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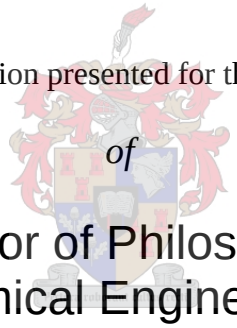
Development of a bioprocess for the production of an aquaculture biological agent

by

Rajesh Lalloo



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DECLARATION

I , the undersigned hereby declare that the work contained in this document is of my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

The experiments in this thesis constitute work carried out by the candidate unless otherwise stated and complies with the stipulations set out, for the degree of Doctor of Philosophy in Process Engineering, by the University of Stellenbosch.

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ABSTRACT

Biological agents offer several opportunities to address the many challenges faced in intensive reticulated aquaculture. We therefore isolated and selected *Bacillus* spp. as potential biological agents, because this group has demonstrated an array of biological activities of possible benefit to aquaculture. They also display advantages in terms of robustness during bioprocessing and end product application.

Natural isolates obtained from *Cyprinus carpio,* selected as a model high-value ornamental fish species, and associated culture environments, were purified and assessed for in vitro efficacy based on the inhibition of growth of pathogenic *Aeromonas hydrophila* and the decrease in concentrations of ammonium, nitrite, nitrate and phosphate ions, typically found as waste products in aquaculture systems. Based on suitability for aquaculture application, isolates B001, B002 and B003 were selected and further evaluated in vitro and in an in vivo trial with *C. carpio*. Inhibition of *Aer. hydrophila* growth and a decrease in concentrations of waste ions were demonstrated in these studies. Based on 16S RNA sequence homology, the isolates were identified as *Bacillus subtilis*, *B. cereus* and *B. licheniformis*, respectively. High sequence homology between *B. subtilis* and *B. anthracis* necessitated further safety studies on the best isolate, *B. cereus* NRRL100132 (B002). The isolate was shown not to contain the anthrax virulence genes pOX1, pOX2 or the *B. cereus* enterotoxin.

Elucidation of the potential modes of action of a biological agent facilitates an understanding of functionality and encourages technology uptake by end users. Competitive exclusion through growth rate and competitive uptake of glucose and iron, the latter facilitated by siderophore production, were shown to be key mechanisms at play in inhibition of *Aer. hydrophila* by the *B. cereus* isolate.

As production cost is an important consideration in development of commercially relevant biological products, we examined the optimization of nutrient supplementation, which has an impact on high-density production of spores by fermentation. Corn steep liquor (CSL) was identified as a lower cost and more effective nutrient source in comparison to conventional nutrient substrates, in particular yeast extract and nutrient broth. The improved sporulation performance of *B. cereus* could be related to the increased availability of free amino acids,

carbohydrates, and minerals in CSL, which had a positive effect on organism growth and sporulation efficiency. The impact of nutrient concentration on spore yield and productivity was modelled to develop a tool for selection of optimal conditions. Excellent correlation with actual laboratory fermentation data was demonstrated. A cost analysis revealed that production using liquid phytase treated and ultra-filtered CSL was less expensive than spray dried CSL and supported cultivation of *B. cereus* spores at densities higher than 1×10^{10} CFU ml^{-1}.

Adoption of biological agents in commercial applications is lacking, due to limitations in process and product development that address key end user product requirements such as cost, efficacy, shelf life and convenience. The development of suitable spore recovery, drying, formulation and tablet production process steps was thus performed. Key criteria used for downstream process unit evaluation included spore viability, recovery, spore balance closure, spore re-germination, product intermediate stability, end product stability and efficacy. A process flow sheet comprising vertical tube centrifugation, fluidised bed agglomeration and tablet pressing yielded an attractive product. The formulation included corn steep liquor and glucose to enhance subsequent spore re-germination. Viable spore recovery and spore balance closure across each of the process units was high (>70% and >99% respectively), with improvement in recovery possible by adoption of continuous processing at large scale. Spore re-germination was 97%, whilst a product half-life in excess of 5 years was estimated based on thermal resistance curves. The process resulted in a commercially attractive product and affordable variable cost of production.

Functionality of the product, incorporating the *B. cereus* isolate, was investigated across a range of physiological conditions, including salinity, pH and temperature, based on rearing of *C. carpio.* Temperature had a significant influence on germination, specific growth rate and increase in cell number of *B. cereus*, whilst salinity and pH did not have any measurable effect on growth. Controlled studies in bioreactors and modelling of the data to the Arrhenius function indicated the existence of high and low growth temperature domains. The rates of pathogenic *Aer. hydrophila* suppression and decrease in waste ion concentrations (ammonium, nitrite, nitrate and phosphate) were translated into a linear predictive indicator of efficacy of the *B. cereus* isolate at different temperatures.

This study has resulted in development of an upstream and downstream process for production of a new *B. cereus* isolate (NRRL 100132) which was shown to be safe, stable, functional, robust and cost effective for application in aquaculture.

OPSOMMING

Biologiese middels bied verskeie maniere om die veelvoudige uitdagings van intensiewe netsgewyse akwakultuur aan te spreek. Gevolglik het ons uitgesoekte *Bacillus* spesies as potensiële biologiese middels geïsoleer, omdat hierdie groep verskeie biologiese aktiwiteite demonstreer wat van potensiële waarde kan wees in akwakultuur. Die groep toon ook voordele in terme van robuustheid gedurende bioprosessering en eind-toepassings.

Natuurlike bakteriële isolate vanuit *Cyprinus carpio* geassosieerde kultuur omgewings, geselekteer as 'n hoë-waarde model ornamentele spesie, is gesuiwer. Die in vitro doeltreffendheid van die isolate is bepaal gebasseerd op die groei inhibisie van patogeniese *Aeromons hydrophila* asook die afname in konsentrasies van ammonium, nitriete, nitrate en fosfaat ione wat as tipiese afval produkte gevind word in akwakultuur sisteme. Isolate B001, B002 en B003 is geselekteer op grond van geskiktheid en verder evalueer in in vitro en in vivo proewe met *C. carpio*. Groei inhibisie van *Aer. hydrophila* asook 'n afname in konsentrasies van afval ione was tydens die studies gedemonstreer is. Die isolate is identifiseer as *Bacillus subtilis, B. cereus* en *B. licheniformis*, respektiewelik, op grond van 16S RNS volgorde homologie. Die hoë volgorde homologie tussen *B. subtilis* en *B. anthracis* het verdere veiligheidstudies op die beste isolaat, *B. cereus* NRRL100132 (B002) genoodsaak. Die isolaat het nie die antraks virulensie plasmied pOX1, pOX2 of die *B. cereus* enterotoksien getoon nie.

Uitklaring van die potensiële meganismes van aksie van biologiese middels fasiliteer 'n begrip van funksionaliteit en moedig tegnologie aanvaarding deur eind-gebruikers aan. Mededingende uitsluiting deur groeitempo en mededingende opname van glukose asook die produksie van siderofore is bewys as sleutel meganismes betrokke in die inhibisie van *Aer. hydrophila* deur die *B. cereus* isolaat.

Aangesien koste 'n belangrike oorweging is in die ontwikkeling van kommersiële toepaslike biologiese produkte, is die optimisering van voedingstof aanvullings wat 'n impak het op hoë-digtheid produksie van spore deur fermentasie ondersoek. Week-vloeistof van mielie prosessering (CSL) is identifiseer as 'n lae koste en effektiewe voedingsbron in vergelyking met konvensionele voeding substrate, veral gisekstrak en voedingsboeljon. Die verbeterde sporulering prestasie van *B. cereus* kon toegeskryf word aan die verhoogde beskikbaarheid van vrye

aminosure, koolhidrate en minerale in CSL, wat 'n postitewe effek op organisme groei en sporulerings effektiwiteit getoon het. Die impak van voedingstof konsentrasie op spoor opbrengs en produktiwiteit is gemodelleer om 'n werktuig vir die selektering van optimale kondisies te ontwikkel. Uitstekende korrelasie met werklike laboratorium data is gedemonstreer. Koste analises het getoon dat produksie deur middel van vloeibare fitase-behandelde en ultra-filtreerde CSL goedkoper is as sproei-gedroogde CSL en ondersteun verder die kultivering van *B. cereus* spore teen digthede hoër as 1 x 10^{10} kolonie vormende eenhede.ml^{-1}.

Die opname van biologiese middels in kommersiële toepassings skiet tekort as gevolg van beperkinge in proses en produk ontwikkeling wat belangrike eind-gebruiker vereistes soos koste, doeltreffendheid, rak leeftyd en gerieflikheid aanspreek. Die ontwikkeling van toepaslike prosesse vir spoor herwinning, droging, formulering en tablet produksie is gevolglik uitgevoer. Belangrike maatstawwe wat gebruik is vir stroomaf proseseenheid-ontwikkeling het lewensvatbaarheid, herwinning, spoor balans sluiting, spoor her-ontkieming, intermediêre produk stabiliteit, eindproduk stabiliteit en doeltreffendheid ingesluit. 'n Proses vloeidiagram bestaande uit vertikale buis sentrifugasie, vloeibare bed agglomerasie en tablet persing het 'n aantreklike produk voortgebring. Die formulering het ook CSL en glukose ingesluit om gevolglike spoor her-ontkieming te verbeter. Lewensvatbare spoor herwinning en spoor balans sluiting oor elke proses eenheid was hoog (>70% en 99% respektiewelik) met verbetering in herwinning wat moontlik gemaak is deur die gebruik van aaneenlopende prosessering op groot skaal. Spoor her-ontkieming was 97%, terwyl produk halfleeftyd langer as 5 jaar beraam is, gebasseer op termiese weerstand grafieke. Die proses het gelei tot 'n kommersiële aantreklike produk asook bekostigbare veranderbare produksie koste.

Die funksionaliteit van die tablet-produk met die ingeslote *B. cereus* isolaat is ondersoek oor 'n reeks fisiologiese kondisies insluitend soutgehalte, pH en temperatuur, gebasseer op die kultivering van *C. carpio*. Temperatuur het 'n betreklike invloed op ontkieming, spesifieke groeitempo en toename in sel hoeveelheid van *B. cereus* gehad, terwyl soutgehalte en pH nie enige meetbare effek op groei gehad het nie. Gekontrolleerde studies in bioreaktors en modellering van die data op die Arrhenius funksie het hoë en lae groei temperatuur domeins gewys. Die tempo van patogeniese *Aer. hydrophila* onderdrukking en afname in konsentrasies van afval-ione (ammonium, nitriete, nitrate en fosfaat) is herlei na 'n linière voorspellende aanwysing van effektiwiteit van *B. cereus* isolate by verskillende temperature.

Die studie het gelei tot die ontwikkeling van stroomop- en stroomaf-prosesse vir die produksie van 'n nuwe *B. cereus* isolaat (NRRL 100132) wat bewys is as veilig, stabiel, funksioneel, robuust en koste effektiewe vir toepassing in akwakultuur.

This Thesis Is Dedicated

to

Dr Winston Leukes

My dear friend and co-supervisor of this study, who unfortunately was called to rest, before completion of his work. May your wisdom, shine brightly on all emerging scientists the world over, from the heavens above.

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Chapter 1 Introduction

1.1 Introduction

Global shortages in seafood resources have driven growth of aquaculture as an economic activity, predominantly in developing countries (Kesarcodi-Watson et al. 2008; Chinabut et al. 2006). Because of space and resource constraints traditional aquaculture has been intensified into reticulated systems with high stocking densities of the cultured species (Robertson et al. 1999; Balcazar et al. 2006). These systems result in an artificial environment, which has a propensity for supporting the growth of pathogenic bacteria and the accumulation of waste metabolites in the environment (Moriarity 1999).

The rearing of ornamental species has proven to be a lucrative market and this study therefore used ornamental *Cyprinus carpio* (carp) as a model species due to the niche market possibilities for low volume, high value product entry. The attractiveness of this species as a useful representative of the many challenges faced in aquaculture, coupled to the potential extension of the envisaged technology for use in other emerging aquaculture initiatives further substantiated this choice.

Disease outbreak caused by bacterial pathogens is a complex phenomenon associated with stressful environmental conditions such as poor water quality coupled to the prevalence of disease causing agents which ultimately can result in mass mortality and significant loss to the industry (Jeney and Jeney 1995; Irie et al. 2005). Selection for desirable characteristics by breeders of ornamental species have also reduced the vigour in breeding lines, making fish less hardy and more susceptible to disease (Soevenyi et al. 1988). Of particular importance is the prevalence of bacterial ulcer disease, which results in damage to the appearance of the specimen and often leads to death (Fijan 1972). *Aeromonas hydrophila* is one of the main pathogenic micro-organisms responsible for bacterial ulcer disease of carp (Jeney and Jeney 1995; Austin and Austin 1999). The main source of waste accumulation is through hypernutrification as a result of excessive feeding rates (Liao and Mayo 1974; Boyd 1985) and high nutrient dietary composition (Shimeno et al. 1997), both of which are common phenomena in intensive

ornamental carp culture systems. High levels of nitrogenous and phosphorous waste accumulation predispose fish to infestation by parasites and pathogens and pose a threat to the environment (Liao and Mayo 1974; Jeney et al. 1992; Jana and Jana 2003).

Conventional methods of dealing with disease include chemical and antibiotic treatments. There are several disadvantages to these forms of treatment, such as consumer resistance to the use of chemicals, and the negative impact of chemical usage on the environment and biological filtration systems (Barker 2000). Furthermore, the use of chemotherapeutics as treatments and prophylactics has resulted in increased resistance and virulence of pathogenic organisms such as *Aeromonas* spp. (Moriarity 1999; Skjermo and Vadstein 1999; Jana and Jana 2003). The global extent of these challenges present significant opportunity for development of alternate solutions for disease control and enhancement of water quality (Shotts et al. 1980).

Micro-organisms have major roles in pond culture, particularly with respect to productivity, nutrient cycling, nutrition of the cultured animals, water quality, disease control and environmental impact of effluent (Moriarity 1997; Sanders 2003; Hong 2005). Bacterial additives demonstrate potential to improve water quality, reduce pathogen load and reduce mortality and have thus emerged in modern day aquaculture as alternatives to chemicals and antibiotics (Jana and Jana 2003, Hong et al. 2005). Many bacterial strains have a significant algaecidal effect (Rico-Mora et al. 1998), thus bacterial reduction of algal growth would be advantageous when undesired algal blooms develop or in the case of ornamental fish culture where water clarity is an aesthetic requirement (Verschuere et al. 2000).

Biological agents such as gram positive *Bacillus* spp. offer an attractive solution to the challenges facing modern aquaculture. Advantages of this genus include the ability to grow rapidly, tolerate a wide range of physiological conditions and the ability to sporulate. Some *Bacillus* spp. have been evaluated as biological treatment agents in several studies, with uses including the improvement of water quality as well as the reduction of pathogens (Queiroz and Boyd 1998; Gatesoupe 1999; Verschuere et al. 2000; Irianto and Austin 2002; Hong et al. 2005). Furthermore, *Bacillus* spp. are found in marine sediments, are naturally ingested by animals and have an advantage in that they are unlikely to acquire genes for antibiotic resistance or virulence from gram negative pathogens such as *Aeromonas* spp. (Moriarity 1999). The robustness of the spores of *Bacillus* spp. are also amenable to simple and cost effective production processes and

the end product is stable for long periods (Hong et al. 2005). Because of the many advantages for application of *Bacillus* spp. in aquaculture, this project focused exclusively on isolation and exploitation of biological agents from this genus.

As disease is the outcome of a delicate interaction between the host, the disease-causing agent, such as a pathogen, and external conditions such as water quality, it was imperative to examine pathogen inhibition in concert with enhanced water quality when developing biological agents of value to aquaculture. This project therefore focused on providing solutions to the many challenges faced in global aquaculture, by rational application of the current state of the art in biotechnology and bioprocess engineering. Product development begins with selection of the appropriate biological starting material which must be shown to be safe and present opportunities for commercialization based on a group of strategically important desirable characteristics. This is followed by an understanding of the biological functionality prior to development of bioprocess technology that ensures competitive production and processing to realise a functional end product (Schisler et al. 2004). The suitability of the end product in a form that commands customer acceptance and the tolerance of the product to storage and environmental conditions during application must be demonstrated.

The scope of this project therefore spanned isolation and selection of putative biological agents in accordance with pre-defined criteria that could potentially address the challenges faced in aquaculture, coupled to identification and bio-safety assessment of promising isolates. The key effects, including pathogen inhibition and a decrease in the concentration of waste metabolites such as ammonium, nitrite, nitrate and phosphate, were verified during in vitro co-culture studies and using in vivo model systems containing *C. carpio*. The mode of action of the best isolate was also elucidated, as understanding functionality was an important requirement for technology adoption. Upstream and downstream process unit operations were developed to produce this biological agent by maximising key production based indicators such as productivity, yield and recovery, while minimising cost of production. The main focus of the fermentation process development was on protein source optimization to enhance spore production. The downstream process integrated simple unit operations to enhance product recovery and minimise cost of production while realising a product form with demonstrable stability. The robustness, tolerance and functionality of the biological agent across the range of key physiological ranges encountered in the rearing of *C. carpio* further showed the potential of the isolate for application in

aquaculture of this species. The knowledge developed during this project has enhanced competitive production of a new biological agent. The applications knowledge has further enhanced adoption of such technology by the aquaculture industry, thus providing modern day solutions to the many challenges faced by this important emerging industry.

Chapter 2 Literature Review

2.1 Aquaculture as an economic activity

Aquaculture is a growing industry associated with a concomitant reduction in growth in the capture fisheries industry. The global aquaculture market is estimated at 39 million tons with a market value of ~$50 billion. The market has experienced an annual growth rate of ~13% since 1998 (FAO 1999). The key impetus for growth of the market is global food security and a wider resistance towards resource exploitation through over-harvesting of natural waters. Aquaculture production is dominated by developing countries, predominantly in Asia. The methods of practice of aquaculture have evolved into intensive reticulated systems in contrast to traditional extensive systems due to restrictions in availability of land as well as increased environmental awareness against pollution.

The global aquaculture market comprises two major segments, which are the ornamental and edible segments respectively. Of the freshwater market, ornamental aquaculture is valued at ~$200 million and edible aquaculture the major portion of the balance. Each of these segments is driven by unique market dynamics. The food market is a mass market with high volumes and a relatively lower return per unit mass. Environmental impact is a key issue for these large-scale operations. The consumer drives the aquaculture practice and product quality and branding.
The product must address consumer food concerns and must at least be as desirable as naturally harvested products. The ornamental market shares some of the dynamics of the mass aquaculture market. The value per unit fish mass is however orders of magnitude higher. Because of the high desirability for near perfect specimens, culling proportions are high, resulting in a very strong impetus to “grow-on” and minimise mortality of un-culled specimens.

2.1.1 Dynamics of product offerings to the aquaculture industry

Some of the main product inputs into the aquaculture industry include feed and treatments to improve water quality and treat diseases. Each of the two segments of the market has common niche requirements in terms of product effect, which are improvement of water quality, nutrition and disease prevention. Although the requirement for the net effect of these niches is similar in

the two segments, the product form, application technology, efficacy, stability and packaging requirements are unique. The edible aquaculture market segment requires affordable products for extensive use. Packaging and branding must target mass success, with overall improvement in yield of the aquaculture operation, ultimately meeting consumer approval to food consumption standards. The ornamental sector of the aquaculture market is focused at lower volume niche products that command higher value. Another unique characteristic of this market is that hobbyists dominate about 80% of market demand and spend almost two times the value of the specimen on associated products (nutrition and health). The hobbyists market demands species-specific products with reputable technical information and branding. Products into these markets command high price premiums.

2.1.2 A model species for process and product development

Ornamental carp (koi), *Cyprinus carpio*, are widely reared by aqua culturists for supply to the hobbyist market. There is a preference for intensive reticulated culture systems (Liao and Mayo, 1974), whereby high growth rate and high stocking density are major requirements. This species is thus prone to water quality and disease challenges. As the specimen value of ornamental carp is substantially higher than edible carp, the health and survival of ornamental carp is an exceedingly important requirement for both hobbyists and culturists. Bacterial ulcer disease typically causes lesions on the fish, which render high value specimens valueless, causing significant loss to breeders and hobbyists. This is thus an excellent model species for the development of a biological agent as it affords the opportunity for high value, low volume niche market product entry. As the end user requirements are extremely stringent, satisfying these requirements creates a unique opportunity for novel technology interventions in process and product development, with attractive financial reward for the small and medium emerging knowledge intensive industry in South Africa.

Figure 1 Intensive aquaculture of ornamental carp (insert showing high stocking densities)

2.2 Current challenges of the aquaculture industry

Key challenges to the development and growth of aquaculture as an economic activity are limited water resources and the environmental impact of aqua-farming methods. To address these challenges water is re-cycled and farming activities are intensified, resulting in an increase in stocking density, deterioration in water quality, increased incidence of disease, poor feed to body mass conversion efficiencies and higher mortality rates. The net result is reduced yield. Annual loss to the market is estimated at 40% due to disease, water quality and nutrition.

2.2.1 Disease in aquaculture

Definitions of disease include an unhealthy condition and infection with a pathogen. Disease is a complex phenomenon, leading to some form of measurable damage to the host (Austin and Austin, 1999) Outbreaks of disease either begin suddenly, progress rapidly often with high mortalities, and disappear with equal rapidity (acute disease) or develop more slowly with less severity, but persist for greater periods (chronic disease).

Fish disease is the outcome of a delicate interaction between the host, the disease-causing agent, such as a pathogen, and external conditions such as unsuitable changes in the environment, poor hygiene and overcrowding. Before the occurrence of clinical signs of disease, there may be demonstrable damage to, or weakening of, the host. Disease outbreak is generally associated with

a primary invasion, such as by parasites or mechanical injury, coupled to stressful environmental conditions such as changing temperature and poor water quality (Jeney and Jeney, 1995). The prevalence of infectious agents can result in mass mortality causing significant losses to aquaculture operations (Irie et al. 2005).

Fish diseases such as rotting fins, ulcerations of the skin and mortality rate are more prevalent when fluctuation in temperature causes immune modulation, which results in inferior disease resistance (Le Morvan et al. 1996; Engelsma et al. 2003). The initial weakening process may involve an array of stress factors such as water quality, parasite load or a natural physiological state (e.g. during the reproductive phase) in the life cycle of the fish (Austin and Austin 1999). Disease is not necessarily caused by action of a single bacterial taxon, as representatives of many bacterial taxa have at one time or another been associated with fish diseases. However, not all of these bacteria constitute primary pathogens, as many can be categorized as opportunistic pathogens, which colonize and cause disease in already damaged hosts.

Aer. hydrophila and *Pseudomonas* spp. are predominantly involved in causing fish diseases (Chalmers et al. 2003). Many of these bacterial pathogens are members of the normal microflora of water and/or fish. Other pathogens have been associated only with clinical diseases or covertly infected (asymptomatic) fish.

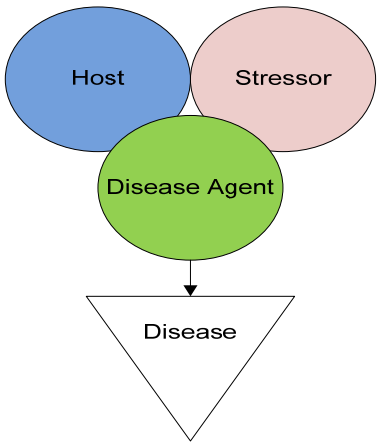

Figure 2 Interactive factors influencing disease in aquaculture (modified from Austin 1999)

2.2.2 Diseases prevalent in ornamental Cyprinus carpio

The rearing of ornamental carp in a reticulated system results in an artificial environment which has a propensity for the accumulation of waste metabolites and promotes growth of pathogenic bacteria. Environmental factors play a key role in the onset of disease which is reported as being a consequence of the interaction between the host, environmental stress and prevalence of disease causing agents (Paperna 1991; Jeney and Jeney 1995; Austin and Austin 1999). Strict selection for desirable characteristics by breeders in this ornamental species has also reduced the vigour in breeding lines, making fish less hardy and more susceptible to disease (Barwick 2003).

The major causative agents of disease in *C. carpio* are bacteria of the Genus *Aeromonas* (Jeney and Jeney 1995). Of particular importance in ornamental carp is the prevalence of bacterial ulcer disease, which results in damage to the appearance of the specimen and in severe cases, mortality. The disease is prevalent in spring and associated with environmental change to warmer temperatures. This period is characterised by an increase in activity of pathogenic bacteria and parasites. Fluctuation in temperature causes transient immunomodulation, which can result in inferior disease resistance (Le Morvan 1996; Engelsma 2003).

Figure 3 Bacterial ulcer disease manifested on ornamental *C. carpio*

In general ulcer disease of cyprinids has occurred in widely separated geographical locations across the globe (Shotts et al. 1980). The disease is a sub-acute to chronic contagious skin disease (Bootsma et al. 1977). Further symptoms of the disease include lethargy, loss of appetite and orientation, including abnormal swimming behaviour. Haemorrhagic septicaemia occurs with

infections caused by a wide range of pathogens (Austin and Austin 1999) resulting in open ulcerated lesions and haemorrhages (Sakai et al. 1989; Lansdell et al. 1993). In addition, fin and tail rot and the loss of scales may be seen, including localized haemorrhages particularly in the gills and vent, exophthalmia and abdominal distension (Austin and Austin 1999). Furunculosis is recognised by the presence of lesions resembling boils or furuncles, in the musculature. The acute form of these diseases is of sudden onset, and the fish usually die within 2-3 days (McCarthy 1975; Bejerano et al. 1979; Morrison et al. 1981). The infection often starts at the site of injury to the epidermis. A hemorrhagic inflammatory process then develops between the epidermis and the dermis. This red inflammatory zone gradually extends as the infection spreads. The breakdown of tissue leads to the formation of a central ulcer, which may occur in any location on the body surface, although it is most frequently located on the flanks. Secondary invasion of the ulcer by fungi or other bacteria is common. Unlike furunculosis, which usually occurs at water temperature above 16˚C, carp erythrodermatitis may occur at all water temperatures (Austin and Austin 1999). The disease normally results in mortality, but if the fish recover, contraction of scar tissue collagen can result in serious deformity, reducing the commercial value of the fish and causing major losses to the industry (Fijan 1972).

Table 1 Predominant bacterial pathogens causing disease of *Cyprinus carpio* (modified from Austin and Austin 1999)

Pathogen	**Disease**
Aeromonas hydrophila	Haemorrhagic septicaemia, motile *Aeromonas* septicaemia, redsore disease, fin rot
Aeromonas salmonicida	Furunculosis, carp erythrodermatitis, ulcer disease
Pseudomonas fluorescens	Generalized septicaemia
Pseudomonas pseudoalcaligenes	Skin ulceration

The main pathogenic micro-organisms involved in bacterial ulcer disease of carp are *Aer. hydrophila, Aer. salmonicida*, and to a lesser extent *P. flourescens* (Jeney and Jeney 1995; Austin and Austin 1999). The presence of *Aer. hydrophila* is associated with haemorrhagic septicaemia in carp by production of haemolysin, cytotoxins and enterotoxins which cause tissue necrosis resulting in ulcers, dropsy and abdominal oedema (Jeney and Jeney 1995). *Aer. salmonicida* was specifically associated with ulcerative erythrodermatitis and furunculosis of ornamental carp (Jeney and Jeney 1995; Austin and Austin 1999; Matoyama 1999). *P. flourescens* which is ubiquitous in fresh water and is generally regarded as a secondary invader of damaged tissue, has

been associated with outbreaks in carp exacerbating the damage caused by ulcer disease (Shewan 1960; Otte 1963; Csaba 1981; Allen 1983). There is therefore merit in reducing the prevalence of bacteria such as *Aer. hydrophila*, *Aer. salmonicida* and *P. flourescens* in water systems used to rear ornamental carp.

2.2.3 Water quality

Reticulated systems for intensive culture result in substantial amounts of particulate organic and soluble inorganic excretory waste, due to increased stocking density (Jana and Jana 2003). The main source of this metabolic waste is through hyper-nutrification as a result of excessive feeding rates (Liao and Mayo 1974; Boyd 1985; Farzanfar 2006) and high nutrient dietary composition (Shimeno 1997), both of which are a common phenomena in ornamental carp systems and have a significant influence on the survival, growth and reproduction of fish (Jana and Jana 2003). Nitrogen and phosphorous waste accumulation in ornamental carp systems pose a threat to the environment and can predispose fish to infestation by parasites and pathogens due to a reduction in immunity (Liao and Mayo 1974; Jana and Jana 2003). Water quality is sub-optimal due to attenuation of water bioremediation activity of beneficial microbes in the spring season, which further exacerbates disease propensity during this time.

Ammonia is a primary metabolic waste of fish (Ng 1992) and is excreted through the gills through branchial diffusion (Grommen 2002), produced by bacterial ammoniafication from uneaten food and faeces (Grommen 2002; Gross 2003) and released from the mineralization of sediment (Jimenez-Montoealegre 2005). Ammonia is oxidised to nitrite and finally to nitrate through the process of nitrification, with ammonia and nitrite being the most toxic to fish. Nitrite can also be produced through the process of denitrification (Grommen 2002).

Ammonia concentrations above 0.3 $mg.l^{-1}$, have been reported to be toxic to fish (Larmoyeux and Piper 1973; Boyd and Tucker 1998), with hyperplasia of gill tissue, gill necrosis due to pathogenesis, growth rate reduction and pathological evidence of kidney and liver damage occurring with an increase in concentration (Jeney et al. 1992). The net result of high exposure to ammonia is epithelial lifting of gill filaments, resulting in respiratory impairment and mortality (Frances 2000).

Nitrite is usually present at low concentrations in natural systems, except when there is an imbalance, because it is a common intermediate in both nitrification and denitrification, catabolic ammoniafication and nitrate assimilation (Sakai 1997). Through denitrification, nitrite can be produced as an intermediate in the conversion of nitrate to nitric oxide, nitrous oxide and nitrogen gas (Ferguson 1994). Nitrite is considered harmful to fish at levels of 0.15 $mg.l^{-1}$ through conversion of haemoglobin to methaemoglobin in blood, resulting in inhibition of oxygen transport and mortality due to brown blood disease (Liao and Mayo 1974). Increased concentrations of nitrite also significantly affected weight gain, specific growth rate and food conversion efficiency (Frances 1998).

Dietary phosphorous is an essential component of high nutrient feeds as it improves weight gain and feed conversion ratio, but is however poorly utilized in carp due to the absence of an acidic stomach and because phosphate is often bound to phytic acid in vegetable protein (Kim et al. 1998). Ingested phosphorous is therefore lost in faeces and results in poor water quality with increased algal growth and eutrophication (Auer 1986, Kaushik 1995). Furthermore, in many ornamental fish systems, the excessive presence of algae is aesthetically displeasing and also results in blockages to reticulated filtration systems.

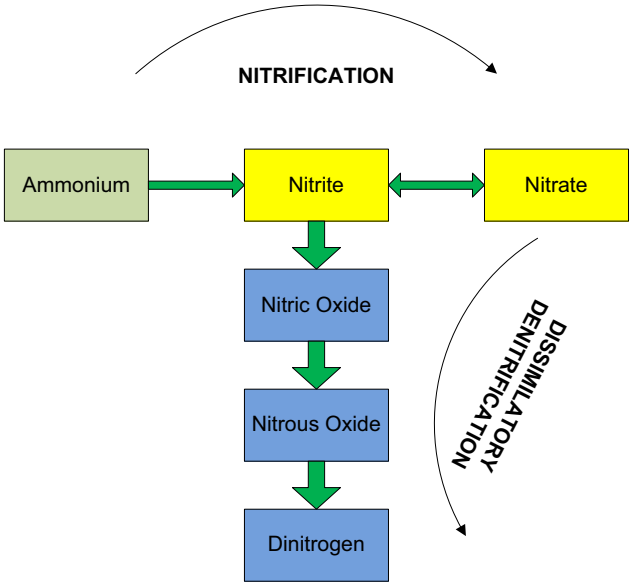

Figure 4 Nitrification and denitrification cycle

2.3 Conventional approaches to aquaculture challenges

The rearing of ornamental carp in a reticulated system results in a highly artificial environment which has a propensity for the accumulation of waste metabolites and promotes growth of pathogenic bacteria. Management considerations during aquaculture are nutrition, water quality, physical parameters and pathogen and disease control (Moriarity 1998). Chemicals used in aquaculture include a wide range of topical disinfectants, organophosphates, antimicrobials and parasiticides to deal with disease and water quality (de Kinkelin 1992; Vershuere et al. 2000). Water quality is traditionally managed through conventional reticulated filtration systems, which are sensitive to process fluctuations and can result in mass mortality when such systems crash.

2.3.1 Use of chemicals in aquaculture

Antimicrobial agents are extensively used as treatments during a disease outbreak or at prophylactic doses. This leads to increased virulence and antibiotic resistance of pathogenic organisms which then require high doses of existing drugs or new drugs to control disease (Moriarity 1999; Skjermo and Vadstein 1999; Jana and Jana 2003). The presence of higher drug concentrations, and an ever increasing spectrum of chemical residues, can result in detrimental effects to consumers and the environment (Barker 2000). These chemicals also have a negative impact on the aquaculture filtration systems themselves, resulting in a deterioration in water quality. Chemicals are often recalcitrant, persisting for several days to months, and can result in alterations to naturally occurring bacterial populations (Sze 2000; Jana and Jana 2003). The resistance to usage of chemicals is substantiated by the ever increasing list of banned substances reducing treatment options for aquaculture (Hong 2005).

2.3.2 Conventional bio-filtration

Normally the oxidation of ammonia to the more benign nitrate ion is through ammonia and nitrite oxidising obligate chemoautotrophs such as *Nitrosomonas* and *Nitrobacter* spp. which are slow growing and sensitive to fluctuations in environmental conditions (Focht and Verstraete 1977; Sakai, 1997). Removal of nitrate still remains a challenge. In intensive operations this requires a higher capital investment for larger scale filtration, but system fluctuations because of the

sensitivity of natural filter bacteria often lead to accumulation of ammonium, nitrite, nitrate and phosphate causing toxicity to fish. Although these residues can be reduced by the addition of fresh water, the effluent purges containing high levels of these compounds into natural river and sea waters also results in a deterioration of the environment and can lead to algal blooms, which may be detrimental to natural ecosystems (Kaushik 1995).

2.4 Biological solutions as alternatives to aquaculture challenges

Given the challenges in conventional aquaculture practise, alternative methods for disease control and enhancement of water quality are desperately required. Micro-organisms have major roles in pond culture, particularly with respect to productivity, nutrient cycling, nutrition of the cultured animals, water quality, disease control and environmental impact of effluent (Moriarty 1997). Beneficial microbes can be used to alter or regulate the composition of bacterial flora in a water system and thus reduce the load of pathogens (Skjermo and Vadstein 1999; Jana and Jana 2003). Microbial based biological additives also benefit aquaculture by improving water quality, through accelerated mineralization and nitrification. This results in reduction in algal growth and acceleration in sediment decomposition (Fast and Menasveta 2000; Gomez-Gil et al. 2000). The marketing of biological and "organic certified" solutions for enhancement of fish health has also gained consumer acceptance. These agents also confer an added advantage of natural integration into existing ecosystems and present opportunities for multi-effect product development which are attractive to end users. The use of such beneficial microbes is a more appropriate remedy than the use of chemicals (Moriarty 1999). The success of this strategy however depends on an understanding of the ecological processes and the agents responsible for disease coupled to a knowledge of the beneficial characteristics of bacteria to be used as biological agents (Moriarty 1999).

2.4.1 Biological agents

Microbial webs are an integral part of aquaculture and have a direct impact on productivity especially in intensive culture operations. The quality of water and health of the cultured species

is governed by activities of microbes which have a great diversity with different roles and interactions in the ecosystem (Moriarty 1998).

A probiotic can be defined as a cultured product or live microbial feed supplement, which beneficially affects the host by improving its intestinal balance (Fuller 1987). The important components of this definition reflect the need for a living microorganism and application to the host as a feed supplement. A broader definition is that of a live microbial supplement, which beneficially affects the host animal by improving its microbial balance (Gram et al. 1999). In a third proposed definition, a biological agent is any microbial preparation or the components of microbial cells with a beneficial effect on the health of the host (Salminen et al. 1999). It is thus apparent that there are variations in the actual understanding of the terminology (Irianto and Austin 2002). Based on the observation that organisms are capable of temporarily modifying the bacterial composition of water and sediment, it was suggested that the definition should include the addition of live naturally occurring bacteria to tanks and ponds (Maeda et al. 1997). Verschuere et al. (2000) presented a wider and useful description, given the broad spectrum effects of microbial consortia used in aquaculture. He described a biological agent as a live microbial adjunct, which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment.

There are distinct uses of bacterial supplements in aquaculture as probiotics, bio-augmentation, biocontrol and bioremediation agents (Gatesoupe 1999). Probiotics are normally associated with feed and digestion. Bio-augmentation refers to the augmentation of the environment and or the microbes to result in enhanced fish health. In some instances, “biocontrol” also transcends the boundary between bio-augmentation, and the exclusion of pathogens, but a stricter definition is that the strains are antagonistic to pathogens (Maeda et al. 1997). Bioremediation refers to the breakdown of pollutants or waste by microbes (Moriarty 1998; Moriarty 1999).

The range of biological treatments examined for use in aquaculture has encompassed both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, unicellular algae, enzyme preparations and plant extracts. Microbes have been used successfully in artificial feed, live feed,

in bio-filtration and in water (Irianto and Austin, 2002). Most biological treatments used in aquaculture belong to the genera *Lactobacillus*, *Vibrio*, *Bacillus*, or *Pseudomonas*, although other genera have been applied to a lesser extent (Verschuere et al. 2000).

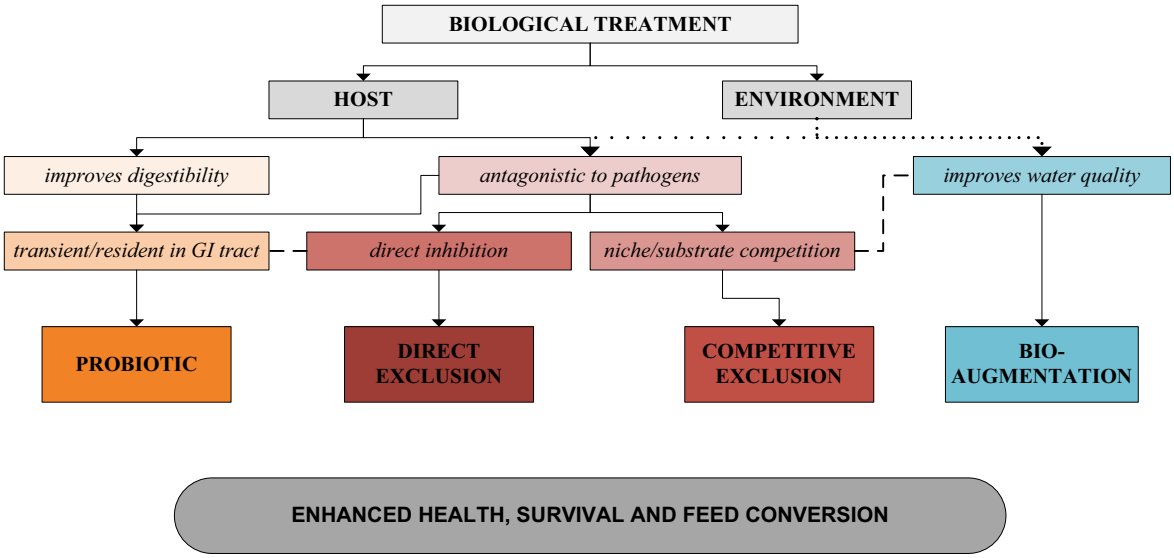

Figure 5 Schematic representation of the effects of biological agents in addressing aquaculture challenges

2.4.2 Modes of action of biological agents

Mechanisms of probiosis include competition for adhesion sites, immune stimulation, synthesis of antimicrobials, competitive exclusion, bioaugmentation and bioremediation (Vanbelle 1990; Verschuere et al. 2000; Sanders 2003; Hong 2005). Although many biological treatments have emerged over the last decade, the approach has been empirical and the exact modes of action were rarely elucidated, negatively affecting technology adoption and implementation in aquaculture (Verschuere et al. 2000).

One possible mechanism for preventing colonization by pathogens is competition for adhesion sites on gut or other tissue surfaces (Vanbelle 1990). It is known that the ability to adhere to enteric mucus and cell wall surfaces is necessary for bacteria to become established in fish intestines (Westerdahl et al. 1991; Olsson et al. 1992). Since bacterial adhesion is important during the initial stage of pathogenic infection (Krovacek et al. 1987), competition of adhesion

receptors with pathogens might be the first probiotic effect (Montes and Pugh 1993). Adhesion capacity and growth on intestinal or skin mucus has been demonstrated in vitro for fish pathogens (Krovacek et al. 1987; Garcia et al. 1997).

Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistant to infections (Raa 1996). Fish larvae, shrimps, and other invertebrates have immune systems that are less well developed than adult fish and are dependent primarily on non-specific immune responses for their resistance to infection (Söderhall and Cerenius 1998). Bacterial compounds may act as immune-stimulants in fish and shrimp (Sakai 1996), but it is not clear whether bacteria have a confirmed beneficial effect on the immune response of cultured aquatic species (Verschuere et al. 2000).

Microbial populations may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations, which can alter inter-population relationships. The presence of bacteria producing inhibitory substances is thought to constitute a barrier against the proliferation of opportunistic pathogens. In general, the antibacterial effect of bacteria is due to the production of antibiotics, bacteriocins, siderophores, enzymes, hydrogen peroxide or alteration of pH by the production of organic acids, ammonia or diacetyl (Verschuere et al. 2000). Many authors assign the inhibitory effects detected in in vitro antagonism tests to bacteriocins or antibiotics without looking for any other causes. It has been argued that observed growth inhibition, could in many cases, be accounted for by primary metabolites or simply by a decrease in pH (Verschuere et al. 2000). At this stage, however, the association between amensalistic activity and in vivo probiotic activity is very weak and circumstantial. Typically, a correlation is made between the in vitro ability of probiotics to inhibit pathogens and the in vivo protection of the cultured aquatic species, but in none of the studies reviewed by Verschuere et al. (2000), has it been shown unequivocally that the production of inhibitory compounds is the cause of the in vivo probiotic activity of the strains. Hence, future research in this field is required.

Theoretically, competition for chemicals or available energy may determine how different microbial populations coexist in the same ecosystem, but to date there have been no comprehensive studies on this subject (Ringø and Gatesoupe 1998). Competitive exclusion is an ecological process that allows manipulation of the composition of bacterial species in water, sediment or the host itself by competitive assimilation of nutrients and /or an intrinsically higher

growth rate (Moriarity 1999; Sanders 2003; Hong et al. 2005). The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs competing for organic substrates as both carbon and energy sources, thus competitive utilization of these substrates can attenuate target microorganisms. A bacterial strain selected for its active growth in organic-poor substrates, prevented the establishment of a *V. alginolyticus* infection in vivo. Since the inoculated strain had no in vitro inhibitory effect on the pathogen it was thought to be a consequence of competitive exclusion (Rico-Mora et al. 1999). In another example, in vitro antagonism tests did not show production of extracellular inhibitory compounds, yet living cells were required to protect Artemia against pathogenic *V. alginolyticus*. It was suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy (Verschuere et al. 2000).

Virtually all microorganisms require iron for growth. Siderophores are low molecular weight (< 1,500), ferric ion-specific chelating agents (Schwyn and Neilands 1987) that can dissolve precipitated iron thus making it available for microbial growth. The ecological significance of siderophores resides in their capacity to scavenge an essential nutrient from the environment and deprive competitors from it. The requirement for iron is high for many pathogens in highly iron limited environments (Wooldridge and Williams 1993). In a challenge test with pathogenic *V. anguillarum*, salmon mortality increased linearly with dietary iron content (Gatesoupe et al. 1997). Siderophore-producing *P. fluorescens* AH2 was inhibitory to several gram-positive and gram-negative bacteria, particularly when iron availability was limited (Gram et al. 1999). In vitro co-culture tests revealed that the growth of *V. anguillarum* was inhibited by the filter-sterilized supernatants from iron-limited cultures of *P. fluorescens* AH2 but not from iron-replete cultures. The mortality of rainbow trout juveniles due to *V. anguillarum* infection was decreased by 46% when the culture was treated by *P. fluorescens* AH2 in vivo. Harmless bacteria which can produce siderophores could be used as probiotics to compete with pathogens whose pathogenicity is known to be due to siderophore production and competition for iron, or to out-compete organisms requiring ferric iron from solution (Verschuere et al. 2000). Similar to the production of inhibitory compounds, the evidence for the participation of competition for chemicals or available energy and, more specifically, of free iron or siderophores in the mode of action of probiotics is still circumstantial (Verschuere et al. 2000).

Water quality improvement has been recorded in studies during the addition of biological agents. Gram-positive bacteria are generally more efficient in converting organic matter to CO_2 than are gram-negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime. By maintaining higher levels of these gram-positive bacteria in the production pond, farmers can minimize the build-up of dissolved and particulate organic carbon during the culture cycle while promoting more stable phytoplankton blooms through the increased production of CO_2 (Verschuere et al. 2000). Nitrite accumulation may be caused by imbalanced activities of nitrate and nitrite reductase and inhibition of nitrite reductase by oxygen. Bio-communities however, usually contain bacteria with different nitrate and nitrite reductase activities, enhancing the denitrification efficiency of the overall bio-community (Matiensen 1999). Although the specific nitrification activity of heterotrophic bacteria is generally lower than that of chemoautotrophs, the overall impact on denitrification could be greater due to the higher cell numbers of heterotrophic bacteria and their robustness to process fluctuations. There is therefore merit in utilizing biological agents for nitrification and phosphate bioremediation to improve water quality in aquaculture (Sakai 1996; Verschuere et al. 2000). The bioremediation effect could lower nitrate and phosphate concentrations in aquaculture systems, thus reducing key nutrients required for algal growth. Many bacterial strains have been shown to have a significant algaecidal effect on various species of micro algae (Munro et al. 1995; Fukami et al. 1998; Verschuere et al. 2000). This effect is valuable in ornamental fish rearing, where algal blooms may be aesthetically displeasing and changes in oxygen concentration due to algal cellular respiration are undesirable. When probiotic bacteria are selected to be used in a culture environment comprising algae, their possible interaction must be taken into account when the mode of action is investigated (Verschuere et al. 2000).

2.4.3 Bacillus spp. as attractive biological agents

The use of *Bacillus* species in aquaculture and research into this field is expanding widely, especially in countries with intensive farming of fish and shellfish (Sanders 2003; Hong et al. 2005). Bacilli have been used as components of biocontrol products and are often composed of mixtures of species, which have an antagonistic effect on pathogens (Hong et al. 2005). They are ubiquitous in sediments and are naturally ingested by animals (Moriarty 1999). An advantage to using *Bacillus* spp. is that they are unlikely to use genes for antibiotic resistance or virulence

from Gram negative organisms such as *Vibrio* and *Aeromonas* spp. (Moriarty 1999). Other key positive characteristics of this genus are the ability to replicate rapidly, tolerate a multitude of environmental conditions and impact a broad range of beneficial effects that can improve aquaculture productivity (Hong et al. 2005). Additionally the ability of *Bacilli* to sporulate allows for the downstream processing and formulation of shelf stable products. Several spore forming bacteria are sold worldwide as components of products for human and animal use such as *Bacillus coagulans*, *B. subtilis*, *B. clausii*, *B. cereus* and *B. toyoi* (Sanders 2003).

There has been some demonstration of *Bacillu*s based products in aquaculture. *Bacillus* strain IP5832 spores fed to turbot larvae resulted in a decrease in the *Vibrionaceae* population with significant improvement in weight gain and survivability of the larvae (Gatesoupe 1999). It was also shown that *Bacillus* improved food absorption by enhancing protease levels and resulted in improved growth. A decrease in the number of suspected pathogenic bacteria in the gut was also observed (Irianto and Austin 2002). The survival and net production of channel catfish was improved in a farm trial, but the mode of action was not specified (Queiroz and Boyd 1998). It was found that *Penaeus monodon* larvae fed *Bacillus* S11 fortified Artemia, had significantly shorter development times and fewer disease problems than did larvae reared without the *Bacillus* strain. Survival was also significantly improved when challenged by a pathogenic *V. harveyi* strain D331, in treated groups compared to untreated controls (Rengpipat et al. 1998). Moriarty (1998) concluded, based on his studies on several farms in Indonesia that the use of *Bacillus* in penaeid culture ponds, enhanced the production of shrimps by preventing mortality normally caused by luminescent *Vibrio* spp. A cost-benefit analysis of the use of *Bacillus* in aquaculture showed a clear benefit to the use of these biological agents.

Bacillus spp. also contributes to nitrogen removal in spite of the classical belief that this is predominated by autotrophic bacteria (Abou Seada and Ottow 1985; Robertson and Kuenen 1990; Sakai et al. 1997; Martiensen and Schöps 1999; Su et al. 2001; Kim et al. 2005; Lin et al. 2006). During the denitrification process, nitrate is reduced via nitrite and nitric oxide to nitrous oxide or nitrogen gas by facultative heterotrophic bacteria (Ferguson 1994). Some members of this group, such as *B. subtilis* and *B. cereus,* have the versatility to grow aerobically, facultatively and anaerobically under certain conditions allowing for switches in nitrogen metabolism that facilitate both nitrification and denitrification (Sakai 1996; Nakano 1998; Martiensen and Schöps 1999). Nitrite is usually present at low concentrations in natural systems, except when there is an

imbalance, because it is a common intermediate in both nitrification and denitrification, catabolic ammoniafication and nitrate assimilation (Sakai 1997). The pattern of nitrite metabolism by *B. subtilis* I-41 was demonstrated as exceptional among strains which showed switching of nitrite and nitrate metabolism (Sakai 1997). Nitrite oxidation might be common, rather than an exception, in heterotrophic bacteria such as *Bacillus* spp. (Sakai 1996). Culture of Bacilli may potentially exert these effects via a multitude of mechanisms such as bioaccumulation, bio-assimilation, nitrification and dissimilatory nitrate reduction. The improvement in bio-availability of bound phosphate, through solubilisation, is also thought to facilitate removal of phosphate and reduce the propensity of algal blooms (Illmer and Schinner 1995; Kaushik 1995).

Table 2 Summary of studies on *Bacillus* based biological treatments

Identity of probiotic	**Used on**	**Method of application**	**Reference**
Bacillus sp. S11	*Penaeus. monodon*	Premixed with feed	Rengpipat et al, 1998
Bacillus sp. 48	*Centropomus undecimalis*	Added to water	Kennedy et al,1998
Bacillus sp.	Penaeids	In water	Moriarty, 1998
B. megaterium, B. polymyxa, B. licheniformis, *B. subtilis*	Channel catfish	In water	Queiroz & Boyd, 1998
Mixed culture, mostly *Bacillus* spp.	*Brachionus plicatilis*	Mixed with water	Hirata et al, 1998

2.4.4 Advantages of spore formers as biological agents

Bacillus spp. has the ability to form endospores which are rigid structures that are capable of surviving under harsh conditions. Spores are considered metabolically inert, but can be used as biological agents due to the many advantages of this form, over vegetative cells. These include their higher resistance to external factors such as mechanical force, desiccation, solar radiation and high temperatures (Wolken 2003). Because of this resistance to environmental stress, spores are attractive for commercial application as they can endure harsher processing steps during

production and are resilient to fluctuations in processes where they are applied, thus ensuring better survival and effect than vegetative cells (Sanders 2003). Products containing spores can be stored in a stable form for long periods under challenging conditions normally prevalent on aquaculture farms (Hong et al. 2005; Ugoji et al. 2006). *Bacillus* spores are found in ponds, lakes and rivers and many aquatic species will naturally ingest these microbes. They generally exist in symbiotic relationships with their host (Hong et al. 2005). The ability to germinate selectively in response to external triggers is advantageous as biological agents applied in aquaculture, as they have the ability to recover the characteristics of a metabolically active cell in response to specific nutrients, when these effects are required (Wolken 2003; Moir 2006). Some bioconversion activities in spores are absent from, or are much reduced in, the vegetative cells of the same organism. These attributes make them well suited to various applications (Wolken 2003).

2.5 Isolation, Screening and Selection of candidate biological treatment agents

There is an elegant logic in isolating putative biological treatments from the host or the environment in which the treatment organisms are likely to exert a beneficial effect, but there is no unequivocal indication that these isolates perform better than isolates completely alien to the cultured species, or originating from a different habitat (Verschuere et al. 2000). A combination of methods and incubation conditions need to be used to achieve pure cultures of target organisms. To an extent, the range of media to be used is governed by personal choice and experience (Austin and Austin 1999). Many bacteria that are residents of soil and aquatic habitats low in nutrients have difficulty growing in rich media. Also, many potential contaminants cannot compete in dilute media, so the shortage of nutrients becomes a selective factor. In aquatic animals, the digestive tract and the surrounding water, is an important source of bacteria (Gatesoupe 1999). In order to appropriately select biological agents it is essential to understand the mechanisms of action and to define selection criteria for potential microbes. Classical isolation screening and selection rationale may include collection of background information, acquisition of isolates, purification of isolates and evaluation based on pre-determined criteria in vitro and in vivo (Gomez-Gil et al. 2000). Good pre-selection criteria could be the viability of the potential probiotic within the host and/or within its culture environment, adherence to host surfaces and the ability to prevent infection by pathogenic bacteria. Other selection criteria include biosafety considerations, methods of production and processing, the method of

administering the probiotic and the robustness of the biological agent to the location where the microorganisms are expected to be active.

2.5.1 Isolation of biological agents

When selecting desirable biological agents, enrichment techniques should be employed that make it possible to exploit the differential characteristics of target isolates in mixed microbial populations. *Bacillus* spp. are isolated almost ubiquitously from soil, water, mud, sediment, dust, air and the surfaces and organs of aquatic animals (Sanders 2003). Specifically they have been isolated from fish, crustaceans, bivalves and shrimps and have been found in the microflora of the gills, skin and intestinal tract (Gatesoupe 1999; Hong et al. 2005). One effective strategy being used in developing countries is the isolation of *Bacillus* spp. from commercial ponds and then using selected isolates as commercial products (Hong et al. 2005).

The Bacilli are classified under endospore forming Gram-positive rods and cocci and isolation procedures must selectively enrich for this group of organisms while excluding other genera in the same group. Methods used for isolating various *Bacillus* strains were based mainly on resistance of their endospores to elevated temperatures. Foldes, (2000) used a technique whereby cells were blended with a special enrichment medium, which also induced vegetative cells to sporulate followed by incubation to allow formation of mature spores in large quantities. The isolation involved heat treatment for the selection of spores of *Bacillus* species. Ethanol is a useful disinfectant and dehydration agent to use for isolation of strains of Bacilli as it kills vegetative cells in a sample whereas the more resistant endospores survive. Additionally, the resistance of Bacilli to the antibiotic polymyxin B will enable selection of this group of bacteria whilst eliminating most Gram-negative bacteria. Cells can be characterised by microscopic appearance, gram stain, the catalase and other tests (Foldes 2000).

2.5.2 In vitro screening and selection of aquaculture biological agents

The application of in vitro tests to screen isolated bacterial strains presupposes well-known modes of action to select appropriate tests. The in vitro production of inhibitory compounds toward known pathogens of a species of interest has often been used in the selection of putative

biological agents (Rengpipat et al. 1998; Verschuere et al. 2000). In aquaculture, bioremediation or bioaugmentation also forms part of the selection criteria and the selected isolate must function under appropriate environmental conditions (Moriarty 1999). The effects of ideal biological agents must thus span improved disease resistance, growth, water quality of the culture system and a general improvement in health (Gomez-Gil et al. 2000).

To appropriately select biological agents, it is essential to understand the mechanisms of action and to define selection criteria for potential probiotics (Huis in't Veld 1994). Many bacteria have been exploited as biological agents but the selection has been based mainly on empirical observations, rather than scientific data (Gomez-Gil et al. 2000). The use of the target organism in the screening procedures provides a stronger basis for selection of beneficial antagonists (Verschuere et al. 2000).

A common way to screen candidate biological agents is to perform in vitro antagonism tests, in which pathogens are exposed to antagonists in culture medium (Nogami and Maeda 1992; Olsson et al.1992; Sugita et al. 1996; Riquelme et al. 1997; Gibson et al. 1998; Gram et al. 1999). Assays for the production of inhibitory compounds and siderophores, or the competition for nutrients, are some common strategies that have been used (Dopazo et al. 1988; Olsson et al., 1992; Smith and Davey 1993; Jöborn et al. 1997; Gram et al. 1999). Results of in vitro antagonism tests should however be interpreted with caution, as growth media and conditions can influence the effects observed which may differ from the actual activity in vivo (Mayer-Harting et al. 1972; Olsson et al. 1992). The pre-selection of candidate biological agents based on in vitro antagonism tests has however led to the finding of many effective probionts and is a useful first step in selection (Gibson et al. 1998). Growth inhibition may not always be a consequence of the production of inhibitory substances, such as antibiotics, as inhibition caused by other mechanisms must also be considered during in vitro screening tests (Ten Brink et al. 1987; Bergh 1995). The important area of screening for bioremediation effects of aquaculture probiotics has regrettably not been well reported to date.

2.5.3 Demonstration of efficacy related to desirable attributes of selected isolates

The rearing of an aquaculture species of interest on small scale model systems allows more certainty in selection of many candidate biological agents (Munro et al. 1995; Verschuere et al. 2000). Thus the next important step once candidate biological agents have been selected is confirmation of effects using in vivo tests. These tests may measure various effects including antagonism by including an experimental infection with a representative pathogen. Pathogens can be administered via the diet, through immersion, by injection or via the culture water (Gildberg and Mikkelsen 1998). To determine the effects of a specific bacterial strain on a cultured organism, the elimination of other microbes from the culture system may be necessary (Bull and Slater 1982). This approach can also be used to examine other effects on water quality and impact on other trophic levels, such as algae (Munro et al. 1995; Fukami et al. 1997; Suminto and Hirayama 1997).

With in vivo challenge tests, the effects should be studied to determine changes in population dynamics of the antagonist and the pathogen and to observe other effects on the culture system. Of importance, are the unintended negative effects on the target species, interference of filtration efficiency in reticulated culture systems and impact on other trophic levels in the system (Gildberg and Mikkelsen 1998; Verschuere et al. 2000). As an example, a clear decrease in the level of the pathogen *V. tubiashii* in culture of oysters was observed when *Aeromonas* culture media was added with a probiotic strain, but the putative antagonist itself could not be detected in the culture after only four days. This example shows the importance of measuring interactions, including mortality or disease, after experimental infection and to include appropriate controls in study designs (Gibson et al. 1998).

2.5.4 Other considerations during selection of biological agents

Putative strains showing well-established biological effects in vitro and in vivo need to be tested for suitability regarding other consequences of biological treatment. Additional criteria such as biosafety considerations, methods of production and processing, the method of administering the probiotic and the location where the microorganisms are expected to be active are important considerations (Huis in't Veld 1994). An isolate cannot be used as a probiotic unless it has been

confirmed that there is no pathogenicity to the host or detrimental effects to humans or the environment (Verschuere et al. 2000). The target species should be challenged under normal or stress conditions with the candidate biological agent. As an example, Austin and Austin (1999) tested their candidate biological agent by injecting Atlantic salmon followed by histopathological examination of the kidneys, spleen and muscles. Pathogenecity to different life stages of the target species should also be considered. Verschuere et al. (2000) tested their probiotics on artemia to verify that the defence systems of the shrimps were able to cope with the intrusion. Similarly, unicellular algae may be inhibited or even stimulated by bacterial isolates (Munro et al. 1995). Effective legislation, if any, should be taken into account before commercial application is commenced. Finally, a cost-benefit analysis will determine whether the probiotics could be applied in practice or not (Verschuere et al. 2000).

2.6 Bio-production of Biological Agents

Large scale production of probiotics is an essential step towards their application in the aquaculture industry (Preetha et al. 2006). The cultivation of microorganisms at a large scale is influenced by various factors such as the composition of the media, as well as physical and chemical variables (Patel et al. 2009). The most common approach towards improvement of biological agents has been through engineering of a selected strain, but other options such as the nutritional and physicochemical parameters of the fermentation process need to be optimized because of public resistance to genetically modified organisms (Lawford and Rousseau 1997). The composition of a fermentation medium has always been a vital component in the optimization of growth conditions, because it affects productivity and overall cost of the production process (Lee 2005). It has been suggested that economical and commercially available media should be investigated to reduce the bio-production costs (Lawford and Rousseau, 1997; Kona et al. 2001). Media formulation and optimization are key considerations in development of bioprocesses that can produce affordable aquaculture biological agents, yet limited progress has been made in this area to satisfy market opportunities for affordable commercial aquaculture products (Irianto and Austin 2002; Preetha et al. 2006). With increased cell yield, productivity and cost reduction, during upstream bio-process development, the fermentation production process can be made feasible and economically attractive for application of aquaculture products

(Gouda et al. 2001). Another key consideration is that scale up of production must not compromise product efficacy or amenability to stabilization and formulation (Schisler et al. 2004)

2.6.1 High cell density cultivation of Bacillus spp.

Although *Bacillus* biological agents are widely used in aquaculture, there are limited studies on their production and little is known about the impact of nutrient supplementation on high-density production of bacterial spores by fermentation (Monteiro et al. 2005; Prabakaran et al. 2007). Carbon and nitrogen sources generally play a dominant role in the productivity of a fermentation process as these nutrients are directly linked with the production of the biological agent (Lopez et al. 1981; Zhang and Greasham 1999). According to current understanding, the development pathway leading from a vegetative cell to a spore is triggered by depletion of carbon, nitrogen, phosphate or essential micronutrients (Liu et al. 1994; Nicholson et al. 2000; Sonenshein 2000). A suitable medium must thus support vegetative growth and also the production of spores (Nickerson and Bulla 1974). The highest values reported for *B. cereus* spore concentration in fermentation are typically around $2x10^9$ $CFU.ml^{-1}$ (Chang et al. 2007; Prabakaran et al. 2007). It has been widely documented that nutrient sources influence the growth, spore production and synthesis of commercially useful metabolites in this species (Rättö et al. 1992; Payot et al. 1999; Gouda et al. 2001).

The type of carbon source and the carbon to nitrogen ratio plays an important role in microbial growth (Gandhi et al. 1998). It has been observed that *B. subtilis* uses glucose as its major carbon source and the efficiency of carbon utilisation towards biomass formation is low when the glucose concentration exceeds ~10g/l in batch culture (Vuolanto et al. 2001). The production of by-products is increased in the presence of excess glucose resulting in reduced yields of biomass, which is undesirable when producing biological agents (Vuolanto et al. 2001). Certain over-flow metabolites can also inhibit cell growth (Vuolanto et al. 2001). Monterio et al. (2005) also observed that an increase in glucose concentration up to $5g.l^{-1}$ led to an increase in the vegetative cell and spore concentration of *B. subtilis*, while higher sugar concentrations inhibited sporulation. It is therefore of great importance to regulate carbon availability to optimise growth and sporulation parameters precisely (Patel et al. 2009). It is also noteworthy that glucose

consumption rate depends significantly on factors such as pH and oxygen sufficiency. Mass transfer parameters such as agitation and aeration are thus important in maximising vegetative cell growth, without inducing a premature onset of sporulation (Calik et al. 2003). In most studies, glucose was found to be the best carbon substrate for the production of Bacilli and their spores (Calik and Ozdamar 2000).

Various protein substrates have been tested for the growth and synthesis of commercially useful metabolites by *Bacillus* spp. (Srivastava and Baruah 1986; Rättö et al. 1992; Payot et al. 1999; Gouda et al. 2001). It has furthermore been widely documented that protein sources influence spore production in this species. (Nickerson and Bulla 1974; Shinke et al. 1977; Sharpe and Bulla 1978; Goldberg et al. 1980). Commonly used nitrogen based nutrient sources include a wide range of peptones, extracts and hydrolysates, many of which are expensive for industrial scale manufacture of large volume products and have negative market acceptance if they contain animal by-products (Nohata and Kurane 1997; Vuolanto et al. 2001). Media formulated to support high productivities are predominantly formulated with inexpensive complex nitrogen sources (Miller and Churchill 1986; Zhang and Greasham 1999).

Although yeast extract, peptones and meat extracts have been shown to improve growth rate as good sources of protein, vitamins and co-factors (Payot et al. 1999), there have been reports suggesting that metabolite, and particularly spore production, are often better when corn steep liqour (CSL) is used (Nickerson and Bulla 1974; Srivastava and Baruah 1986; Shikata et al. 1990; Kuppusamy and Balaram 1991; Gouda et al. 2001; Prabakaran et al. 2007). CSL contains a wide range of macro and micro elements known to be important for spore production (Shi and Zhu 2007).

Apart from the nature of protein source, the concentration in culture media also affects growth and spore production (Chandra et al. 1980). *B. subtilis* spore productivity increased, but spore yield decreased, with an increase in CSL concentration (Goldberg et al. 1980). The yield of spores on carbohydrate increased with increasing concentration of CSL, suggesting that a higher protein to carbon ratio was preferable for growth of *B. subtilis* (Vuolanto et al. 2001) and *B. licheniformis* (Mao et al. 1992). High levels of CSL supplementation (~60g.l^{-1}) however resulted

in slow growth, cell lysis and poor spore formation as sporulation efficiency is known to be low following poor growth (Nickerson and Bulla 1974; Purushothaman et al. 2001; Silveira et al. 2001). Sporulation takes longer in high cell density cultivations, thus resulting in a compromise between spore concentration and productivity (Liu et al. 1994).

A major advantage is that CSL is available in an ultra-filtered phytase treated variant in South Africa, which is cost competitive and has processability advantages in both up-stream and down-stream process unit operations (Mao et al. 1992; Silveira et al. 2001). Precipitation and mass transfer issues are reduced when using this form, due to hydrolysis of phytic acid and removal of solids through the ultra-filtration process, which is of importance in mass transfer required for high density cultivation (Liu et al. 1994; Monteiro et al. 2005). As this CSL is not spray dried, as is typical of the conventional type, degradation of vitamins and key nutrients is minimised which improves growth performance (Payot et al. 1999). Of peripheral benefit is the use of a corn wet processing waste which improves value addition and reduces environmental pollution normally caused by such materials (Gouda et al. 2001).

2.6.2 Production of spores

The key challenge in spore production is to maximize sporulation from a high density vegetative cell culture (Nicholson et al. 2000; Monteiro et al. 2005). Environmental signals for sporulation include the role of culture density dependant peptides, oxygen availability and nutrient limitation of carbon, nitrogen or phosphorous (Sonenshein 2000). The life-cycle of a spore forming bacteria consists of four stages, namely; vegetative growth, sporulation, germination and outgrowth (Nicholson et al. 2000; Almedia et al. 2006). Cells enter a sporulation pathway, which involves three differentiating cell types, namely the predivisional cell, mother cell and the forespore, in response to nutrient limitation (Wang et al. 2006). The forespore undergoes dehydration, while the cortex is produced between the two membranes that separate the mother cell and the forespore. Eventually the mature spore is released when the mother cell lyses. This mature spore has the ability of remaining dormant for long periods of time (Wang et al. 2006). The most important sporulation related transcriptional regulator is Spo0A which is phosphorylated via a complex network of interactions in response to nutrient limitation (Sonenshein 2000; Errington

2003). Furthermore, genes controlled in the Res system are induced under anaerobic growth conditions which contribute to the sporulation cascade (Msadek 1999). Low phosphate concentration results in the earlier onset of sporulation due to the response of the Pho system to phosphate starvation (Msadek 1999). Magnesium sulphate, calcium carbonate and phosphate all stimulate sporulation (Shi and Zhu 2007), whereas divalent cations (particularly Ca^{2+}) assist in dehydration and mineralization of the spore (Errington 2003). According to Monterio et al. (2005), the sporulation efficiency for *B. subtilis* was found to be independent of the pH values within the range of 6.9-9.0. For several *Bacillus* spp. sporulation is highly related to O_2 supply and it has been reported that non-limited oxygen conditions during the growth phase are important to realise high spore yields (Dingman and Stahly 1983; Avignone-Rossa et al. 1992).

2.7 Downstream processing and product formulation

Important criteria influencing the commercial use of biological products are cost, efficacy, shelf life and convenience to the end user (Amer and Utkhede 2000; Keller et al. 2001). Fermentation broth comprises a mixture of components and the spore product needs to be recovered efficiently to be utilized in subsequent processing and formulations steps (Tsun et al. 1999; Rowe and Margaritis 2004). The downstream process has a major influence on product commercialization as the major constraint is embedded in harvesting and formulation costs (Brar et al. 2006; Prabakaran et al. 2007). This includes key aspects such as maximising recovery and preservation of viability during processing, while ensuring a product that meets with end user requirements such as stability, consistency, easy application, efficacy and affordability (Schisler et al. 2004; Brar et al. 2006). The durability of *Bacillus* spores allows consideration of process options that may be more severe than that for less resistant microbes (Emmert and Handelsman 1999; Driks 2004). Although the downstream process, which bridges the fermentation and field application, is an extremely important prerequisite for commercialization of biological agents, published literature regarding downstream processing and formulation for commercially available products is very limited (Schisler et al. 2004; Brar et al. 2006). Thus robust economical choices of process steps and ingredients, dictated by the end product characteristics, are necessary to improve the commercial success of new biological products (Brar et al. 2006). The main objective for downstream processing is to minimise the number of unit operations involved in the process, thus reducing overall process and validation costs, while also simplifying ease and economy of

process automation (Brar et al. 2006). An additional consideration is the final anticipated form of the end product which has implications on the choice of process options and the final product while meeting customer expectations (Lydersen et al. 1994).

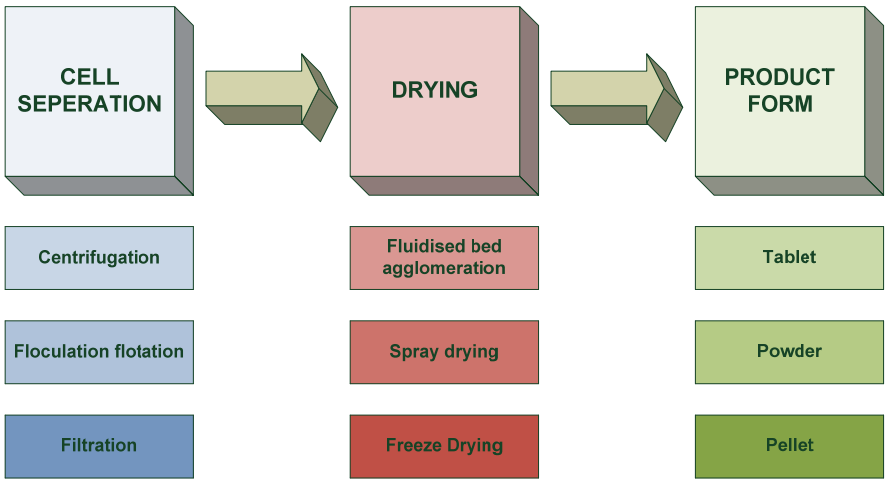

Figure 6 Schematic illustration of potential downstream process unit options

2.7.1 Separation of spores from fermentation broth

Harvesting efficacy governs the marketability of a product, affecting potency and aiding in further processing during formulation development. The goal of the recovery process is to produce a product of acceptable quality, in compliance with any regulatory and safety requirements, at an acceptable cost (Lydersen et al. 1994). Process options for cell harvesting from fermentation broth include microfiltration, sedimentation, flocculation and ultrafiltration (Luna-Solano et al. 2005; Prabakaran et al. 2007). Flocculation and flotation using surface action or electrical charge have been reported to be inefficient in the separation of bacterial cells (Lydersen et al. 1994). Although there have been some positive reports for harvesting using ultra-filtration (Prabakaran et al. 2007), the most widely used process remains centrifugation for *Bacillus* spores (Puziss et al. 1963; Zamola et al. 1981; Rojas et al. 1996).

Centrifugation is the most viable alternative for spore harvesting of biological agents resulting in recoveries of ~ 99% (Torres-Anjel and Hedrick 1970; Brar et al. 2006). Factors influencing

recovery during cell separation include the accumulation of solids in the machine resulting in product losses and the number of passes required to realise a suitable recovery (Torres-Anjel and Hedrick 1970; Prabakaran et al. 2007). Slower flow rates were found to be less efficient in spore removal when compared to faster rates and it has also been reported that recovery, but not productivity, can be compromised with high spore load (Torres-Anjel and Hedrick 1970). For the separation of bacterial spores at large scale, centrifugation options include disc stack and tube centrifuges because of their high g forces, whereas basket and decanter centrifuges are less appropriate (Riviere 1977). The tube-type centrifuge usually consists of a slender tube that is capable of delivering high centrifugal forces. The centrifugal force is 10 000 to 20 000 x g which is much higher than the decanter and disc-stack type machines. Tubular centrifuges are easy to operate and have lower capital and running costs due to their simplicity (Lydersen et al. 1994). In the case of *Bacillus* spores the product intermediate is anticipated to be a high cell concentration paste, therefore tube centrifugation is a useful process that can yield a lower moisture paste thus minimizing the energy required in subsequent drying steps (Riviere 1977; Berovic 1998).

2.7.2 Product formulation options

Formulation is a crucial link between production and application and dictates processability, economy, shelf life, efficacy, ease of application and provision of a product form that commands customer appeal. Intelligent formulations also allow innovation in application techniques using unique combinations of active ingredients, adjuvants or inerts (de Medeiros et al. 2005; Brar et al. 2006). Formulations can be broadly classified into dry solids in various forms and liquid suspensions or emulsions (Rhodes 1993). The inclusion of additives that enhance processability eco-friendliness and customer acceptance of the end product are also important considerations (Brar et al. 2006). A considerable influence on activity and impact to consumers and the environment can be attributed to the type of substance added to a formulation, and it is therefore imperative to ensure that there are no negative consequences (Werner et al. 1993). In the case of a spore product, the formulation needs to encompass ingredients that aid germination and growth of the spore in its intended application. Sugars and proteins are normally the key nutrients supporting germination and further provide a protective layer for the spores, preventing death and assisting recovery of injured cells during processing (Costa et al. 2001; Larena et al. 2003; Brar et al. 2006). Addition of nutrients was also shown by others to improve storage of a *P. fluorescens*

F113 strain (Moene-Loccoz et al. 1999) and a *B. megaterium* (Wiwattanapatapee et al. 2004) used in biocontrol. Appropriate formulations can facilitate easier processing and influence the stability and appeal of an end product in large scale production (Amer and Utkhede 2000).

2.7.3 Processing options for dry product forms

Fresh cells as biological agents are not applicable for routine use due to limited stability (Wiwattanapatapee et al. 2004). Drying is a widely employed unit operation in processing of bioactive materials such as biological suspensions containing whole cells and spores. Preservation of the functionality of useful micro-organisms during drying is essential because it directly affects the final quality, stability, ease of application and market value of the end product (Chen and Patel 2007). During development of a drying process, a key playoff exists between reducing drying period and energy consumption, while maximising product quality and maintaining a suitable product form (Yuzgec et al. 2006).

Potential options for commercial processes to stabilize biological products include refrigeration, freezing, freeze drying, spray drying and low temperature fluidised bed agglomeration. Refrigerated and frozen cultures occupy large storage volumes and demand higher storage and shipment costs in contrast to dry cultures which are an economic and practical alternative (Klein and Lortal 1999). Low temperature drying processes such as freeze drying are suitable for higher value, heat labile bioproducts, but is costly, time consuming and discontinuous for bulk production compared with moderate temperature drying processes (Werner et al. 1993; Knorr 1998). Spray drying processes are widely used for large scale drying of products (Werner et al. 1993). Higher drying temperatures decrease the viability of microbes and spores faster than lower drying temperatures, but the latter requires longer drying times (Bayrock and Ingeldew 1997). Although spores are significantly more resistant to heat than vegetative cells (Setlow 2006), spray drying requires high temperatures to facilitate water evaporation, which can cause irreversible changes to structural and functional integrity of the biological agent and reduce viability and activity of organisms (Tamez-Guerra et al. 1996; Chen and Patel 2007). Spray drying also has a high energy demand requiring 2500 to 10 000 $J.g^{-1}$ of evaporated water (Luna-Solano et al. 2005).

There are several reports on the use of agglomeration as a commercially viable process option for moderate temperature drying of biological material, mainly due to excellent mass and heat transfer characteristics (Bayrock and Ingeldew 1997; Grabowski et al. 1997; Larena et al. 2003). During agglomeration, a mixture is atomised to form droplets at lower temperature (typically 30-40^{o}C) which results in coating of the spores on the surface of carrier particles. Cells are subjected to evaporative cooling during the warming up and constant-rate drying periods and therefore have a substantially lower temperature than the drying air, resulting in increased viability (Bayrock and Ingeldew 1997). Advantages of fluid bed drying over freeze and spray drying include lower investment and maintenance costs, ease of large scale continuous production, rapid exchange of heat, minimising heat damage, rapid mixing providing near isothermal conditions and uniform end product (Bayrock and Ingledew 1998; Mille et al. 2004; Luna-Solano et al. 2005). Agglomeration also results in a homogenous distribution of spores on the carrier, which influences end product consistency. The variability in agglomerate size also enhances flowability of the resultant powder for subsequent process steps. Other advantages of a fluidized bed dryer over other equipment include; large scale continuous production, easy handling of feed and product, a lack of mechanical moving parts, rapid exchange of heat between gas and particles, shorter drying times and uniform particle moisture content. For this reason fluidized bed drying has become an accepted method for large scale production of heat labile biological materials such as spores (Grabowski et al. 1997)

Spores of *B. cereus* are moderately heat resistant (Houska et al. 2007) and fluidised bed drying at lower temperature results in better cell viability (Larena et al. 2003; Brar et al. 2006), due to the control of temperature and osmotic pressure change (Mille et al. 2004). *Bacillus* spores are well protected from dry heat by the spore structure and two major small acid soluble DNA binding proteins, α and β. (Setlow and Setlow 1995). The protein exosporium is a large loose fitting structure found on spores of some species, in particular those of the *B. cereus* group which is responsible for heat resistance of these spores (Setlow 2006).

The use of nutrient based carriers such as whey powder and corn starch has previously been demonstrated (Dandraun et al. 1994; Mille et al. 2004). It has been reported that smaller particle

size carriers (50 - 100μm) enhanced bacterial survival more than large particle sizes (Dandraun et al. 1994). Spray dried corn steep liquor (CSL) is an ideal carrier for agglomeration, as it does not pose a serious health risk through dust formation and has a spore activation benefit in comparison to inert carriers (Brar et al. 2006). Although there is a large variation in hydrophobicity among spores of different *Bacillus* species (Doyle et al. 1984; Rönner et al. 1990), *B. cereus* is more than twice as hydrophobic as *B.licheniformis* or *B. subtilis* (Rönner et al. 1990; Anderson et al. 1995). *B. cereus* has a loosely associated exosporium as the outermost layer, which is composed mainly of proteins lipids and phospholipids (Warth 1978). This structure together with hair like protrusions extending from the spore surface have been implicated in enhancing adhesion to carrier surfaces (Busscher and Weerkamp 1987; Rönner et al. 1990).

2.7.4 Considerations for end product form and stability

Bacillus spores offer prolonged shelf life and their robustness allows the consideration of an array of end product forms. *Bacillus* spores have been formulated into a number of unique product forms, from adhesion onto simple carriers to packaging into tablets and capsules. *B. subtilis* was stable for 45 days at ambient temperature in a simple mixture of vermiculite, kaolin and bacterial broth carriers (Amer and Utkhede 2000). Wiwattanapatapee et al. (2004) showed the beneficial physical properties of their pelleted *B. megaterium* end product, including bacterial release during application, whereas top coating of suitable supports using a spore suspension has also been demonstrated (Biourges et al. 1998). A *B. megaterium* product was also prepared by an extrusion-spheronisation process (Wiwattanapatapee et al. 2004). An alternative product technology is encapsulation, which provides protection from environmental conditions and offers enhanced stability and the possibility of slow release. Use of carbohydrate rich biopolymeric gels and polymers as encapsulation agents has been demonstrated, but encapsulation matrices are expensive and not ideal for biological agents (McGuire et al. 1990).

Tablet formulations have advantages such as uniformity, stability, easy transportation and field applicability (de Medeiros et al. 2005). A tablet containing *B. cereus* spores was successfully produced by direct compression of a powder blend containing magnesium stearate at 2% (de Medeiros et al. 2005). A concern during tablet production is the inactivation of spores by pressure

and frictional heat during compression and de-compression (Margosch et al. 2004; Mathys et al. 2008). Biourges et al. (1998) demonstrated a loss of greater than 99% of *B. cereus* spores during extrusion, related to pressure and heat. The mechanism of inactivation of bacterial spores by heat and pressure is as yet unresolved (Mathys et al. 2008), but it has been postulated that the thick proteinaceous spore coat could play a role in resistance to pressure (Setlow 1999). There is however also evidence that *B. cereus* can withstand pressures up to ~50mPa, without any significant impact on viability (Aoyama et al. 2005).

Apart from production of an end product through a downstream process, the stability and consistency of product intermediates and the end product are a crucial requirement for successful commercialization (Prabakaran and Hoti 2008; Keller et al. 2001). A loss of bioactivity results in other process complications and imparts a direct increase in production cost, because most biological agents are sold per unit bioactivity (Chen and Patel 2007). The lag time between process operations can vary due to process integration and scheduling during manufacture, thus storage conditions and the addition of specific stabilizers and biocidal chemicals may be required to prevent vegetative growth or contamination in product intermediates (Soper and Ward 1981). It has also been reported that a mixture of spores and vegetative cells tend to be less stable in *Bacillus* based products, thus high sporulation efficiencies in fermentation are an important factor influencing product stability (Wiwattanapatapee et al. 2004). The problems of stability during processing, storage and after application have stalled development of biological products (Tsuji 1997). Accelerated aging studies based on the methodology of death rate plots at different temperatures to generate thermal resistance curves are useful for predicting stability (Ramaswamy 1997). Temperature dependant half life plots can be generated to predict stability of product intermediates and the end product. This approach has however only been used to a limited extent, to determine the stability of *Bacillus* based biological agents (Puziss et al. 1963; Wiwattanapatapee et al. 2004).

2.8 Application of Biological Agents

A key challenge for usefulness of biological agents is the survival of micro-organisms in the environment to which they are applied. These biological agents must be tolerant to the prevailing

environmental conditions in which they are expected to perform, often dictated by the species being cultured for a specific aquaculture application (Gross 2003). Several methods of addition of biological agents to the host or its ambient environment exist, with each application method presenting unique challenges to the survival and efficacy of the biological agent (Smith and Davey 1993; Austin et al. 1995; Gomez-Gil et al. 1998; Verschuere et al. 1999). A biological agent must provide actual benefit to the host, be able to survive in the environment of the intended application and should be stable and viable during prolonged storage (Irianto and Austin 2002). Other factors such as natural deterioration and washout of the biological agent may necessitate the ongoing addition of the treatments to maintain the positive effect (Verschuere et al. 2000). Information on the robustness and functionality of biological agents in response to environmental conditions such as salinity, pH and temperature are however limited. Changes in these conditions influence spore germination, cell growth, survival and functionality of *Bacillus* spp. as aquaculture biological agents (Budde et al. 2006).

A key consideration for the application of *Bacillus* based biological agents is that the spores need to germinate and grow such that the characteristics of a metabolically active cell can be recovered (Moir 2006). The replication of vegetative cells can further enhance the bioactivity in the intended application. Spores lose their dormant properties when conditions are favourable in the presence of specific germinants such as nutrients (Vary 1973). However, the germinant has to penetrate the outer coat and cortex layers of the spore before coming into contact with specific germinant receptors (Hornstra et al. 2006; Moir 2006). The germination of spores is a sensitive transition state involving the initiation of metabolism (Stewart et al. 1981). Towards the application of spores as aquaculture biological agents, determination of their functionality as antagonists to disease or for improvement in water quality under the physiological ranges to be encountered in the aquaculture system is thus an important requisite.

Changes in growth conditions such as temperature constitute a key factor that influences cell growth and survival of *Bacillus* spp. in their habitats. *B. subtilis* has the ability of sustaining growth in a wide temperature range from approximately 11ºC (Nicholson et al. 1996) to 52ºC (Holtman and Bremer 2004). When the growth temperature for *B. subtilis* is increased rapidly, changes in gene expression occur. This is known as a heat shock response. A cold shock response is elicited when the temperature is dropped down to 15ºC from 37ºC (Budde et al. 2006). *B. cereus* is apparently not well adapted to cold temperatures and the metabolic rate decreases

drastically below 13^{o}C (Choma et al. 2000). A useful method for the elucidation of temperature domains for prediction of functionality of a biological agent is by examining conformance of efficacy measures to Arhenius and Ratkowsky functions (Ratkowsky et al. 1983; Choma et al. 2000).

The vegetative cells of *B. cereus* are more sensitive to acidic conditions than spores. However, like many other cells, vegetative cells of *B. cereus* have the ability to induce an acid tolerance response (Thomassin et al. 2006). The mechanisms of resistance to acidic conditions involve three factors i.e. (i) F_0F_1 ATPase and glutamate decarboxylase (ii) metabolic modifications and (iii) protein synthesis to protect and repair macromolecules (Thomassin et al. 2006). *B. cereus* spores are generally tolerant to the salinity and pH extremes typically encountered in the culture of ornamental *C. carpio* (Leguerinel et al. 2000, Jobin et al. 2002). It is noteworthy that the efficacy of biological agents in response to physiological ranges must be assessed in line with the dynamics of the target species and the aquaculture system. As an example, reduced activity of a biological agent at lower temperature does not necessarily indicate a failure of the biological agent to perform, as lower temperature could translate to a lower intake of feed, waste metabolite generation and pathogen propensity in the aquaculture system.

Chapter 3 Research and Development Study

3.1 Problem statement

Modern day ornamental aquaculture is predominantly practiced in intensive reticulated culture systems by both culturists and hobbyists. Damage and losses during culturing, harvesting, transportation and rearing of fish result in major losses to the industry and hobbyist. In the ornamental market, the hobbyist is always in search of perfect specimens, which are often entered into shows and competitions. The specimen value is thus defined by its flawless ornamental appeal and any damage or blemish to the specimen can significantly de-value it. Disease in ornamental fish is a result of interactions between the fish species, the environment and the disease causing agent, resulting in damage and mortality. Water quality and pathogen load within culture systems are key contributors to the prevalence of disease. Conventional methods for control and treatment of diseases include the use of a wide array of anti-bacterial and chemical compounds. General practice is to use these at prophylactic doses for control and to increase the dosage for treatment in response to a disease outbreak. High concentrations of wastes in the water are normally controlled by effluent purging resulting in wastage of water and potential damage to the environment. The major disadvantages of these types of treatment regimes are:-

- Widespread use of sub-lethal doses, resulting in the development of resistant strains of the pathogens, which become more difficult to eradicate.
- Release of scheduled drugs, toxic chemical compounds and wastes into the environment
- Non-target specific mode of action resulting in the mortality of other harmless or beneficial microflora and microfauna in the biosystem
- Mortality of fish due to repeated use of high antibiotic dosages.
- Poor stability of treatments
- High cost of treatment
- Product and treatment forms not specifically designed for application in aquaculture.
- A lack of species specific holistic solutions for the end user.

3.2 Opportunities for Research and Development

Biological agents offer potential to address these challenges in a holistic manner, with lower negative impact on people and the environment. The adoption of these technologies has however been limited, mainly due to limitations in appropriate research and development that demonstrate functionality and safety, coupled to bioprocess development that delivers stable, cost competitive and innovative products.

3.2.1 Isolation screening, identification, safety and mode of action of biological agents

The use of beneficial bacteria in aquaculture has received considerable interest and has several advantages to conventional practise (Moriarty 1999; Sanders et al. 2003; Hong et al. 2005). The type and application of a biological agent must be synchronised to the type of culture method, the species cultivated and the economics (Moriarty 1998). There is no single technique for the isolation of biological agents, while appropriate methods for isolation of putative biological agents require further development to ensure a higher success rate. Furthermore the range of media used, the environments targeted and the alignment of studies towards a particular fish species, is governed by personal choice and experience, rather than a directed approach towards isolation of putative biological agents from environments with a high likelihood of yielding organisms of value (Austin and Austin 1999). The proposed research in this regard, focuses on the isolation of organisms from South Africa's biodiversity and the development of targeted isolation protocols for selection of putative biological agents of the genus *Bacillus.* This genus offers a multitude of advantages for exploitation as biological agents in aquaculture. The isolation strategy, based on pre-defined characteristics and a targeted rationale, is expected to yield positive isolates in a limited number of environmental samples, rather than extensive screening of large biomes. This will ensure speedy progress of technology development from isolate to product.

Although several isolates have been screened for positive effects on cultured species (Queiroz and Boyd 1998), the published evidence for water quality improvement is poor, except for nitrification (Verschuere et al. 2000). Many authors have assigned inhibitory effects detected in in vitro antagonism tests to bacteriocins or antibiotics without explicitly investigating the modes

of action, as growth inhibition could in many cases, be accounted for by a multitude of effects (Verschuere et al. 2000). The pre-selection of candidate probionts based on in vitro antagonism tests often yields effective probionts (Gibson et al. 1998), but there has been much uncertainty because of a lack of proper studies to show efficacy, safety and the basis for functionality, which has hampered adoption of this technology (Ringø and Gatesoupe 1999). It has not been demonstrated unequivocally that there are representative in vitro assays which can ensure appropriate selection of useful biological agents (Verschuere et al. 2000). It would therefore be useful if bacterial characteristics were identified for the selection of beneficial bacteria, but work in this area is limited, and the selection is predominantly based on trial and error (Gomez-Gil et al. 2000). There is thus a significant gap in the current state of knowledge, as predefined selection characteristics associated with a collection of desirable traits of consequence to a particular species and culture environment, have not been exploited for the targeting of appropriate screening protocols to yield useful biological agents. This lack of research has been circumvented in the aquaculture industry by the use of generic based probiotics used in animal husbandry, which have not provided holistic and effective solutions. The assessment of isolates for synergistic and holistic benefits to the cultured species of interest, relating to pathogen inhibition and water quality, has furthermore been neglected in the research and development of aquaculture biological agents. Our strategy will be to exploit pre-defined selection criteria that can yield a high ratio of positive isolates with desirable characteristics, while minimising extensive screening campaigns.

A bacterial culture cannot be used as a biological agent, unless it has been confirmed that no pathogenic effects occur in the host, there is no significant negative environmental impact or pathogenicity to humans (Verschuere et al. 2000). This phase of research can be combined with small-scale in vivo tests under monoxenic conditions, to eliminate other interactions with the already established microbiota, and should also be examined in vivo to assess any potential negative impact on the fish species of interest. Attention should also be paid to proper identification and characterization of the biological agents and molecular tools may be the most appropriate for this kind of application (Verschuere et al. 2000). Many of the biological agents in application have however been poorly characterized, particularly those of the genus *Bacillus*, predominantly due to the difficulty in distinguishing species of the *B. cereus* group of organisms (Carlson et al. 1994; Phelps and McKillip 2002; Helgason et al. 2004; Rasko et al. 2004). The lack of appropriate identification and safety assessment of putative biological agents presents

significant risk to application of such technologies and requires appropriate research to ensure safe application of these microbes.

3.2.2 Development of upstream and downstream processes for high productivity of Bacillus spores and formulation into products that meet commercial end user requirements

The mass production of organisms is an important factor in successful development and implementation of biological agents in aquaculture. The benefit to cost ratio is an important consideration for commercial end users and determines whether the biological agents can be usefully applied in practice (Verschuere et al. 2000). There are several studies on the production of metabolites using *Bacillus* spp. although studies on the production of biological agents are limited. There is a significant gap in current knowledge particularly in high cell density cultivation of *Bacillus* spores as an end product of a fermentation process. Development of appropriate stabilization technology for the maintenance of viability and novel downstream processing that result in appropriate product forms of biological agents are also limited. These bioprocess development considerations are important to ensure a feasible production process that is economically viable and requires further impetus. It is thus necessary to develop processes and monitoring tools to control the production and application of bacterial cultures for use as biological agents on a large scale, with appropriate quality control to avoid contamination by other bacteria and to ensure that the key effects of the biological agents are maintained through the production process and embodied in the end product (Verschuere et al. 2000).

3.2.3 Evaluation of the suitability of putative biological agents to function in the dynamic environments in which they are to be applied

A key challenge for usefulness of biological agents is the survival of micro-organisms in the environment to which they are applied. These biological agents must be tolerant to the conditions in which they are expected to perform (Gross et al. 2003). This is especially important in the application of spore formers, as germination, growth and efficacy of the biological agents under a range of environmental conditions associated with culture practices of the species of interest are limited. Research is also sparse regarding appropriate activation technology for the germination of spores once applied to the culture systems. There has been a greater emphasis on laboratory

and in vitro findings, but in many cases the full potential of these studies have not been fully exploited to predict performance in vivo or to generate data that would be useful to end users regarding functionality. In many cases the focus has been on in vivo end point effects, such as mortality or growth rate, without elucidating the underlying features and functionality of the specific biological agent in these studies. Consequently, information is often limited for commercial application of biological treatments to aquaculture (Irianto and Austin 2002). Thus a collection of key variables of importance to the effects of the biological agent should be measured in manageable trials with appropriate controls.

3.3 Objectives of Study

The key objective of the project was to research and develop bioprocess technology that will result in innovative products to effect biological solutions that improve water quality, reduce pathogen load and prevent negative environmental impact. The products must address a multitude of problems encountered and must ultimately reduce the use of harsh chemicals and antibiotics in aquaculture. The research and development should result in integrated species specific biological solutions to the aquaculturist, with a focus on decreasing both the pathogen load and the concentrations of key waste ions such as ammonium, nitrite, nitrate and phosphate, ultimately decreasing disease prevalence and mortality. The process for production of the biological agents should result in safe, stable and easy to apply commercial end products. The products must be cost competitive in international markets and confer a competitive advantage to the end user. The product should target high value, niche markets, such as ornamental carp. The project aims to deliver a process for the production of biological products that will: -

- Reduce the pathogen load in water.
- Ensure improved water quality.
- Ensure stability and ease of use under demanding industry conditions
- Function in a wide range of physiological conditions
- Reduce the routine usage of harsh chemicals and antibiotics by integrated management practises using biological agents
- Use robust, safe and multi-effect naturally occurring isolates of bacteria

- Ensure successful commercialization through innovative bioprocess development with a high effect (benefit) to cost ratio to the end user

3.4 Aims of the study

The objectives of the research were realized by fulfilling the following aims: -

- To isolate, screen, select, identify and assess the safety of putative biological treatments from indigenous environments in South Africa (Chapter 4)
- To elucidate the key modes of action of a selected biological agent (Chapter 5)
- To research and develop appropriate methods and bioprocess unit operations for high density production of a selected isolate by fermentation (Chapter 6)
- To develop a downstream process including formulation of the biological agent into a stable, robust and functional product prototype (Chapter 7)
- To research and develop product application technology, including activation technology and assessment of the robustness of the biological agents to environmental conditions (Chapter 8)

Chapter 4 Publication 1

Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish

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ORIGINAL ARTICLE

Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish

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aquaculture, *Bacillus* spp., bacterial pathogens, biological agent, water quality.

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Abstract

Aims: To isolate, select and evaluate *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish.
Methods and Results: Natural isolates obtained from mud sediment and *Cyprinus carpio* were purified and assessed *in vitro* for efficacy based on the inhibition of growth of pathogenic *Aeromonas hydrophila* and the decrease in concentrations of ammonium, nitrite, nitrate and phosphate ions. Based on suitability to predefined characteristics, the isolates B001, B002 and B003 were selected and evaluated *in vitro* in the presence of *Aer. hydrophila* and in a preliminary *in vivo* trial with *C. carpio*. The inhibitory effect on pathogen growth and the decrease in concentrations of waste ions was demonstrated. Based on 16S RNA sequence homology, the isolates were identified as *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis*, respectively. Isolate B002 did not contain the anthrax virulence plasmids pOX1, pOX2 or the *B. cereus* enterotoxin.
Conclusions: Selected isolates effected synergistic reduction in pathogen load and the concentrations of waste ions *in vitro* and *in vivo* and are safe for use in ornamental aquaculture.
Significance and Impact of the Study: A new approach for assessment of biological agents was demonstrated and has yielded putative isolates for development into aquaculture products.

Introduction

Culture of fish in reticulated systems results in waste accumulation, disease proliferation and negative environmental impact (Liao and Mayo 1974; Boyd 1985; Shimeno *et al.* 1997). Interaction between the host, environmental stress and disease-causing agents contributes to the onset of disease (Paperna 1991; Jeney and Jeney 1995; Austin and Austin 1999; Moriarity 1999), resulting in usage of chemicals and anti-microbials, which alter natural populations, damage the environment and increase resistance and virulence of pathogenic micro-organisms (de Kinkelin and Michel 1992; Gatesoupe 1999; Moriarity 1999; Skjermo and Vadstein 1999; Sze 2000; Jana and Jana 2003). Alternative methods for disease control and enhancement of water quality are therefore required (de Kinkelin and Michel 1992; Barker 2000; Sze 2000). Bacterial amendments have potential to improve fish health by improving water quality and reducing pathogen load (Fast and Menasveta 2000; Gomez-Gil *et al.* 2000; Jana and Jana 2003; Hong *et al.* 2005). However, the usefulness of biological agents is dependant on their survival in the environment (Gross *et al.* 2003). Spores of the genus *Bacillus* have advantages over vegetative cells, because they are stable for long periods, can be formulated into useful commercial products, are widely used as biological agents, possess antagonistic effects on pathogens and are naturally ingested by animals (Hong *et al.* 2005). *Bacillus* spp. are furthermore unlikely to use genes for antibiotic resistance or virulence from Gram-negative micro-organisms such as *Aeromonas* spp. (Moriarity 1999). The objectives of this study were to isolate microbes of the genus *Bacillus* from

natural environments in South Africa and to determine their suitability towards enhancement in water quality, using *Cyprinus carpio* (ornamental carp) as a model species, because the specimen value of ornamental carp is high and survival of ornamental carp is important for both hobbyists and culturists. Ulcerative erythrodermatitis caused by *Aeromonas hydrophila* is a disease prevalent in this species, which can result in damage to the appearance of the specimen and mortality (Jeney and Jeney 1995; Austin and Austin 1999). Furthermore, nitrogen- and phosphorous-based waste accumulations pose a threat to fish health and the environment (Jana and Jana 2003) and result in stress which aggravates infestation by parasites and pathogens (Liao and Mayo 1974; Jeney *et al.* 1992; Ng *et al.* 1992; Grommen *et al.* 2002; Gross *et al.* 2003; Jimenez-Montealegre *et al.* 2005). In the present study, selection criteria were based on the ability of isolated microbes of the genus *Bacillus* to decrease concentrations of both pathogenic bacteria and the ions of waste metabolism, typically produced by *C. carpio*. Selected putative biological agents were evaluated under simulated pond water conditions *in vitro* and *in vivo* and subjected to identification and safety tests. Safety assessment in selection studies of biological agents is limited (Verschuere *et al.* 2000; Hong *et al.* 2005; Balcázar *et al.* 2006) and there is thus an impetus for thorough evaluation of the required characteristics and appropriate safety assessment of putative biological agents prior to development into commercial products.

Materials and methods

Isolation of putative biological agents of the genus *Bacillus*

A method was developed based on previous research (Holt *et al.* 1994) and was validated using known organisms inoculated into sterile soil samples, wherein a full recovery of test organisms was achieved (data not presented). Mud samples and live *C. carpio* were obtained from dams used for the rearing of this fish species in the Gauteng Region, South Africa. Mud sediment was collected from the bottom of the dam in a presterilized McCartney bottle. Mucus was collected from the skin layer by scraping with a sterile loop and gut contents were collected by squeezing the abdomen of the specimen to release the excrement from the gut. Each of the mud sediment samples, skin mucus samples or gut content samples (1 g suspended into 3 g of 0·9% m/v NaCl solution) was added into a presterilized McCartney bottle containing nutrient broth (9 ml) and incubated for 24 h at 30°C followed by incubation at 45°C for 10 min in a convection oven to activate the sporulation process. Ethanol (50% v/v) was added to a volume of 20 ml to each of the bottles which were incubated at 20°C for 1 h. The contents were centrifuged at 10 000 ***g***, the supernatants decanted and the resultant pellets incubated at 105°C in a convection oven for 5 min. The dry pellets were reconstituted into 20 ml of sterile physiological saline and serially diluted to 10^{-4} in 10^{-1} increments. Thereafter, aliquots (0·1 ml) of each of the serial dilutions were spread onto nutrient agar plates supplemented with polymyxin B (10 mg l^{-1}; Merck, Darmstadt, Germany). The plates were incubated for 24 h at 30°C. Single colonies isolated from these plates, were purified and subjected to Gram staining, API identification (API 50 CHB/CHE, Biomérieux, Marcy-l'Etoile, France) and an assessment of catalase activity (Washington 1981).

Isolate and pathogen storage

Isolates were grown in culture media (0·8% m/v yeast extract, 0·005% m/v $MnSO_4$, 0·01% m/v $CaCl_2$ and 0·03% m/v $MgSO_4{\cdot}7H_2O$). The pathogenic organism, *Aer. hydrophila* (ATCC 7966), was obtained from the American Type Culture Collection (www.atcc.org) and grown according to Kielwein *et al.* (1969) and Kielwein (1971). Cultures were cryo-preserved using sterile glycerol (25% v/v) as described by Meza *et al.* 2004. Media components were obtained from Merck (Darmstadt, Germany). These cryo-preserved cultures were used as starter inocula for all experiments.

Isolate selection based on *in vitro* assessments

The *in vitro* tests comprised inhibition of pathogenic *Aer. hydrophila*, determination of specific growth rate and determination of the decrease in the concentrations of ammonium, nitrite, nitrate and phosphate ions in water. Inhibition of *Aer. hydrophila* by each of the isolates was assessed by plate well inhibition assays (Bauer *et al.* 1959). Whole broth of each isolate was preincubated in Tryptic Soy Broth (Foldes *et al.* 2000) at 30°C for 24 h. Aliquots (0·1 ml) were added per well to nutrient agar plates prespread with *Aer. hydrophila* and incubated at 30°C for 24 h, followed by measurement of zones of inhibition. Specific growth rate (μ) and rate of decrease in ion concentration were determined by inoculation of each of the isolates into synthetic pond water (0·0085% m/v KNO_3, 0·006% m/v $NaNO_2$, 0·0093% m/v $(NH_4)_2SO_4$, 0·0038% m/v H_3PO_4, 0·1% m/v yeast extract and 0·1% m/v glucose) which was composed to amplify detection of effects that would typically be found in conventional pond water, and was prepared by dissolution of the ingredients in tap water, pH adjustment to 7·00 using NH_4OH (25% m/v) and sterilization through a 0·22 μm sterile

filter. Synthetic pond water (100 ml) was decanted into presterilized 500 ml Erlenmeyer flasks. Each flask was inoculated with one cryovial of each isolate and incubated at 30°C on a rotary shaker at 220 rev min^{-1} (Innova 2300, New Brunswick Scientific, Edison, NJ, USA). Each flask was aseptically sampled (5 ml) prior to inoculation and thereafter two hourly up until the stationary growth phase was observed. The specific growth rate (μ) was determined from $OD_{660\ nm}$ measurements (Genesys 20 spectrophotometer, Spectronic, USA) for data points conforming to high linearity ($r^2 > 0{\cdot}9$) of a plot of $\ln(OD_{660\ nm})$ against time. Samples were analysed for ion concentrations of ammonium (Reflectoquant, Cat. No. 1·16892·0001, Merck, Darmstadt, Germany), and nitrate, nitrite and phosphate which were measured by ion exchange chromatography (Morales *et al.* 2000) using Ion Chromatography (Dionex, Sunnyvale, USA) with an anion precolumn and anion separator column (Dionex AG14 and AS14, Sunnyvale, USA). The rate of decrease in the concentrations of ammonium, nitrite, nitrate and phosphate ions by each isolate in synthetic pond water was determined by using data points conforming to high linearity ($r^2 > 0{\cdot}9$) of a plot of ion concentration against time. Statistical comparison of data was by two-tailed *t*-tests assuming equal variance.

Verification of performance of selected isolates by *in vitro* co-cultivation with pathogenic *Aeromonas hydrophila*

Selection criteria for isolates were grouped into growth rate, inhibition of pathogen and decrease of waste ion concentration, and the response values normalized as a relative percentage of the maximum for each grouping to provide an overall suitability index, which indicated the cumulative desirability for each of the isolates for the criteria tested. Each of the selected isolates (B001, B002 and B003) and a control organism (B007; selected on the basis of low growth, low ion removal rates, and inability to inhibit pathogen growth), were co-inoculated with *Aer. hydrophila* into synthetic pond water and cultivated as described previously. Combinations of the selected isolates (B001 + B002, B001 + B003, B002 + B003, B001 + B002 + B003) were similarly tested. The viable cell count of *Aer. hydrophila* was determined by serial dilution and plating on selective agar (Kielwein *et al.* 1969; Kielwein 1971), whereas the viable cell count of *Bacillus* spp. was assessed on Nutrient Agar plates supplemented with Polymyxin B (Donovan 1958) as described previously. All trials were conducted in triplicate. Specific growth rate (based on viable cell count) and rate of decrease in ion concentration were determined as previously described.

Identification of selected isolates by genetic evaluation – 16S RNA sequences

Amplification of the 16S gene of each selected isolate (B001, B002 and B003) was performed (University of Cape Town, DNA Sequencing Laboratory, Cape Town, South Africa) using five sets of forward and reverse overlapping sequence primers, which allowed sequencing of the entire length of the double-stranded DNA (approx. 1423 bp). The five sets of forward (^{f}) and reverse (^{r}) primers (5′–3′) were as follows (Alm *et al.* 1996).

^{f1}AGAGTTTGATCTGGCTCAG ^{r1}GTATTACCGCGGCTGCTGGCAC

^{f2}ACTCCTACGGGAGGCAGCAG ^{r2}GGACTACCGGGTATATCTAATCC

^{f3}GCCAGCAGCCGCGGTAATAC ^{r3}CACGAGCTGACGACACCATGC

^{f4}GGATTAGATCCCGGTAGTCC ^{r4}CCATTGTAGACGTGTGAGCCC

^{f5}GCATGGTGTCGTCAGCTCGTG ^{r5}ACGGTACCTTGTTACGACTT

Sequencing was carried out using a DYEnamic ET Dye Terminator Cycle Sequencing kit for MegaBACE based on traditional dideoxynucleotide chain termination chemistry (Lane 1991). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). PCR cleanup was performed using Qiaquick PCR purification kits (Qiagen, Cat. No. 28104, Hilden, Germany), High Pure PCR Product purification kits (Roche Applied Science, Cat. No. 1 732 668, IN, USA) or using Post Reaction Purification columns (Sigma, Cat. No. S-5059, St Louis, MO, USA) as per manufacturer's instructions. Sequence alignments were performed using BLASTN, available on the NCBI server (http://www.ncbi.nlm.nih.gov).

Safety assessment of selected isolates

Anthrax detection was performed using a BioThreat Alert™ kit (Tetracore Inc, Rockville, USA) according to manufacturer's instructions. Additionally, presence of *B. anthracis* virulence plasmids (pOX1 and pOX2) in isolate B002 was examined using the LightCycler *Bacillus anthracis* Detection kit (Roche Applied Science, Cat. No. 03303411001, Basel, Switzerland), which allows specific amplification of *capB* and *pagA* genes. Total genomic DNA extraction of *B. cereus* was performed using a High Pure PCR Template Preparation kit (Roche Applied Science, Cat. No.11796828001, Basel, Switzerland). Real-Time PCR was performed using a LightCycler 2·0 (PerkinElmer, MA, USA) according to the manufacturer's instructions which enabled on-line evaluation of

amplification efficiency as well as melting curve analysis to access the accuracy of individual PCR preparations and reactions. Positive controls contained *capB* and *pagA* amplicons as template DNA (50–100 ng) and the negative controls contained PCR water. Two separate reactions were performed using specific primers targeting amplification of genes encoding capB and pagA proteins and using template DNA (50–100 ng) from the *B. cereus* isolate (B002). Evaluation of *B. cereus* enterotoxin production was performed using the Oxoid *Bacillus cereus* diarrhoeal toxin kit (BCET-RPLA, Oxoid Ltd, UK) according to manufacturer's instructions.

Preliminary assessment of efficacy of selected isolates *in vivo*

A combination of the isolates B001, B002 and B003 were tested *in vivo* to verify the effects observed *in vitro*, whereby pathogen inhibition and decreases in the concentrations of waste ions excreted by *C. carpio* were investigated. The experiment was carried out in glass aquaria (100 l) comprising triplicate control and test systems. Each aquarium was equipped with three air diffusers, a base filter and a reticulated filter chamber. Filter chambers were packed with biological filter matting (1·22 g l^{-1}). The water recirculation rate and air flow rate were 6·0 and 1·8 l per litre of tank volume per hour, respectively. Each aquarium was purged every 3 days (5% v/v) and tap water was added to maintain the starting volume. Each aquarium was stocked with 11 juvenile *C. carpio* with an average mass of 3·49 ± 0·13 g and average length of 59·80 ± 1·07 mm per specimen. The stocking density in each aquarium was 0·38 ± 0·01 g l^{-1}. Fishes were fed commercial koi pellets (3 mm diameter, ∼35% m/m protein) twice daily at a rate of 5% of the initial total body mass, per day, for the duration of the trial. The pH was maintained between 7·0 and 8·0 and was adjusted with either HCl (1% m/m) or $CaCO_3$ (2 mol l^{-1}). The temperature was maintained in the range 20 ± 1°C using an aquarium heater (150 W). Temperature and dissolved oxygen were measured daily using a Multi 350i multimeter (WTW, Weilheim, Germany). The system was not cleaned for the duration of the trial. Control aquaria were dosed with a water placebo, whereas test aquaria were treated with a mixture (1 ml each) of equal proportions of the isolates B001, B002 and B003 at a dosage rate of 1×10^5 CFU l^{-1} at 7-day intervals. *Aeromonas hydrophila* was dosed at the same level to all aquaria. The dosage rates of the isolates and *Aer. hydrophila* were based on previous studies examining dosage and efficacy response *in vitro* (data not presented), wherein 1×10^5 CFU l^{-1} was determined to be a suitable dosage. The dosage regime could furthermore be realistically applied in commercial systems for *Bacillus*-based products. The control and test aquaria were sampled every second day and the water samples analysed for total count of *Bacillus* spp., *Aer. hydrophila* and oxygen, ammonium, nitrite, nitrate and phosphate concentrations. The test and control treatments were compared over the 80-day period by examining the frequency of occurrences, where the concentrations of key measurables were significantly lower ($P < 0·05$) in the test than the control treatment because an overall analysis of averages is unreliable in naturally fluctuating systems. At the end of the trial period, the fishes were removed from the aquaria, the sludge and slime contents washed into the water phase and homogenized prior to the collection of three randomly selected samples. All samples were analysed as described previously.

Results

Isolation, selection and evaluation of isolates *in vitro*

Nine isolates belonging to the genus *Bacillus* were isolated by the procedure described. Six of the isolates were obtained from mud sediment of a dam used for the rearing of *C. carpio*, two from the intestinal content and one from the skin mucus layer of *C. carpio*. All isolates obtained were spore forming, Gram-positive and catalase positive rods and identified as *Bacillus* spp.

Results for evaluation of growth rate, pathogen inhibition and decrease in the concentrations of waste ions *in vitro* are tabulated in Table 1. The highest growth rates were observed for the isolates B001, B002 and B003, with the remaining isolates demonstrating substantially lower growth rates. Only the isolates B002 and B003 inhibited the growth of *Aer. hydrophila* in plate well inhibition assays. The isolate B001 reduced the concentrations of all four of the ions measured, whereas the isolates B002 and B003 reduced the concentrations of all the ions except phosphate. The remaining isolates reduced the concentrations of two or less of the four ions measured. The suitability index, based on growth rate, pathogen inhibition and rate of decrease in ion concentration indicated that the isolates B002, B001 and B003 displayed suitability indices of 84·1%, 44·1% and 41·6%, respectively, which were significantly higher ($P < 0·01$) than any of the other isolates tested. Based on these results, the isolates B001, B002 and B003 were selected as candidate putative biological agents and subjected to further study. The isolates B001, B002 and B003 were therefore deposited at the Netherlands Culture Collection of Bacteria as NCCB 100131, 100132 and 100133, respectively. Isolate B007 resulted in the lowest suitability index and was selected as a control organism for these studies.

Table 1 Summary of responses of isolates against criteria measured *in vitro*

Isolate No.	Specific growth rate μ (h^{-1})	Inhibition zone diameter	Ion reduction rate (mg l^{-1} h^{-1})			
			Ammonium	Nitrite	Nitrate	Phosphate
Control	0·000	NI	NR	NR	NR	NR
B001	1·400	NI	1·000	0·606	3·550	7·205
B002	1·609	+(16 mm)	1·371	2·025	8·575	NR
B003	1·211	+(18 mm)	0·514	1·138	1·900	NR
B004	0·702	NI	0·486	NR	NR	5·750
B005	0·659	NI	0·429	NR	NR	NR
B006	0·616	NI	1·329	NR	NR	9·275
B007	0·162	NI	0·565	NR	NR	NR
B008	0·235	NI	0·619	NR	NR	NR
B009	0·181	NI	1·071	0·988	NR	NR

NI, no inhibition; NR, no reduction.

Table 2 Comparison of combinations of isolates against single isolates selected *in vitro*

Isolate No.	Pathogen growth rate (h^{-1})	Ion reduction rate (mg l^{-1} h^{-1})			
		Ammonium	Nitrite	Nitrate	Phosphate
Control B007	0·437	0·077	0·056	0·014	0·230
B001	0·240	0·860	2·314	1·440	3·412
B002	−0·199	1·160	5·300	2·860	1·188
B003	−0·173	0·594	1·944	2·622	1·146
B001 + B002	−0·571	1·900	5·667	2·183	1·888
B001 + B003	−0·465	1·145	4·830	1·683	1·457
B002 + B003	−0·572	1·000	4·910	2·467	1·322
B001 + B002 + B003	−0·622	2·313	6·730	3·150	3·430

Co-cultivation of the isolates B002 or B003 with pathogenic *Aer. hydrophila* in synthetic pond water resulted in complete growth inhibition of the pathogenic bacteria (Table 2). The growth rate of *Aer. hydrophila* was −0·199 and −0·173 when co-cultivated with isolates B002 and B003, respectively. All the test isolates grew favourably. Isolate B001 attenuated pathogen growth rate (0·238 h^{-1}) in comparison with the control organism B007 (0·606 h^{-1}) (Table 2). Isolate B002 resulted in the highest rate of decrease of ammonia, nitrate and nitrite concentrations, whereas isolate B001 demonstrated the highest rate of decrease of phosphate concentration. All the three selected isolates had a significantly higher ($P < 0·01$) rate of decrease of all ions, in comparison with the control isolate B007, which demonstrated a negligible decrease in concentration of any of the ions measured (Table 2). Additionally, the combination of all three of the selected isolates (B001 + B002 + B003) resulted in a significantly lower ($P < 0·01$) pathogen growth rate and higher rate of decrease in ion concentration when compared with single and paired combinations of isolates (Table 2). The percentage composition of B001, B002 and B003 was 33%, 28% and 29%, respectively, at the end point of this test.

Identification and safety assessment of selected isolates

Based on the preliminary identification by API 50 CHB/CHE, the selected isolates B001, B002 and B003 were classified as *B. subtilis*, *B. mycoides* and *B. licheniformis*, respectively. As a result of inaccuracies at species level in the API system, the identification of the isolates was verified by 16S RNA sequence homology. The results of the 16S RNA identification of the isolates B001 and B003 matched the tentative identification by API 50 CHB/CHE and were classified as *B. subtilis* (99%) and *B. licheniformis* (98%), respectively. Isolate B002 was reclassified as *B. cereus* based on the results of the BLAST search which corresponded to a match of 100%.

The close relationship between *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides* is well documented (Leonard *et al*. 1998). Identical 16S rRNA sequences for *B. anthracis* and *B. cereus* have also been reported (Ash and Collins 1992). Results of the rapid qualitative test (Bioalert™) revealed that none of the three isolates selected were positive for anthrax toxins (data not show). Molecular level differentiation (Real-time PCR) specifically targeting amplification of plasmid-borne virulence factor genes clearly showed that the *B. cereus* isolate (B002) did not contain the genes encoding any of the virulence plasmids (pOX1 and/or pOX2). Amplification of these virulent genes in positive control reactions using *capB* and *pagA* amplicons as template DNA was observed. Furthermore, results of the melting curve analysis were positive for DNA unfolding and folding, thus indicating successful PCR reactions. This result further confirmed the identification of the isolate B002 as *B. cereus*, which warranted further confirmation that the isolate did not produce the *B. cereus* diarrhoeal enterotoxin. The isolate

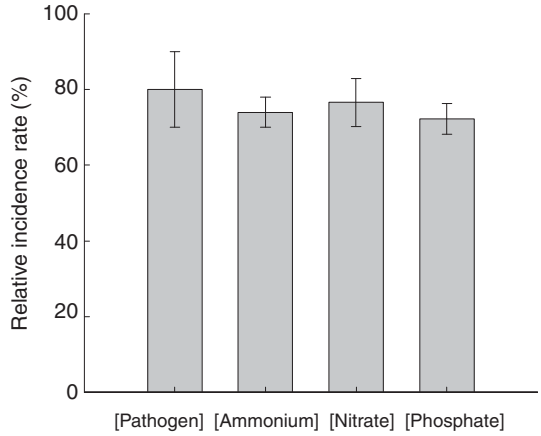

Figure 1 Incidence rate (%) where concentrations of key responses were lower in test than in control treatments *in vivo*.

B002 was found to be negative for the presence of this enterotoxin (data not shown).

Preliminary assessment of efficacy of selected isolates *in vivo*

Based on the results of the *in vitro* study and the biosafety assessment, the isolates B001, B002 and B003 were examined *in vivo*. The data analysed over the 80-day time period are presented as a frequency distribution of occurrences in the test treatments in comparison with the control treatments in Fig. 1. Pathogen, ammonium, nitrite and nitrate concentrations were significantly lower in test treatments when compared with control treatments in 80%, 74%, 77% and 72% of the occurrences measured ($n = 120$, $P < 0{\cdot}05$), respectively. Nitrite was not detectable in any of the treatments for the duration of the trial. There were no mortalities or observable incidents of disease and there were no significant differences in oxygen concentration ($P = 0{\cdot}391$), fish mass ($P = 0{\cdot}522$) or length ($P = 0{\cdot}276$) gain. The results of pathogen and ion concentrations measured in control and test treatments at the terminal point of the *in vivo* trial (Fig. 2) were similar to the frequency distribution data over the 80-day period, wherein pathogen, ammonium, nitrate and phosphate concentrations were significantly lower in test treatments when compared with the control treatments ($P < 0{\cdot}001$). The cumulative concentration of *Bacillus* spp. was significantly higher in the test treatment ($P < 0{\cdot}001$), whereas the pathogen concentration was correspondingly lower when compared with the control treatment. In all cases, the percentage composition of the isolates B001, B002 and B003 approximated that observed in the *in vitro* study.

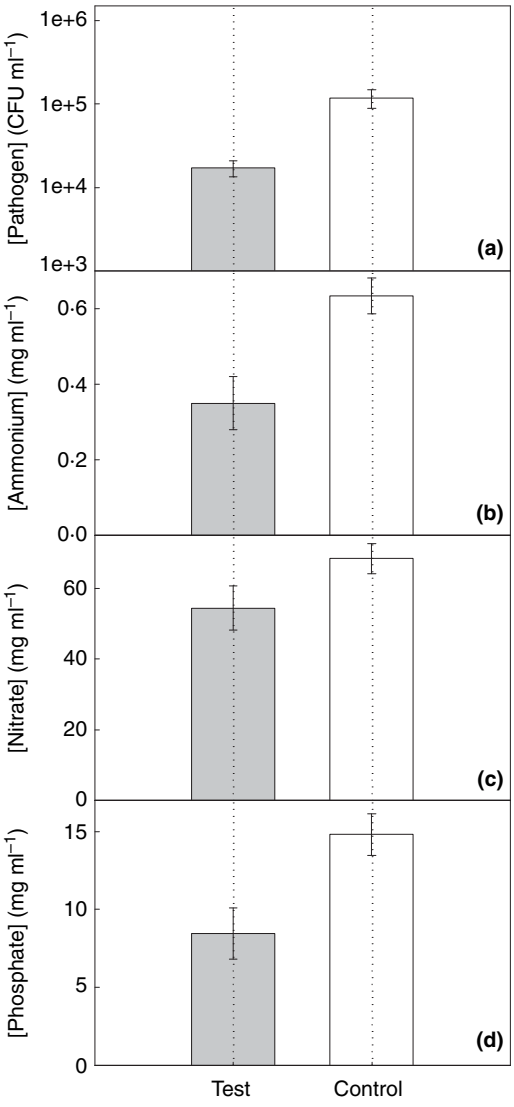

Figure 2 End point comparisons of test and control concentrations of key responses *in vivo* (a) pathogen, (b) ammonium, (c) nitrate and (d) phosphate.

Discussion

Application of spore-forming bacteria as biological agents for improving water quality and reducing disease offers a number of advantages (Sanders *et al.* 2003; Wolken *et al.* 2003) and a number of spore-forming biological agents are sold worldwide for animal use (Sanders *et al.* 2003). The isolation procedure yielded nine *Bacillus* isolates and similar approaches based mainly on resistance of endospores to elevated temperatures have elsewhere been reported for isolation of *Bacillus* spp. (Foldes *et al.* 2000).

Attenuation of growth of *Aer. hydrophila* by the isolate B001, inhibition of growth by the isolates B002 and B003 and a decrease in pathogen cell number when a mixed

culture (B001 + B002 + B003) was tested in synthetic pond water (Table 2) could potentially be ascribed to the mechanism of competitive exclusion (Sanders *et al.* 2003; Hong *et al.* 2005), because the average growth rates of the selected isolates were in excess of 1·5 times than that of *Aer. hydrophila* (data not shown), when compared with the control treatment (B007). These isolates therefore demonstrate potential for reduction of *Aer. hydrophila* in aquaculture systems (Vanbelle *et al.* 1990; Matoyama *et al.* 1999; Moriarity 1999). Furthermore, the isolates B001, B002 and B003 decreased the concentrations of ammonia, nitrite and nitrate ions in synthetic pond water (Table 2). The isolate B001 decreased the ammonia concentration more rapidly than the nitrite concentration, whereas the isolates B002 and B003 decreased the concentration of nitrite more rapidly than that of ammonia, indicating that application of B002 and B003 could prevent nitrite accumulation. All three isolates were capable of decreasing the concentrations of nitrates at rates exceeding that of ammonia and nitrite, thus indicating a potential synergistic benefit for use of all three isolates as biological agents. In this study, the selected isolates demonstrated significantly higher rates of decrease in waste ion concentrations ($P < 0{\cdot}01$) than the control organism and this phenomenon was also previously observed in *B. subtilus*, *B. cereus* and *B. licheniformis* (Kim *et al.* 2005). The mixed culture of the selected isolates may potentially exert these effects via a multitude of mechanisms such as bioaccumulation, bio-assimilation, nitrification and dissimilatory nitrate reduction. Although nitrogen removal is classically predominated by autotrophic bacteria in natural systems, there have been several reports suggesting a contribution by heterotrophic bacteria in this regard (Abou Seada and Ottow 1985; Robertson and Kuenen 1990; Sakai *et al.* 1996; Sakai *et al.* 1997; Martienssen and Schöps 1999; Su *et al.* 2001; Kim *et al.* 2005; Lin *et al.* 2006). Isolate B001 decreased the concentration of phosphate ions in primary studies (Table 1), but all the selected isolates decreased phosphate concentration in the presence of the pathogen (Table 2), which may have been caused by improved bio-availability of bound phosphate, through solubilization (Illmer and Schinner 1995). This attribute is important as phosphate and nitrate accumulation can result in algal blooms in culture systems (Kaushik 1995).

In addition to quantification of the efficacy of the selected isolates, the identification and safety assessment is an important requirement fulfilled by this study. The identification of organisms within the *B. cereus* group is difficult because of the genetic similarity between *B. cereus*, *B. anthracis* and *B. thuringiensis* (Carlson *et al.* 1994; Phelps and McKillip 2002; Helgason *et al.* 2004; Rasko *et al.* 2004). In the present study, 16S rRNA-based identification of *B. cereus* was limited by the nearly identical 16S rRNA sequences of the group members (Ash *et al.* 1991; Ash and Collins 1992). Definitive identification and safety of B002 was therefore based on the absence of key virulence genes, such as the *B. anthracis* lethal toxin complex (pOX1) and poly-D-glutamic acid capsule (pOX2) (Okinaka *et al.* 1999). Furthermore, the absence of anthrax toxin and strain dependent *B. cereus* enterotoxin in isolate B002 (Turnbull 1999) confirms the safety of this isolate for use as a potential biological agent.

The findings of the preliminary *in vivo* trial were similar to those of the *in vitro* tests, wherein there was an attenuation of prevalence of *Aer. hydrophila* and a decrease in the concentrations of ammonium, nitrate and phosphate ions by the mixed culture of the three selected isolates over the 80-day trial period (Fig. 1) and at the end point of the trial (Fig. 2). The treatment did not result in a negative impact on oxygen sufficiency, growth or health of the test specimens, which is an important consideration for potential application of the isolates. Although the interaction of the putative biological agents with the larger bio-community when tested *in vivo* could have resulted in a complex ecological system, the observations were similar to those in the *in vitro* studies. The selection of isolates *in vitro* based on grouped characteristics through suitability indices have resulted in synergistic holistic improvements in water quality *in vivo*. Other researches have also reported that the addition of beneficial bacteria can enhance the health of animals by effecting a holistic improvement in waste ion removal and pathogen reduction (Larmoyeux and Piper 1973; Liao and Mayo 1974; Jeney and Jeney 1995; Shimeno *et al.* 1997; Boyd and Tucker 1998; Frances *et al.* 1998; Frances *et al.* 2000). Results have clearly indicated the synergistic positive effect of the selected isolates of *Bacillus* spp. on pathogen inhibition and water quality *in vitro* and *in vivo*. The selected isolates should be further evaluated in larger scale *in vivo* trials and an in-depth assessment of the mode of action regarding pathogen inhibition and removal of waste ions should be made, prior to progress towards commercial products.

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Chapter 5 Publication 2

Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent

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ORIGINAL ARTICLE

Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent

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Abstract

Aims: To determine the contribution of potential modes of action of a *Bacillus cereus* aquaculture biological control agent in inhibition of the fish pathogen, *Aeromonas hydrophila*.

Methods and Results: When *B. cereus* was tested in plate well inhibition studies, no production of antimicrobial compounds was detected. *Bacillus cereus* had a high growth rate (0·96 h^{-1}), whereas *Aer. hydrophila* concentration decreased by *c.* 70% in co-culture experiments. In nutrient limitation studies, *B. cereus* had a significantly higher growth rate when cultured under glucose ($P < 0·05$) and iron ($P < 0·01$) limitation in comparison with *Aer. hydrophila.* *Bacillus cereus* glucose (0·30 g l^{-1} h^{-1}) and iron (0·60 mg l^{-1} h^{-1}) uptake rates were also significantly higher ($P < 0·01$) than the *Aer. hydrophila* glucose (0·14 g l^{-1} h^{-1}) and iron (0·43 mg l^{-1} h^{-1}) uptake rates. Iron uptake was facilitated by siderophore production shown in time profile studies where relative siderophore production was *c.* 60% through the late exponential and sporulation phases.

Conclusions: Competitive exclusion by higher growth rate, competition for organic carbon and iron, facilitated by siderophore production, could be identified as mechanisms of pathogen growth inhibition by *B. cereus.*

Significance and Impact of the Study: This study is the first elucidation of the mechanism of action of our novel *B. cereus* biological agent in growth attenuation of pathogenic *Aer. hydrophila*. This study enhances the application knowledge and attractiveness for adoption of *B. cereus* NRRL 100132 for exploitation in aquaculture.

Introduction

Global aquaculture is challenged by poor water quality and the outbreak of diseases (Jeney and Jeney 1995; Moriarty 1999). The use of conventional chemotherapies has resulted in the increased virulence of pathogenic strains, negative environmental impact and is often met with consumer resistance (Verschuere *et al.* 2000). Exploitation of beneficial bacteria as biological agents has potential advantages to address aquaculture challenges by improving water quality and reducing disease propensity caused by pathogenic bacteria (Fast and Menasveta 2000; Gomez-Gill *et al.* 2000; Jana and Jana 2003; Hong *et al.* 2005). Water quality and infection by pathogenic *Aeromonas hydrophila* are major challenges in the highly lucrative aquaculture of *Cyprinus carpio*.

A novel *Bacillus cereus* (NRRL 100132) strain was previously isolated as a biological agent for *C. carpio* and its outstanding capability in enhancing water quality and reducing *Aer. hydrophila* growth was demonstrated in both *in-vitro* and *in-vivo* studies (Lalloo *et al.* 2007). This *B. cereus* is a water additive and was shown to be safe for use. The functionality of the micro-organism was also demonstrated across a range of physiological conditions,

prevalent in aquaculture (Lalloo *et al.* 2008). Spore-forming *Bacillus* spp. are attractive as biological control agents as they possess antagonistic effects on pathogens, can improve water quality and are ubiquitous in natural environments (Wolken *et al.* 2003; Hong *et al.* 2005). Spores are physiologically robust and can be formulated into stable commercial products which are tolerant to the environmental conditions required in their application (Gross *et al.* 2003; Lalloo *et al.* 2009).

The success of strategies using biological agents and adoption of this technology by the aquaculture industry depends on an understanding of the beneficial characteristics and mechanism of action (Verschuere *et al.* 2000; Vine *et al.* 2006). However, studies showing the mode of action for antagonism of *Aer. hydrophila* by *Bacillus* spp. are limited, while no studies on the mode of action of *B. cereus* as a biological agent against this pathogen have been reported (Kumar *et al.* 2006; Newaj-Fyzul *et al.* 2007). Potential mechanisms of biological agents against pathogens include competition for adhesion sites, production of enzymes, immune stimulation, synthesis of antimicrobials, competitive exclusion and bioremediation (Verschuere *et al.* 2000; Sanders 2003; Hong *et al.* 2005). The basis of competitive exclusion is through competition for chemicals or for available energy or by intrinsic growth rate advantage (Verschuere *et al.* 2000; Holzapfel *et al.* 2001; Irianto and Austin 2002; Hong *et al.* 2005). Many of these mechanisms only apply to probiotics added to feed, but the latter three are relevant to waterborne additives such as *B. cereus*.

The bioremediation capability for ammonium, nitrite, nitrate and phosphate waste removal by *B. cereus* NRRL 100132 was well elucidated previously (Lalloo *et al.* 2007). Likely modes of action by our *B. cereus* isolate in antagonism of *Aer. hydrophila* are the production of inhibitory compounds and competitive exclusion. Fastidious heterotrophs such as *Bacillus* spp. often demonstrate a high utilization of organic carbon (Verschuere *et al.* 2000). Some are also capable of synthesizing low molecular weight chelating compounds called siderophores which facilitate competitive uptake of iron for growth (Verschuere *et al.* 2000; Winkelmann 2002). As both carbon and iron are essential requirements for growth by most organisms, limitations can result in growth attenuation (Braun and Killmann 1999). In this study, we investigated the contribution of direct inhibition by production of extracellular inhibitory compounds and competitive exclusion through growth rate advantage, competition for key nutrients such as organic carbon and iron as potential modes of action involved in the inhibition of the important fish pathogen, *Aer. hydrophila* by our novel *B. cereus* aquaculture biological agent.

Materials and methods

Micro-organisms used in the study

Our novel isolate *B. cereus* (NRRL 100132) and a test pathogen Aer. *hydrophila* (ATCC 7966) was cultured and stored as described previously (Lalloo *et al.* 2007).

Detection of antimicrobial activity of *Bacillus cereus* NRRL 100132

The production of antimicrobial compounds by *B. cereus* NRRL 100132 was assessed by culturing the strain in 2 l Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) as previously described (Lalloo *et al.* 2009). Airflow was maintained at 1 v v^{-1} m^{-1}, and agitation speed was ramped from 500 rev min^{-1} to a maximum of 1000 rev min^{-1} to maintain oxygen saturation above 30%. All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

Fermenters were sampled during early exponential, mid exponential and the sporulation phase. The growing culture (fermentation broth sample), intracellular cell fraction and extracellular supernatant were evaluated for the presence of inhibitory compounds. The extracellular fraction was the resultant supernatant after centrifugation of the whole broth at 13 000 ***g***. The resultant cell pellet was washed, re-suspended in saline (0·9% m v^{-1} NaCl) and ultra-sonicated at a frequency of 20 kHz s^{-1} at 192 watts on ice for 12 min (12 × 48 s cycles of sonication with a 12-s pause between cycles) and then re-centrifuged. The supernatant of this cell preparation was used as the intracellular fraction. Cell preparations (100 μl) of growing culture, intracellular fraction or extracellular supernatants were loaded into wells (10 mm) on nutrient agar plates prespread with *Aer. hydrophila* (ATCC 7966) culture. Plates were incubated (12 h, 32°C) and visualized for zones of inhibition.

Co-culture of *Bacillus cereus* and *Aeromonas hydrophila* in shake flasks

Stored cryo-cultures (2 ml) of *Aer. hydrophila* and *B. cereus*, prepared according to Meza *et al.* (2004), were used to inoculate triplicate 1-l Erlenmeyer flasks, containing synthetic pond water (SPW) growth medium and the culture flasks incubated (Lalloo *et al.* 2007). Similarly, a negative control co-culture study devoid of iron was conducted. Samples were taken two hourly, and cell counts were performed using a Thoma® bacterial counting chamber (Hawksley & Sons, London, UK) for both organisms.

Comparison of growth rate between *Bacillus cereus* and *Aeromonas hydrophila* under nutrient limitation

The impact of nutrient limitation on growth of *B. cereus* or *Aer. hydrophila* was assessed by lowering the concentration of one media component (glucose, nitrite, nitrate, ammonia, iron or phosphate) in SPW to 10% of base case. De-ionized water was the negative control and SPW was the positive control.

Media were prepared by combining amino acid, vitamin, trace element, nutrient and ion solutions. Each media formulation contained 20 μl of an amino acid solution (45 mg l^{-1} each of the following: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine), 20 μl of a vitamin solution (Lalloo *et al.* 2009) and 20 μl of a trace element solution ($CaCl_2$ 3·4 mg l^{-1}, $MgCl_2{\cdot}4H_2O$ 2·6 mg l^{-1}, H_3BO_3 5·0 mg l^{-1}, Na2$MoO_4{\cdot}2H_2O$ 0·3 mg l^{-1}, $CoCl_2{\cdot}6H_2O$ 0·4 mg l^{-1}). The nutrient solution (glucose 10·0 g l^{-1}) and ion solution [$NaNO_2$ 0·6 g l^{-1}, KNO_3 0·85 g l^{-1}, $FeC_6H_6O_7$ 0·16 g l^{-1}, $(NH_4)_2SO_4$ 0·93 g l^{-1} and H_3PO_4 3·8 g l^{-1}] were added as 20 μl aliquots to the media. Once all media components were added, the volume of each well was made up to 200 μl with de-ionized water. All solutions were sterilized by filtration through 0·22-μm filters.

Cultures of *B. cereus* NRRL 100132 or *Aer. hydrophila* (ATCC 7966) were grown (Lalloo *et al.* 2007) to 1×10^5 cells per ml, and an inoculum volume of 10 μl was used to inoculate the respective micro-titre wells (six wells per organism per test). Plates were incubated at 32°C for 24 h on a microtitre plate shaker set at 100 rev min^{-1}, and absorbance was measured and recorded every hour at 660 nm (Abs_{660}) using a BioTek Power wave^{HT} microtitre plate reader (BioTek Instruments Inc., Vermont, USA). Growth rates were determined from plots of the natural logarithm of Abs_{660} over time, conforming to linearity ($r^2 > 0{\cdot}9$). The growth rates obtained for both *B. cereus* and *Aer. hydrophila* were compared (ANOVA) to assess the impact of the individual component limitations on the growth of the two organisms (Table 1).

Table 1 Assessment of different cell preparations for the growth attenuation of *Aeromonas hydrophila* by *Bacillus cereus*

	Growing culture	Intracellular fraction	Extracellular supernatant
Mid exponential phase	+	–	–
Early stationary phase	+	–	–
Sporulation phase	+	–	–

–, No inhibition observed; +, presence of inhibition.

Measurement of glucose and iron uptake rates

Cryopreserved cultures of *B. cereus* or *Aer. hydrophila* were used to inoculate 1-l Erlenmeyer flasks containing 100 ml of sterile SPW in triplicate and incubated as previously described. Samples were taken on an hourly basis and analysed for iron and glucose concentrations. Iron concentrations were determined using a Spectroquant® kit 1.14549.0001 (Merck). Glucose concentrations were determined using an HPIC (CarboPac™, PA1 column; Dionex, Sunnyvale, MA, USA). Uptake rates were calculated from plots of concentration of iron or glucose against time for each micro-organism.

Measurement of siderophore production

Bacillus cereus (NRRL 100132) was used to inoculate 100 ml of sterile SPW in 1-l Erlenmeyer flasks and incubated as previously described. Flasks were sampled two hourly, and the cell and spore concentrations were determined, from which the sporulation ratio was calculated (Monteiro *et al.* 2005). Qualitative siderophore production using a modified chrome azurol S (CAS) assay (Milagres *et al.* 1999) and semi-quantitative siderophore production using the CAS universal siderophore assay (Schwyn and Neilands 1987) were assessed. The qualitative assessment of siderophore production in the culture medium was visualized by a colour change from blue to orange on modified CAS agar plates. For the semi-quantitative assay, the amount of siderophore present in the test sample was reported as a percentage relative to a control sample of which the siderophore concentration was known.

Results

Inhibition of growth by production of an antibacterial compound

Zones of inhibition of *Aer. hydrophila* growth were observed during the exponential, early stationary and sporulation phases when viable cells were tested in plate well assays. However, plate well assays testing intracellular extracts or extracellular supernatants did not show any antagonism of *Aer. hydrophila* by *B. cereus* during the entire growth cycle (Table 1).

Investigation into competitive exclusion in co-culture studies

Co-culture experiments were conducted by cultivating *B. cereus* and *Aer. hydrophila* together in shake flasks. *Bacillus cereus* displayed a typical growth profile

($\mu = 0{\cdot}96$), but there was a drastic decrease in the cell density of the pathogenic *Aer. hydrophila* population. When *B. cereus* cell concentration peaked, the pathogen had decreased by more than 70% of the starting concentration (Fig. 1a). In co-culture studies devoid of iron (Fig. 1b), a drastic decrease in *Aer. hydrophila* cell numbers was observed. *Bacillus cereus* cell number also decreased, although the initial death rate was more gradual than that of *Aeromonas hydrophila*.

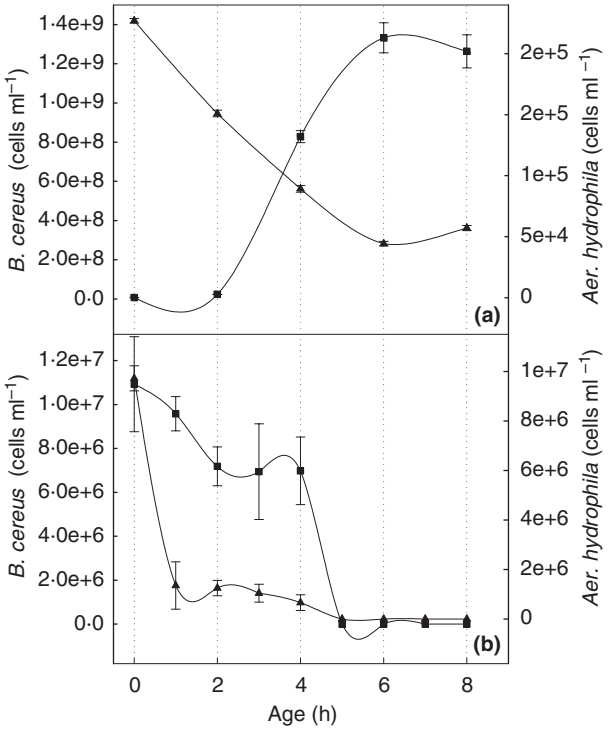

Figure 1 Cell concentration during co-cultivation of *Bacillus cereus* (■) with *Aeromonas hydrophila* (▲) in SPW (a) and SPW devoid of iron (b). SPW, synthetic pond water.

Effect of individual nutrient components on antagonism against the pathogen measured by differential growth rates

Bacillus cereus had a significantly higher growth rate in comparison with *Aer. hydrophila* when cultivated in SPW as a positive control ($P = 0{\cdot}003$), SPW with low iron concentration ($P < 0{\cdot}001$) and SPW with low glucose concentration ($P < 0{\cdot}05$) (Table 2). When media contained reduced concentrations of ammonia, nitrite or nitrate, there was no significant difference in growth between the two organisms ($P > 0{\cdot}05$) (Table 2). Neither of the micro-organisms grew in treatments where phosphate was limited (Table 2).

Evaluation of iron and glucose uptake rates by *Bacillus cereus* and *Aeromonas hydrophila*

During separate batch cultivations under identical conditions, *B. cereus* and *Aer. hydrophila* demonstrated classical exponential growth curves. Trends for glucose uptake from the growth media were linear ($r^2 > 0{\cdot}9$), but not for iron uptake by either of the micro-organisms (Fig. 2). *Bacillus cereus* had an overall iron uptake rate of 0·60 mg l^{-1} h^{-1} and a glucose uptake rate of 0·30 g l^{-1} h^{-1}. These uptake rates were significantly higher ($P < 0{\cdot}01$) than that of *Aer. hydrophila* for iron (0·43 mg l^{-1} h^{-1}) and glucose (0·14 g l^{-1} h^{-1}), respectively.

Evaluation of the production of siderophores

In the qualitative siderophore plate assay, *B. cereus* colony-forming units with orange halos were observed during the exponential growth and sporulation phases (data not shown). This observation was confirmed in the *B. cereus* culture study, where siderophore production was assessed. A maximum growth rate of 0·7 h^{-1} and cell concentration of *c.* $7{\cdot}00 \times 10^7$ cells per ml was achieved (Fig. 3a). The culture reached a high sporulation ratio at *c.* 12 h of growth (Fig. 3b). There was a gradual increase

Table 2 Growth rate assessment of *Bacillus cereus* and *Aeromonas hydrophila* cultivated under nutrient limitation

Treatment	*B. cereus* μ_{max}	Std. dev	*Aer. hydrophila* μ_{max}	Std. dev	Difference in growth rate	*P*-value
Synthetic pond water	0·041	0·001	0·032	0·000	0·009	0·003
De-ionized water	0·000	0·000	0·000	0·002	0·000	0·374
Low glucose	0·033	0·001	0·031	0·001	0·002	0·045
Low nitrite	0·035	0·004	0·031	0·006	0·004	0·473
Low nitrate	0·032	0·001	0·036	0·004	−0·003	0·210
Low ammonia	0·033	0·002	0·036	0·002	−0·003	0·266
Low iron	0·044	0·001	0·033	0·002	0·011	0·001
Low phosphate	0·000	0·000	0·000	0·000	0·000	$^{n}/_{a}$

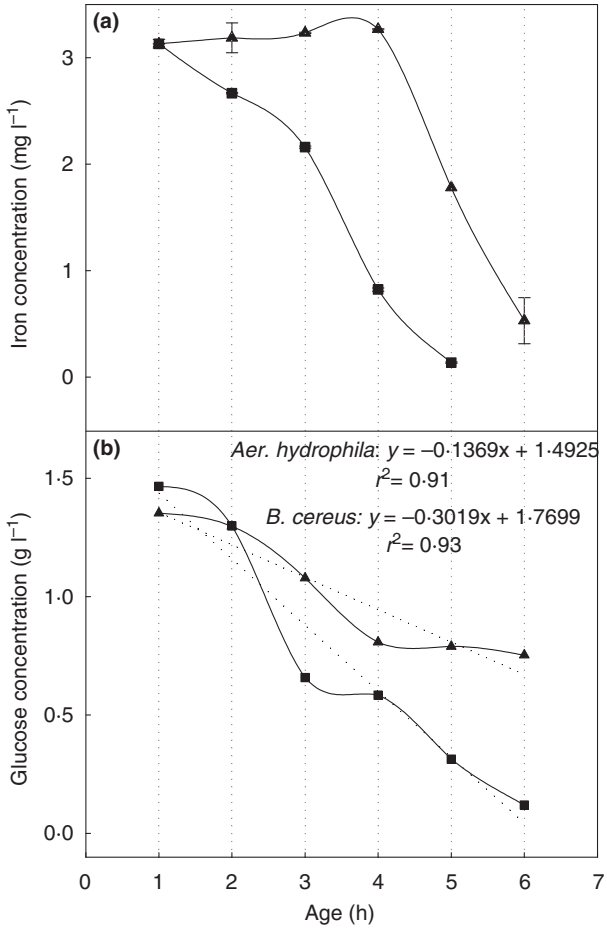

Figure 2 Iron (a) and glucose (b) uptake rates by *Bacillus cereus* (■) and *Aeromonas hydrophila* (▲).

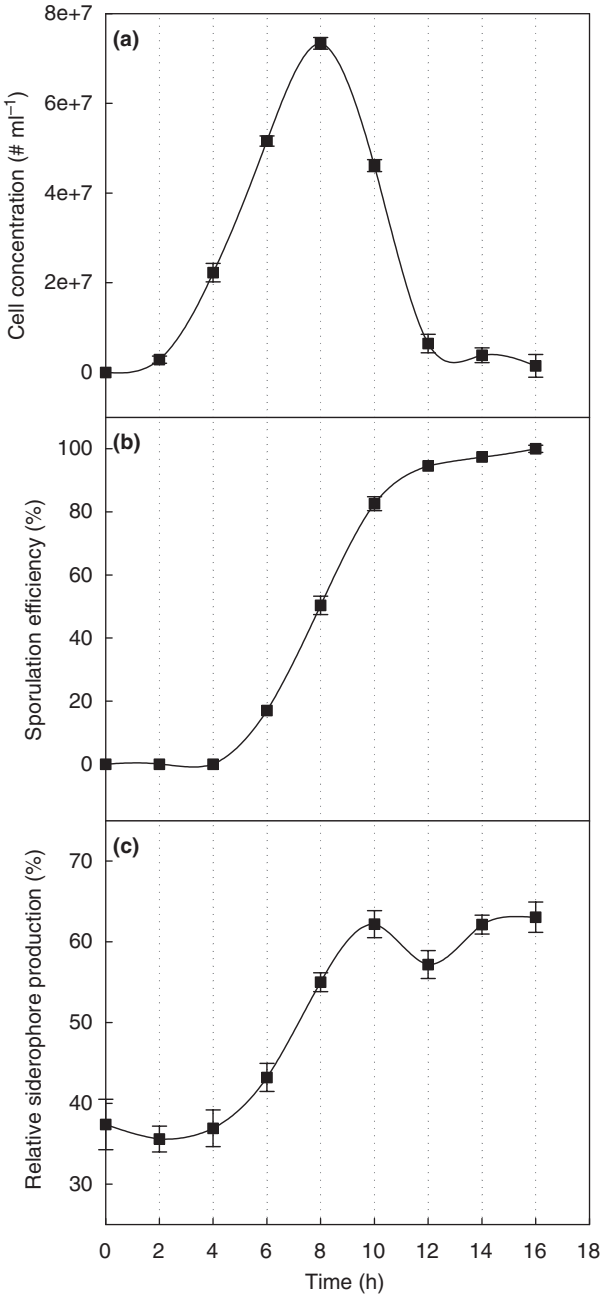

Figure 3 Growth data based on cell concentration (a), sporulation ratio (b) and relative siderophore production (c) by *Bacillus cereus* cultivated in synthetic pond water.

in the production of siderophores during the course of the cultivation (Fig. 3c), reaching a maximum relative sideropohore production of 65% as the culture entered the stationary phase. After completion of sporulation, the siderophore concentration remained at a constant high level.

Discussion

The mode of action of a novel *B. cereus* isolate as a biological agent in aquaculture for the inhibition of pathogenic *Aer. hydrophila* was investigated. The production of antimicrobial compounds by *B. cereus* was excluded as a mode of action based on the absence of growth inhibition of pathogenic *Aer. hydrophila* by intracellular or extracellular fractions of *B. cereus* (Table 1). In contrast, actively growing *B. cereus* cells caused growth inhibition of *Aer. hydrophila*. Although production of antimicrobial compounds is a common mode of action exploited for attenuation of a selected target pathogen in an environment (Fredrickson and Stephanopoulos 1981; Hong *et al.* 2005), this mechanism did not apply to *B. cereus* NRRL 100132. Similar to our findings, Brunt and Austin (2005) showed that their *Bacillus* obtained from the digestive tract of carp also inhibited the growth of pathogenic *Lactococcus garvieae* and *Streptococcus iniae* without showing any signs of antibiosis, thus indicating an alternate

mode of action other than production of antimicrobial compounds.

Competitive exclusion through an intrinsically higher growth rate and competitive uptake of essential nutrients was identified as a mode of action involved in the antagonism of *Aer. hydrophila* by *B. cereus*, based on co-culture data (Fig. 1). Co-cultivation of *B. cereus* together with *Aer. hydrophila* in SPW resulted in a decline of more than 70% in the cell density of the pathogenic organisms in a remarkably short time period (Fig. 1a).

Competitive exclusion was partly attributed to a substantially higher growth rate of *B. cereus* ($0{\cdot}96\ h^{-1}$) in comparison with *Aer. hydrophila,* where cell death was observed. These findings further confirmed our previous work where pathogen decline was proven in *in vitro* and *in vivo* studies, when *B. cereus* was administered as a biological agent (Lalloo *et al.* 2007). Several previous studies have reported higher growth rate as a likely mechanism of biological agents in the inhibition of other micro-organisms (Moriarty 1998; Pinchuk *et al.* 2001; Patterson and Burkholder 2003).

In addition to the intrinsically higher growth rate, competition for the essential nutrients, glucose and iron, contributed to the mechanism of competitive exclusion of *Aer. hydrophila* by *B. cereus*. Competitive exclusion by an intrinsically higher growth rate is often linked to competitive uptake of essential nutrients such as iron and glucose (Rico-Mora *et al.* 1998; Verschuere *et al.* 2000). As *B. cereus* and *Aer. hydrophila* are both heterotrophic, competition for organic substrates as both carbon and energy sources could be expected, although this mode of action for the inhibition of *Aer. hydrophila* by *B. cereus* has not been demonstrated previously (Verschuere *et al.* 2000). In nutrient limitation studies, *B. cereus* had a significantly higher growth rate than *Aer. hydrophila* in both SPW and SPW with limited glucose or iron (Table 2). We further confirmed these observations in glucose and iron uptake studies (Fig. 2), which indicated a significantly higher uptake ($P < 0{\cdot}001$) of glucose ($0{\cdot}30\ g\ l^{-1}\ h^{-1}$) and iron ($0{\cdot}60\ mg\ l^{-1}\ h^{-1}$) by *B. cereus* in comparison with *Aer. hydrophila* for glucose ($0{\cdot}14\ g\ l^{-1}\ h^{-1}$) and iron ($0{\cdot}43\ mg\ l^{-1}\ h^{-1}$), respectively. When *Aer. hydrophila* iron uptake rates were evaluated, a 4-h lag was observed, whereas *B. cereus* uptake was immediate (Fig. 2). Furthermore, when grown in co-culture media devoid of iron (Fig. 1b), death of the biological agent and the pathogen confirmed the essential requirement for iron by both micro-organisms. Results also indicated that *B. cereus* had a slower death rate and was thus more resilient to iron deficiency than *Aer. hydrophila*. These results indicated that competition through higher growth coupled with the competitive uptake of glucose and iron were key modes of action for antagonism by *B. cereus* (Verschuere *et al.* 2000; Patel *et al.* 2009).

The mechanism of competitive exclusion by competition for iron uptake was facilitated by siderophore production by the *B. cereus* isolate. The strain of *B. cereus* exhibited a growth-associated increase in siderophore concentration during the exponential phase of growth (Fig. 3c). Most importantly, the siderophores remained in the medium during and postsporulation. These results correlated with the work conducted by Patel *et al.* (2009), where siderophore production increased during the exponential phase of growth and remained stable during the sporulation phase, with a similar level of siderophore production to the present *B. cereus* isolate. A qualitative assay revealed a large number of colony-forming units with orange halos (data not shown), confirming the presence of siderophores (Milagres *et al.* 1999). Prior research conducted by Park *et al.* (2005) and Wilson *et al.* (2006) also specifically demonstrated the ability of *B. cereus* to produce siderophores. Studies carried out by Smith and Davey (1993) and Gram *et al.* (1999) demonstrated a positive correlation between the production of siderophores and a decrease in pathogen prevalence. Although *Aer. hydrophila* is itself capable of synthesizing low molecular weight siderophores, termed 'amonabactins', production of the siderophore is thought to be inducible and regulated by extracellular iron concentration (Chart and Trust 1983). *Bacillus cereus* was able to produce siderophores immediately at the start of batch culture (Fig. 3), thereby decreasing the iron concentration to very low levels within the first 5 h (Fig. 2) thus starving *Aer. hydrophila* of iron.

The modes of action for attenuation of growth of pathogenic *Aer. hydrophila* by the *B. cereus* isolate, in particular competitive exclusion by growth rate, competition for essential nutrients such as glucose and iron, and siderophore production, increase its attractiveness as a probiotic and biological agent for aquaculture. The siderophore-producing capability of the *B. cereus* isolate addresses the severe shortage of probiotics able to facilitate competitive exclusion based on iron competition (Patel *et al.* 2009). The absence of antimicrobial activity is beneficial for application of the *B. cereus* isolate as a biological agent, as the presence of antimicrobial substances in aquaculture systems is undesirable because of increased virulence in disease-causing pathogens, negative acceptance by consumers and carryover to the environment (Barker 2000; Jana and Jana 2003). Lack of information on modes of action of biological agents limits the adoption of biological solutions to address the challenges of aquaculture, ultimately perpetuating the use of chemotherapeutic agents (Moriarty 1997; Moriarty 1998; Balcázar *et al.* 2006). The modes of action described here,

combined with previously demonstrated *in vitro* and *in vivo* functionality, the ability to reduce the concentration of waste ions in reticulated aquaculture, physiological tolerance to environmental conditions and bio-safety (Lalloo *et al.* 2007, 2008) renders the *B. cereus* isolate NRRL 100132 as an ideal biological agent to address the many challenges facing modern day intensive aquaculture.

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Chapter 6 Publication 3

High-density spore production of a *B. cereus* aquaculture biological agent by nutrient supplementation

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

High-density spore production of a *B. cereus* aquaculture biological agent by nutrient supplementation

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Abstract Previous studies have demonstrated the efficacy of our *Bacillus cereus* isolate (NRRL 100132) in reducing concentrations of nitrogenous wastes and inhibiting growth of fish pathogens. In vivo efficacy and tolerance to a range of physiological conditions in systems used to rear *Cyprinus carpio* make this isolate an excellent candidate for aquaculture applications. Production cost is an important consideration in development of commercially relevant biological products, and this study examines the optimization of nutrient supplementation, which has an impact on high-density production of spores by fermentation. Corn steep liquor (CSL) was identified as a lower cost and more effective nutrient source in comparison to conventional nutrient substrates, in particular yeast extract and nutrient broth. The improved sporulation performance of *B. cereus* could be related to the increased availability of free amino acids, carbohydrates, and minerals in CSL, which had a positive effect on sporulation efficiency. The impact of nutrient concentration on spore yield and productivity was modeled to develop a tool for optimization of nutrient concentration in fermentation. An excellent fit of the model was confirmed in laboratory fermentation studies. A cost comparison revealed that production using liquid phytase and ultrafiltered-treated CSL was less expensive than spray-dried CSL and supported cultivation of *B. cereus* spores at densities higher than 1×10^{10} CFU ml^{-1}.

Keywords *Bacillus cereus* · Fermentation · Biological agent · Corn steep liquor · Aquaculture

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Introduction

Bacillus spp. are exploited for a wide range of applications, from the synthesis of metabolites to the production of whole-cell biological agents used in human health, biological control, and aquaculture (Rengipat et al. 2000; Sanders et al. 2003). *Bacillus* spp. are ideal as additives in aquaculture because they occur naturally in sediments, are ingested by animals, and are unlikely to acquire antibiotic resistance or virulence genes from fish pathogens such as *Aeromonas* spp. (Moriarity 1999). *Bacillus* spores also have several advantages over vegetative cells as aquaculture additives, including resistance to toxic compounds, temperature extremes, desiccation, and radiation (Wolken et al. 2003). This allows the formulation of stable products (Hong et al. 2005; Ugoji et al. 2006).

Although *Bacillus* biological agents are widely used in aquaculture, there are limited studies on their production and little is known about the impact of nutrient supplementation on the high-density production of bacterial spores by fermentation (Monteiro et al. 2005; Prabakaran et al. 2007). The fermentation medium influences the nutritional and physiochemical environment and directly affects productivity and process economics (Zhang and Greasham 1999). According to current understanding, the development pathway leading from a vegetative cell to a spore is triggered by depletion of either carbon, nitrogen, phosphate, or essential micronutrients (Liu et al. 1994; Nicholson et al.

2000; Sonenshein 2000). A suitable medium must thus support vegetative growth and also the production of spores (Nickerson and Bulla 1974). The most important sporulation-related transcriptional regulator is Spo0A which is phosphorylated via a complex network of interactions in response to nutrient limitation (Errington 2003; Sonenshein 2000).

Media formulation and optimization are key considerations in development of bioprocesses that can produce affordable aquaculture biological agents, yet limited progress has been made in this area to satisfy market opportunities for affordable commercial aquaculture products (Irianto and Austin 2002; Preetha et al. 2006). In previous in vitro studies, our *Bacillus cereus* (NRRL 100132) was shown to inhibit the fish pathogen, *Aeromonas hydrophila*, and to decrease the concentrations of ammonia, nitrite, nitrate, and phosphate waste ions (Lalloo et al. 2007). The same properties were also demonstrated in vivo, in systems used to rear ornamental koi carp, *Cyprinus carpio*, as a model species (Lalloo et al. 2007). This isolate tolerated a wide range of physiological parameters, making it an excellent candidate for aquaculture applications (Fast and Menasveta 2000; Guetsky et al. 2002; Lalloo et al. 2008). No studies have however been done on this organism regarding development of a bioprocess to produce the biological agent for commercial application in aquaculture.

It has been widely documented that nutrient sources influence the growth, spore production, and synthesis of commercially useful metabolites in this species (Gouda et al. 2001; Payot et al. 1998; Rättö et al. 1992). Commonly used nutrient sources include a wide range of peptones, extracts, and hydrolysates, many of which are expensive for industrial-scale manufacture of large-volume products and have negative market acceptance as animal by-products (Nohata and Kurane 1997; Vuolanto et al. 2001). Nutrient supplementation was the focus of this study as the type and concentration of nutrient has a major impact on the production cost, process, and consumer consideration of products (Zhang and Greasham 1999).

The present study aimed to maximize spore production by ensuring a high level of sporulation from a high-density vegetative cell culture (Monteiro et al. 2005; Nicholson et al. 2000). High levels of spore production were achieved by replacing the conventional nutrient substrates with those used in industry to enhance vegetative cell growth and subsequent spore production. We tested spray-dried corn steep liquor (CSL_{SD}) and liquid phytase-treated and ultrafiltered corn steep liquor (CSL_{LPUT}) at different concentrations and modeled the optima based on spore concentration, productivity, and yield coefficients. The modeled optima were confirmed by fermentations run under the same conditions. The high *B. cereus* spore yield and productivity attained in this study with relatively cheap nutrient supplementation makes commercial production feasible.

Materials and methods

Microorganisms and inocula

A cryopreserved *B. cereus* culture (NRRL 100132) containing $\sim 1 \times 10^7$ CFU ml^{-1} viable cells was used as inoculum (Lalloo et al. 2007), prepared according to Meza et al. (2004). All materials used in the present study were obtained from Merck (Darmstadt, Germany), unless otherwise specified.

Comparison of laboratory and commercial nutrient substrates in shake-flask studies

Nutrient substrates tested were yeast extract, nutrient broth, CSL_{SD} (Roquette, Lestrem, France), and CSL_{LPUT} (African Products, Johannesburg, South Africa), to evaluate the impact of nutrient supplementation on production of *B. cereus*. All experiments were supplemented with nutrient equivalent to 5 g l^{-1} total protein, to limit microorganism growth and thus ensure sufficiency of oxygen in flask experiments. Each of the relevant nutrient sources was added (based on protein content, determined by total Kjeldahl nitrogen) into 1,000 ml Erlenmeyer flasks containing macronutrients (citric acid 0.01, $(NH_4)_2SO_4$ 0.06, $Ca(NO_3)_2$ 0.00472, $MgSO_4 \cdot 7H_2O$ 0.03, $MnSO_4 \cdot 4H_2O$ 0.0004, $FeSO_4 \cdot 7H_2O$ 0.00032, KCl 0.02 and H_3PO_4 0.025 g l^{-1}) and 190 ml of deionized water. The pH of the media was adjusted to 7.0 using either 20% m v^{-1} NaOH or 10% m v^{-1} H_2SO_4. The flasks were sterilized at 121°C for 15 min and cooled to ambient temperature. Vitamins (thiamine 95.2, biotin 12.0, calcium pantothenate 95.2, ascorbic acid 95.2, niacin 95.2, pyridoxine 95.2 mg l^{-1}) were dissolved in 10 ml of deionized water and aseptically filter sterilized (0.22 μm) into each flask. The contents of a single cryovial of *B. cereus* were added to each flask, which was incubated at 30°C and 180 rpm in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ, USA) until stationary phase, as determined by $OD_{660\ nm}$ measurements (Genesys 20, Thermo Scientific, Waltham, MA, USA).

Fermentation studies to determine and validate optimum CSL supplementation levels

The contents of a single cryovial of *B. cereus* was inoculated into 700 ml tryptone soy broth (30 g l^{-1}) contained in a 1.8 l Fernbach flask incubated at 30°C and 220 rpm for 8 h in an orbital shaker (Innova 2300, New

Brunswick Scientific, Edison, NJ, USA). A single flask was used to inoculate each fermenter. All fermentation experiments were conducted in 15 l Biostat C fermenters (Sartorius BBI Systems, Melsungen, Germany) operated at 10-l working volume. Macronutrients (as described previously), antifoam (1 ml l^{-1}, Pluriol P2000, BASF, Ludwigshafen, Germany), and either CSL_{SD} or CSL_{LPUT} were added to the initial charge at varying protein concentrations (10–60 g l^{-1}) and made up to 8 l with deionized water. The initial charge medium was sterilized in the vessel followed by addition of a separately sterilized glucose solution (47 g l^{-1}, 60% m m^{-1}). Vitamins were added as for shake-flask cultures, followed by the addition of water to make up the volume to 9.3 l. The fermentation temperature was maintained at 30°C, pressure at 50 kPa using a back pressure controller, pH at 7.0 using 25% v v^{-1} NH_4OH or 20% v v^{-1} H_2SO_4, and aeration and impeller speed ramped over 5 h from 1 to 2 v v^{-1} m^{-1} and 500–1,200 rpm, respectively, from the start of the fermentation. Measurable dissolved oxygen was maintained above 30% saturation. At sugar depletion, glucose was fed (5–7 g l^{-1} h^{-1}) to maintain a residual glucose concentration of 5 g l^{-1} until the sporulation ratio equaled 50%. The total glucose fed to each fermentation varied, based on the type and concentration of nutrient tested. The fermentation was stopped when the sporulation ratio exceeded 90%. Analysis of the fermentation exhaust gases were conducted using an Uras 10E gas analyzer which allowed online calculation of oxygen utilization and carbon dioxide evolution rates by MFCS software (Sartorius BBI Systems, Melsungen, Germany).

Analyses and calculations

Viable cell counts were determined by spreading serially diluted samples of *B. cereus* onto nutrient agar plates. Glucose concentration was measured using an HPIC (CarboPac™ PA1 column, Dionex, MA, USA). Sporulation ratio, which is the ratio of spores to the total cell concentration, was measured by microscopic counting of cells and spores using a Thoma counting slide (Hawksley and Sons, London, UK) according to Monteiro et al. (2005). Cell productivity was determined for data points conforming to high linearity (r^2>0.9) of a plot of viable cell concentration against time (Nori et al. 1983). Yield coefficients were calculated based on data points conforming to high linearity (r^2>0.9) of plots of viable cell count against either total protein, carbohydrate, or oxygen consumed (Papanikolaou and Aggelis 2002). Responses (viable spore concentration, spore productivity, yield on protein, carbohydrate, and oxygen) from the fermentation studies were analyzed statistically (analysis of variance) using the optimization function of Design Expert-6 software (Stat-Ease, Inc., Minneapolis, MN, USA), to determine the optimum supplementation concentration of CSL_{SD} and CSL_{LPUT}. Material cost was determined by cumulating the cost for each media category expressed in euro. The component cost contribution was calculated as the percentage ratio of each media component over the total cost. The unit cost was expressed as euro per 1×10^9 CFU, which was the tested dosage of the biological agent per 10 m^3 (Lalloo et al. 2007).

Results

Comparison of commercial nutrient substrates to laboratory-based nutrient substrates

Two types of corn steep liquor (CSL_{SD} and CSL_{LPUT}) were compared to yeast extract and nutrient broth in shake-flasks cultures, wherein each of the nutrient sources were supplemented to the equivalent concentration of 5 g l^{-1} total protein (Table 1). The coefficient of variation of triplicate results was <10%. CSL_{SD} and CSL_{LPUT} resulted in a ~46- and ~300-fold increase, respectively, in spore concentration, productivity, and yield on protein when compared to yeast extract or nutrient broth. Differences in sporulation ratio were insignificant between the different types of nutrient sources tested (Table 1). CSL_{LPUT} was better in all of the responses measured in comparison to CSL_{SD}.

Determination of the optimum concentration of CSL_{SD} and CSL_{LPUT} supplementation for fed-batch production of *B. cereus* spores

Fed-batch fermentation studies were conducted to determine the concentration of CSL_{SD} and CSL_{LPUT} supplementation in the range of 10 to 60 g l^{-1} based on total protein that maximized spore production (Fig. 1). Spore production

Table 1 Key responses measured during selection of candidate nutrient sources for the production of *B. cereus* (n=3, coefficient of variation <10%)

Protein source	Sporulation ratio (%)	Viable spore # (CFU ml^{-1})	Spore productivity (CFU ml^{-1} h^{-1})	Spore yield on protein (CFU g^{-1})
Yeast extract	97	6.88×10^6	3.82×10^3	1.39×10^9
Nutrient broth	99	5.46×10^7	3.03×10^4	1.10×10^{10}
CSL_{SD}	95	1.42×10^9	7.86×10^5	2.98×10^{11}
CSL_{LPUT}	98	8.82×10^9	4.90×10^6	1.80×10^{12}

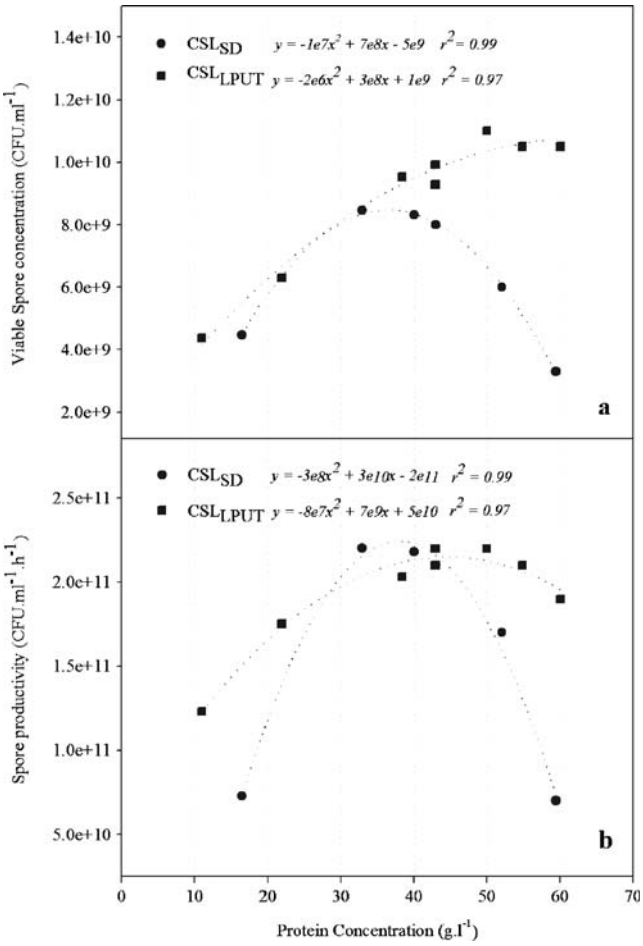

Fig. 1 *B. cereus* viable spore concentrations (**a**) and spore productivity (**b**) when different concentrations of CSL_{SD} or CSL_{LPUT} based on total protein were tested

was measured in terms of spore concentration, productivity, and yields on protein, carbohydrate, and oxygen. The spore concentration and productivity of *B. cereus* fitted a second-order polynomial quadratic graph ($r^2>0.95$, $p<0.05$ for all terms) when plotted against a range of concentrations tested for each of the CSL types (Fig. 1). The maximum spore concentration was 1.1×10^{10} CFU ml^{-1} when CSL_{LPUT} was supplemented at 50 g l^{-1} protein in comparison to a maximum of 8.5×10^{9} CFU ml^{-1} when CSL_{SD} was supplemented at 33 g l^{-1} protein (Fig. 1a). Maximum productivity (Fig. 1b) was however identical when CSL_{SD} was supplemented at 40 g l^{-1} or CSL_{LPUT} was supplemented at 55 g l^{-1} protein (2.2×10^{11} CFU ml^{-1} h^{-1}).

The yield of *B. cereus* spores based on supplemented protein, for both CSL_{SD} and CSL_{LPUT}, fitted a quadratic second-order polynomial graph ($r^2>0.95$, $p<0.05$ for all terms) across the range of protein concentrations tested (Fig. 2a). The difference in yield coefficients between each of the nutrient sources was negligible across the concentration range tested. The yields of spores on carbohydrate substrate and oxygen (Fig. 2) were fitted to quadratic second-order polynomial graphs ($r^2>0.95$, $p<0.05$ for all terms). Spore yield on carbohydrate increased with an increasing concentration of CSL_{SD} to a maximum of 1.56×10^{11} CFU g^{-1} at the 60 g l^{-1} protein supplementation level. CSL_{LPUT} supplementation resulted in a maximum yield on

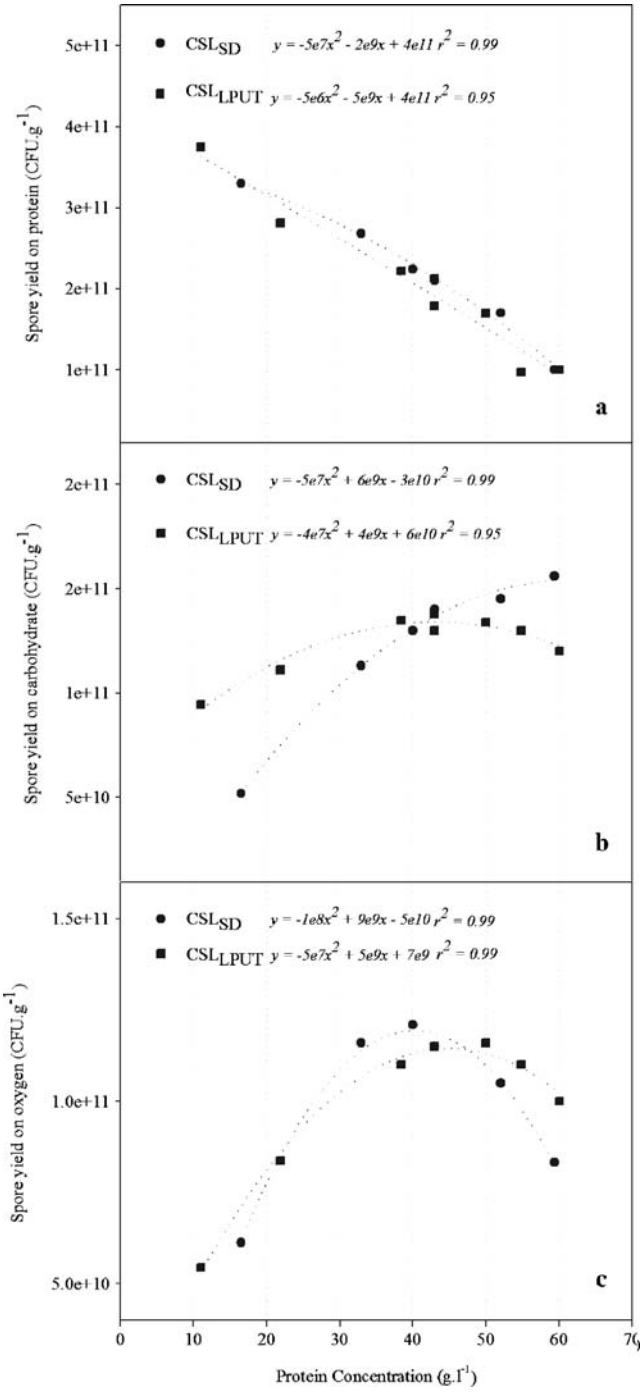

Fig. 2 *B. cereus* yield on protein (**a**), yield on carbohydrate (**b**), and yield on oxygen (**c**) when different concentrations of CSL_{SD} or CSL_{LPUT} based on total protein were tested

carbohydrate of 1.34×10^{11} CFU g^{-1}, at 50 g l^{-1} protein (Fig. 2b). The best yields on oxygen were achieved when CSL_{SD} was supplemented at 40 g l^{-1} (1.2×10^{11} CFU g^{-1}) in comparison to 1.16×10^{11} CFU g^{-1} when CSL_{LPUT} was supplemented at 50 g l^{-1} protein (Fig. 2c).

Determination of combined optima of the five responses using optimization software indicated a calculated optimum concentration of 34 and 48 g l^{-1} protein for CSL_{SD} and CSL_{LPUT}, respectively. These responses were determined from fitted quadratic models that were statistically significant ($p<0.05$). Testing of the mathematically modeled concentration optima for each of the CSL types resulted in a good correlation between the modeled responses and those determined from actual fermentation data (Table 2). The coefficient of variation was less than 10% in all of the responses measured, for actual triplicate test fermentations and when these data were compared to the mathematical optima (Table 2). Testing of the optima also indicated that CSL_{LPUT} resulted in improved spore concentration, whereas CSL_{SD} resulted in improved yield on protein. The productivity and yield of spores on carbohydrate and oxygen were similar irrespective of the CSL type used (Table 2). Material cost of production was therefore calculated at the optimal supplementation level for each CSL type. CSL_{LPUT} resulted in a material cost of production that was 40% lower than CSL_{SD} (Table 3).

Discussion

The present study demonstrated that corn steep liquor was a preferred nutrient substrate for the production of *Bacillus* spores, in comparison to conventional nutrient substrates. The use of both CSL_{SD} and CSL_{LPUT} resulted in a higher spore concentration, productivity, and spore yield on protein in comparison to yeast extract and nutrient broth (Table 1). Although yeast extract has been shown to improve the vegetative growth rate, as it contains protein, vitamins, and cofactors (Payot et al. 1998), other reports have suggested that spore production is improved when CSL is used, as high vegetative cell growth is not always ideal for spore production (Gouda et al. 2001; Nickerson and Bulla 1974; Sharpe and Bulla 1978; Shikata et al. 1990; Srivasta and Baruah 1986). Increased product yields in various processes have been reported when CSL was used to replace conventional nutrient sources (Kuppusamy and Balaraman 1991; Prabakaran et al. 2007), possibly due to a more suitable protein profile of this complex substrate.

Modeling the optima of several key indicators of *B. cereus* spore production, coupled to confirmation in actual fermentation studies, provided a robust method for optimization of the concentrations of the two CSL types in fed-batch culture. This enabled improvement of spore concentration, volumetric productivity, and yield, which are key requirements to cost-effective production of aquaculture biological agents (Riesenberg and Guthke 1999; Vuolanto et al. 2001). The maximum *B. cereus* spore concentration obtained in the present study was approximately five times greater (1×10^{10} CFU ml^{-1}) than previous studies (Chang et al. 2008; Prabakaran et al. 2007) and slightly higher than that reported by Monteiro et al. (2005) for production of *Bacillus subtilis* spores. This represented the highest spore concentration reported to date for this aquaculture biological agent. The present study also examined the yield of spores on carbohydrate, protein, and oxygen as these are key cost and performance drivers in fermentation. The yield of spores on carbohydrate increased with increasing concentration of both types of CSL, suggesting that a higher protein to carbohydrate ratio was preferable, as was also observed in *B. subtilis* (Vuolanto et al. 2001) and *Bacillus licheniformis* (Mao et al. 1992).

The enhanced *B. cereus* sporulation performance in CSL could be related to the improved availability of free amino acids, in comparison to conventional nutrient substrates. Protein is a key factor for sporulation (Shi and Zhu 2007) and plays a role in the growth of vegetative cells, the sporulation process, to protect DNA and to form a multilayered proteinaceous coat outside the cortex of the spore (Errington 2003). Major differences in spore productivity between cultures were mostly related to the quantity of vegetative cells produced, as sporulation efficiency was similar in all of the protein sources tested (>95%, Table 1).

Table 2 Validation of modeled optima of key responses by experimental data for CSL_{SD} and CSL_{LPUT}, respectively (*n=3, coefficient of variation <10%)

Key responses measured	Units	CSL_{SD}			CSL_{LPUT}		
		Model	Actual	CV %	Model	Actual	CV %
Viable spore concentration	CFU ml^{-1}	8.47×10^{9}	8.36×10^{9}	0.9	1.02×10^{10}	1.12×10^{10}	6.7
Productivity	CFU l^{-1} h^{-1}	2.20×10^{11}	2.15×10^{11}	1.6	2.14×10^{11}	1.96×10^{11}	6.2
Yield on protein	CFU g^{-1}	2.45×10^{11}	2.60×10^{11}	4.1	1.65×10^{11}	1.56×10^{11}	3.8
Yield on carbohydrate	CFU g^{-1}	1.17×10^{11}	1.13×10^{11}	2.4	1.32×10^{11}	1.15×10^{11}	9.5
Yield on oxygen	CFU g^{-1}	1.18×10^{11}	1.16×10^{11}	1.1	1.33×10^{11}	1.16×10^{11}	9.8

Table 3 Comparison of the material cost of production using CSL_{SD} and CSL_{LPUT} for the production of *B. cereus* based on prices at July 2008

Material components	CSL_{SD}		CSL_{LPUT}	
	Cost (euro l^{-1})	Component cost contribution (%)	Cost (euro l^{-1})	Component cost contribution (%)
Salts and antifoam	0.02	12.75	0.02	14.89
CSL	0.09	53.69	0.07	45.10
Carbon substrate	0.01	6.42	0.01	8.48
Vitamins and additives	0.05	27.14	0.05	31.54
Total	0.17	100.00	0.15	100.00
Spores produced (# l^{-1})	8.61×10^{12}		1.23×10^{13}	
Cost (euro per 1×10^{9} CFU)	1.99×10^{-5}		1.20×10^{-5}	

CSL is a good source of the entire spectrum of essential amino acids, with glutamate, which is important in cell growth and sporulation, present at high concentration (He et al. 2004; Schilling et al. 2007). A compositional analysis of the nutrient sources at the 5 g l^{-1} protein supplementation level revealed that the total quantity of amino acids available was approximately three times greater in CSL_{LPUT} than in other protein sources (data not shown). The higher concentration of free amino acids in CSL_{LPUT}, where the mass fraction of low-molecular-weight substances are concentrated by ultrafiltration, resulted in higher vegetative cell growth and spore production than CSL_{SD} as the concentration of CSL increased (Fig. 1). In flasks supplemented with CSL_{SD}, the onset of sporulation was earlier than with CSL_{LPUT}, possibly due to limitation in amino acid availability. Limitation of amino acids, particularly glycine, glutamic acid, aspartic acid, isoleucine, or methionine, results in a decrease in intracellular concentration of purine nucleotides which derepress sporulation genes (Lopez et al. 1981; Ochi et al. 1981; Sonenshein 2000).

CSL was useful for spore production, not only because of protein and amino acid components but also because of the sugars and organic acids present in this substrate. Both types of CSL contained a higher level of reducing sugars and organic acids than the conventional nutrient substrates, which apparently benefited vegetative biomass production and sporulation. A limitation in available carbohydrate and organic acids apparently stimulated the early onset of sporulation at lower cell titers in the shake-flask studies when using conventional nutrient sources. Sugars and organic acids participate in the complex interplay of energetic requirements for biomass production and protein turnover during sporulation (Liu et al. 1994). Citrate and succinate were shown to increase growth rate and cell mass production (Schilling et al. 2007) as the portion of glucose-6-phosphate that feeds into the pentose phosphate pathway is increased when organic acids are present (Schilling et al. 2007). Glucose limitation results in decreased pyruvate and this, or its metabolites, is needed during the growth phase for good sporulation to occur (Dingman and Stahly 1983).

CSL_{LPUT} was preferred to CSL_{SD} for fed-batch fermentation due to a higher biomass growth, sporulation, and eventual viable spore concentration (Table 2), which could be related to the higher levels of free amino acids, reducing sugars, ammonium sulfate, phosphate, and other salts in CSL_{LPUT}. CSL_{LPUT} supported the highest spore concentration, while peak spore productivity and yield on CSL_{LPUT} were similar when compared to CSL_{SD}, although the concentration required for maximum productivity of CSL_{LPUT} was ~10 g l^{-1} protein higher than CSL_{SD} (Table 2). Ammonium sulfate and $MgSO_4$ were shown to be important variables for spore production (Shi and Zhu 2007). Magnesium sulfate, $CaCO_3$, and phosphate stimulated sporulation (Shi and Zhu 2007), whereas divalent cations (particularly Ca^{2+}) assist in dehydration and mineralization of the spore (Errington 2003). The lower levels of phosphate in CSL_{SD} when compared to CSL_{LPUT} could explain the earlier onset of sporulation in CSL_{SD} due to the response of the Pho system to phosphate starvation (Msadek 1999). Yield of *B. cereus* spores on oxygen was marginally better on CSL_{SD} than on CSL_{LPUT}, when conditions of oxygen sufficiency were maintained as this is important to realize high spore yields in *Bacilli* (Avignonne-Rossa et al. 1992; Dingman and Stahly 1983). The degradation of vitamins and key nutrients in the spray-drying process could also explain the improvement in growth performance of CSL_{LPUT} when compared to the other dried protein sources tested (Payot et al. 1998).

CSL_{SD} supplementation at concentrations above the optimum levels resulted in a severe attenuation in spore concentration and productivity due to reduced growth and sporulation efficiency. This may have resulted from reduced mass transfer of oxygen into the cell due to the high concentration of solids in the reactor, when using CSL_{SD}. Precipitation and mass transfer issues are however reduced when using CSL_{LPUT} due to hydrolysis of phytic acid and removal of solids through the ultrafiltration process. Liu et al. (1994) also reported that media components cause precipitation and mass transfer problems in high-density cultivation and a negative impact on spore concentration

was reported when dissolved oxygen dropped below 30% (Monteiro et al. 2005). Furthermore, genes controlled in the Res system are induced under anaerobic growth conditions which contribute to the sporulation cascade (Msadek 1999). In our study, supplementation with both types of CSL above 70 g l^{-1} protein resulted in slow growth, cell lysis, and no spore formation (data not shown), which was similar to observations of other researchers (Purushothaman et al. 2001; Silveira et al. 2001). Sporulation efficiency is known to be low following poor growth (Nickerson and Bulla 1974). Sporulation takes longer in high-cell-density cultivations, thus resulting in a compromise between spore concentration and productivity (Liu et al. 1994).

The present study concluded that CSL_{LPUT} was the preferred nutrient source for the production of the *B. cereus* aquaculture biological agent, based on reduced material cost and improved fermentation performance (Table 3), which improved the commercial attractiveness of *B. cereus* as an aquaculture biological agent (Verschuere et al. 2000). Cost-effective media based on locally available raw materials is an important consideration for lower-value products such as aquaculture biological agents (Prabakaran et al. 2007). Furthermore, CSL_{LPUT} is a phytase-treated liquid of plant origin that is cost competitive and has advantages in upstream and downstream processability (Mao et al. 1992; Silveira et al. 2001). Our bioprocess development using this substrate increases the economic potential of large-scale bioproduction and consumer acceptance of the *B. cereus* aquaculture biological agent and provides a rationale for production of other *Bacillus* spore products.

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Chapter 7 Publication 4

A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent

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Abstract Biological products offer advantages over chemotherapeutics in aquaculture. Adoption in commercial application is lacking due to limitations in process and product development that address key end user product requirements such as cost, efficacy, shelf life and convenience. In previous studies, we have reported on the efficacy, physiological robustness and low-cost spore production of a *Bacillus cereus* isolate (NRRL 100132). This study examines the development of suitable spore recovery, drying, formulation and tablet production from the fermentation product. Key criteria used for such downstream process unit evaluation included spore viability, recovery, spore balance, spore re-germination, product intermediate stability, end product stability and efficacy. A process flow sheet comprising vertical tube centrifugation, fluidised bed agglomeration and tablet pressing yielded a suitable product. The formulation included corn steep liquor and glucose to enhance subsequent spore re-germination. Viable spore recovery and spore balance closure across each of the process units was high (>70% and >99% respectively), with improvement in recovery possible by adoption of continuous processing at large scale. Spore re-germination was 97%, whilst a product half-life in excess of 5 years was estimated based on thermal resistance curves. The process resulted in a commercially attractive product and suitable variable cost of production.

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Introduction

The use of biological agents has gained popularity in aquaculture as an alternative to chemotherapeutics, which are more costly, damaging to the environment and often met with consumer resistance (Sanders et al. 2003). Although biological agents are an attractive alternative in improving fish health through disease attenuation and water quality enhancement, proper technology development has been limited, preventing wider adoption of this technology (Moriarity 1999). Important criteria influencing the commercial use of biological products are cost, efficacy, shelf life and convenience to the end user (Amer and Utkhede 2000; Keller et al. 2001). Apart from the fermentative production, the downstream process has a major influence on product commercialization because it influences these product characteristics (Prabakaran and Hoti 2008; Rowe and Margaritis 2004; Tsun et al. 1999). In response to these challenges and the global growth in intensive reticulated aquaculture due to dwindling natural reserves, we developed a novel downstream process for our *Bacillus cereus* (NRRL 100132) biological agent which resulted in a spore product suitable for aquaculture application, by minimising the number of unit operations, maximising the overall process yield and reducing overall process costs whilst also simplifying commercial implementation.

Bacillus spp. offer the required advantages of biological agents in aquaculture because they are ubiquitous, can be formulated into stable products and are unlikely to use genes for antibiotic resistance from common Gram-negative pathogenic organisms (Gatesoupe 1999; Hong et al. 2005;

Sanders et al. 2003). The durability of *Bacillus* spores furthermore allows consideration of robust downstream process options (Driks 2004; Emmert and Handelsman 1999).

Development of a biological product containing *Bacillus* spores begins with microbial screening, followed by development of bioprocess technology that ensures competitive production and downstream processing (Schisler et al. 2004). To this effect, our isolated *B. cereus* (NRRL 100132) was shown to inhibit the fish pathogen, *Aeromonas hydrophila*, and to decrease the concentrations of ammonia, nitrite, nitrate and phosphate waste ions during in vitro and in vivo studies using ornamental *Cyprinus carpio* as a model species (Lalloo et al. 2007). This *B. cereus* isolate also tolerated a wide range of physiological parameters (Lalloo et al. 2008), making it an excellent candidate for aquaculture applications (Fast and Menasveta 2000; Guetsky et al. 2002). A successful fermentation process for high-density spore production of this microorganism, which resulted in an attractive material cost of production, has also been developed (Lalloo et al. 2009).

Although an efficient downstream process is a key requirement for commercialisation of biological agents, published data regarding downstream process development and formulation for commercially available biological products are very limited (Brar et al. 2006; Schisler et al. 2004). This step dictates processability, economy, shelf life, efficacy, eco-friendliness, ease of application and provision of a product form that commands customer appeal (Brar et al. 2006; de Medeiros et al. 2005). As robust economical choices of process steps and ingredients dictated by the end product characteristics are necessary to improve the commercial success of new biological products (Brar et al. 2006), our development addresses this knowledge gap and further enhances the commercial adoption of biological agents in aquaculture.

Materials and methods

A process flow sheet was conceptualised and tested for the production of a tablet end product containing *B. cereus* NRRL 100132 spores as an active biological agent (Fig. 1).

Organism production

B. cereus NRRL 100132 was cultured in 15-l Biostat C fermenters (Sartorius BBI Systems, Melsungen, Germany) as previously described (Lalloo et al. 2009), and the harvested broth containing bacterial spores as the active biological agent was used in experiments for development of a downstream process.

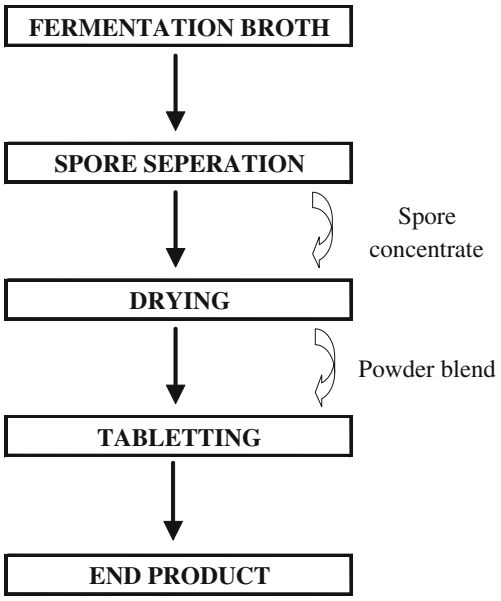

Fig. 1 Conceptual downstream process flow sheet for production of a tablet product

Spore separation from fermentation broth

A centrifugation process was developed for harvesting of the bacterial spores (biological agent) from the fermentation broth. Disc stack (Westfalia, SA1, GEA, Germany) and vertical tube (Sharples AS16 V, Paris, France) centrifuges were evaluated as alternatives for this process unit operation. The operating rational of these centrifuges has been described by Van Dam-Mieras et al. (1995) and Rivière (1977). Similar batches of starting broth (10l, 1.3×10^{13} CFU l^{-1}) were used to minimise variance in the comparative study of the two centrifuges. The broth feed was continuously agitated during centrifugation using an overhead stirrer (Heidolph RZR 2102, Kelheim, Germany), to prevent settling of the biomass. Broth flow rates and the de-sludge time (disc stack only) were selected on the basis of previous operational experience with the equipment. Mass, volume and spore concentration were measured for the broth feed, supernatant and resultant biomass slurry.

Fermentation broth was pumped at 12 l h^{-1} (Watson Marlow 505U, Cornwall, England) into the inlet of the disc stack centrifuge operated at 11,000×*g*. The bowl pressure was maintained at 100 kPa by adjusting the backpressure valve, and the bowl was de-sludged every 4.5 min to collect the biomass paste. Fermentation broth was similarly pumped at 25 l h^{-1} into the inlet of the vertical tube centrifuge operated at 23,000×*g*. After the entire volume of broth was pumped into either of the centrifuges, it was allowed to spin for a further 5 min to maximise sedimentation. The bowl contents were removed by a final de-

sludge (disc stack) or manually from the tube (vertical tube) and re-suspended into 0.15% $m{\cdot}v^{-1}$ potassium sorbate buffer equivalent to half the initial broth volume, resulting in a spore slurry. Any residual biomass was purged and ascribed to the loss fraction.

Fluidised bed coating of carrier with *B. cereus* spores

To produce a dried product, the spore slurry from the vertical tube centrifuge was used as the feed for the fluidised bed coating operation. In a screening test, yeast powder (Microbial Solutions, Kya Sands, South Africa) or spray-dried corn steep liquor (CSL, Solulys, Roquette, Lestrem, France), milled to sizes ranging from 50 to 500 μm, was used as the carrier material for fluid bed coating and tested at ratios of 1:2 and 1: 4 spore slurry to carrier. The appropriate carrier (10 or 20 kg depending on ratio) was added to the fluidised bed drier (PAC FBD 15, Johannesburg, South Africa) and fluidised using an inlet air flow of $1 v{\cdot}v^{-1}{\cdot}m^{-1}$ and automatically controlled air inlet temperature to maintain internal agglomeration temperature at 40°C. The internal pressure was maintained below 0.1 kPa (gauge). The carrier material was allowed to fluidise until the internal temperature was constant. The spore slurry (5 l) was then pumped into the fluidised bed drier via an atomising spray nozzle using a peristaltic pump (Watson Marlow 101U, Cornwall, England) at a rate of 300 g h^{-1} and the atomizer air spray pressure set at 200 kPa (gauge). The fluidised bed drier was allowed to fluidize for a further 15 min to evaporate excess moisture. The product was removed from the fluidised bed drier, weighed and assayed for viable spore concentration. Powder remaining in the agglomerator and bag filter was similarly measured as the loss fraction.

Formulation of key ingredients

A formulation comprising dry powder ingredients and the bacterial spores was developed to yield a tablet product as dictated by customer preference. The formulation comprised CSL coated with spores and glucose (based on optimum ratio in germination and growth studies), polyvinylpyrrolidone (2% $m{\cdot}m^{-1}$, Kollidon, BASF, Ludwigshafen, Germany), magnesium stearate (2% $m{\cdot}m^{-1}$, Merck, Darmstadt, Germany) and Idicol blue (0.0006% $m{\cdot}m^{-1}$, Dye Chem, Johannesburg, South Africa). The chemical ingredients are typically used in tablet formulations, with the inclusion of glucose and CSL as nutrients for germination and growth of the spores during product application. The powder mixture was blended to yield a homogenous distribution of spores using a ribbon blender (Anderson Engineering, Pietermaritzburg, South Africa) for 10 min.

Different ratios of CSL and dextrose monohydrate (glucose) were tested in culture studies to examine the impact of these nutrients on germination and growth of the *B. cereus* product. Glucose to CSL ratios ranging from 0:100 to 100:0 were dissolved in de-ionised water (1 l) equivalent to 1×10^{-4} g l^{-1} total ingredient, which mimicked the final application dosage (0.1 g m^{-3}). The solution was filter-sterilised into a 2-l Erlenmeyer flask, inoculated with 1×10^{5} CFU ml^{-1} of *B. cereus* and incubated at 30°C and 180 rpm in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, USA). Germination ratio, viable cell number and growth rate were determined (Lalloo et al. 2009) and analysed statistically (ANOVA) using the optimisation function of Design Expert-6 software (Stat-Ease, Inc., Minneapolis, USA), to determine the optimum ratio of glucose to CSL that would support spore germination and growth. The impact of Kollidon, Idicol Blue or magnesium stearate on spore viability was similarly tested at double the formulation dosage to confirm the lack of toxicity to *B. cereus* spores.

Production of a tablet end product containing *B. cereus* spores

A tablet was produced from the powder mixture containing spores. The formulated powder blend was added to the hopper of a Manesty E2 tablet press (Manesty, Sussex, England). The mixture was compressed into tablets using an 11-mm circular punch and die set. The compression force and depth were adjusted to result in a firm pill of ~1.0 g in mass.

Calculation of spore recovery and spore balance closure

The mass of the feed, harvest and loss fractions of each key process step was determined, and triplicate samples were analysed for both viable spore counts (Lalloo et al. 2009) and moisture content, using a moisture balance (Mettler Toledo, HR83 Halogen, Switzerland). These measurements allowed for an assessment of spore recovery which was expressed as the percentage yield of viable spores in the harvest relative to the feed fraction. The spore balance closure was the total spores in the harvest and loss fractions expressed as a percentage ratio of the spores in the feed.

Assessment of viability and stability of product intermediates

The viability and stability of product intermediates are important considerations that influence process scheduling and scale of equipment. The product intermediates from the centrifugation (spore slurry) and agglomeration (powder blend) process units were assessed for stability. Sample aliquots (100 ml) were stored at 4°C, 22°C and 32°C for a

period of 42 days. Samples were removed at regular intervals over this period and analysed for viable spore count (Lalloo et al. 2009). The viability of each product intermediate was compared within treatments and across treatments by statistical analysis of variance.

Assessment of viability and stability of end product

The viability and stability of the end product were assessed as this is an important consideration for end users. Tablets (ten each) from three separate production batches were randomly selected and assessed for viable spore concentration on nutrient agar culture plates and for growth and germination in synthetic pond water (Lalloo et al. 2007). Tablets were also assessed for shelf life stability (viable spore concentration) based on the methodology of death rate plots at different temperatures to generate a thermal resistance curve (Hosahalli et al. 1997). A temperature-dependant product half-life plot was generated to predict shelf stability. Actual samples retrieved from the market were also tested for viable spore concentration over a 5-year period.

Assessment of material cost of production

The downstream material cost was determined by cumulating the cost for each ingredient expressed in Euro. The component cost contribution was calculated as the percentage ratio of ingredient cost over the total cost. The total unit cost per tablet was expressed as the cumulative cost of the fermentation (Lalloo et al. 2009) and downstream material cost.

Results

Spore recovery and spore balance closure across process unit operations

The recovery and mass balance closures for key process unit operations in the downstream process flow sheet (Fig. 1) are presented in Table 1. Spore harvesting from the fermentation broth was evaluated through disc stack and rotating vertical tube centrifugation. Both options resulted in minimal loss of spores to the supernatant fraction (<1%), but the disc stack centrifuge only resulted in an overall recovery of 40% in comparison to 71% when using the vertical tube centrifuge. The major part of the loss fraction was retained in the bowl or vertical tube of the respective centrifuges. The cell separation process unit operation using the Sharples centrifuge resulted in a viable spore balance closure of 100%.

CSL was shown previously to support germination and subsequent growth of spores and was better than yeast extract (Lalloo et al. 2008). CSL and yeast powder were also compared as carriers for fluidised bed agglomeration in screening experiments. The resultant recovery of spores through the fluidized bed agglomeration process was only 92% for yeast powder in comparison to 99% for CSL. In screening experiments, the impact of CSL particle size on viable spore recovery through the agglomeration process resulted in recoveries of ~81%, 82%, 84% and 95% at 50-, 100-, 200- and 500-μm particle sizes respectively, at a ratio of 1:2 spore slurry to carrier. When the carrier ratio was doubled, recoveries increased by an average of ~4%. The average recovery of viable spores through the fluidised bed agglomeration process was 99% when CSL was used as a carrier in the actual process (Table 1). The viable spore balance closure through the agglomeration process using CSL was 99.8%. The spore concentration in the resultant agglomerate was extremely consistent (co-efficient of variation <8%), when different batches were randomly tested.

The powder blend was successfully compressed into tablets resulting in a recovery of 78.6% and a viable spore balance closure of 99.7% during this process unit operation (Table 1). The tablet product was found to be suitable in qualitative assessments of surface quality, hardness, friability and dissolution in water (data not shown).

Formulation of key ingredients

The pill product form required additives that would support germination of the spores into vegetative cells when hydrated and ensure the formation of an appropriate dry tablet. The growth rate, increase in vegetative cells and

Table 1 Recovery and mass balance closure for key processing unit operations

Process unit operation	Selected operation type	Total spores in CFU	Total spores out CFU	Total spores in loss fractions CFU	Recovery %	Balance closure %
Spore separation	Vertical tube centrifugation	1.34E+14	9.50E+13	3.90E+13	70.9	100.0
Drying	Fluidised bed agglomeration	9.50E+13	9.42E+13	5.70E+11	99.2	99.8
Tablet production	Automatic tablet press	9.42E+13	7.41E+13	1.99E+13	78.6	99.7

germination ratio were therefore evaluated at different glucose to CSL ratios (Fig. 2). These responses resulted in suitable models at the 90% confidence level. Simultaneous optimisation of the responses indicated an optimum glucose:CSL ratio of 22:78, with desirability co-efficient of 0.99. Inclusion of a dye at the required dosage level to impart an appropriate colour (0.0006% $m{\cdot}m^{-1}$) and at twice this level did not result in any significant negative impact on spore viability ($p>0.80$). Kollidon and magnesium stearate added at 2% $m{\cdot}m^{-1}$ each, based on screening

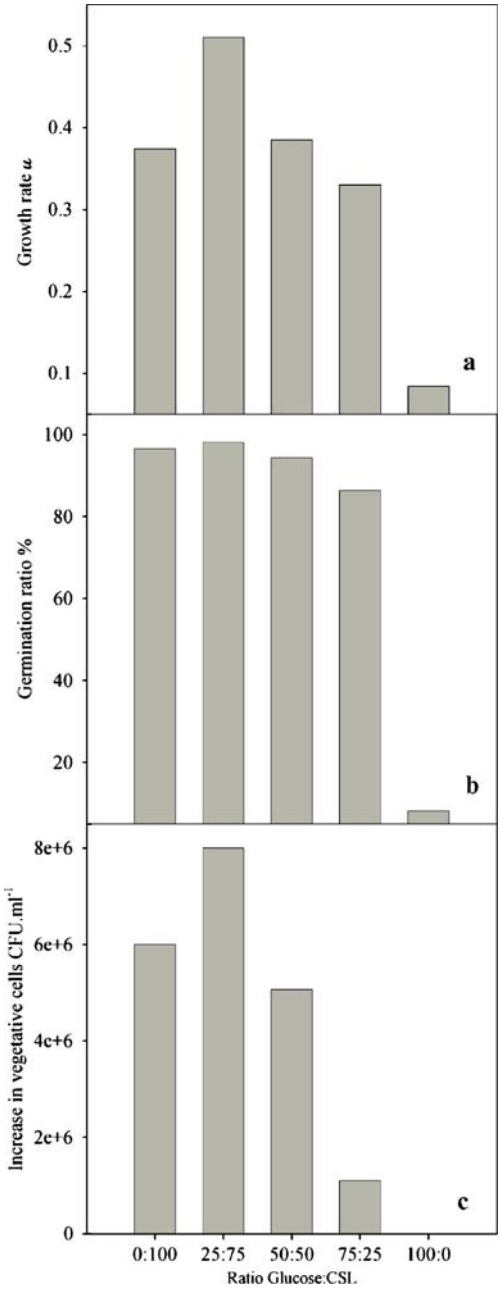

Fig. 2 **a**–**c** Influence of glucose to CSL ratio in supporting germination and growth

experiments (data not shown), did not show any toxicity and resulted in a suitable pill product. The final powder mixture was formulated (CSL 76.5, glucose 19.5, Idicol blue 0.0006, Kollidon 2.0 and magnesium stearate 2.0% $m{\cdot}m^{-1}$) and blended into a consistent mixture.

Stability of product intermediates in process flow sheet

The spore concentrate and powder blend were the two product intermediates in the overall downstream process flow sheet (Fig. 1). These product intermediate forms were stable over a 42-day test period (Fig. 3) without significant loss in viability of spores (co-efficient of variation <10%, $n=7$). Storage under refrigeration (4°C), controlled ambient environment (22°C) and warmer industrial environments (32°C) did not significantly affect viability ($p>0.9$; Fig. 3).

Evaluation of end product

The tablet end product was tested in simulated pond water to assess germination and growth of the *B. cereus* spores (Fig. 4), as in vitro and in vivo efficacy in model systems containing actual *C. carpio* had been shown previously (Lalloo et al. 2007). The average germination efficiency, growth rate and increase in viable cells from a starting population of 1×10^5 CFU ml^{-1} was 97%, 0.87 and 1.8×10^7CFU ml^{-1}, respectively. The co-efficient of variation of end product test samples within batches and across batches was less than 10% for all of the variables tested. Spores were evenly distributed in the tablet, and the tablet dissolution rate was ~0.08 g h^{-1} (data not shown).

The end product was also tested for shelf life stability. The survival curves (data not shown) of *B. cereus* spores at 4°C, 30°C, 60°C and 90°C resulted in a linear thermal resistance curve (Fig. 5; $r^2=0.998$). The data were used to develop a half-life-predictive model at varying storage temperature, which indicated that the product would have a half-life of ~5 years at a typical shelf storage temperature (20°C). This was also verified by actual measurement of product samples from the market over a 5-year period, whereby no samples contained less than 1×10^9CFU g^{-1} of viable *B. cereus* spores.

Assessment of material cost of production

The material component cost for the downstream process is presented in Table 2. The total material cost for the downstream process was 9.25×10^{-4} Euro per tablet, which was predominated by the cost of CSL (>66%). The total material cost of the tablet, inclusive of the fermentation process (Lalloo et al. 2009), was 9.49×10^{-4} Euro.

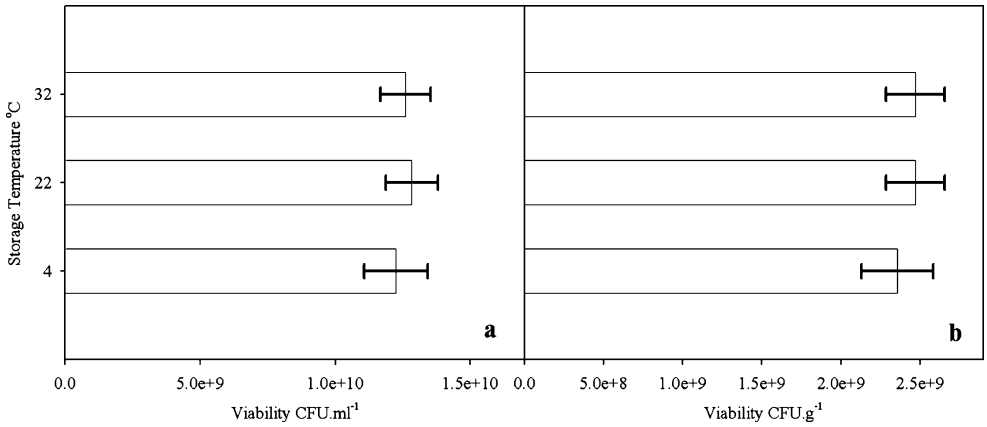

Fig. 3 Viability of product intermediates **a** spore concentrate and **b** powder blend at different temperatures

Discussion

Apart from production of microbial biomass through fermentation, the downstream process is an important consideration in ensuring a robust process yielding useable end product. Maximisation of recovery and viability during processing, whilst ensuring a product that meets with end user requirements such as stability, consistency, easy application, efficacy and affordability, are key objectives (Brar et al. 2006; Schisler et al. 2004). We conceptualised a flow sheet (Fig. 1) and developed a downstream process that yielded a tablet containing a functional and novel *B. cereus* biological agent. The recovery, viability and stability through our process flow sheet, including end product evaluation for consistency, functionality, stability and material cost of production, were shown.

Vertical tube centrifugation resulted in a better recovery (71%) in comparison to disc stack centrifugation (40%) when harvesting *B. cereus* spores. In both cases, losses to supernatant fractions were minimal, but the recovery of cell pastes was less efficient in batch mode, due to residual biomass holdup in the machine. The excessive accumulation of solids, even in centrifugation with automated desludging, has also been reported by others (Prabakaran and Hoti 2008; Torres-Anjel and Hedrick 1970). Importantly, the loss of spore viability was negligible as the spore balance closure was ~100%, and the recovery can thus be improved by continuous operation in a commercial process with frequent purging of the spore paste. As our fermentation broth contained the highest *Bacillus* spore concentration reported in the literature at the time (Lalloo et al. 2009), recovery may have been compromised by high spore loading, yet productivity was excellent (Torres-Anjel and Hedrick 1970). Similar to findings by Torres-Anjel and Hedrick (1970), our centrifugation did not require additional centrifugation cycles, as loss to the supernatant fraction was negligible on the first cycle. Amongst all advances, centrifugation appears to be the most viable step for removal of *Bacillus* spores (Brar et al. 2006; Rojas et al. 1996; Zamola et al. 1981). Alternative approaches such as flocculation requires post-separation removal of chemical additives. Cross-flow filtration is prone to fouling, especially due to the high protein load in our fermentation broth and the release of intracellular material during sporulation, thus increasing costs and negatively affecting process throughput.

The recovery during coating and drying of *B. cereus* NRRL 100132 spore slurry through a fluidised bed agglomerator, containing an atomising spray nozzle, when using CSL as a carrier, resulted in a viable spore recovery exceeding 99%. Such preservation of the functionality of biological products during drying directly benefits the quality and marketability of the end product (Chen and Patel 2007). Alternate options for commercial processes include refrigerated or frozen cultures, but these products incur higher storage and shipment costs in contrast to our dry tablet. (Klein and Lortal 1999; Werner et al. 1993). Spray drying may have been an option for our process (Werner et al. 1993) but, although spores are more resistant to heat than vegetative cells (Setlow 2006), this process could result in viability loss due to irreversible changes in structural and functional integrity of the spore (Chen and Patel 2007; Tamez-Guerra et al. 1996). In contrast, viability loss was minimal through our fluid bed agglomeration process, and this technology has additional advantages such as lower investment and maintenance costs, ease of large-scale continuous production, rapid exchange of heat-minimising heat damage, rapid mixing providing near isothermal conditions and uniform end product (Bayrock and Ingledew 1997; Larena et al. 2003; Luna-Solano et al. 2005; Mille et al. 2004).

The high recovery of *B. cereus* spores through our agglomeration process may be due to the protection from heat by the spore protein exosporium and two major small acid-soluble DNA binding proteins α and β, which are a characteristic of spores of the *B. cereus* group (Brar et al. 2006; Larena et al. 2003; Setlow and Setlow 1995). High recovery in drying could also be attributed to adhesion of *B.*

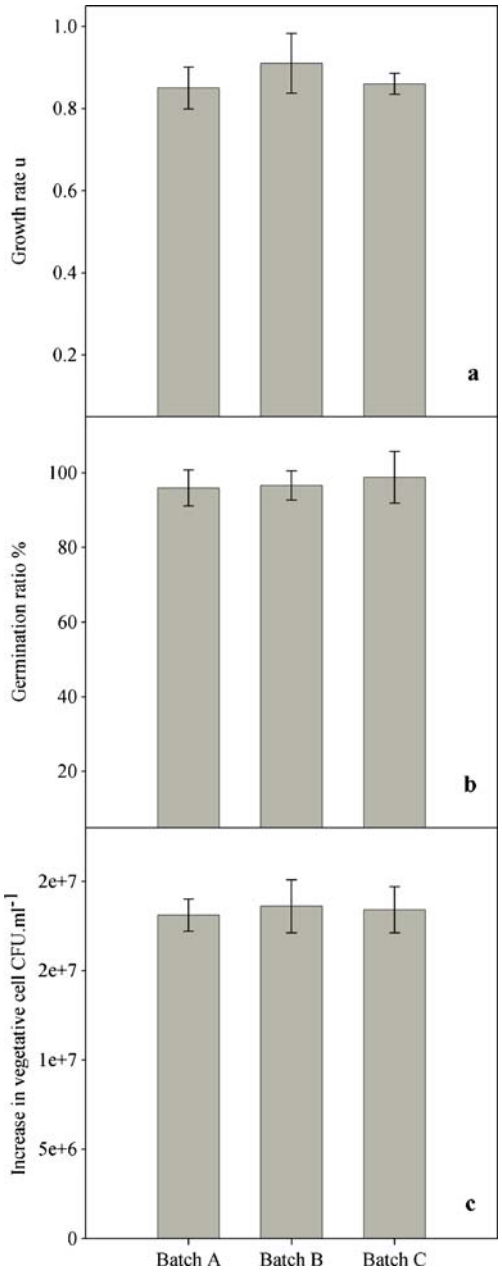

Fig. 4 **a**–**c** Germination and growth of *B. cereus* in the tablet end product (CV<10% across batches *A*, *B* and *C*)

cereus spores to the CSL carrier, demonstrated by a minimal loss of fine particles and lack of fouling of the agglomerator bag filters. Spore adhesion to the carrier surface may have been enhanced by the hydrophobicity of *B. cereus* and the hair-like protrusions on the spore surface, as was demonstrated previously (Busscher and Weerkamp 1987; Rönner et al. 1990). The use of CSL as a carrier in our agglomeration process was advantageous as dust formation was minimal, preventing passage through the vent filter bags and any potential health risks. CSL is also affordable and provides nutrients for spore activation in contrast to inert carriers. Although it has been reported that smaller particle size carriers (50–100 μm) enhanced bacterial survival better than large particle sizes (Dandurand et al. 1994), we did not observe a loss of viability when using CSL with a 500-μm particle size. An added advantage of our process was the resultant homogenous distribution of spores on the carrier, thought to positively influence end product consistency (Fig. 4). The variability in agglomerate size also enhanced flowability of the resultant powder for subsequent process steps, such as tablet production.

The inclusion of CSL and glucose as nutrients in our formulation, after optimising the ratio of these two ingredients (Fig. 2), supported maximum spore germination and growth of *B. cereus* (Fig. 4), whilst still allowing for the production of a suitable tablet product. CSL was better than yeast powder in supporting germination and growth of spores (Lalloo et al. 2008) and also resulted in a higher recovery in agglomeration trials (Table 1). Apart from the support of germination and growth, proteins and sugars in CSL apparently provided a protective layer for cells, preventing death and assisting recovery of injured cells during processing, as was demonstrated previously (Brar et al. 2006; Costa et al. 2001; Larena et al. 2003). Addition of nutrients was also shown by others to improve storage of a *Pseudomonas fluorescens* F113 strain (Moënne-Loccoz et al. 1999) and a *Bacillus megaterium* (Wiwattanapatapee et al. 2004) used in biocontrol applications. The addition of magnesium stearate, Kollidon and Idicol blue facilitated production of a stable and appealing end product through a simple process amenable to large-scale production As a considerable influence on activity can be attributed to the type of substance added to a formulation (Werner et al. 1993), we tested these ingredients at double the formulation level and found no negative impact on spore viability. The

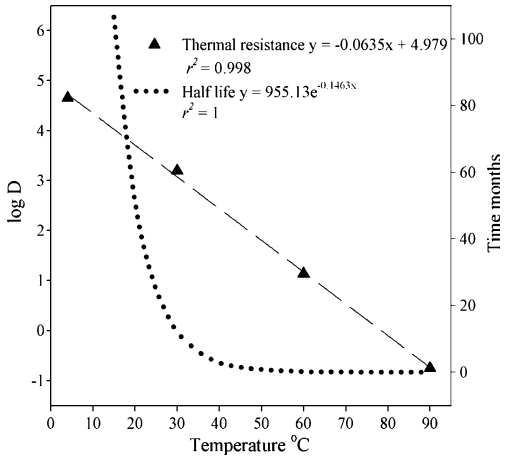

Fig. 5 Thermal resistance and half-life plots showing end product stability

Table 2 Material cost of production for downstream process and overall process to end product

Material components	Cost (EURO per tablet)	Component cost contribution (%)
CSL	6.30×10^{-4}	68.11
Glucose	1.10×10^{-4}	11.89
Idicol blue	1.00×10^{-5}	1.19
Kollidon	1.50×10^{-4}	16.65
Magnesium stearate	2.00×10^{-5}	2.16
Total DSP material cost	9.30×10^{-4}	100.00
Material cost per tablet FERM	2.40×10^{-5}	2.53
Material cost per tablet DSP	9.25×10^{-4}	97.47
Total material cost per tablet	9.49×10^{-4}	100.00

dual functionality of CSL as a carrier for fluidised bed coating of spores and as a nutrient for spore germination is a novel approach for aquaculture biological agent production.

B. cereus spores were successfully entrained in a tablet end product, resulting in a recovery of ~80%, with the major loss fraction contained in powder fines, which can be re-worked in a continuous commercial process. This product form had advantages such as uniformity, stability, easy transportation and field applicability. Similar to our formulation, de Medeiros et al. (2005) were also able to successfully produce a tablet containing *B. cereus* by direct compression of a powder blend containing magnesium stearate at 2.0% $m{\cdot}m^{-1}$. A concern during tablet production is the inactivation of spores by pressure and frictional heat during compression and de-compression (Margosch et al. 2004; Mathys et al. 2008). The mechanism of inactivation of bacterial spores by heat and pressure is as yet unresolved, but it has been postulated that the thick proteinaceous spore coat could play a role in resistance to pressure (Mathys et al. 2008; Setlow 2006). *B. cereus* has been shown to withstand pressures up to ~50 mPa (Aoyama et al. 2005), and in our tablet process, there was minimal loss of viability (99% spore balance closure). We also observed that the homogenous distribution of spores within the tablet resulted in a release of spores and activation nutrients proportional to the tablet dissolution rate (~0.08 $g\,h^{-1}$). In contrast, a post-production top-coated product would not have facilitated homogenous distribution (Biourge et al. 1998).

Intermediate products in the process for the production of the *B. cereus* biological agent, namely the biomass slurry resulting from the centrifugation step and the powder blend after agglomeration, were both sufficiently stable for up to 42 days at refrigeration, ambient and industrial processing temperatures (Fig. 3), thereby avoiding the need for additional biocidals for stability. Apart from suitable recoveries and balance closures through downstream processing, the stability of product intermediates is an important consideration in developing a robust process, even though the lag time between process operations is typically under 12 h. Soper and Ward (1981) reported that addition of specific biocidal chemicals may be required to prevent growth in a centrifuge slurry, but we achieved a stable spore slurry by re-suspending our spore paste in a mild sorbate buffer, to prevent any unintended carryover of biocidal activity into the end product. The powder blend was stable due to low moisture content (< 5% $m{\cdot}m^{-1}$). The high sporulation ratio achieved during fermentation development (Lalloo et al. 2009) apparently contributed to the stability of the intermediate spore products in the process, as a mixture of spores and vegetative cells tend to be less stable (Wiwattanapatapee et al. 2004).

The final *B. cereus* tablet spore product was stable at 20°C for more than 5 years when formulated at 2×10^{9} CFU g^{-1} and retained its stability and biological activity under real market conditions. Although *Bacillus* spores generally allow for development of products with prolonged shelf life, stability times typically range between 1 and 12 months only (Amer and Utkhede 2000; Puziss et al. 1963; Wiwattanapatapee et al. 2004). The *B. cereus* tablet end product was consistent between batches and germinated and grew well in simulated pond water conditions (Fig. 4). We had previously shown the excellent functionality of this biological agent in vivo (Lalloo et al. 2007) and furthermore elucidated the robustness to physiological ranges encountered in application of this product (Lalloo et al. 2008).

We demonstrated an attractive material cost of production of our *B. cereus* biological agent in fermentation (Lalloo et al. 2009), but the actual constraint is mainly embedded in downstream processing costs for biological products (Brar et al. 2006). Our tablet product resulted in an attractive total material cost for both the upstream and downstream process of only 9.49×10^{-4} Euro per tablet, which treats 10 m^3 of pond water. The fully absorbed cost is ~0.05 Euro per tablet for a small facility producing ~3 million tablets per annum. Our costs were minimised by selection of simple yet robust process steps that delivered high recoveries in the downstream process (Table 1), whilst ensuring stability of product intermediates and the end product (Figs. 3 and 5). We furthermore produced a

compact tablet product that minimised post-production transport and storage costs, without a requirement for a cold chain. This is the first comprehensive report of the full downstream process flow sheet for the production of a novel aquaculture biological agent. The integration of the process flow sheet to synergise process unit operations is an innovative approach that simplified production, reduced cost and resulted in an end product that surpassed current quality standards in terms of stability of aquaculture biological agents. Our tablet end product met with customer preference for convenience, quality and functionality, substantiated by sustained market presence spanning 5 years to date.

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Chapter 8 Publication 5

Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in aquaculture

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in aquaculture

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Abstract The potential of a *Bacillus cereus* isolate (NRRL 100132) as a biological agent for aquaculture has been demonstrated in vitro and in vivo. The functionality of this isolate across a range of physiological conditions, including salinity, pH and temperature, based on rearing of high-value ornamental *Cyprinus carpio*, was investigated. Temperature had a significant influence on germination, specific growth rate and increase in cell number of *B. cereus* in shake-flask cultures, whilst salinity and pH did not have a measurable effect on growth. Controlled studies in bioreactors and modelling of the data to the Arrhenius function indicated the existence of high and low growth temperature domains. The rates of pathogenic *Aeromonas hydrophila* suppression and decrease in waste ion concentrations (ammonium, nitrite, nitrate and phosphate) were translated into a linear predictive indicator of efficacy of the *B. cereus* isolate at different temperatures. The present study confirmed the robustness of the *B. cereus* isolate (NRRL 100132) as a putative biological agent for aquaculture and further demonstrated a novel method for the assessment of in vitro biological efficacy as a function of temperature.

Keywords *Bacillus cereus* · pH · Salinity · Temperature · Aquaculture · *Cyprinus carpio*

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Introduction

The success of modern aquaculture is driven by intensive reticulated culture systems (Liao and Mayo 1974), whereby high growth rate and high stocking density are major requirements. However, this practice results in the onset of fish disease and environmental pollution (Paperna 1991). The rearing of ornamental *Cyprinus carpio* (koi) is a rapidly growing, high-value industry, and the health and survival of ornamental carp is thus an important requirement for both hobbyists and culturists.

Carp are prone to a wide range of diseases with one of the major causative agents being *Aeromonas hydrophila*, which results in the outbreak of bacterial ulcer diseases by haemorrhagic septicaemia (Jeney and Jeney 1995; Austin and Austin 1999). Because mass mortality can occur if there is a prevalence of infectious agents (Irie et al. 2005), reducing the prevalence of bacteria such as *A. hydrophila* in water systems used to rear ornamental carp is required. Fish disease is normally a consequence of the interaction between the host, environmental stress elements and disease causing agents (Jeney and Jeney 1995; Austin and Austin 1999). The deterioration of water quality as a result of metabolic waste accumulation (ammonia, nitrite, nitrate and phosphate) should thus be considered (Jana and Jana 2003). The main source of metabolic waste is through excessive feeding rates (Liao and Mayo 1974; Boyd 1985) and dietary composition (Shimeno et al. 1997; Kim et al. 1998), which is amplified in ornamental carp systems due to the use of high-nutrient diets. Waste metabolites are removed via a multitude of mechanisms such as bio-assimilation, nitrification and dissimilatory nitrate reduction (Lalloo et al. 2007). Modern day ornamental carp culture systems also use reticulated filtration systems with innovative vortex, self-cleaning moving bed adsorbent material,

ultraviolet and ozone sterilizers, which allow for the removal of excess particulate matter, bacterial cells and organic material. Addition of fresh water, to decrease concentrations of waste metabolites, causes effluent purges resulting in negative environmental impact.

Conventional methods, such as disinfectants and chemotherapeutics, have been used to treat bacterial disease; however, these result in the development of resistance in pathogenic organisms and chemical residues with detrimental effects to end users and the environment (de Kinkelin and Michel 1992; Moriarity 1999; Barker 2000; Sze 2000; Verschuere et al. 2000; Jana and Jana 2003).

Bacterial amendments have emerged as an appropriate alternative to the use of antibiotics in aquaculture (Vanbelle et al. 1990; Hong et al. 2005) and have demonstrated the potential to improve water quality, decrease pathogen load and reduce fish mortality (Moriarity 1999; Skjermo and Vadstein 1999; Fast and Menasveta 2000; Jana and Jana 2003). Gram-positive *Bacillus* species are an attractive option as a bacterial amendment for aquaculture as these organisms are found naturally in sediments, are ingested by animals and are unlikely to use genes for antibiotic resistance or virulence from gram-negative organisms such as *Aeromonas* spp. (Moriarity 1999). Furthermore, bacterial spores of the genus *Bacillus* have several advantages compared to vegetative cells, such as resistance to toxic compounds, temperature extremes, desiccation and radiation (Wolken et al. 2003), thus allowing for the formulation of stable products (Hong et al. 2005; Ugoji et al. 2006). Several spore-forming bacteria, such as *B. coagulans*, *B. subtilis*, *B. clausii*, *B. cereus* and *B. toyoi*, are already exploited as components of products for human and animal use (Sanders et al. 2003).

A natural isolate of *Bacillus cereus* (NRRL 100132) was shown previously to inhibit pathogenic *A. hydrophila* and to decrease the concentrations of ammonia, nitrite, nitrate and phosphate ions in previous in vitro and in vivo studies (Lalloo et al. 2007). The application of biological agents requires demonstration of the tolerance of the microorganism to the conditions in which they must perform (Moriarity 1999; Gross et al. 2003), in particular their ability to germinate and grow (Wolken et al. 2003). The functionality of a biological agent is often affected by physiological conditions such as salinity, pH and temperature, and biological agents must remain functional and effective over a wide range of harsh physiological conditions (Guetsky et al. 2002). The benefits of bacterial additives to water and the tolerance of biological agents to the physiological conditions of the environment in which they are applied have not been sufficiently demonstrated (Fast and Menasveta 2000) due to the lack of in vitro investigation of the interaction between key physiological variables. This is the first reported study of the effects of key physiological variables on the functionality of a putative biological agent targeted for use in the rearing of ornamental *C. carpio*.

Materials and methods

Microorganisms and inocula

The *B. cereus* (NRRL 100132) isolate (Lalloo et al. 2007) was cultured in medium containing 0.8% m v^{-1} yeast extract, 0.005% m v^{-1} $MnSO_4$, 0.01% m v^{-1} $CaCl_2$ and 0.03% m v^{-1} $MgSO_4{\cdot}7H_2O$ for 24h at 30°C, pH7.0 and 180rpm in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, USA). *A. hydrophila* (ATCC 7966), a fish pathogen, was similarly cultured on selective media (Kielwein et al. 1969; Kielwein 1971). Both cultures were obtained in cryopreserved form from cell banks and prepared according to Meza et al. (2004). Resultant cultures were used as inocula for experiments. All materials used in the present study were obtained from Merck (Darmstadt, Germany), unless otherwise specified.

Cultivation and media

Cultivation of *B. cereus* in shake-flasks over a range of salinity, pH and temperature were performed according to a Box Behnken matrix experimental design, followed by statistical analysis of the data using the Design Expert-6 software (Stat-Ease, Minneapolis, USA). The ranges for the numeric factors conformed to the tolerable ranges for the rearing of ornamental *C. carpio* (Lammens 2004), whereby NaCl between 0% and 1% m m^{-1}, pH between 6 to 9 and temperatures between 4°C and 30°C were tested. Shake-flask experiments were conducted in activation media (0.075% m m^{-1} spray-dried corn steep liquor and 0.025% m m^{-1} dextrose monohydrate) developed previously (data not presented), and supplemented with NaCl according to the experimental design. Spray-dried corn steep liquor was obtained from Roquette (Lestrem, France). The pH of the media was adjusted to the desired value using either 20% m v^{-1} NaOH or 10% m v^{-1} H_2SO_4. Media was prepared, inoculated and incubated at the desired temperatures as described previously (Lalloo et al. 2007). Experiments were sampled two hourly. Specific growth rate (μ), germination ratio and increase in viable cell number were the responses measured.

Cultivation of *B. cereus* across a range of temperatures (4°C, 13°C, 16°C, 20°C, 25°C and 30°C) was conducted in Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany). The activation media was prepared to a volume of 1,400ml in the fermenter and sterilized at 121°C for 30min. The pH of the media was adjusted in situ

to 7.5 with 20% m v^{-1} NaOH, followed by inoculation with *B. cereus* culture to result in an initial concentration of 1 × 10^5CFU ml^{-1}. The airflow was maintained at 1v v^{-1} m^{-1} and agitation at 300rpm to ensure oxygen saturation relative to ambient conditions. The reactor was sampled four hourly. Specific growth rate (μ), germination ratio and increase in viable cell number were measured.

Shake-flask evaluation of the efficacy of *B. cereus* across a range of temperatures was also performed in filter sterilized synthetic pond water (0.0085% m v^{-1} KNO_3, 0.006% m v^{-1} $NaNO_2$, 0.0093% m v^{-1} $(NH_4)_2SO_4$, 0.0038% m v^{-1} H_3PO_4, 0.1% m v^{-1} yeast extract and 0.1% m v^{-1} glucose) to mimic conditions in rearing ponds for *C. carpio*. Each shake-flask was co-inoculated with *B. cereus* and *A. hydrophila* to an effective final concentration of approximately 1.0 × 10^5CFU ml^{-1} for each microorganism except for the control flasks which only contained *A. hydrophila*. Flasks were sampled two hourly and analysed for viable cell number of *A. hydrophila* and *B. cereus* and ammonium, nitrite, nitrate and phosphate concentrations.

Analyses and calculations

All experiments were conducted in triplicate. The specific growth rate (μ) was determined from OD_{660nm} measurements (Genesys 20, Thermo Scientific, Waltham, USA) for data points conforming to high linearity ($r^2 > 0.9$) of a plot of ln (OD_{660nm}) against time. Viable cell counts were determined by spreading serially diluted samples of *B. cereus* onto nutrient agar plates and *A. hydrophila* onto plates containing media selective for the growth of *Aeromonas* spp. (Kielwein et al. 1969; Kielwein 1971), followed by incubation at 30°C for 24h and enumeration of colony-forming units. The increase in cell number was quantified by difference in viable cell counts between final and initial samples. Germination ratio, which is an indication of the percentage of vegetative cells within the culture, was calculated as the ratio between the vegetative cell concentration and total cell concentration (Monteiro et al. 2005) as determined by microscopic counting of cells and spores using a Thoma counting slide (Hawksley and Sons, London, UK). Ammonia was analysed using a Reflectoquant (Catalogue No. 1.16892.0001, Merck, Darmstadt, Germany), and nitrate, nitrite and phosphate ion concentrations were measured by ion chromatography (Morales et al. 2000) using an Ion Chromatography System (Dionex, Sunnyvale, USA) with an anion pre-column and anion separator column (Dionex AG14 and AS14, Sunnyvale, USA). The rates of decrease in the concentration of ammonium, nitrite, nitrate and phosphate ions were determined by using data points conforming to high linearity ($r^2 > 0.9$) of plots of ion concentration against time. An Arrhenius plot was generated by plotting the ln function of maximum specific growth rate against the reciprocal of the absolute temperature (K). The square root of maximum growth rate was plotted against the absolute

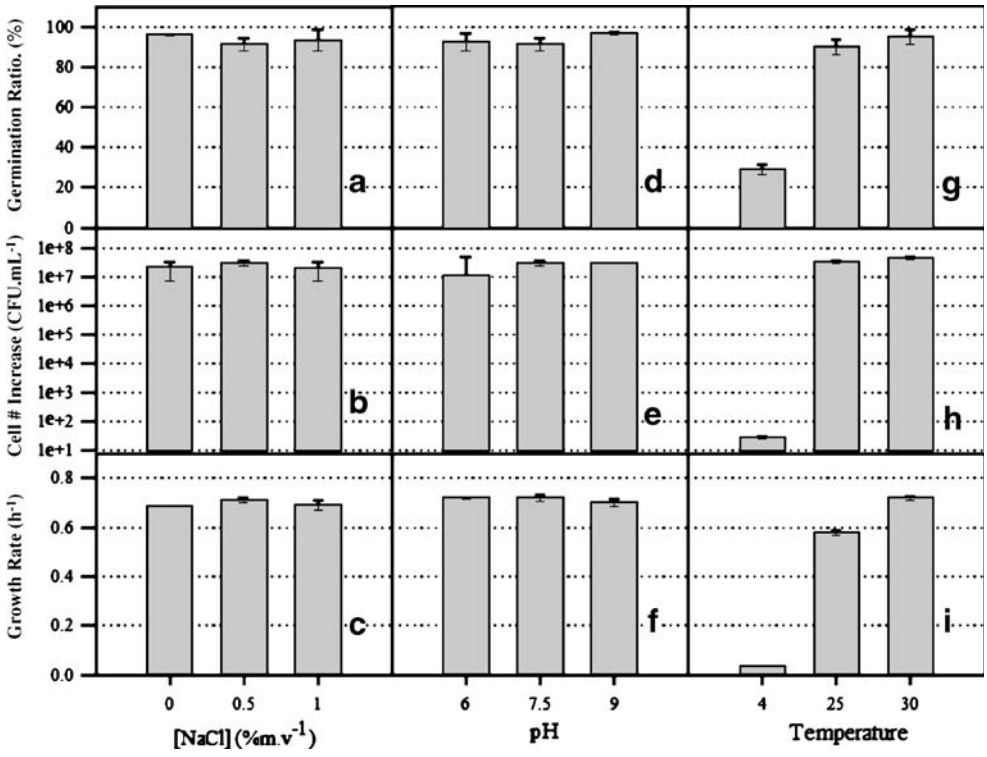

Fig. 1 Growth rate, cell number increase and spore germination in response to [NaCl], pH and temperature in matrix studies during shake-flask cultivation

temperature (K) to examine conformance of the data to the Ratkowsky function (Ratkowsky et al. 1983; Choma et al. 2000). The normalised efficiency of the biological agent was calculated by determination of the relative percentage values using the linear equations for the actual rates of decrease in concentration of waste ions and *A. hydrophila* with the rate at 25°C (optimum temperature for rearing of ornamental *C. carpio*) being equated to 100% efficiency.

Results

Effect of temperature, salinity and pH on the growth of *B. cereus*

The effects of temperature, salinity and pH on spore germination and cell growth of the putative biological agent *B. cereus* during shake-flask cultivation in activation medium are presented in Fig. 1. Changes in temperature demonstrated a significant impact on germination ratio ($p < 0.0001$), increase in cell number ($p < 0.01$) and specific growth rate ($p < 0.001$) of the biological agent. Decreasing the temperature from 30°C to 4°C resulted in a drastic decline in germination ratio from 95% to 29%, viable cell number from 4.6×10^7 to 3×10^1CFU ml^{-1} and specific growth rate from 0.718 to 0.035h^{-1}. However, there was no significant impact of salinity or pH on the responses measured within the minimum–maximum of the ranges tested ($p > 0.1$).

The impact of temperature on *B. cereus* growth was further investigated under well-controlled conditions in bioreactors (Fig. 2). The germination ratio was lowest at 4°C (28.8%) and increased substantially up to 13°C (83.2%). The further increase in germination ratio at temperatures between 13°C and 30°C was negligible, resulting in an average germination ratio of 88.5 ± 4.9%. A rapid increase in cell number was observed between 4°C and 20°C, whilst higher cultivation temperature did not significantly increase cell number ($p = 0.29$). The correlation between temperature and specific growth rate could be modelled using a linear function ($\mu = 0.027t - 0.1052$; $r^2 = 0.99$), which could be used to estimate the specific growth rate of the biological agent at any given temperature. The growth rate response to temperature was also modelled to the Arrhenius and Ratkowsky functions (Fig. 3). The Arrhenius plot resulted in a lower linear regression of the complete data set ($r^2 = 0.92$), whilst separate linear regressions for each of the low- and high-temperature data domains were highly significant ($r^2 = 0.99$). The growth rate data also conformed to the Ratkowsky function ($r^2 = 0.99$), indicating that the growth rate response of the biological agent to temperature is similar to that of other microorganisms.

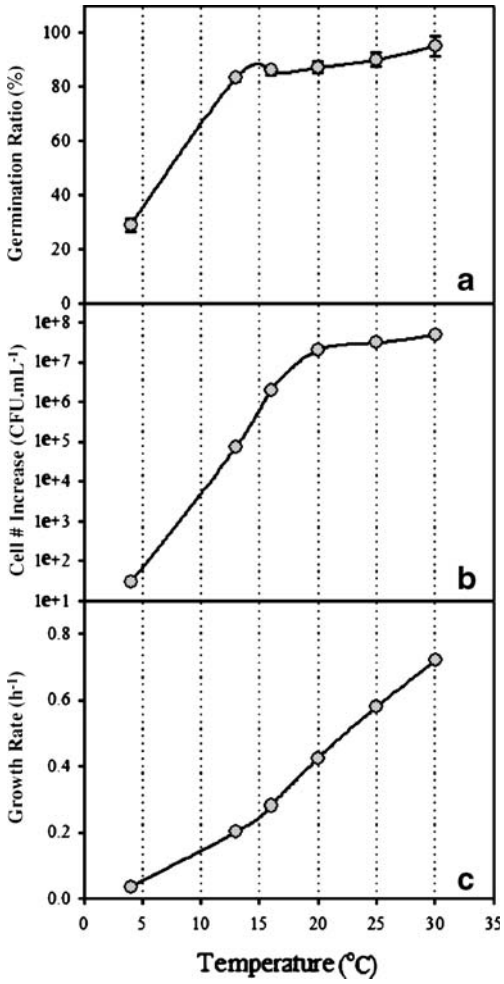

Fig. 2 Growth rate, cell number increase and spore germination in response to temperature in bioreactor cultivation

Effect of temperature on the functionality of *B. cereus* as a biological agent

The impact of temperature on the efficacy of *B. cereus* as a biological agent for aquaculture, measured as the decrease in concentrations of pathogenic *A. hydrophila* and ammonium, nitrite, nitrate and phosphate waste ions, was investigated at 13°C, 20°C and 30°C in shake-flasks using synthetic pond water. These conditions mimic the environments in rearing ponds for *C. carpio* and conformed to the low-, mid- and high-temperature domains for *B. cereus* growth as determined from the Arrhenius plots. Co-culturing in the presence of pathogenic *A. hydrophila* resulted in *B. cereus* growth rates of 0.424, 0.579 and 0.718h^{-1} at 13°C, 20°C and 30°C, respectively (Fig. 4). Although the *A. hydrophila* growth rate in the control treatments (absence of *B. cereus*) was also reduced at lower temperature, a marked decrease in *A. hydrophila* cell number was observed during *B. cereus* co-culture (Fig. 5).

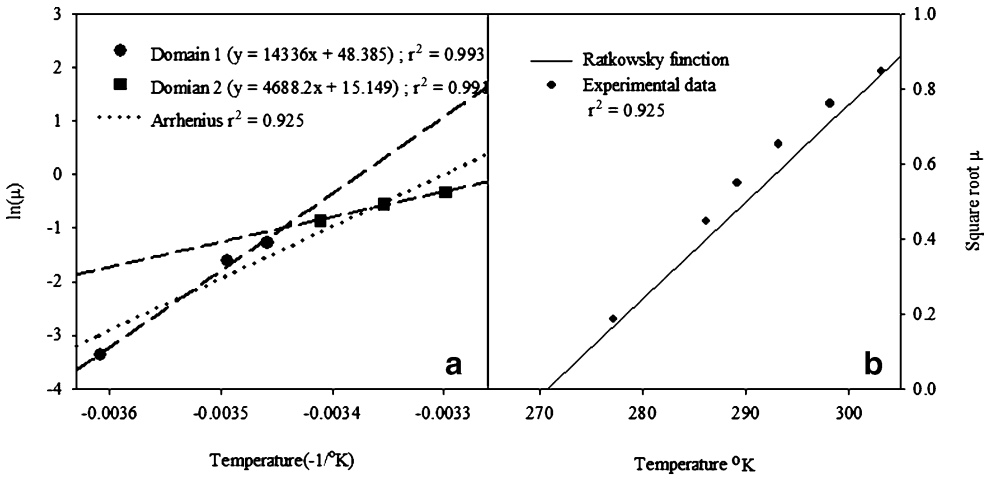

Fig. 3 Arrhenius and Ratkowsky plots in response to temperature

The *B. cereus* biological agent, therefore, reduced the *A. hydrophila* growth at all the temperature points tested. The difference in viable cell number of *A. hydrophila* between control and test treatments at the endpoint of the study was 3.5×10^5, 6.9×10^5 and 9.7×10^5CFU ml^{-1} at 13°C, 20°C and 30°C, respectively, confirming that the reduction in *A. hydrohila* cell number is attributed to the actual presence of *B. cereus*. Significant rates of decrease in the concentrations of ammonium, nitrite, nitrate and phosphate waste ions were observed across the range of temperatures studied, as a result of *B. cereus* co-culture (Fig. 6).

The rate of decrease in concentrations of *A. hydrophila* and ammonium, nitrite, nitrate and phosphate ions could be correlated to the cultivation temperature in a linear manner (r^2>0.98; Fig. 6a), resulting in equations that could predict the efficacy of the biological agent against each of these criteria. A more useful indicator of efficacy of the biological agent at different temperatures was determined by normalisation of the rates of decrease to a temperature equivalence point (25°C being the optimum temperature for rearing of ornamental *C. carpio*), thus resulting in a single straight line equation ($R_{nr} = 6.691t - 67.461$; $r^2 = 0.99$) for prediction of the efficacy of the biological agent across different temperatures (Fig. 6b).

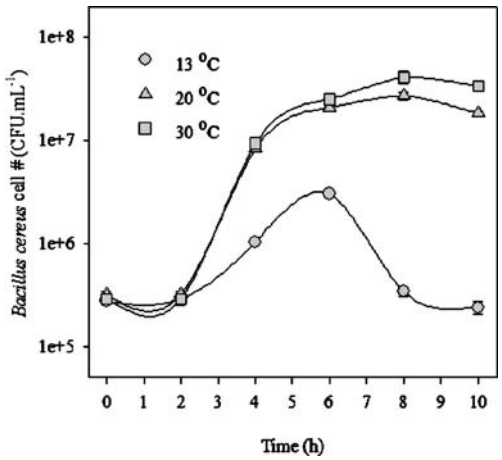

Fig. 4 Growth of *B. cereus* in co-culture with *A. hydrophila* at low-, mid- and high-temperature domains in shake-flask culture (n = 3)

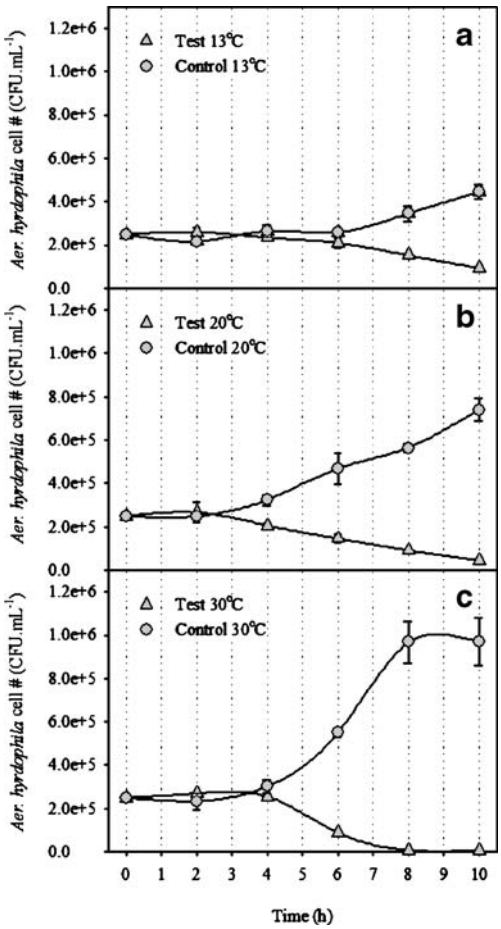

Fig. 5 Growth of *A. hydrophila* in co-culture with and without *B. cereus* at low-, mid- and high-temperature domains in shake-flask culture (n = 3)

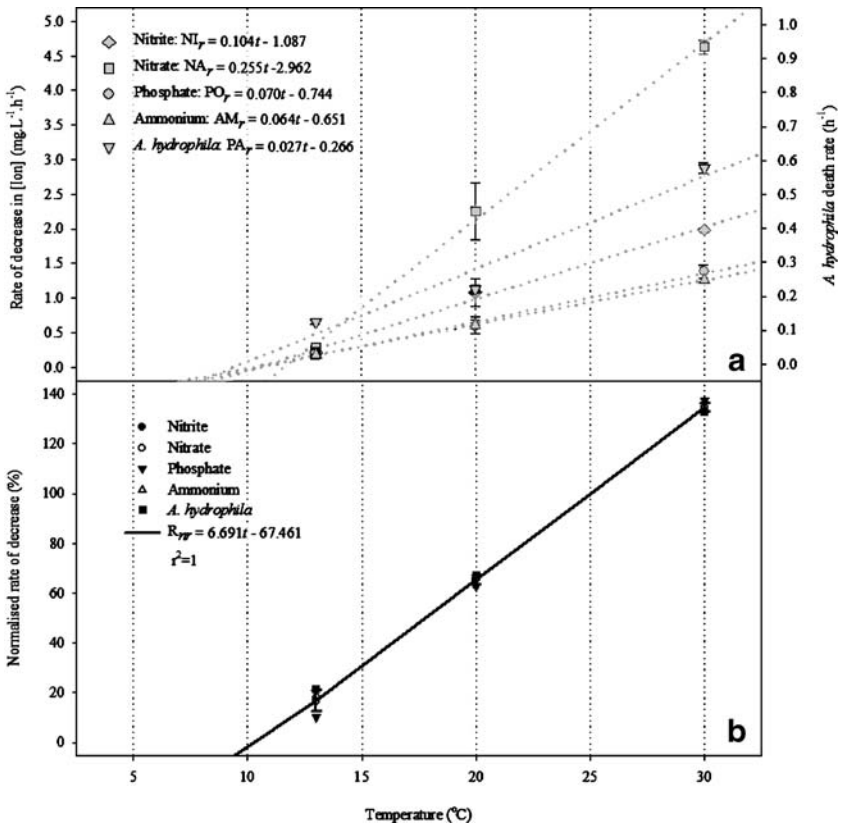

Fig. 6 Rate of decrease in pathogen and waste ion concentrations in response to temperature

Discussion

There is sufficient evidence of the benefits associated with the use of spore-forming bacteria, such as *Bacillus* spp., as biological agents for improving water quality and reducing disease in aquaculture (Gomez-Gil et al. 2000; Irianto and Austin 2002; Rengipat et al. 2000; Sanders et al. 2003; Wolken et al. 2003; Vaseeharan and Ramamsamy 2003; Hong et al. 2005; Lalloo et al. 2007). *B. cereus* (NRRL 100132) was previously isolated and selected over other isolates based on in vitro and in vivo tests (Lalloo et al. 2007) using a rationale similar to other researchers (Gram et al. 1999; Vaseeharan and Ramamsamy 2003). This putative biological agent demonstrated the potential for commercial use in culture of ornamental *C. carpio* (Lalloo et al. 2007). Information on the suitability and robustness of putative biological agents in response to environmental conditions such as salinity, pH and temperature are limited due to the difficulty in measuring interactive effects in vivo and a lack of in vitro studies, yet changes in these conditions influence cell growth, survival and functionality of *Bacillus* spp. as biological agents (Fast and Menasveta 2000; Budde et al. 2006). The functionality of the *B. cereus* NRRL 100132 isolate across the extreme ranges of culture conditions for *C. carpio* was therefore investigated. Oxygen was not considered as it is generally maintained at ambient saturation conditions in carp rearing systems, whilst an in vivo study did not indicate any negative impact of the biological agent on oxygen concentration (Lalloo et al. 2007).

The present study has demonstrated that the growth of the *B. cereus* biological agent could be maintained across the range of pH and salinity typically applied to the rearing of *C. carpio*, whilst changes in temperature had a significant impact on spore germination and vegetative cell growth (Fig. 1). The absence of interaction between salinity (NaCl concentration), pH and the growth of a different strain of *B. cereus*, within the ranges tested in the present study, has been reported previously (Chorin et al. 1997; Leguerinel et al. 2000; Jobin et al. 2002).

Spore germination, growth rate and increase in cell number of *B. cereus* were low at 4°C but increased substantially above 13°C (Fig. 2). Germination was, however, observed at 4°C in contrast to the study by Chorin et al. (1997) where no growth was observable at this temperature using different strains of *B. cereus*. Cell number increased significantly up to 20°C with limited increase thereafter, indicating a maximum cell yield on the growth media independent of the temperature increase from 20°C to 30°C. *Bacillus subtilis* was also shown to sustain viability below 11°C (Nicholson et al. 2000) and cold shock responses have been furthermore observed below

these temperatures (Budde et al. 2006). The growth rate data was indicative of a thermokinetic relationship with increasing temperature (Fig. 2c). When modelled to the Arrhenius function, low- and high-temperature domains were observable (Fig. 3a), which conformed to changing metabolic growth rates between the low and high ranges, respectively. A similar observation has been previously reported for *B. cereus* TZ415 where the critical switch point was 13°C (Choma et al. 2000). The experimental data showed a linear correlation to the Ratkowsky function (Fig. 3b), indicating that the model is useful as a predictive tool of the growth rate for *B. cereus* (NRRL 100132) across a temperature range. In contrast to the Arrhenius model, however, the temperature domains could not be predicted by the Ratkowsky function, thus demonstrating the usefulness of the Arrhenius function in predicting the growth of biological agents in vitro.

Attenuation of the growth rate of the *B. cereus* biological agent at lower temperatures did not translate directly into a lack of functionality because acceptable rates of pathogen suppression and removal of waste metabolites across the range of temperature was observed. Furthermore, the metabolic rate of *C. carpio* is significantly reduced at lower temperatures, which translates to a reduced intake of feed, waste metabolite generation and concomitant decrease in pathogen propensity (Lammens 2004). The present study also demonstrated a reduction in the growth rate of pathogenic *A. hydrophila* at low temperature. Attenuation of pathogen growth by *B. cereus* increased with increasing temperature (Fig. 6), indicating that the functionality of the biological agent was growth-associated and could potentially be ascribed to the mechanism of competitive exclusion (Sanders et al. 2003; Hong et al. 2005). A separate study on *Bacillus* isolates also revealed a relationship between temperature and the efficacy of biological agents relating to the production of inhibitory metabolites (Foldes et al. 2000). The decreases in concentrations of waste ions were also enhanced at higher temperatures. Nitrogen removal is classically ascribed to autotrophic bacteria in natural systems, but there have been several reports suggesting a contribution by heterotrophic bacteria in this regard (Robertson and Kuenen 1990; Sakai et al. 1996; Sakai et al. 1997; Martienssen and Schöps 1999; Su et al. 2001; Kim et al. 2005; Lin et al. 2006).

The present study has demonstrated a novel method for the assessment and prediction of the functionality of biological agents in an in vitro system across a temperature range. Results of the in vitro assay of the *B. cereus* isolate for disease control and water quality enhancement in the rearing of ornamental *C. carpio* were in agreement with an in vivo assessment using live ornamental *C. carpio* in a previous study (Lalloo et al. 2007). Other researches have also reported that the addition of beneficial bacteria can enhance the health of animals by effecting a holistic improvement in waste ion removal and pathogen reduction (Larmoyeux and Piper 1973; Liao and Mayo 1974; Jeney and Jeney 1995; Shimeno et al. 1997; Boyd and Tucker 1998; Frances et al. 2000). By normalising the rates of decrease of pathogenic *A. hydrophila*, ammonium, nitrite, nitrate and phosphate relative to the optimum temperature (25°C) for growth of *C. carpio* (Metz et al. 2003), a useful predictive model was generated which expresses the holistic functionality of the biological agent across a temperature range.

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Chapter 9 Conclusions

The primary objective of the project was to develop a bioprocess technology that would produce an innovative biological product to address the many challenges encountered in modern day reticulated aquaculture. The research and development had to provide biological solutions that included reduction in pathogen load and key waste ions, such as ammonium, nitrite, nitrate and phosphate of benefit to the aquaculture industry. The resultant production process and end products should be commercially attractive in terms of safety, implementation, robustness and cost.

9.1 Study conclusions

The objective of the project was met by fulfilling all of the aims through innovative research and bioprocess development, that addressed existing technology gaps and through the creation of new knowledge. The major conclusions addressing the aims are summarised here:-

9.1.1 Isolation, screening, selection, identification and safety assessment of putative biological agents

An isolation procedure incorporating current best practice with appropriate improvements was successful in exclusively isolating pure cultures of *Bacillus* spp. from culture pond sediment and the intestine and skin mucus layers of *C. carpio*. Screening of isolates, based on in vitro tests for predefined characteristics such as growth rate, inhibition of pathogenic *Aer. hydrophila* and decrease in concentration of waste ions yielded nine putative biological agents of interest. Based on the targeted screening strategy, positive isolates were obtained in the first isolation campaign. The rationale can be useful for rapidly finding a greater array of biological agents in further isolation campaigns. By derivation of a relative suitability index, incorporating these criteria, the desirability of each isolate to the intended application in aquaculture of *C. carpio* was determined. This resulted in the selection of isolates B002, B001 and B003 with suitability indices of 84.1%, 44.1% and 41.6% respectively, which were significantly higher than those of any of the other isolates tested ($p<0.001$). These isolates were identified by 16sRNA sequence homology as *B. subtilis* (B001, NRRL 100131), *B. cereus* (B002, NRRL 100132 and *B.*

licheniformis (B003, NRRL 100133). In vitro co-cultivation of these isolates in synthetic pond water resulted in inhibition of *Aer. hydrophila*. Similarly in vivo tests in model systems containing live *C. carpio* confirmed a significant positive effect on decreasing pathogen and waste ion concentrations when compared to control studies ($n=120$, $p<0.05$). *B. cereus* displayed the best characteristics of the putative biological agents and was selected for further research and development. As *B. cereus* is closely related to *B. anthracis*, biosafety tests were performed and these confirmed the absence of the anthrax toxin and the key virulence genes pOX1 and pOX2. The absence of the *B. cereus* diarrhoeal enterotoxin was also shown, confirming the safety of this isolate for use in aquaculture.

9.1.2 Elucidation of the modes of action of B. cereus as an aquaculture biological agent

Aer. hydrophila was inhibited by actively growing cells of *B. cereus* in plate well inhibition and co-culture assays, while intracellular or extracellular extracts did not result in inhibition of the pathogen, thus confirming that antimicrobial production was not a mode of action. Competitive exclusion was elucidated as a mode of action when *B. cereus* grew at a high growth rate ($0.96.h^{-1}$) whereas the *Aer. hydrophila* population decreased by >70% in a co-culture study. Nutrient limitation screening studies revealed that growth rate, glucose and iron were involved as mechanisms in competitive exclusion. It was shown that growth rate and uptake rates for glucose and iron were significantly higher ($p<0.05$) for *B. cereus* in comparison to *Aer. hydrophila.* The production of siderophores facilitating competitive iron uptake by *B. cereus* was also confirmed in time profile studies.

9.1.3 Development of an upstream fermentation process for high density production of B. cereus spores

High density spore cultivation of *B. cereus* was successfully achieved through appropriate development of a fermentation process. As carbohydrate utilization in *Bacillus* spp. has been well researched, the study focused on protein based nutrient sources, which greatly impact on the cost of production. Corn steep liquor (CSL) was shown to be a better nutrient supplement when compared to conventional laboratory based nutrient substrates. The concentration of spray dried (CSL_{SD}) and liquid phytase treated and ultrafiltered CSL (CSL_{LPUT}) was successfully optimised

by mathematical modelling of commercially relevant responses such as spore concentration, productivity and yield co-efficients. The modelled data correlated well with actual fermentation data (CV<10%). The mathematical optima for the concentration of each type of CSL, expressed as protein concentration were 34g.l^{-1} and 48g.l^{-1} for CSL_{SD} and CSL_{LPUT} respectively. Although CSL_{SD} resulted in higher yield, whereas CSL_{LPUT} resulted in higher spore concentration at their respective optima, CSL_{LPUT} was shown to be the superior nutrient substrate based on a material cost comparison and other process advantages. CSL_{LPUT} resulted in a spore concentration of 1.1x10^{10} CFU.ml^{-1}, which is the highest concentration reported for a *B. cereus* aquaculture biological agent to date. The fermentation process developed resulted in a material cost of production of ~0.02 EURO cents per 1x10^{9}viable spores.

9.1.4 Development of an integrated downstream process including formulation of the biological agent into a stable and functional end product

A conceptual process flow sheet was tested for production of a stable end product. Vertical tube centrifugation was the best process option for harvesting of spores from fermentation broth and resulted in a recovery of 71%, with a spore balance closure of 100%. CSL was superior as a carrier in fluidized bed agglomeration in comparison to yeast extract and resulted in a spore recovery of 99%. The resultant agglomerate was successfully formulated into a processable powder mixture, which resulted in a recovery of 79% during tablet production. The novel powder formulation contained kollidon and magnesium stearate to facilitate tablet production, glucose and CSL to aid post production germination of spores and a dye to enhance aesthetic appeal. The ratio of glucose to CSL (22:78) was pre-determined based on an optimization of key growth responses. Product intermediates in the process flow sheet (spore concentrate and powder blend) were shown to be stable under refrigerated, ambient and warmer industrial temperatures, without any significant loss in spore viability (n=7, CV<10%). The end product was shown to be consistent within and across batches (CV<10%) and the spores germinated (97%) and grew well (0.87 h^{-1}). The half life of the end product exceeded five years based on thermal resistance data and actual measurement of product samples. This stability greatly exceeded the typical shelf life of 1-12 months for existing *Bacillus* based products. The final integrated upstream and downstream process resulted in an attractive material cost of less than 1 EURO cents per tablet containing 1x10^{9} viable spores.

9.1.5 Assessment of the functionality of the biological agent across physiological ranges

The germination and growth of *B. cereus* was maintained across the salinity and pH tolerance ranges encountered in aquaculture of *C. carpio.* Temperature variation however, demonstrated a significant impact on germination ratio, growth rate and increase in cell number ($p<0.01$ for all responses). Spore germination and growth were low at $4^{o}C$ but increased substantially above $13^{o}C$. Experimental data correlated linearly to the Ratkowsky function and hot and cold temperature domains were predicted by the Arrhenius function. The effect of temperature, on the functionality of the biological agent in reducing the concentrations of pathogenic *Aer. hydrophila* and typical waste metabolites in aquaculture (ammonium, nitrite, nitrate and phosphate), was successfully elucidated. A predictive model of the functionality of the biological agent in response to temperature was developed.

9.2 Implications of study

The successful completion of this study has resulted in the following implications and impact:-

- A new rational approach for isolation and selection of putative biological agents of the genus *Bacillus*. The study advanced existing isolation and screening procedures enabling a higher selectivity of new biological agents, thus enabling the current and future isolation of novel *Bacillus* based biological agents for aquaculture in limited isolation campaigns.
- The discovery of novel isolates from South Africa's rich and as yet not fully elucidated biodiversity, based on pre-defined characteristics desirable for aquaculture. Demonstration of an array of attributes such as inhibition of the fish pathogen *Aer. hydrophila* and a decrease in concentration of typical waste metabolites both in vitro and in vivo. Proper identification and demonstration of bio-safety further enhanced attractiveness of a multi-effect technology for application in aquaculture.
- The successful selection of high performance isolates, such as *B. cereus* (NRRL100132) presents an alternate solution to health and environmental challenges in aquaculture by decreasing the conventional use of chemotherapeutic agents, reducing negative impact to end users and the environment and minimising the development of virulence in pathogenic micro-organisms.

- Elucidation of the mode of action and functionality in response to physiological variables has enhanced the level of technology understanding of the benefits and performance of the specific biological agent. This reduces traditional scepticism of the use of biological alternatives and enhances technology adoption by end users.
- The development of a novel fermentation process that has yielded the highest *B. cereus* spore density to date, while exploiting local waste materials such as corn steep liquor as a nutrient source, has resulted in new knowledge in this area as the process development aspects of spore production have been neglected as an area of research. The resultant fermentation process yielded high spore concentration, at high productivity rate and high conversion efficiency of vegetative cells to viable spores, with an extremely attractive cost of production that will stimulate the use of biological solutions as a feasible option. The process technology can also be adapted to other applications where spores are the product form, such as agricultural bio-control agents, biocatalysts and food and feed probiotics.
- The development of a novel downstream process, integrating suitable unit operations for spore harvesting, drying and tablet production has yielded an attractive and easy to apply tablet end product. The process flowsheet and formulation allows simple and robust processability with a high recovery of bioactivity. This presents a new and affordable product option to end users in ornamental aquaculture, and has addressed commercial concerns such as stability during storage and distribution.

Figure 7 End product packaged for global distribution

9.3 Future perspectives

- The isolation and screening rationale should be further exploited to yield a wide array of new and improved biological agents.
- The end product should be more extensively tested under in-vivo conditions in extensive farm trials.
- An alternate liquid product form has been prepared for general pond husbandry and in the use of fresh water ornamental fish tanks. This product form is cheaper and more amenable for use where small doses are required. Further testing of the efficacy of this product form for these applications, is necessary.
- Extension of the current technology to other freshwater applications. Evaluation of the technology has commenced for application in aquaculture of edible carp and tilapia. Preliminary results in small scale model in vivo systems are positive and further work is required to elucidate the actual benefits in yield, health, environmental impact and food quality under true farm conditions.
- Extension of technology to marine aquaculture. The traditional practise of extensive land based aquaculture of marine species is under pressure, due to limitations in space requirements of flow through systems and global energy constraints. To this effect, current operations are transitioning towards partial of fully reticulated culture systems.

The products have been tested in vitro and were shown to be functional under marine conditions. Future in vivo testing in culture of local species of interest, such as abalone and stumpnose, is being planned.

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