.

3.1.3. Bioassay of isolates against selected fungi

The bacterial isolates from the culture collection and soil were cultivated in Tryptone Soy Broth (TSB). Shake flask cultivations were performed by transferring one cryo-vial of each individual bacterial isolate into a 3% $v.v^{-1}$ TSB medium with a total volume of 700mL in a 1.8L Fern Bach flask. Flasks were subsequently incubated at 32°C for 12-18hrs at 200rpm in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ).

The antagonism potential of the isolates from the culture collection and the soil was tested against a spectrum of fungi (appendix 6a & b) that had been cultivated on PDA. The antagonist test was done using either a fungal suspension or fungal disk. A fungal suspension was prepared by cutting a $3mm^2$ cube from a fully grown fungal plate and suspending the disk in 9mL of sterile peptone. After proper mixing, 100µL of suspension was aseptically transferred to wells bored by a sterile cork-borer on the adjacent positions of the PDA plate. Fungal disk application was performed by aseptically transferring $3mm^2$ disks from a fully grown fungal plate to fresh PDA plates. The fungal disks were placed at opposite corners of the plate as per figure 3.1.3.1.

[13] CSIR Biosciences, P.O Box 395, Pretoria, 0001.
[14] ARC Roodeplaat, Private Bag X 293, Pretoria, 0001.

Figure 3.2 .3.1.: Plate demonstration of ***in vitro*** screening of *Bacillus* isolate against test fungi

The plates were subsequently inoculated with 0.1mL of the bacterial isolates cultivated in TSB. Plates were incubated at 25ºC for up to 10 days with continuous monitoring of fungal inhibition by measuring the clearing zone diameters.

3.1.4. Selection, identification and characterization of bacterial antagonist

The result obtained in the screening for antagonism against numerous fungal pathogens resulted in identification of putative isolates with great potential for development as biological agents. This would therefore provide an opportunity for the development of a process for the realization of a product with antifungal properties. An isolate that showed wide spread inhibition was selected for further investigation and identified to ensure GRAS status of the product.

A *Bacillus* isolate that showed antagonism against all tested fungi was cultivated in plate count agar (PCA) (Biolab, 1024507) ensuring a pure culture. It was subsequently sent to the University of Cape Town[15] for molecular identification using 16s RNA gene sequencing to confirm its identity. The organism was prepared by streaking on PCA for three consecutive series to ensure pure colonies. Once pure colonies were confirmed the plate was sealed and sent for identification. The nucleotide sequence results were

[15] University of Cape Town, Department of Molecular Biology and Cell biology, Private Bag, Rodenbosch, 7701, Cape Town.

submitted to the GenBank database and compared to other closely related sequences in order to obtain identification.

The effectiveness of the identified isolate in inhibiting known pathogens was compared with a known systematic fungicide, Benomyl. A range of Benomyl concentrations were tested for inhibition against two *Verticillium dahliae* racemates and two *Fusarium* spp (*F. solani* and *F. oxysporum*). The comparison was made with a previously screened and identified isolate, *Bacillus subtilis*. Plate count agar (PCA) containing Benomyl was prepared by dissolving 10.4g PCA with the volume targeted to be 390 ml in a Schott bottle. The contents were sterilized for 20 minutes at 121ºC. Once the contents were cooled, 14mg of Benomyl dissolved in 10ml distilled water was filter sterilized into the PCA solution with a 0.22 µm sterile filter. The final concentration of Benomyl in the agar was determined to be 35mg.L^{-1}. The plates were prepared as per section 3.1.3. by transferring only one 3mm^2 fungal disk to the centre of a PDA plate. The plate was incubated at 28°C and monitored for growth. Plates to test different concentrations were prepared by spreading Benomyl solution on to the surface of the plates. The Benomyl solutions were prepared by dissolving 10mg (25mg.L^{-1}), 14mg (35mg.L^{-1}) and 20mg (50mg.L^{-1}) of Benomyl in 400ml of distilled water. The final solutions were filter sterilized prior to surface addition onto PDA plates. A volume of 100µL of each concentration was aseptically spread onto the agar surface and allowed to dry for about 5 minutes prior to the addition of test fungi. Fungal cultures in 3mm^2 disks were placed in the centre of PDA, incubated at 28ºC and growth monitored over time. The development of the fungal isolate on the Petri dish was measured over two weeks and compared to controls.

As the isolates mode of action had not been characterized, *B. subtilis* was grown on two different cultivation media and final product variants screened for antifungal activity. The same isolate was cultured as per section 2.1.2 and stabilized as per section 2.1.3. The stabilized product was kept at 4ºC and it was also tested for antifungal properties. The growth media tested was the sporulation media and TSB prepared as per Section 2.1.1.

Both media were inoculated with contents of a cryo-vial of *B. subtilis* per flask and incubated at 32ºC with an agitation speed of 200 rpm on an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ), 5ml samples were aseptically taken for testing at designated times. To prepare the supernatant, 2mL of sample was transferred to sterile eppendorf tubes and centrifuged at 10 000 x g. For whole broths and the resulting supernatants, a 100µL from each was aseptically spread on the surface of a PDA plate and allowed to dry for 5 minutes. A disk of the designated fungal pathogen was transferred to the plate and incubated at 28ºC, the growth of the fungus was monitored over time. To test the effect of temperature on the antifungal properties, the broths and supernatants were sterilized at 121ºC for 20 minutes (Anzy JSD400, Getinge USA, Inc, Rochester, NY). After the contents were cooled they were spread onto PDA plates as described above and allowed to dry for 5 minutes. The disk with the fungal culture was subsequently inoculated onto plates and these were incubated at 28°C. The growth of the fungus was monitored over time.

3.2. Results

3.2.1. Primary screening of isolates against selected fungi.

Table 3.2.1.3.: Inhibition of fungal growth by *Bacillus* isolates as indicated by the presence of clearing zones, average measurement given in mm.

Test organisms	Fungal isolates								
	Fusarium solani	*Stenocarpella maydis*	*Fusarium moniliforme*	*Fusarium* spp.	*Fusarium oxysporum*	*Aspergillus flavus*	*Aspergillus clavatus*	*Alternaria alternata*	Panama AVC 145
B3. *B. licheniformis*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
C3 *B. licheniformis*	No inhibition	No inhibition	No inhibition	10mm	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
A3 *B. polymyxa*	10.25mm	9mm	10.25mm	15.81mm	7.75mm	No inhibition	No inhibition	No inhibition	10mm
B2 *B. chitinosporus*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
B1 *B. polymyxa*	8.75mm	10mm	11.25mm	13mm	10mm	No inhibition	No inhibition	17.5mm	No inhibition
C2 *B. megaterium*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
C4 *B. laterosporus*	No inhibition	No inhibition	7.25mm	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
C4 *B .licheniformis*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
B4 *B. chitinosporus*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
A1 B1 sg	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
C4 *B. chitinosporus*	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
A2 *B. subtilis*	15.5mm	15.5mm	17mm	18.25mm	17.5mm	17.25mm	10.5mm	20mm	15.5mm

The *Bacillus* isolates that were tested for *in vitro* antagonism demonstrated variable results. Only isolate A2 demonstrated total inhibition of all test fungal strains, the other bacterial isolates were either inhibiting part of the fungal spectrum (i.e. B1 & A3, *B. polymyxa*) or showed no inhibition at all.

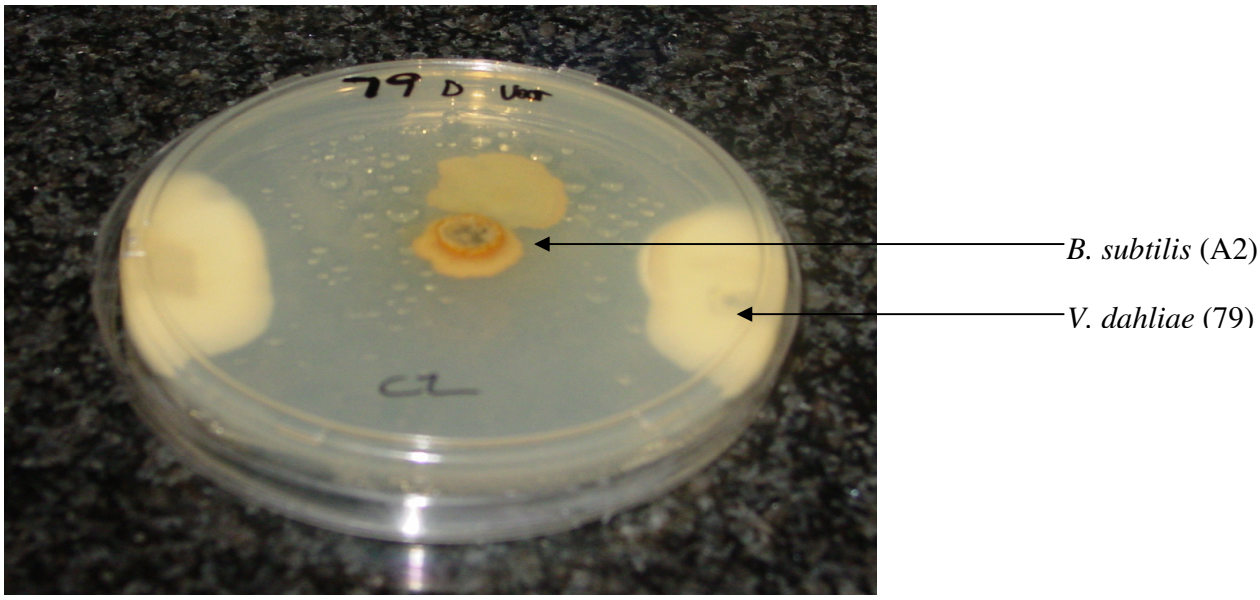


Figure 3.3.1.1.: *In vitro* inhibition of *Verticillium dahliae* (79) by isolate A2 (*B. subtilis*) as demonstrated by the uneven growth of the fungus.

Inhibition of the two *Verticillium dahliae* racemates by the isolate A2 is clearly demonstrated in figures 3.2.1.1 and 3.2.1.2

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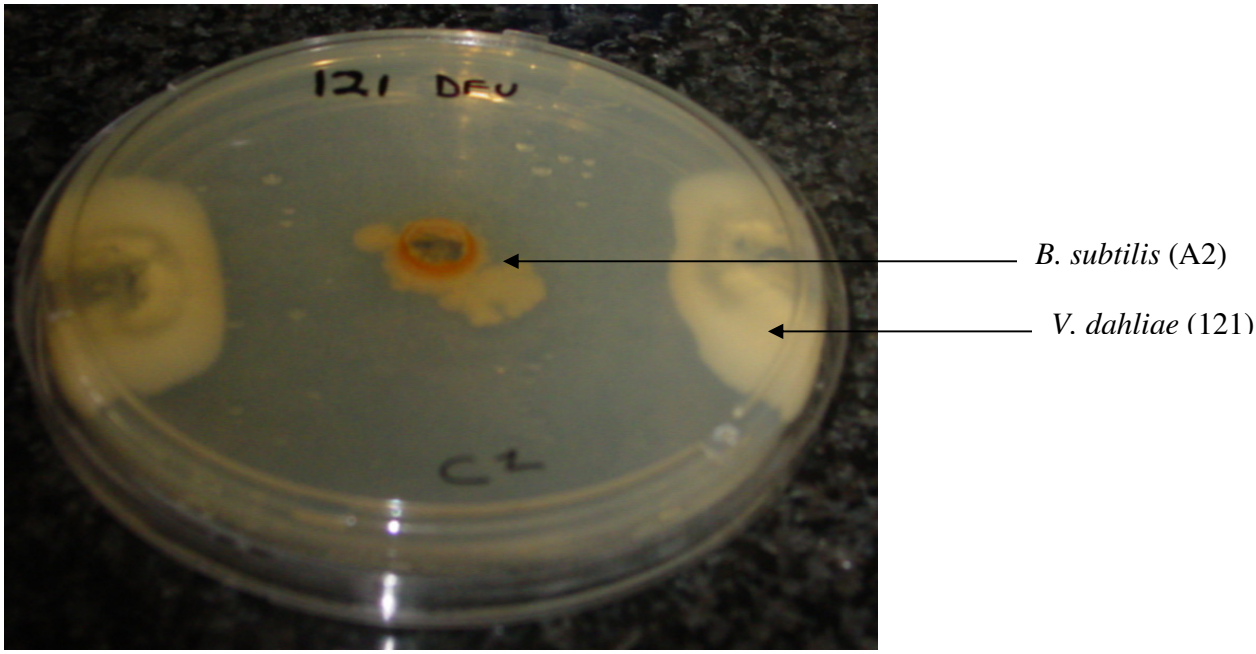

Figure 4.2.1.2.: *In vitro* inhibition of *Verticillium dahliae* (121) by isolate A2 (*B. subtilis)* as demonstrated by the uneven growth of the fungus.

The ability of isolate A2 to inhibit a broad spectrum of fungi including the two *V. dahliae* positioned the isolate as an ideal candidate for potential development as a biological control agent.

3.2.2. Bacterial isolation

A total of 7 isolates were obtained during the isolation process from the soil but only four demonstrated antifungal properties against the screened fungi and were further investigated.

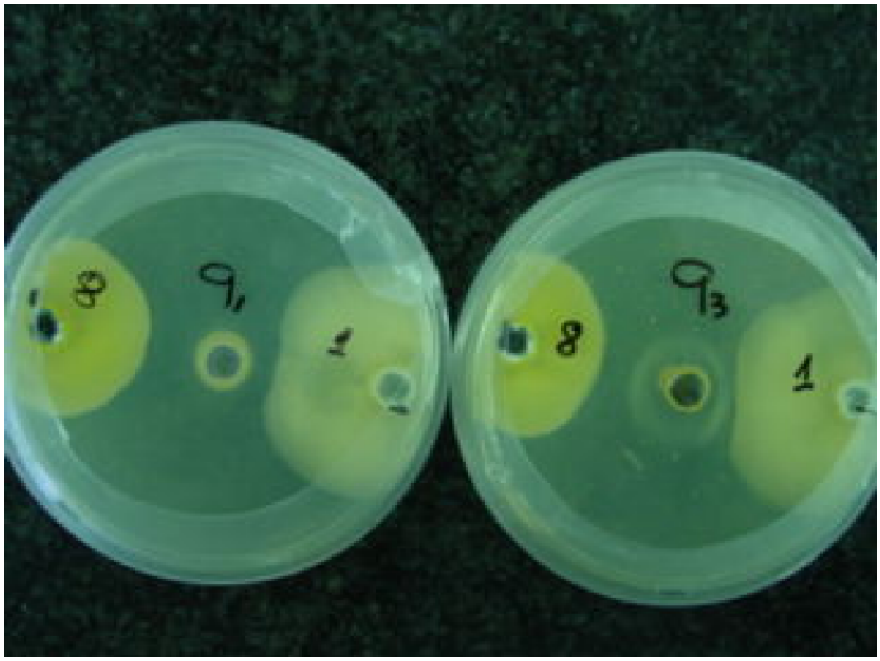

Figure 3.2.2.1.: *In vitro* inhibition of fungal isolate *Aspergillus clavatus* (1) and *Penicillum expansum* (8) (Appendix 6b) by bacterial isolate 1 and 3 from KZN sample 9.

Figure 3.2.2.1. above distinctively demonstrated the inhibition of two fungal isolates, *Aspergillus clavus* and *Penicillum expansum* by two bacterial soil isolates 9_1 and 9_3 originating from a soil sample taken from a field at La Mercy KZN (Appendix 7).

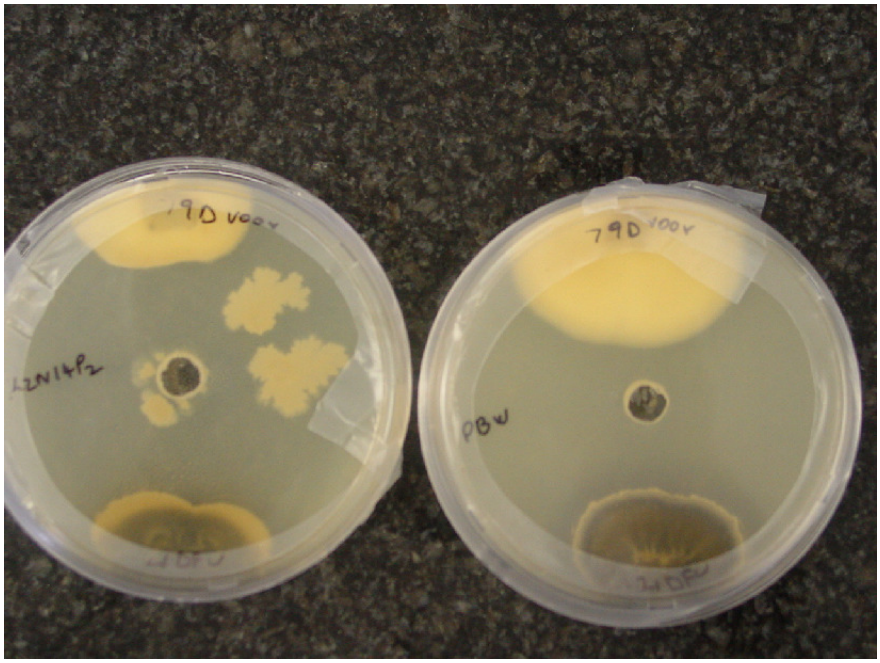

Figure 3.2.2.2.: *In vitro* inhibition of the two *V. dahliae* racemates by bacillus isolate 2 from KZN sample 14 (left) and PBW (right), a chitin producing isolate.

Figure 3.2.2.2. shows bacterial isolate 14_2 exerting antagonism against the two *V. dahliae* isolates in contrast to bacterial isolate PBW which had been demonstrated to produce chitinase but seemed to control one *V. dahliae* (121) isolate.

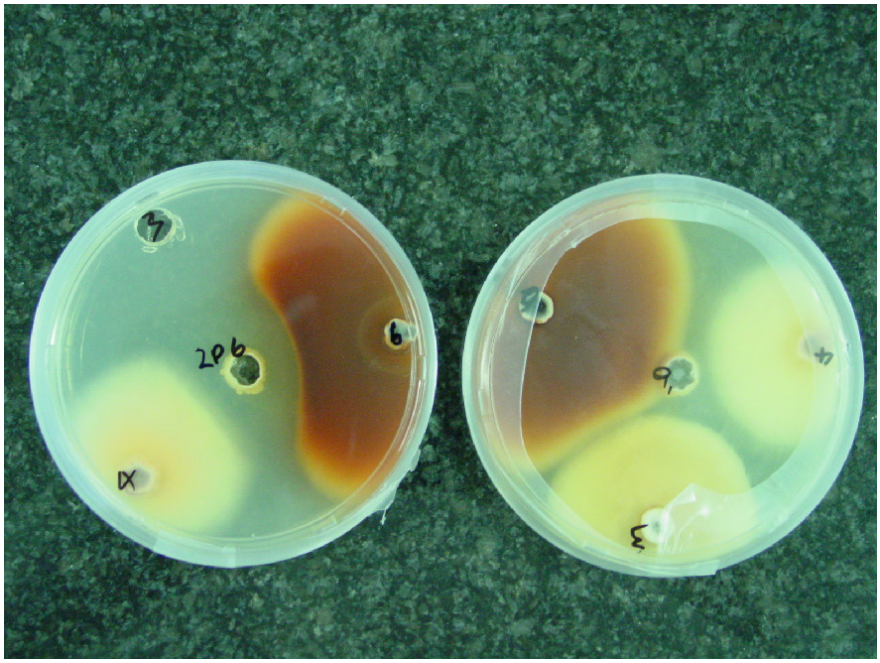

Figure 3.2.2.3.: *In vitro* inhibition of the two fungal *Fusarium* spp. and *Phoma sorghina* by bacterial isolate 6 from KZN sample 2.

Isolate KZN $2p_6$ which originated from a field in Adams Mission KZN demonstrated inhibition against a *Fusarium* sp. (4) and *Phoma sorghina* (6). Isolate KZN 9_1 showed no inhibition against *Phoma sorghina* but some inhibition against *Fusarium* sp. and *Alternaria alternata*.

3.2.3. Identification of bacterial antagonist isolate A2

The results obtained from the primary screening of a number of isolates resulted in the selection of isolate A2 for further evaluation.

The 16S ribosomal RNA sequence of the *Bacillus* isolate A2 was submitted to GenBank and BLAST analysis identified (www.ncbi.nlm.nih.gov/BLAST/). The molecular identification confirmed the isolate as *Bacillus subtilis* strain KL-077 (accession number, AY030331.1) by partial 16S ribosomal RNA sequence alignment (The sequence is a presented in Appendix 8.).

3.2.4. Characterisation and comparison of the bacterial antagonist against a known fungicide.

The effectiveness of the *B. subtilis* isolate was compared with a known systematic fungicide, Benomyl. The test fungi were *Verticillium dahliae* (79 and 121), *Fusarium solani* and *Fusarium oxysporum*. Different concentrations of Benomyl were tested and results are presented.

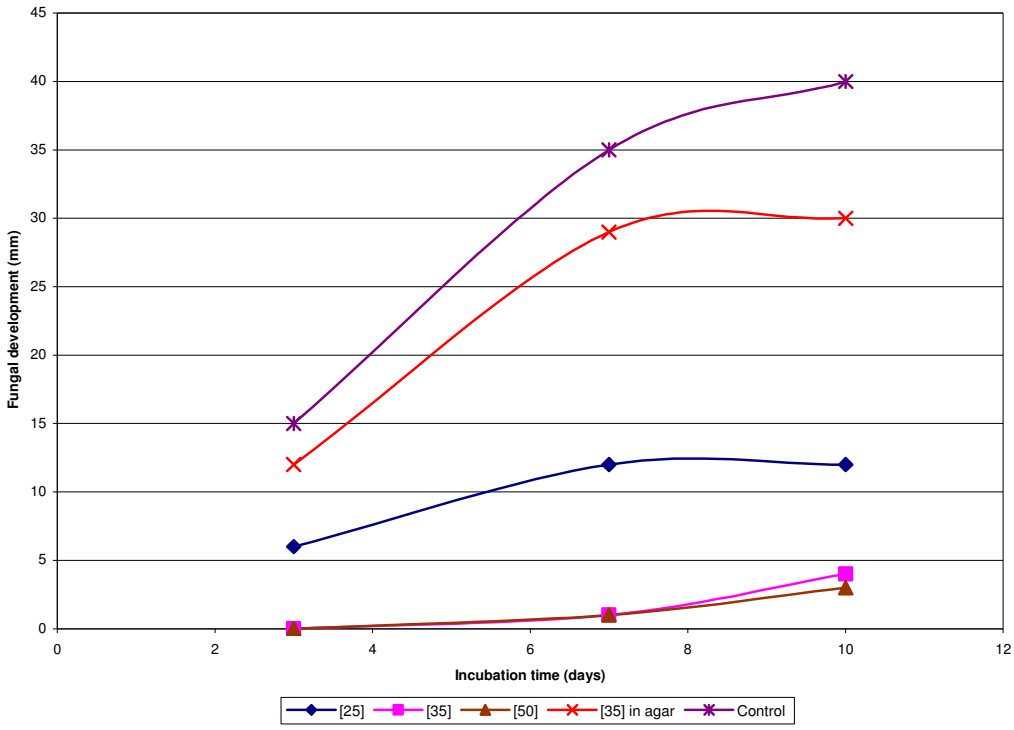

Figure 3.2.4.1.: Effectiveness of Benomyl concentrations ranging from 25mg.L^{-1} to 50mg.L^{-1} against *Fusarium oxysporum* growth as measured in mm.

The higher Benomyl concentrations (Figure 3.2.4.1.) demonstrated better inhibition efficacy of *F. oxysporum* over the test period of ten days. Benomyl concentrations of 35 and 50 mg.L^{-1} showed almost similar trends of fungal development and they seemed to effectively control development. The 35 mg.L^{-1} with Benomyl incorporated into the agar showed poor control compared to the 35 mg.L^{-1} spread on the surface. The fact that

spread solution could be more concentrated on the surface is a likely reason for better fungal inhibition than if it was incorporated into the agar.

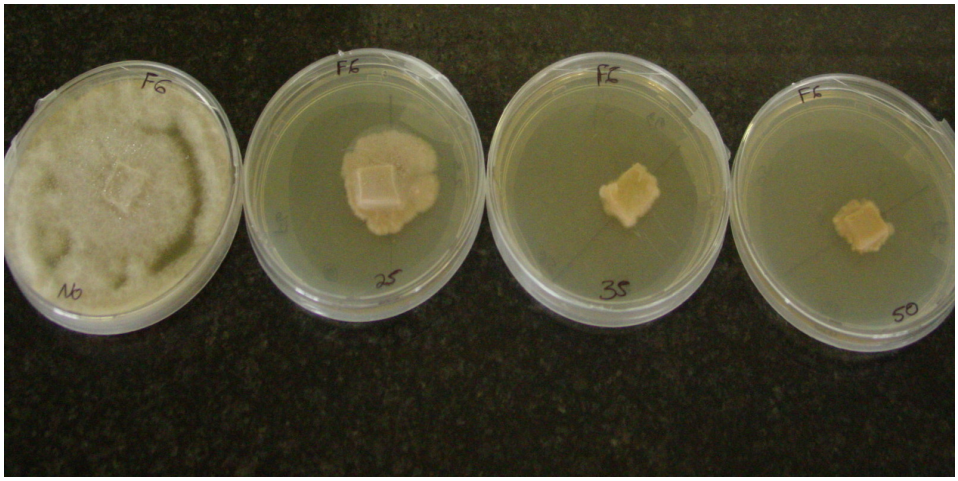

Figure 3.2.4.2.: *In vitro* inhibition of *Fusarium oxysporum* by Benomyl at concentrations 0, 25, 35 and 50 mg.L^{-1}after 7 days incubation.

The plates of *F. oxysporum* demonstrated distinctive control with an increase in Benomyl concentration over the test period of seven days as demonstrated in Figure 3.2.4.2. The test pathogen could therefore be controlled by a Benomyl concentration of between concentrations of 25 mg.L^{-1} and 35 mg.L^{-1}.

The testing of the agrichemical against a different fungal isolate, *Fusarium solani* showed different results. *F. solani* seemed to be more resistant to Benomyl than *F. oxysporum*, as indicated in figure 3.2.4.3.

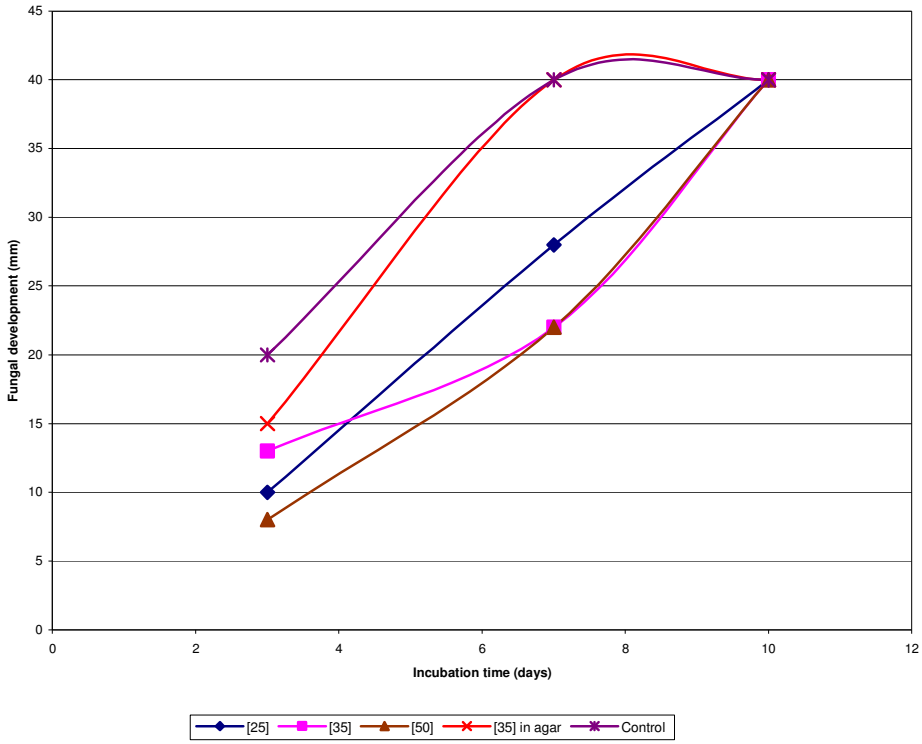

Figure 3.2.4.3.: Effectiveness of Benomyl concentrations ranging from 25-50mg.L^{-1} on the growth of *Fusarium solani*.

Plates with Benomyl incorporated into the agar showed decreased fungal inhibition compared to the plate with same concentration spread on the surface (Figure 3.2.4.3). This is attributed to the concentration gradient of Benomyl being higher in the spread plate. For all tested concentration the fungus grew to same level after 10 days.

Figure 3.2.4.4.: *In vitro* inhibition of *Fusarium solani* at different concentrations (0, 25, 35, 50mg.L^{-1}) of Benomyl after 7 days incubation.

The growth of *Fusarium solani* in the presence of different Benomyl concentrations demonstrated lower fungal development when compared to the control. However no clear differences were observed with the different Benomyl concentrations on overall fungal growth after 7 days of incubation (Figure 3.2.4.4).

Investigation into the characterization of the possible mode of action of the *B. subtilis* isolate yielded interesting results. Student T-tests were conducted to compare the fungal development of heated and unheated *B. subtilis* treatments with the control (being untreated PDA plate inoculated with respective fungi) and p-values above 0.05 were regarded as not significant. Statistical comparison of some *Bacillus* treatments regimes with the highest Benomyl concentration used (50mg.L^{-1}) also determined significance between these treatments, and p-values above 0.05 were regarded as not significant. No significant difference was observed between filter sterilized and unsterilized Benomyl at 50 mg.L^{-1} ($p > 0.05$).

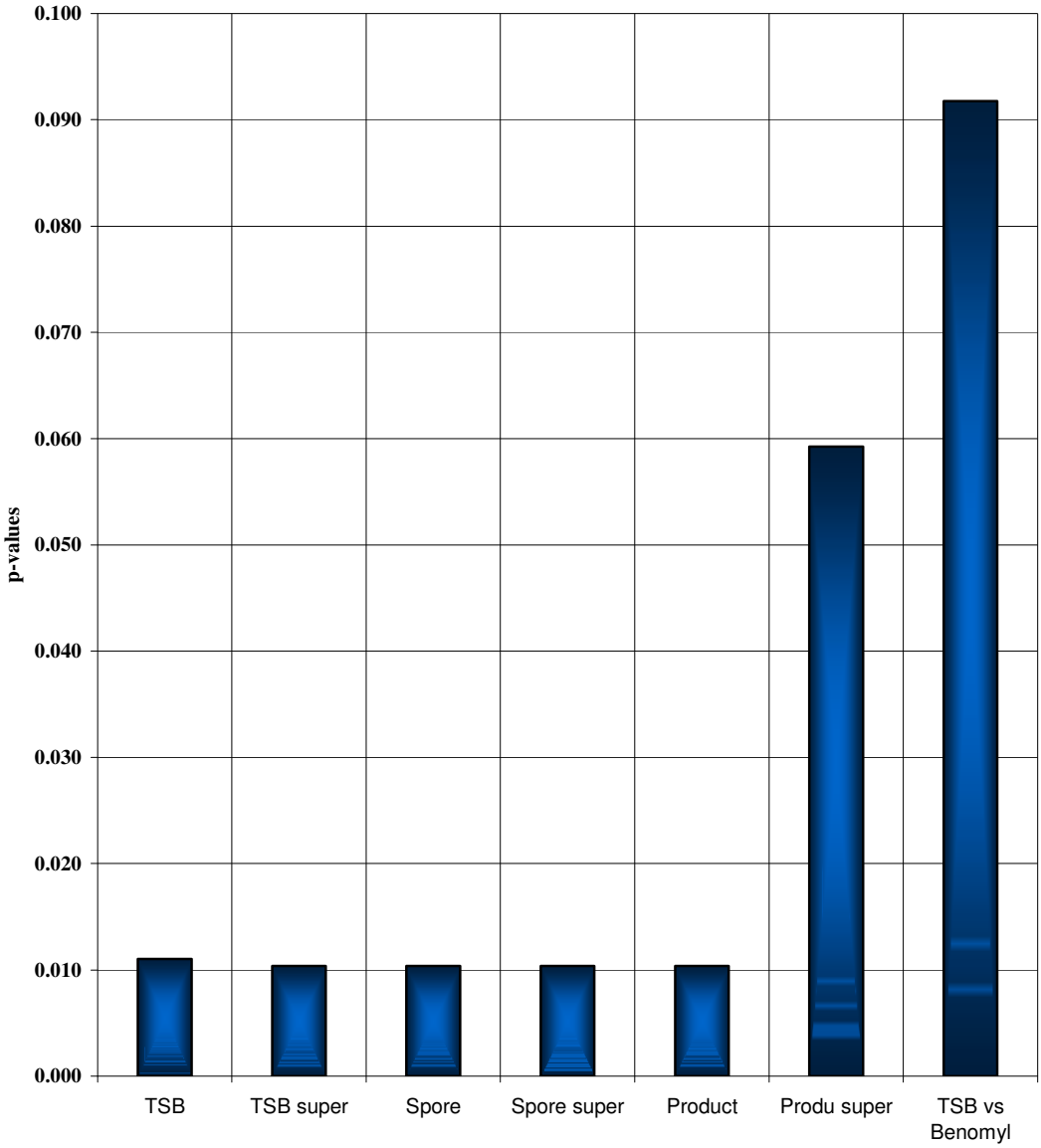

Figure 3.2.4.5.: Comparison of p-values of different unheated treatment regimes (whole broth, sporulation media and their respective supernatants after centrifugation) shows control of *Fusarium oxysporum* growth as compared against Benomyl[50].

Figure 3.2.4.5. and Table 3.2.4.1. present different treatments of *F. oxysporum* with different *B. subtilis* treatment variants demonstrating that *F. oxysporum* was significantly inhibited by TSB cultivated *B. subtilis* in comparison to the control ($p < 0.05$). All other

treatments with the exception of product supernatant, showed significant control of the test fungus ($p < 0.05$), while the product supernatant showed no significant difference from the control ($p > 0.05$). The TSB and Benomyl treatments showed no significant difference in fungal growth indicating that both could significantly inhibit the test fungus.

Table 3.2.4.1.: Analysis of *Fusarium oxysporum* growth when treated with various *Bacillus subtilis* unheated treatment regimes.

	P-Value	Mean	SD
TSB	0.011	1.0	0
TSB supernatant	0.010	0	0
Spore	0.010	0	0
Spore supernatant	0.010	0	0
Product	0.010	0	0
Prod Supernatant	0.059	20.3	4.61
TSB Vs Benomyl[50]	0.092		

It was further confirmed on the t-values table that all tested treatments showed control of fungal growth with the exception of the product supernatant. There was no significant difference between treatment with bacteria cultivated in TSB and 50 $mg.L^{-1}$ Benomyl as the p-value was above 0.05. This confirmed that the bacterial treatments were as effective in controlling fungal growth as 50 $mg.L^{-1}$ Benomyl treatment was. The inability of the stabilised product supernatant to inhibit fungal growth could be due to the dilution of the active compound during the stabilization procedure. The possibility that the organism or a bioactive compound produced by it could have reacted with the stabilization reagent and been deactivated could also not be overlooked.

The heat tolerance of the different unstabilized broths was tested. It was noted with interest that most treatments still inhibited *F. oxysporum* growth even after being sterilized at 121°C.

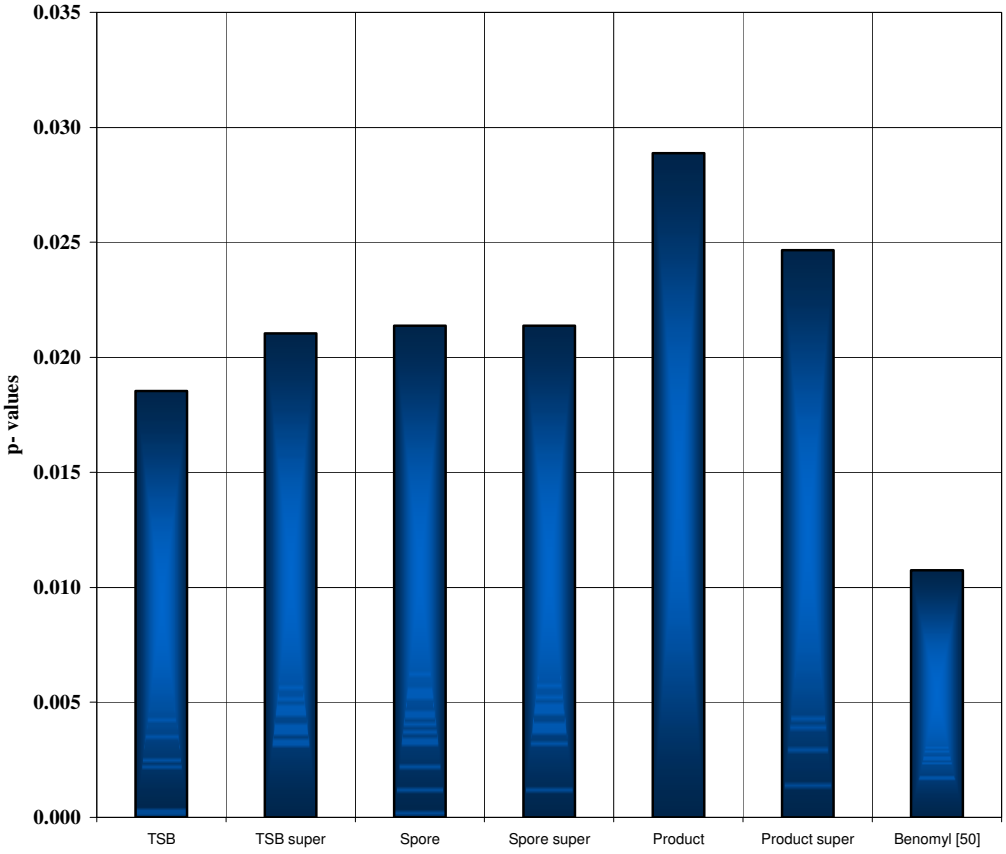

Figure 3.2.4.6.: Comparison of significant values of different heated treatment regimes (whole broth, sporulation media and their respective supernatants) showing control of *Fusarium oxysporum* growth.

All sterilized treatments demonstrated inhibition of *F. oxysporum* development. The determined p-values comparing fungal development on plates with designated treatment against control demonstrated significant control. Such findings demonstrated the ability of the active compound to withstand exposure to high temperatures. This further demonstrated that the bioactive was probably extra cellular as all tested supernatants demonstrated inhibition against *F. oxysporum.* The ability of the heated product supernatant to inhibit fungal was, however, confusing as it was contradicting the observation from the untreated product supernatant. The fact that complexes that are

inhibitory to the test fungus could be formed during the heating process could be one possibility.

Table 3.2.4.2.: Analysis of *Fusarium oxysporum* growth when treated with various *Bacillus subtilis* heated treatment regimes.

	P- value	Mean	SD
TSB	0.019	9.7	0.6
TSB supernatant	0.021	12.3	1.23
Spore	0.021	9.7	0.6
Spore supernatant	0.021	10.0	0.0
Product	0.029	2.0	0.0
Product Supernatant	0.025	18.7	4.0
Benomyl [50]	0.011	0.3	0.6

Table 3.2.4.2. further confirmed that all tested treatment regimes showed control of *F. oxysporum* growth based on p-values below 0.05. The ability of the different broths to withstand excessive heat is interesting especially for the stability of the final product.

Similar experiments were done to test the effect of the different treatment regimes on *F. solani* growth.

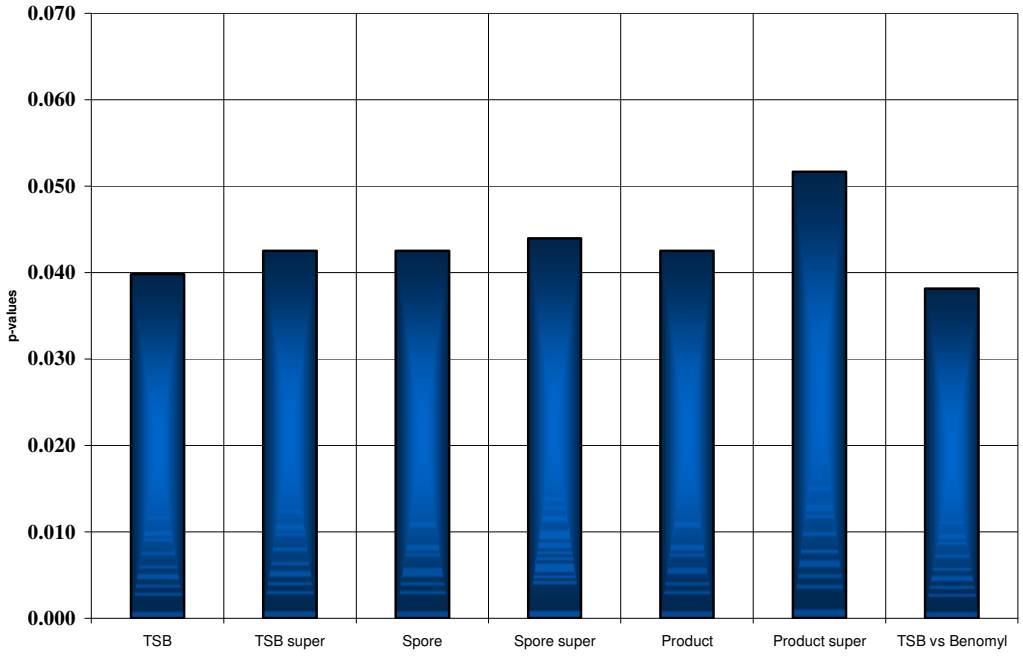

Figure 3.2.4.7.: Comparison of significant values of different unheated treatment regimes (whole broth, sporulation media and their respective supernatants) for control on *Fusarium solani* growth as compared against Benomyl[50].

Growth of *F. solani* was inhibited by all unheated *B. subtilis* treatment regimes as indicated by the significant values which were mostly below 0.05 (Figure 3.2.4.7). The stabilized product supernatant however did not show any significant inhibition of the test fungus *F. solani* ($p > 0.05$). The comparison of bacterial product cultivated in TSB with the highest Benomyl concentration, $50mg.L^{-1}$, showed that there was significant difference between the two treatments. The tested bacterial product inhibited fungal growth while Benomyl did not. This further proved that *B. subtilis* cultivated in TSB had better inhibition potential against *F. solani* than the agrochemical, Benomyl.

Table 3.2.4.3.: Analysis of *Fusarium solani* growth when treated with various *Bacillus subtilis* unheated treatment regimes.

	P- value	Mean	SD
TSB	0.040	0.3	0.6
TSB supernatant	0.043	2.0	0.0
Spore	0.043	2.0	0.0
Spore supernatant	0.044	3.7	0.6
Product	0.043	2.0	0.0
Prod Supernatant	0.052	8.0	0.0
TSB Vs Benomyl[50]	0.038		

The p-values table (Figure 3.2.4.3) demonstrated that most treatment regimes derived from the bacterial isolate were effective against the test fungus *F. solani* with the exception of product supernatant that showed no significant control. A significant difference was recorded between treatment with 50mg.L^{-1} Benomyl and the bacterial product cultivated in TSB.

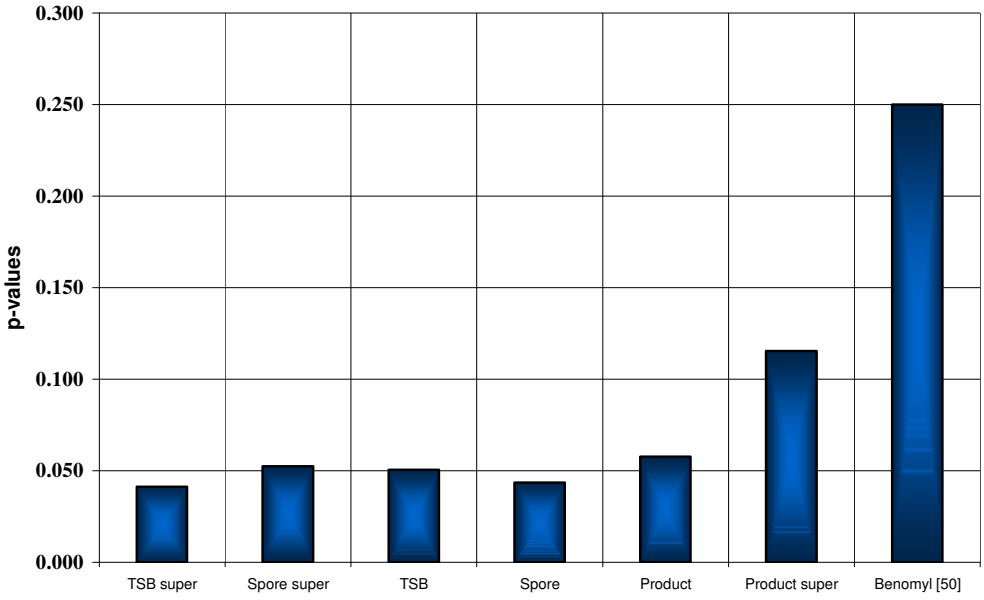

Figure 3.2.4.8.: Comparison of significant values of different heated treatment regimes (whole broth, sporulation media and their respective supernatants) for control on *Fusarium solani* growth.

The non-susceptibility of the test fungus *Fusarium solani*, to Benomyl treatment was further demonstrated in Figure 3.2.4.8. Statistical analysis of fungal development comparing Benomyl treatment and control resulted in a p-value of 0.250 which was not significant. Other treatments derived from the bacterial isolate demonstrated significant inhibition with the exception of stabilized product supernatant that showed no significant difference while spore and TSB supernatant demonstrated marginal significance. The inhibition demonstrated by broth and supernatant to heating at 121°C again suggests production of a bioactive compound as the active ingredient.

Table 3.2.4.4.: Analysis of *Fusarium solani* growth when treated with various *Bacillus subtilis* heated treatment regimes.

	P- value	Mean	SD
Benomyl [50]	0.250	35.0	7.07
TSB supernatant	0.041	12.5	2.12
Spore supernatant	0.053	11.5	0.71
TSB	0.051	10.5	0.71
Spore	0.043	10.0	1.41
Product	0.058	11.0	0.00
Product Supernatant	0.115	20.5	0.71

Observations from Figure 3.2.4.8 were further affirmed by Table 3.2.3.4. Benomyl heated treatment did not show inhibition against the test fungus together with the heated product supernatant.

The tests on the two *Verticillium dahliae* racemates indicated total inhibition of the fungi by all bacterial treatments. Growth was only observed on the control plates therefore there was no statistical analysis conducted. This significant result is a powerful demonstration of the potential of the *B. subtilis* isolate as a possible product for *V. dahliae* control.

3.3. Discussion

The screening of the existing CSIR *Bacillus* culture collection yielded interesting results. Apart from a *Bacillus subtilis* strain that demonstrated inhibition against all tested fungi and a *B. polymyxa* strain (Table 3.2.1.1) that demonstrated inhibition against part of the fungal spectrum tested, none of the other *Bacillus* isolates demonstrated any inhibition. Kavitha *et al.,* (2005) had reported that *B. polymyxa* produced various peptide antibiotics which could exert inhibition against certain fungi, this could be the reason why inhibition was observed against some fungal strains even though not all tested fungal strains were inhibited. Only nine fungal strains were tested during the screening with most strains being from the genus *Fusarium*. It is therefore important that the *Bacillus* isolates that demonstrate effectiveness are tested against wider range fungi and these should include *Basidiomyceta*, *Deuteromycota* and *Oomycota* (Prescott *et al.*, 1996). This is important to establish the potential scope of application of isolates demonstrating fungal inhibition and to understand the degree of influence product application might have on some of the beneficial soil microorganisms. *B. polymyxa* also demonstrated inhibition of some of the tested fungi. For this isolate to be developed into a biological product, it would be important to determine and understand the isolates operational range together with the optimum time of application (Powell *et al.*, 1993).

B. subtilis has been reported to produce an array of compounds that demonstrated antifungal activity, including iturin compounds (Bernal *et al.*, 2002). Iturin compounds constitute a family of lipopeptides that are excreted by *B. subtilis* during cultivation in liquid media. The results of *B. subtilis* inhibition studies demonstrated that this bacterial isolate was also capable of producing antifungal compounds, which may include these iturin compounds and a possibility that not one but a number of these secondary metabolites are produced cannot not be overlooked (Földers *et al.*, 2000). This isolate (A2) therefore demonstrated a potential for development as a biological agent.

The ability of isolate A2 to inhibit a number of fungi generated interest to identifying the isolate. Positive identification of the organism using appropriately accredited methods is a prerequisite for a biological product registration process (Agricultural Remedies Registration Procedure Policy document, 2000), and the organism should not have been recorded as being either a plant, animal or human pathogen for a successful registration. The organism was confirmed by molecular techniques to be a *Bacillus subtilis* strain KL-077.

The process of isolation, screening and selection from soil samples was foreseen to result in more potential antagonists that could be developed for commercialization. This process was however limited to isolates that had antibiosis as the main mode of action. The final isolates from this screening process were therefore not the only potential candidates, but were limited as a result of the selective nature of the technique employed. It should also be noted that only endorspore forming organisms were targeted and therefore other potential isolates that were not of *Bacillus* origin were marginalized e.g. *Pseudomonas* spp. have been reported to be one of the most active and dominant bacteria in the rhizosphere and have been intensively investigated as biocontrol agents (Shoda, 2000; Siddiqui, 2003). The isolation process from the soil samples yielded a number of potential organisms but screening demonstrated that not all had antifungal properties. An average of 6 isolates per sample was obtained and the screening resulted in a total of four isolates that were considered of interest. These isolates had demonstrated antifungal properties against a wider spectrum of test fungi but essentially against *Verticillium* spp. and *Fusarium* spp. that were of interest in this study. The four isolates were cryo-preserved as these were to be further investigated and properly identified. Of the isolated organisms, none demonstrated the same inhibition capabilities as isolate A2. This warranted further investigation of this particular isolate and for potential development into a biological product.

The comparison of *B. subtilis* with a known systemic fungicide, Benomyl, yielded interesting results. Different concentrations of Benomyl were tested against *F. solani*, *F. oxysporum*, *V. dahliae* (79) and *V. dahliae* (121). All tested concentrations of Benomyl

demonstrated inhibitory effects against both *V. dahliae* racemates demonstrating a general susceptibility of these fungal strains to Benomyl.

Fusarium oxysporum showed some level of susceptibility to Benomyl application in contrast to *F. solani* that showed minimal inhibition when comparing the control and Benomyl treated plates (Figure 3.2.4.5, Figure 3.2.4.6, Figure 3.2.4.7 and Figure 3.2.4.8). As *B. subtilis* has been reported to produce an array of compounds that demonstrated antifungal activity (Bernal *et al.*, 2002), different metabolites could be produced by the organism resulting in different inhibiting compounds for both *F. oxysporum* and *F. solani*. The inhibition of *F. oxysporum* by *B. subtilis* cultivated in TSB was determined as not being significantly different to inhibition by 50 $mg.L^{-1}$ Benomyl with a p-value of 0.092, this showed susceptibility of *F. oxysporum* to both Benomyl and *B. subtilis* applications.

The different bacterial treatments also showed different potency in controlling fungal growth. *F. oxysporum* was susceptible to most of the bacterial treatments with the exception of stabilized product supernatant. However, *F. solani* showed even lesser susceptibility to all treatments as there was marginal growth control. A similar trend was also observed for the heat treatments. The fact that the antifungal compound or compounds that had demonstrated inhibition against fungi were still active after being subjected to heat treatment was very interesting. This could mean that the inhibition mechanism may be chemical rather than biological. Most biological compounds e.g. enzymes, will be denatured at higher temperature (Prescott *et al*., 1996), thus rendering them ineffective.

The inability of the stabilized product supernatant to inhibit any of the test fungi was noted with interest. This was even more interesting as the supernatant from other treatments had shown fungal control. The stabilization technique used involves the addition of several ingredients to the final product which could have reacted with the active component. The reality that the stabilization process could have diluted the active compound could also not be overruled.

In conclusion, the isolate indicated favourable anti-fungal activity when compared with the agrichemical, Benomyl. Further development may yield an effective product especially against *Verticillium* wilt as there is no product currently registered in South Africa (Abstract of Agricultural statistics 2002) for this pathogen.

CHAPTER 4

BENCH SCALE DEVELOPMENT OF A BIOLOGICAL CONTROL AGENT ANTAGONISTIC AGAINST FUNGI

4. INTRODUCTION

Pathogenic infections are negative factors that affect the cultivation efficiency and quality of crops. These factors affect both commercial and subsistence farmers (Földes *et al.*, 2000). Agricultural communities are therefore forced to explore remedies to prevent losses while investigating alternatives to the traditional and environmentally harmful chemical application (Gerhardson, 2002; Bargabus, 2003; Yao *et al.*, 2004; Ngugi *et al.*, 2005). Methyl bromide is a soil fumigant (Nemec *et al.*, 1996) that has been used for the control of many resilient plant pathogens viz. tomato rot and crown rot. Methyl bromide is considered extremely harmful to the environment and has been listed as a Class 1 ozone depleting substance by the US Environmental Protection Agency (Mao *et al.*, 1998; Webster *et al.*, 2002), and has been banned in developed countries (Webster *et al.*, 2002).

Microbial based products offer alternatives for control of fungal pathogens and are becoming more widely accepted (Winding *et al.*, 2004). AQ-10 contains *Ampelomyces quisqualis* and targets powdery mildew in grapes. Avogreen, a product with *Bacillus subtilis* as the active ingredient is available for the treatment of fruit spots in avocados (Nel *et al.*, 2003). Serenade is another commercial microbial product formulated from a *B. subtilis* strain which has demonstrated control against mummy berry disease of blueberry caused by the fungus *Monilinia vaccinii-corymbosi* (Ngugi *et al.*, 2005)

Verticillium dahliae is a wilt causing pathogen affecting many cultivars of commercial value. Control measures using agrichemicals have been ineffective due to the formation of microsclerotia which survive in the soil for up to 13 years (Ghaffer, 1986) and there is currently no registered chemical or microbial product that effectively controls the proliferation of this fungus. *Verticillium* wilt is a vascular disease occurring in both irrigated and non-irrigated potato production areas throughout the world. Infection causes loss of turgor in leaves followed by chlorosis and necrosis, resulting in premature senescence of the foliage, thereby shortening the growth period. Depending on the severity, time of occurrence and season, the number and size of progeny tubers may be substantially

reduced. If control is inadequate, penalties on yields can be as high as 50 % in susceptible cultivars and 20-30% in tolerant cultivars as reported in Abstract of Agricultural statistics (2002). A survey conducted by the Agricultural Research Council (ARC) Roodeplaat during January 1995 and December 2000 demonstrated that *V. dahliae* was a major cause of infection in potato fields. Losses amounting to R209 million in potential turnover have been reported as recently as 2002 when *Verticillium* infected 100 000 tons of tomatoes. *V. dahliae* has also been reported to infect a number of other crops namely, brinjal, brussel sprout, cauliflower, cucumber, cabbage and sweet potato.

The screening process presented in Chapter 3 has demonstrated the potential of a *B. subtilis* strain to inhibit the *in vitro* growth of a variety of plant pathogens, some of which cause major losses to the agricultural community. The ability of this particular strain of *B. subtilis* to inhibit *V. dahliae* has generated further interest in the development of the isolate as a potential commercial product.

For such development to be successful, the isolate under investigation should be maintained in such a way to ensure reproducible growth and efficacy. This was achieved by cryo-preservation of the organism (Meza *et al.*, 2003). Cryo-preservation prevents the loss and/or contamination of the culture (Bargabus *et al.*, 2003), while preventing the occurrence of any changes to the organism's biochemical and molecular characteristics.

The objectives of this study were three fold:

1. To preserve the putative biological control agent, *Bacillus subtilis* (A2) and to establish whether the cryo-preserved culture had retained its efficacy and its essential physiological properties.
2. To demonstrate that the isolate could be effectively cultivated to desired levels at bench scale without any adverse effect.
3. To demonstrate that the product had retained its efficacy and could be utilised in green house trials as a biological control product (BCA).

4.1. Materials and Methods

4.1.1. Cryo-presevation and culture maintenance

Bacillus spores have been known to be stable for as long as 9000 years (Renberg and Nilsson, 1992), it was thus deemed feasible to cryo-preserve the isolate, *B. subtilis* in the spore state to ensure long term stability. The isolate was cryo-preserved as had been described in Section 2.1.1. The cryo-preserved culture was further validated and purity confirmed. Growth rates were also determined as described in Section 2.1.1.

The ability of the cryo-preserved cultures to sporulate (Upudhyay and Rai 1986) has to be continually assessed as it is a significant property of Genus *Bacillus*. It was assessed as previously described in Section 2.1.1.

4.1.2. Bioassay of isolate against Verticillium dahliae

Product preparation

The ability of the *B. subtilis* (A2) isolate to inhibit known fungal pathogens as demonstrated *in vitro* (Section 3.1.1) was further confirmed. *Bacillus subtilis* was cultivated in sporulation as discussed in Section 2.1.1. with the final product being stabilized as previously described in Section 2.1.3. The stabilized broth was then tested at the ARC Roodeplaat for antifungal activity against *V. dahliae*. The *V. dahliae* strain used was isolated from infected crops and identified at ARC Roodeplaat, Pretoria.

Antifungal testing

The ability of the antagonist to inhibit *V. dahliae* was conducted by transferring 1ml conidial suspension (concentration: 2.19 x 10^8 CFU.ml^{-1}) to 9 ml of *B. subtilis* (concentration: 2.5 x 10^9 CFU.ml^{-1}) spore product. Sterile water was used as the control. For each suspension a dilution series from 10^{-1} to 10^{-4} was prepared. Dilutions were plated onto PDA after 5 minutes and plates were incubated at 25°C. Colonies formed were counted after 7 days. Number of colonies formed was presented as percentage of the control.

To determine the effect of the product on microsclerotia, a one ml suspension containing microsclerotia of *V. dahliae* (concentration: 3.9 x 10^3 sclerotia.ml^{-1}) was suspended in 9 ml *B. subtilis* (concentration: 2.5 x 10^9 CFU.mL^{-1}) and sterile water respectively. One ml of each suspension was plated onto PDA after 5 minutes. Plates were incubated at 25ºC. Germinating microsclerotia were counted after 7 days. Number of germinating microsclerotia was presented as percentage of the control.

4.1.3. Growth of isolates for biomass production

Bench scale evaluation of medium components and fermentation parameters were conducted in 1.8L Fernbach flasks to demonstrate effective cultivation. The media components were weighed out as per the sporulation recipe (0.8%$m.v^{-1}$ Yeast extract, 0.5% $m.v^{-1}$ Peptone, 0.3% $m.v^{-1}$ Meat extract, 0.005% $m.v^{-1}$ $MnSO_4.4H_2O$, 0.01% $m.v^{-1}$ $CaCl_2.2H_2O$ and 0.03% $m.v^{-1}$ $MgSO_4.7H_2O$) with varying meat extract (Biolab, cat no. HG000BX7, Merck, RSA) concentrations as outlined in table 4.2.3.1 and made up to a final volume of 700ml with RO water in 1.8L Fernbach flasks. Flasks were sterilized for 20 minutes at 121ºC at 100 kPa pressure using an autoclave (Anzy JSD400, Getinge Inc, Rochester, NY).

Table 4.1.3.1.: Composition of media used to compare cultivation of *Bacillus subtilis* with and without meat extract.

Flask allocation	**Study**
Flask 1	*B. subtilis* with meat extract
Flask 2	*B. subtilis* with meat extract
Flask 3	*B. subtilis* without meat extract
Flask 4	*B. subtilis* without meat extract
Flask 5	*B. subtilis* without meat extract

Flasks were inoculated with one cryovial of *B. subtilis* and incubated at 32ºC at 200 rpm on an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ) and sampled at, 12, 24 and 48 hours. Samples were assayed for sporulation efficiency using equation 1. After 48h incubation the flasks were sampled and assayed for CFU counts to determine the final cell concentration.

4.1.4. Stabilization and formulation of final broth

For the product to have commercial value it needs to maintain its efficacy under extended storage periods. Storage at room temperature is preferred to refrigeration as the later adds to the cost and inconvenience of applying the product. The cold chain can also not be ensured and a product that has to be kept at refrigeration temperatures can easily loose its efficacy. The product also needs to be in a formulation which would ensure ease of application for the end user.

Stabilization

The final product from flasks cultivated without meat extract was used for the stabilization experiments. The broth from these flasks was mixed into a single sterile container and a sample was taken to determine the viable cell number on PCA agar plates. The final product was stabilized as had been previously described in Section 2.1.3.

4.1.5. Product efficacy testing

The product was tested for its efficacy prior to process scale-up to ensure that further investigation was conducted on a product that continuously demonstrated product effectiveness. Product efficacy trials were conducted in green house trials at CSIR Biosciences in Pretoria.

Green house trials

Effect of the product against the plant pathogen, *V. dahliae* was tested *in vivo* at green houses at CSIR Scientia campus. Average minimum/maximum temperatures (15/26ºC) and humidity (50%) levels were controlled using an automated humidifier throughout the trial. Natural lighting was used to allow for a day/night cycle. The trial was conducted on two crops, tomatoes (cv. Rooi Kake) and potatoes (cv. UTD). The planting material was sourced by ARC Roodeplaat, Pretoria and a total of 140 tomato seedlings and 140 potato mini tubers were planted in 15cm pots. Four different treatments were planned for each cultivar with each treatment having a total of 25 pots and five pots per replica. The positive control consisted of seedlings and mini tubers planted in potting mix infested with *V. dahliae*, with a negative control being seedlings and mini tubers plated in sterile potting mix. No product or activator was applied to both controls. Treatments A comprised of seedlings and mini tubers planted in pre-sterilized potting mix infested with the relevant pathogen with product being applied continuously once a week from week 1 up to week 5 on the tomato seedlings and application commencing from week 6 up to week 12 on the potato mini tubers due to the delay in mini tuber germination. Treatment B comprised seedlings and mini tubers planted in pre-sterilized potting mix infested with pathogen and product applied once a week from week 1 up to week 4 on tomato seedlings while application on mini tubers was from week 6 up to week 10. Treatment C was designed similar to treatment A with the exception that for Treatment C, the seedlings and mini tuber were first planted in sterilized potting mix with the pathogen being inoculated after one week of planting for both seedlings and mini tubers, product application also commenced during week 1 and progressed to week 5 for the tomato seedlings while product application commenced during week 6 and progressed to week 12 for the mini tubers. Treatment D comprised seedlings and mini tubers planted in sterile potting mix with pathogen inoculation one week after planting and product application commencing during week 1 for the seedling and progressing to week 4 while product application commenced during week 6 up to week 10 for the mini tubers. Product prepared as described in Section 4.1.3. was used for the green house trials. The trial design was targeted to establish the most effective application regime for optimal control of the pathogen.

The dosage regime of product was based on information obtained from Microbial Solutions (PTY) LTD. The dosage volume was calculated on a ratio of 500 ml of product per hectare with 1kg of Biostart® Activator at 1 X 10^9CFU.mL^{-1}. The equivalent dosage for the pot trial was therefore calculated to be 1ml of product with 0.03g activator per pot. Sampling required the destruction of the plant to determine the level of intracellular infection by monitoring the development of *V. dahliae* in stems of test plants Sample assays through cross sectional inspection to determine level of infection and statistical analysis were conducted at ARC Roodeplaat, Pretoria. For the purpose of this trial the impact of the fungal pathogen on roots was not investigated and the counts of the antagonist were not monitored. The results were presented as a percentage of infection levels.

4.2. Results

4.2.1. Cryo-presevation and culture maintenance

Streak plates prepared from 5 % of the working cell bank cryo-vials revealed no contaminating species, thus implying a 100% purity of the entire cell bank. The average spore count in the cryopreserved cultures was 1.6 $X10^{9}$ +/- 7.1 $X10^{8}$ $CFU.mL^{-1}$. Individual counts were within the acceptable range of 1.5 standard deviations above and below the average (Figure 4.2.1.1).

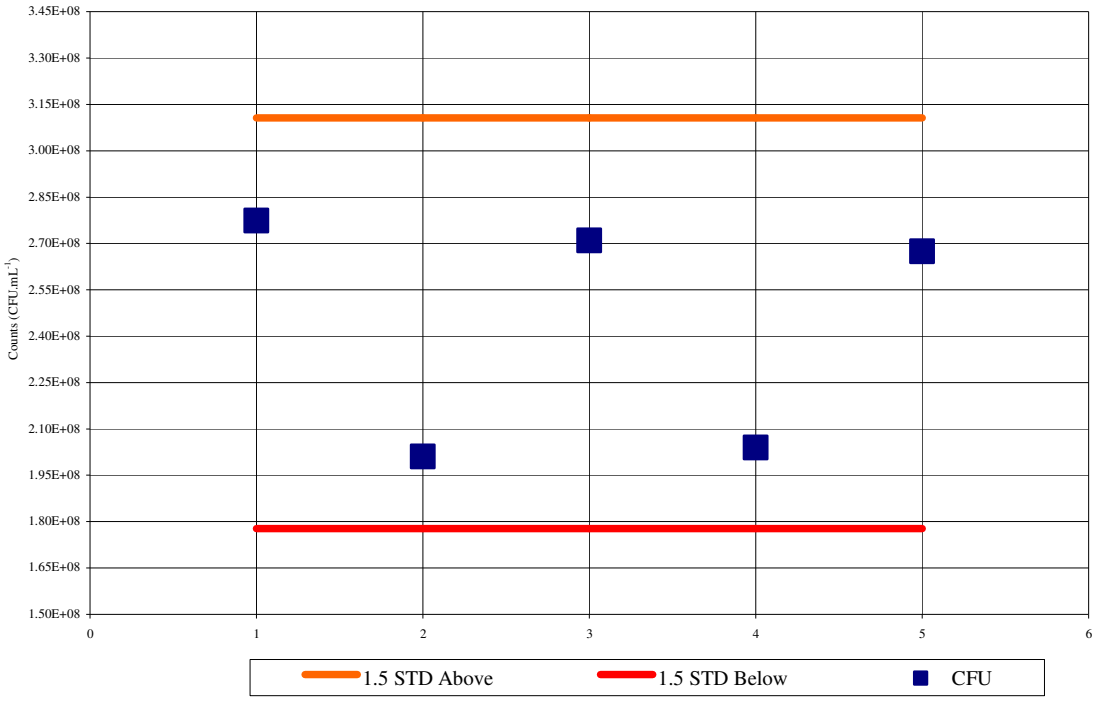

Figure 4.2.1.1.: Spore counts of five cryovials expressed as $CFU.ml^{-1}$ of cryo-preserved *Bacillus subtilis* cultures.

The assessment of sporulation capability demonstrated that the WCB could successfully sporulate to an average sporulation efficiency of 98%+/- 2%. The average cell count after 48 hours incubation in the Fernbach flasks was 2.5 X 10^{9} +/- 1.53 $X10^{8}CFU.mL^{-1}$.

Table 4.2.1.1.: Mean growth rate and doubling time of cryopreserved *Bacillus subtilis*

	Response values
Average growth rate (h)	1.01
SD	0.00
Average doubling time (h)	0.69
SD	0.00
R^2	99.49

The cryo-preserved culture of *B. subtilis* demonstrated an average doubling time of 0.69 hours with an average growth rate of 1.009 hours (Table 4.2.1.1).

The quality of the cryo-preserved WCB is further illustrated in Figure 4.2.1.3 which shows the average growth rate of the *Bacillus subtilis* cultures against time based on OD measurements. The growth curve shows a typical sigmodial growth pattern.

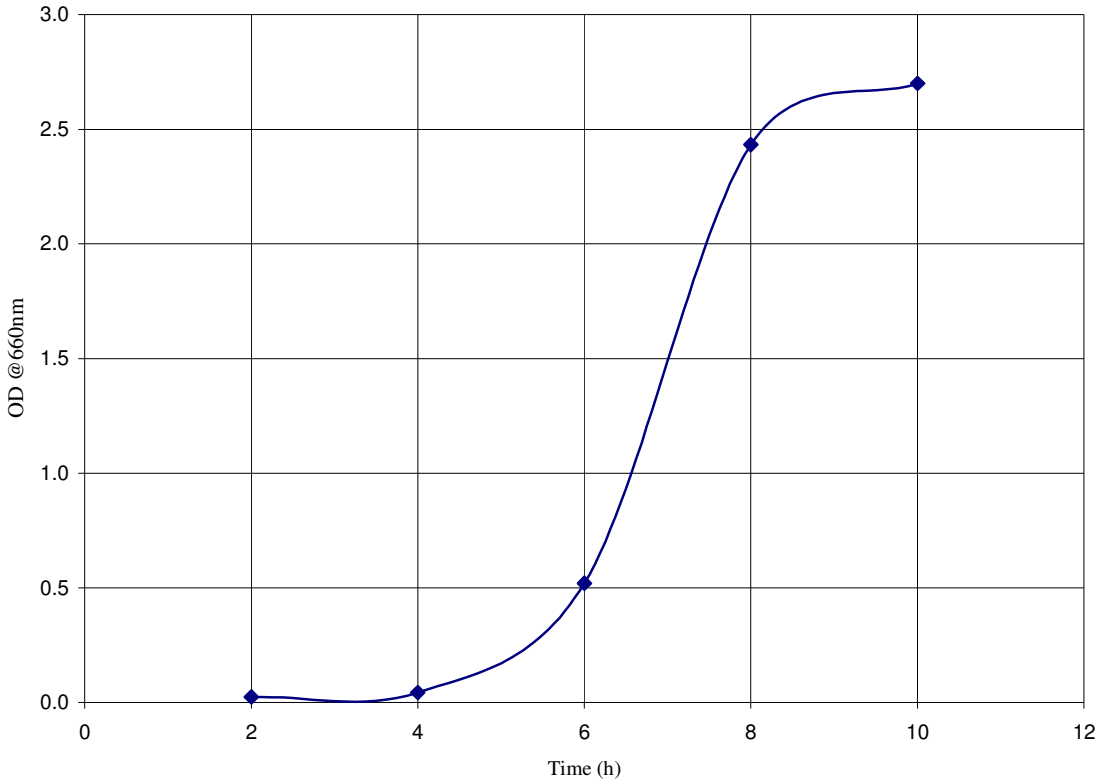

Figure 4.2.1.2.: Growth curve of *Bacillus subtilis* determined in 3% Tryptone Soy Broth.

The *B. subtilis* isolate was successfully cryo-preserved (Table 4.2.1.1, Figures 4.2.1.1 and 4.2.1.2) and was capable of supplying a constant and stable source of the organism which could be revived and cultivated. The maximum growth rate for *B. subtilis* was 1.01 with a 99% fit to the plot of ln OD against time (Section 2.1.1). The lag phase lasted approximately four hours and is representative of the time taken for spores to germinate into vegetative cells on TSB media. It was also evident that the growth of the organism in all test cases was similar in terms of the lag and exponential phases, confirming the consistency of the cell bank and inoculum stage.

4.2.2. Bioassay of isolate against Verticillium dahliae

Bacillus subtilis was tested for antagonistic properties against *V. dahliae*, a phytopathogenic fungus. Positive results were observed as *B. subtilis* demonstrated inhibition against all tested states of the pathogenic fungi. The results are presented as CFU of *V. dahliae* without application of *B. subtilis* and CFU with *B. subtilis* application (Table 4.2.2.1).

Table 4.2.2.1.: Demonstration of inhibition by the test strain, *Bacillus subtilis* against *Verticillium dahliae*.

	Treatment	**Average CFU (conidia)**	**Average Germinating sclerotia**	**Percentage survival**
Conidia	Control	$2.19X10^{7}$		100%
	B. subtilis	$1.33X10^{4}$		0.06%
Microsclerotia	Control		390	100%
	B. subtilis		0	0%

4.2.3. Growth of isolate for biomass production

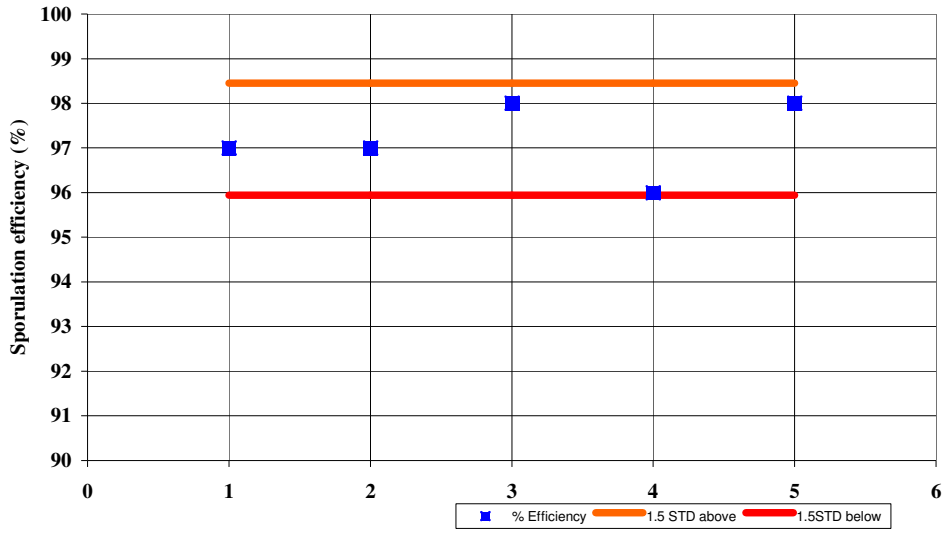

Figure 4.2.3.1.: Sporulation efficiency expressed as % of *Bacillus subtilis* cultivated in media with or without meat extract (with 1-2 representing flasks with meat extract and 3-5 representing flasks without meat extract).

The observations of the comparative media (with and without meat extract) have confirmed the hypothesis that the exclusion of meat extract will not significantly affect the quality of growth of *B. subtilis*. The sporulation efficiencies of all test flasks were in excess of 96 % with an average sporulation efficiency of 97.2 % +/- 0.84% (Figure 4.2.3.1). Sporulation efficiencies > 90% are considered excellent, however this does not indicate the total cell count, it was therefore essential to establish if there was any significant change in the final cell concentration as supported by media without meat extract. Determining the sporulation efficiency of the isolate after media modification was imperative so as to demonstrate that it had retained its ability to sporulate.

Table: 4.2.3.1.: Means of final cell concentrations and sporulation efficiency of *B. subtilis* grown in media with or without meat extract.

	With Meat Extract		**Without Meat Extract**	
	CFU.mL^{-1}	**% efficiency**	**CFU.mL^{-1}**	**% efficiency**
Mean	2.30X 10^8	97	2.55 X 10^8	97.3
SD	6.36 X 10^7	0	3.91 X 10^7	1.15
	TTEST (CFU)	0.3409	**TTEST (% eff)**	0.3333

The t-test performed on both the CFU and sporulation efficiency data showed no significant difference between growth on media with or without meat extract. Omission of meat extract would therefore not result a reduction in product quality (Table 4.2.3.1).

4.2.4. Stabilization and formulation of final broth

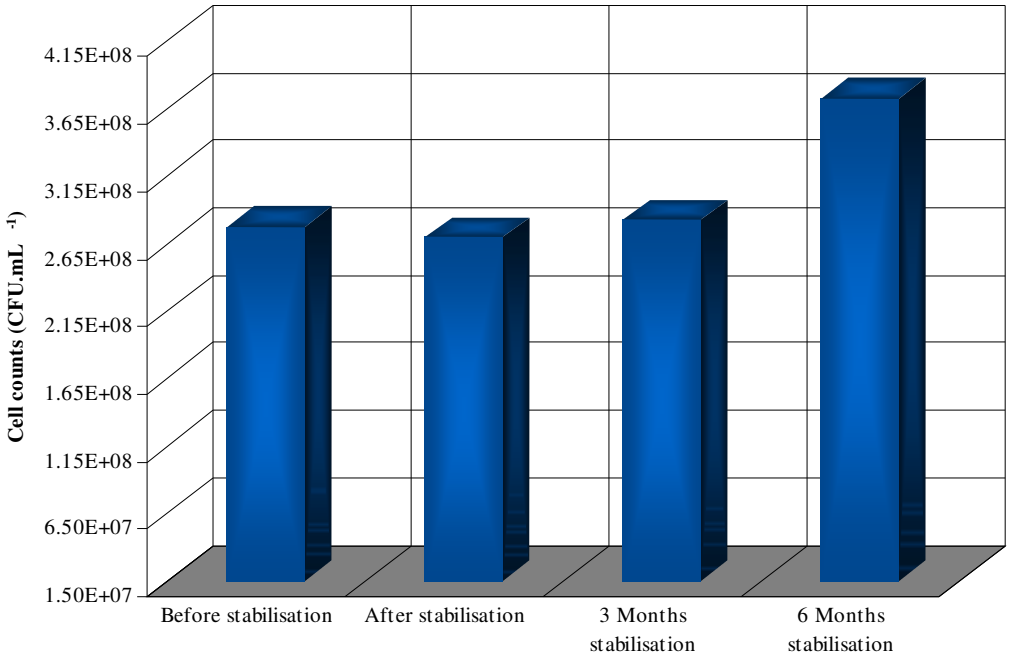

Figure 4.2.4.2.: Final product viability studies conducted on stabilized broth kept in the cold room (4°C) and assayed for viable counts determination.

The CFU count after stabilization was not lower than the count obtained before stabilization, demonstrating a difference of only 0.21% (Figure 4.2.4.2). The average sporulation efficiency was 97.2 % +/- 0.84%.The result for the six months assays showed a 30% increase in the total viable cells enumerated after six months which could be an error introduced by external factors.

4.2.5. Product efficacy testing

The results presented, are averages of assays based on the plants sacrificed over the duration of the green house trials. The results are presented as degrees of infection as determined at ARC Roodeplaat, Pretoria.

Table 4.2.5.1.: Summary of the infection levels for applications on tomato crops presented as percentages.

Treatment	**Tomato** (% infection)
Negative control	0
Positive control	31
Treatment A (1 – 5 weeks)	15
Treatment B (1 – 4 weeks)	23
Treatment C (1 – 5 weeks)	1
Treatment D (1 – 4 weeks)	0

The results in table 4.2.5.1 show the detected level of infection on assayed tomato plants. The negative control showed no infection and the positive control showed 31% infection which was relatively low for a positive control. The infection level of treatment A and B demonstrated that early termination of product application could be detrimental to the crop being cultivated. The antagonist did suppress the development of the pathogen in treatments C and D when it was applied on sterile soil prior to fungal inoculation.

Table 4.2.5.2.: Summary of the infection levels for applications on potato crops presented as percentage.

Treatment	**Potato** (% infection)
Negative control	0
Positive control	40
Treatment A (6 – 12 weeks)	20
Treatment B (6 – 10 weeks)	45.3
Treatment C (6 – 12 weeks)	31.3
Treatment D (6 – 10 weeks)	52.4

The results in table 4.2.5.2 show the detected level of infection on assayed potato plants. The negative control showed no infection as expected while the positive control had a 40% infection level. Treatment A resulted in better control than treatment B and this

further proved that application of the product for longer periods did prevent high infection levels. Interesting results were observed in treatments where the product was applied prior to inoculation with the fungal pathogen. Although treatment C, that had been applied for longer demonstrated better disease suppression than treatment D, these two treatments had relatively higher infection levels than Treatment A and B respectively. The possibility of the activator enhancing the growth and infection of *V. dahliae* needs to be investigated.

4.3. Discussion

The primary objective of cryo-preservation and culture maintenance techniques is to preserve and to maintain the culture's physiological characteristics, while preventing any contamination and changes in the biochemical and morphological characteristics (Meza *et al.*, 2003; Upadhyay and Rai, 1986). This is important for the reproducible availability of high quality culture for research purposes. The cryo-preservation technique employed uses glycerol as a cryoprotectant and has been documented by Baudot and Odagescu (2004) and is a widely used cryo-protectant (Meza *et al.*, 2003). Glycerol has the ability to penetrate the cell and prevent the formation of ice crystals within the cytoplasm (Zohar-Perez *et al.,* 2002). This cryo-preservation technique was effective based on the viable cell recoveries demonstrated after preservation with an average viable count of 1.6 X 10^9 CFU.mL^{-1}. The resulting work cell bank (WCB) was confirmed to be a monoculture and suitable for use as an inoculum source for experimentation. The ability of the newly prepared WCB to sporulate at high sporulation efficiency was determined as an indicator that the isolate had not lost its physiological properties (Meza *et al.,* 2003). The sporulation efficiency is an important parameter as it determines the product concentration and will be continuously tested during all process development stages as change in media composition can affect sporulation (Kolodziej and Slepecky, 1964; Wuytack *et al.*, 1999). Takahashi and MacKenzie (1981) reported on *B. subtilis* isolates that could sporulate in media with a relatively high carbon concentration while Matsuno *et al.,* (1999) indicated that nutritional limitations were the main trigger for *B. subtilis* cells to cease normal cell division and initiate sporulation. Excess glucose in media may result in feed back inhibition of isocitrate dehydrogenase which plays a significant role during the initiation of sporulation (Matsuno *et al.,* 1999). The effect that medium composition has on the sporulation of the isolate under investigation was demonstrated by the fact that the isolate did not sporulate when cultivated in TSB media and sporulation was only achieved when cultivated in a sporulation media containing salts. For the product to be successful, a high sporulation as well as a high percentage germination of the spores is required (Wuytack *et al.*, 1999). This was evaluated by

inoculating the cryo-preserved spores into growth medium. The current WCB reached an average cell count of 2.5 X 10^9 CFU.mL^{-1} when cultivated in growth medium.

The antifungal efficacy of the *B. subtilis* isolate was evaluated after every stage of product development to ensure that the efficacy was not lost during the process development. It was important to prove that the isolate was effective against specific racemates of *V. dahliae* that were identified as being problematic in the target cultivars, namely tomatoes and potatoes. The antifungal evaluation was done on agar plates and demonstrated that the isolate was effective against *V. dahliae*. The *B. subtilis* showed inhibition levels of 99% of the conidial suspension and 100% efficacy against the germination of the microsclerotia. This was exceptionally promising as there is currently no known product that can effectively treat *V. dahliae,* especially as the microsclerotia has the ability to survive for up to 13 years in soil (Ghaffer, 1986). *B. subtilis* is known to produce a range of antifungal compounds (Luna *et al.,* 2002). However the mode of action of the *B. subtilis* isolate to inhibit *V. dahliae* is unknown and needs to be further investigated. The successful results attained in *in vitro* experiments may not necessarily guarantee a similar success in field trials as the experiments are mainly executed under controlled environments while field conditions may be unfavourable to the test product (Zohar-Perez *et al.*, 2002). Even with proven biological applications, the efficacy may vary with a change in location some time due to the change in the physio-chemical condition of the soil (Tenuta and Lazarovits, 2004). Once the dosage regime has been developed in greenhouse trials, field trials will be done to demonstrate product efficacy in different locations and on different soil as part of the registration requirement.

The main purpose of a fermentation process is to maximise productivity using an economical process (Riesenberg and Gurthke, 1999). This can be achieved by adapting the process to locally abundant raw material and by process optimization. During bench scale process development level, the main aim was to demonstrate that the isolate can be produced to desirable cell counts and high sporulation efficiency. The isolate was successfully produced to an average spore concentration of 2.7 X 10^9 CFU.mL^{-1} with 98%+/- 2% sporulation efficiency. A spore based product formulation has better chances

of success in agricultural application as it allows for extended shelf life without refrigeration as well as ease of handling. For this reason, many biocontrol agents are produced in spore forms and in considerably large quantities (Upadhyay and Rai, 1986; Wolken *et al.*, 2003).

The *B. subtilis* isolate has been successfully cultivated in medium containing meat extract as a nitrogen source. The cost implications and recent reluctance to utilize meat based products generated a need to develop a medium without meat extract. Shoda (2000) reported that medium composition has an impact on foam production during liquid cultivation of *B. subtilis.* Although foaming can be controlled with antifoaming agents, excessive foaming has a negative impact on the fermentation as the cells become highly concentrated in the foam and lower growth rates are observed. Statistical analysis indicated that there was no significant difference in total cell count and sporulation efficiency of *B. subtilis* grown with or without meat extract in the medium. Tenuta and Lazarovits (2004) reported that meat and bone extract treatments increased the soil's ability to resist fungal infection. Therefore the omission of meat extract as a media component should be further investigated to establish the impact on the final product's field efficacy.

The effect that the omission of meat extract had on the down stream processing (DSP) was also investigated. A comparison of broths with and without meat extract indicated no significant differences during stabilization and did not result in any significant decrease in total spore counts. Based on these findings the protocol can be used effectively for the stabilization of *B. subtilis*. However only a few data points were generated during the current experiment, it would therefore be essential that further trials are conducted to re-confirm the results. The recovery of viable cell counts from the stabilized broth after six months of storage was 30% higher than the initially stabilized product. The increase in viable cell count after six months could be due to an underestimation of the initial viable count due to clumping of spores that disperses during storage. It is however unclear why such an increase was only observed after six months of storage. Previous stabilization conducted on *B. licheniformis* did not show a similar trend (Chapter 2). .

The final product was tested for efficacy in green house trials on tomatoes and potatoes. *B. subtilis* has previously shown biological activity against several phytopathogenic bacteria and fungi (Luna *et al.*, 2002) and was demonstrated to be effective against *V. dahliae* during the screening procedure (Chapter 3). Testing the isolate in green house trials was done with full understanding that efficacy demonstrated does not necessarily guarantee success in field trials (Zohar-Perez *et al.,* 2002). For successful and consistent application, attention should therefore be given to the physiological state of the product, the expression stage of the active compound and the interactions between the plant, pathogen and biological agent. Knowledge of the environmental conditions the product is to operate in is also important. Shoda (2000) has reported that bacteria require a specific substrate to be available in soil for the production of specific metabolites. This means that a product demonstrating efficacy in one field with a specific cultivar may not have the same efficacy in a different field and different cultivar. Such information outlines the importance of understanding the behaviour of a biological control agent in the environment in which it is expected to perform (Collins and Jacobsen, 2002).

The green house trials supported the results observed *in vitro* and they further demonstrated that the efficacy of the biological agent will differ when applied to different crops. Crops grown in different soil types and under different conditions will result in variations in the root exudates produced (Collins and Jacobsen, 2002). The variation in both the type and concentration of root exudates will influence the soil microbial population. Root exudates are precursors that determine the type of metabolites produced. Tables 4.2.5.1 and 4.2.5.2 demonstrate the variation in disease control *B. subtilis* may have when applied on different crops. More effective control of the fungal pathogen was achieved in the tomato crops as indicated by the lower levels of infection in contrast to the potato crops. The treatment on tomato demonstrated that the application of product at the same time as inoculation with pathogen yielded significantly good results with treatments C and D showing 1% and 0% levels of infection respectively. Such an observation supports the recommendation of product application from planting. Treatment A and B showed higher levels of infection with 15% and 23% plants infected

with the pathogen respectively. The product application for these treatments was one week after planting on the already infected soil mix. The higher levels of infections can therefore be attributed to the delay in application of the *B. subtilis*, resulting in the favourable development of the pathogen and infection of plants. This dosage regime therefore afforded the pathogen time to colonise the seedlings (Paulitz and Bélanger, 2001) and consequently out-competed *B. subtilis*. During product application, a dosage of an activator was also applied. Even though targeted for the germination and growth of *B. subtilis*, these nutrients would have also benefited the conidial fungus and could have promoted rapid growth of the pathogen resulting in the fungus out-competing the *B. subtilis* as it is in a spore form at application. Delayed product application therefore gives a competitive advantage to the pathogen rather than the *B. subtilis*. However, the ability of *B. subtilis* to control the pathogen was evident in treatment C and D where both the antagonist and pathogen were introduced at the same time and *B. subtilis* effectively controlled fungal infection of the plants. The positive control used had a low infection level of 31%, the optimum infection level is 80% or above, such results reflect the complexity of testing under non laboratory conditions. Conditions such as availability of nutrients, moisture or fluctuations in temperature and humidity have an impact on disease development.

Inoculation of the potato crop with *V. dahliae* also resulted in a lower than expected levels of infection in the positive control (40%) indicating that conditions were not optimum for infection. In contrast to what was observed on tomatoes, treatment A showed the lowest level of infection (20%) followed by treatment C (31%). Treatments A and C were both treatments where *B. subtilis* was applied for twelve weeks. The extended application resulted in better control of the pathogen when the *B. subtilis* was applied six weeks after planting of the potatoes in infected soil. Treatment B and D, demonstrated 45% and 52% infection levels respectively, indicating that prolonged application of *B. subtilis* is required for effective control of *V. dahliae* on potatoes.

The results demonstrated that the product should be applied as a prophylactic, taking a preventative approach rather than as a treatment. The recommended application would be

to treat the crop from planting and proceed until late in the developmental stage therefore requiring several applications.

CHAPTER 5

CONCLUSIONS

Global agricultural production is under continuous threat due to diseases, pests and weeds and as much as 50% of losses have been attributed to such challenges. These developments are concurrent with the ever growing global population, demanding more food supplies. The need for the development of alternatives and supplements for the agricultural sector has therefore received great interest, an example of which is Benomyl, a chemical product demonstrating effective control against a variety of pathogenic fungi. These chemicals are however detrimental to other beneficial fungi like mycorrizhal fungi due to their broad spectrum nature, additionally some of these chemical products such as methyl bromide have been implicated in the depletion of the ozone layer and are subsequently being phased out globally. Reports on the detrimental nature of some currently applied chemicals to human health have also been tabled and as a consequence, such chemicals are of great health and environmental concern. There is therefore an urgent need to develop alternatives to fill this ever increasing gap. Biologically based products were identified as possible alternatives to the address these concerns.

Significant benefits could be realised in the application of microorganisms as biofertilizers for growth enhancement and as bio-pesticides for pathogen control. Most of these organisms are resident in the rhizosphere, therefore their application is not regarded as foreign. However, the use of indigenous isolates which are adapted to local conditions is now becoming important, this increased interest has resulted in a drive to have agricultural produce certified and ranked for biosafety which ultimately translates into reduced or no pesticide residue. This has provided an opportunity for the introduction of microbial products as potential alternatives. It is however essential that the contribution of individual isolates constituted in the product is clearly outlined. This is important for purposes of product registration, and the cost implications of their production. This is however not a straight forward task as most organisms survive as a consortia rather than in isolation.

The evaluation of a commercial biological product was conducted as outlined in Chapter 2. The evaluation was based on an already existing commercial product Biostart®, which

is marketed as a growth enhancer. The individual isolates that were major constituents of the product were cryo-preserved and the validation of such cell banks showed that the process had been successful based on the total cell numbers. The tested cryo-preservation technique was therefore successful in preserving *Bacillus* based cultures, however these assays were targeted at quantifying the recovery profile of the preserved culture and did not test for the retention of targeted efficacy. Method development for evaluation of biochemical traits is therefore necessary.

The commercial product had been previously demonstrated to enhance growth of lettuce seedlings. There was however a need to clarify the contribution of each of the three isolates towards the success of the product. The product was previously demonstrated to be an effective growth enhancer when applied as a consortium; however testing of the individual isolates demonstrated no growth enhancement effect. A later trial to confirm the growth enhancement of the consortium, contrastingly demonstrated no growth enhancement, which then posed questions around what the differences in the trials were? The change in the activator composition was suspected to have had a significant influence knowing that growth medium determines the efficacy of the BCA. For this reason, it cannot be conclusively claimed that individual isolates were ineffective, but the observed performance may have been affected by the change in activator. This further concludes that any change in a biological system should be followed by an investigation of the resulting influence. It is therefore important that the effect of the individual isolates be re-investigated, mitigating any influence from a different activator. For the purpose of this study, no further experiments to validate the product's growth enhancement properties were undertaken.

The isolation, screening and preservation of potential isolates are the most vital stage, which may determine the success or failure of a putative product. Also understanding exactly what the target efficacy is, determines the methods to be applied, unfortunately, most modes of action by BCA's may not necessarily be readily assayed using simple laboratory methods. Screening a BCA for properties like solubilization of mineral or ability to induce disease resistance in target crops requires more long term investigation

and specialized techniques. If the methods are not well developed, only organisms with the basic traits can be isolated and selected. An extensive isolation and screening process was undertaken from the CSIR culture collection and from sourced soil samples. The targeted efficacy was the ability of the BCA to inhibit fungal growth *in vitro*. A number of potential isolates showed efficacy during this process, but the selection was limited to organisms with the ability to sporulate and of the genus *Bacillus,* and hence excluded potential agents of any other genus. Contributions of *Pseudomonas* spp. to biological control have been well documented but none were isolated during this process as they were not targeted. The outcome of the isolation procedure resulted in an isolate that demonstrated broad spectrum fungal inhibition, later identified as *Bacillus subtilis* strain KL-077. This isolate was selected for further investigation due to its potential as a biological agent, as well as the fact that this species is mostly indigenous to soil environments, its application may not be regarded as foreign. This will therefore allow easier registration of the product. The broad spectrum nature of the isolate, even though targeted could also have potential negative outcomes, via potential influences on the growth of the mutually beneficial organisms that are soil residents' e.g. mycorrhizal fungi. The impact of product application on the microbial biota has to be properly investigated prior to product registration. This will however be addressed during the registration trials through environmental impact assessments.

An *in vitro* comparison of the *B. subtilis* efficacy in inhibiting known pathogenic fungi against a systematic fungicide, Benomyl demonstrated positive results. This revealed that the isolate could compete favourably with currently available chemical products. The fact that the isolate was natural and had GRAS status, meaning generally regarded as safe, provided the putative product with a competitive edge in penetrating existing markets. Such a status is important in the classification of agricultural products as exposure to products of concern especially to some of immuno-compromised farm personnel. A potential isolate had therefore been identified and it showed great potential benefits, this fulfilled the objective as had been set in Chapter 1.

The capacity of cultivating this organism was demonstrated in Chapter 4 at bench scale. The production technique yielded high spore counts and the isolate was shown to have retained its antifungal properties after cryo-preservation and bench scale production based on *in vitro* inhibition of fungi. Elimination of meat extract as an ingredient did not demonstrate any negative effect on the production and final spore yields. The media composition should therefore be further investigated and optimized as the recipe used may have cost implications in higher production regimes. For fermentation process development, recipe development and optimization will form an integral part to ensure an effective yet economically viable production recipe.

Green house trials to further demonstrate the efficacy of the microbial product were commissioned. Such demonstrations together with field trials are a prerequisite toward successful registration of a microbial product. The product prototype demonstrated levels of inhibition on the targeted phytopathogen, but it was evident that further trials were necessary to conclusively confirm the outcomes of the product application. This further supported that the efficacy of microbial products will be influenced by the choice of crop and the resulting soil conditions. Investigation toward the best application procedure for the product was determined as an important aspect that will determine the necessary formulation of the final product and the application regime.

The studies undertaken were successful in addressing the objectives of developing a putative biological product that may have great effect on crop production systems. This is a preliminary undertaking that requires further investigations to ensure successful development of an active product that has the potential of penetrating the current biological markets.

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APPENDICES

Appendix 1: Fermentation media

Media		
Yeast Extract	24	g .L $^{-1}$
Nutrient broth	15.00	g .L $^{-1}$
Citric acid	0.50	g .L $^{-1}$
$(NH_4)_2SO_4$	3.00	g .L $^{-1}$
$Ca(No_3)_2$	0.24	g .L $^{-1}$
$MgSO_4.7H_2O$	1.50	g .L $^{-1}$
$MnSO_4.4H_2O$	20.00	mg .L $^{-1}$
$FeSO_4.7H_2O$	16.00	mg .L $^{-1}$
Trace element solution	0.00	ml .L $^{-1}$
H_3PO_4 (85%)	0.63	ml .L $^{-1}$
Antifoam	0.1	ml .L $^{-1}$

Appendix 2: Vitamin solution preparation

Vitamins				
Thiamine	4.76	mg .L $^{-1}$	9.52	mg
Biotin	0.6	mg .L $^{-1}$	1.2	mg
Calcium pantothenate	4.76	mg .L $^{-1}$	9.52	mg
Ascorbic acid	4.76	mg .L $^{-1}$	9.52	mg
Niacin	4.76	mg .L $^{-1}$	9.52	mg
Pyridoxine	4.76	mg .L $^{-1}$	9.52	mg
To be filter sterilize into medium				

Appendix 3: Glucose determination by Accutrend method

A broth sample may assayed for glucose concentration using Accutrend® method, this method was mainly developed for continuous measurement of glucose level in blood of diabetic patients but it may also measure glucose in broth but the operator must ensure the compounds that may interfere with correct reading as stated in package insert are absent.

The Accutrend instrument is switched on and wait for the prompt messages, confirm whether the code displayed on screen corresponds with the code on the strips to be used, if not, follow instructions as outlined in the package insert to correct the code. When the instrument prompts, place the strip in the designated strip groove and add a drop of broth in strip pad. Wait for the instrument to read and note the result. Should the instrument give an error message or Hi reading, re-dilute the samples and repeat but ensure not to overfill the strip pad. The final result is presented as mmol/l and the corrected result may be obtained by multiplying the final result by the dilution and by 0.186 to convert the result to g/l.

Glucose molar mass: 180.09 g/mol.

Appendix 4: Tray layout for *in vitro* product efficacy testing.

09	09	09	09	02	02	02	02
01	01	01	01	10	10	10	10
06	06	06	06	15	15	15	15
14	14	14	14	03	03	03	03
08	08	08	08	04	04	04	04
12	12	12	12	13	13	13	13
16	16	16	16	11	11	11	11
05	05	05	05	07	07	07	07
12	12	12	12	03	03	03	03
07	07	07	07	09	09	09	09
01	01	01	01	05	05	05	05
02	02	02	02	08	08	08	08
15	15	15	15	16	16	16	16
11	11	11	11	04	04	04	04
10	10	10	10	14	14	14	14
06	06	06	06	13	13	13	13

Appendix 5: *Bacillus* spp. selected for antagonism screening

Organism designation	**Organism**	**Vial labelling**
A1	*B. licheniformis*	B1 sg
A2	*B. subtilis*	28
A3	*B. chitinosporus*	Wcb3 (6)
B1	*B. polymyxa*	2
B2	*B. chitinosporus*	S.wcb2 (88)
B3	*B. licheniformis*	S.wcb 2 (6)
B4	*B. chitinosporus*	Wcb 3
C2	*B. megaturium*	50
C3	*B. licheniformis*	82
1C4	*B. laterosporus*	Wcb2 (39
2C4	*B. licheniformis*	Wcb2 (96)
C4	*B. chitinosporus*	Wcb2 (57)

Appendix 6a Fungal cultures

Fungal Strains	**Origin**
Fusarium solani	Plant Aid
Fusarium moniliforme	Plant Aid
Fusarium spp.	Plant Aid
Fusarium oxysporum	Plant Aid
Stenocarpella maydis	Plant Aid
Aspergillus flavus	Plant Aid
Aspergillus clavatus	Plant Aid
Alternaria alternata	Plant Aid
Panama AVC 145	CSIR Plant biotech (PTA)

Appendix 6b: Fungal culture designation

Designated no	Fungal isolate
1	*Aspergillus clavus*
2	*Eurotium spp*
3	*Alternaria alternata*
4	*Fusarium* spp.
5	*Eurotium rubrum*
6	*Phoma sorghina*
7	*Eurotium repens*
8	*Penicillum expansum*
9	*Eurotium amstelodami*
10	*Verticillium dahliae (79)*
11	*Verticillium dahliae (121)*

Appendix 7: KZN soil sample designation

Designated no	Location	Crop
1	Adams Mission	Madumbe
2	Adams Mission	Pumpkin
3	Adams Mission	Maize
4	Umkhubi Secondary School	Banana
5	Umkhubi Secondary School	Chillies
6	Umhlanga	Sugar cane
7	Umhlanga filling station	Sugar cane
8	La Mercy	Daniah
9	La Mercy	Chillies
10	La Mercy	Unknown
11	La Mercy	Onion
12	La Mercy	Imbuya
13	La Mercy	Maize
14	Stanger	Banana
15	Darnall	Sugarcane
16	Nyezane	Pawpaw
17	Nyezane	Madumbe
18	Eshowe (Maqhwakazi)	Dam
19	Eshowe (Maqhwakazi)	Cabbage
20	Eshowe (Maqhwakazi)	Spinach
21	Pietermaritzburg	Dennis seedling
22	Pietermaritzburg	After lettuce

Appendix 8: Identification sequence for *B. subtilis*

gi|14009325|gb|AY030331.1| *Bacillus subtilis* strain KL-077 16S ribosomal RNA gene, partial
sequence
Length = 1501

Score = 2833 bits (1429), Expect = 0.0
Identities = 1429/1429 (100%)
Strand = Plus / Plus

```
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