Appl Microbiol Biotechnol (2008) 79:111–118
DOI 10.1007/s00253-008-1403-8

APPLIED MICROBIAL AND CELL PHYSIOLOGY

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

Rajesh Lalloo · Dheepak Maharajh · Johann Görgens · Neil Gardiner

Received: 14 December 2007 / Revised: 6 February 2008 / Accepted: 7 February 2008 / Published online: 4 March 2008
© Springer-Verlag 2008

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*

R. Lalloo (✉) · D. Maharajh · N. Gardiner
CSIR Biosciences,
Private Bag X2,
Modderfontein 1645, South Africa
e-mail: RLalloo@csir.co.za

J. Görgens
Department of Process Engineering, Stellenbosch University,
Private Bag X1,
Stellenbosch 7602, South Africa

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

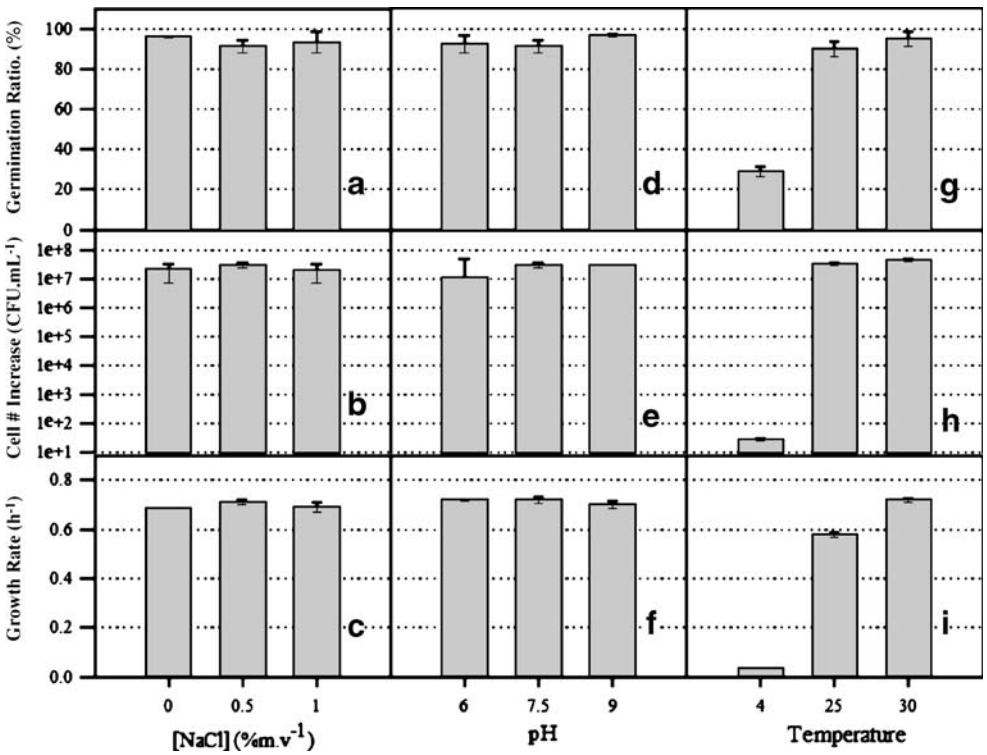

Fig. 1 Growth rate, cell number increase and spore germination in response to [NaCl], pH and temperature in matrix studies during shake-flask cultivation

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.

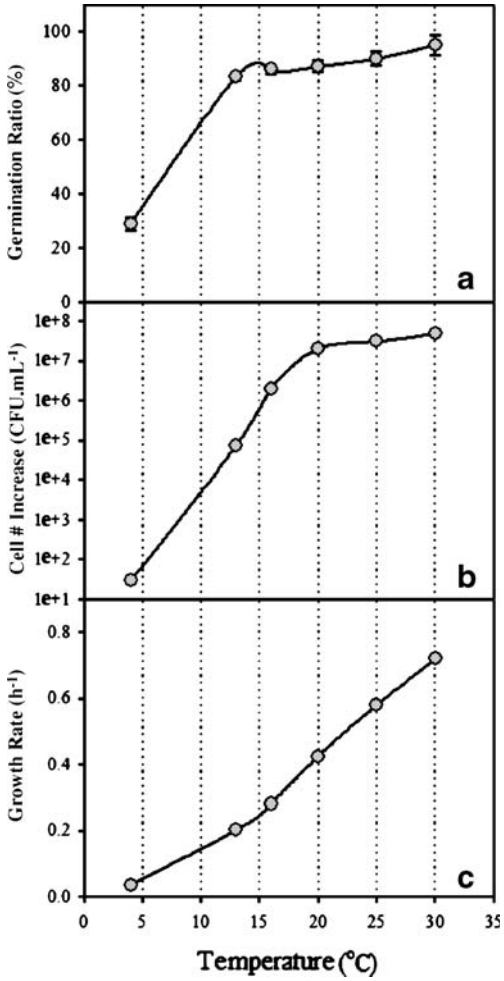

Fig. 2 Growth rate, cell number increase and spore germination in response to temperature in bioreactor cultivation

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).

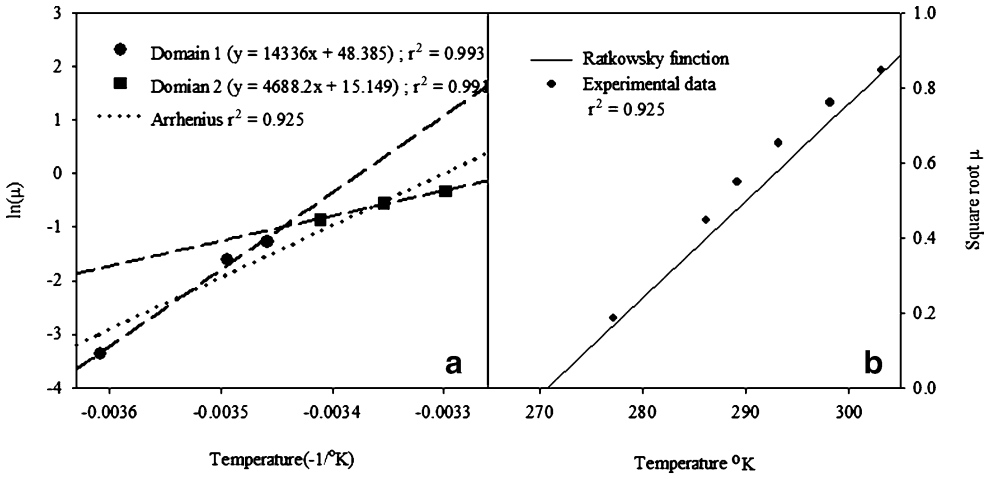

Fig. 3 Arrhenius and Ratkowsky plots in response to temperature

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).

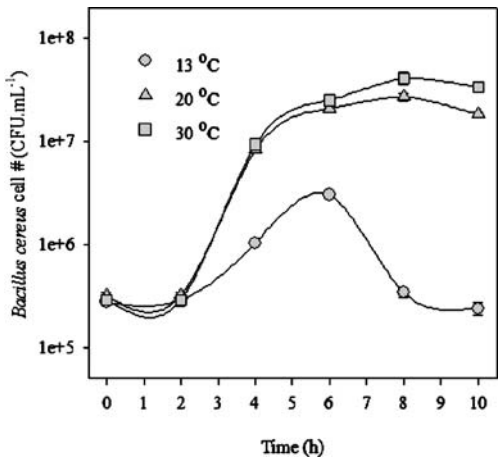

Fig. 4 Growth of *B. cereus* in co-culture with *A. hydrophila* at low-, mid- and high-temperature domains in shake-flask culture (n = 3)

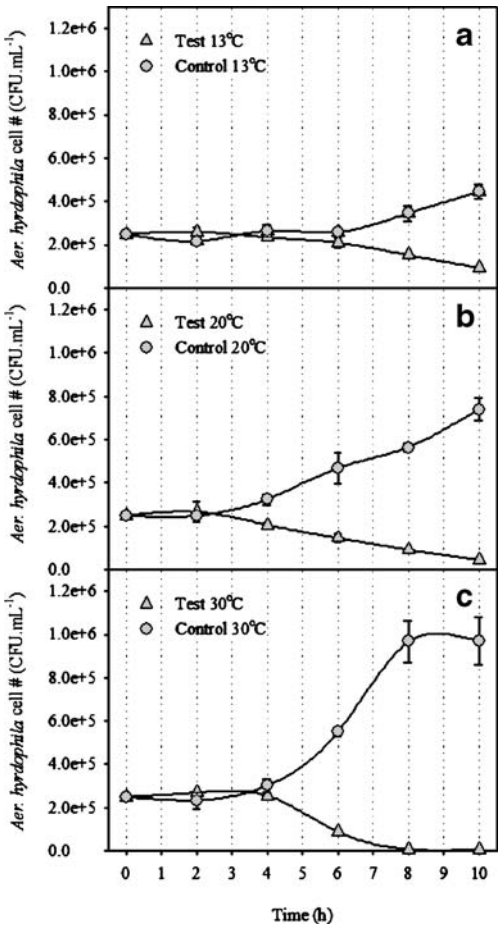

Fig. 5 Growth of *A. hydrophila* in co-culture with and without *B. cereus* at low-, mid- and high-temperature domains in shake-flask culture (n = 3)

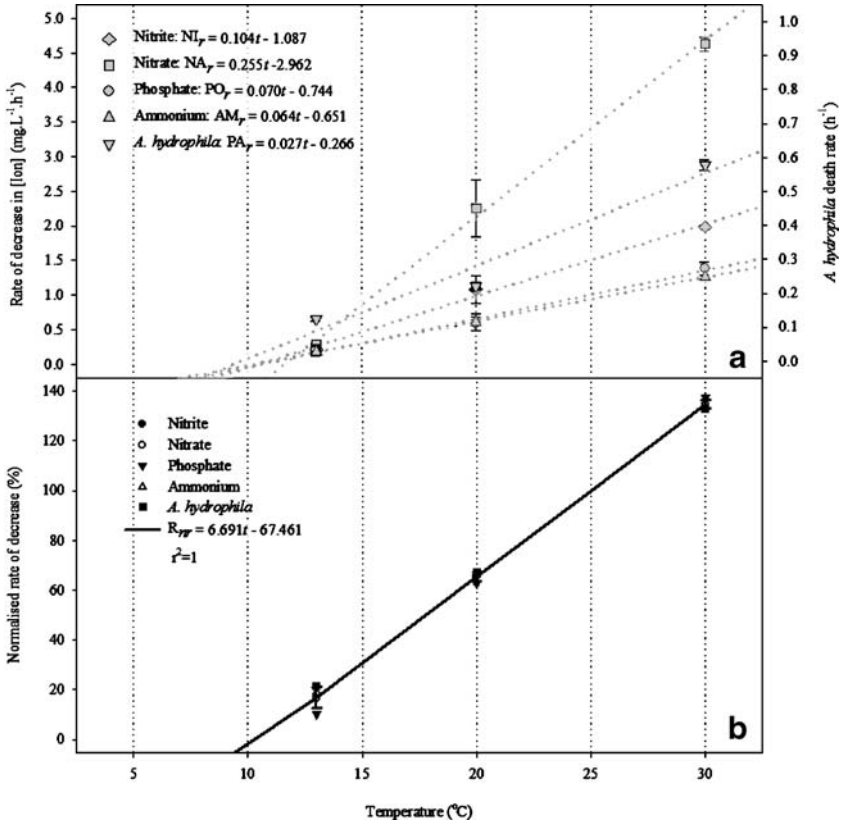

Fig. 6 Rate of decrease in pathogen and waste ion concentrations in response to temperature

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.

Acknowledgements Nodumo Zulu is acknowledged for the technical assistance. This work was supported by the BioPAD Biotechnology Regional Innovation Centre.

References

Austin B, Austin DA (1999) Bacterial fish pathogens. Springer Praxis, Chichester UK

Barker G (2000) Novel methods to reduce disease in aquaculture. Fish Vet J 5:66–71

Boyd CE (1985) Chemical budgets for channel catfish ponds. Trans Am Fish Soc 114:291–298

Boyd CE, Tucker CS (1998) Pond aquaculture water quality management. Kluwer, Boston MA USA

Budde I, Steil L, Scharf C, Völker U, Bremer E (2006) Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. Microbiology 152:831–853

Choma C, Clavel T, Dominguez H, Razafindramboa N, Soumille H, Nguyen-the C, Schmitt P (2000) Effect of temperature on growth characteristics of *Bacillus cereus* TZ415. Int J Food Microbiol 55:73–77

Chorin E, Thuault D, Cléret J, Bourgeois C (1997) Modelling *Bacillus cereus* growth. Int J Food Microbiol 38:229–234

de Kinkelin P, Michel C (1992) The use of drugs in aquaculture. Infofish Int 11:45–49

Fast AW, Menasveta P (2000) Some recent issues and innovations in marine shrimp pond culture. Reviews Fish Sci 8:151–233

Foldes T, Bámhegyi I, Herpai Z, Varga L, Szigeti J (2000) Isolation of *Bacillus* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food borne pathogenic and spoilage micro-organisms. J Appl Microbiol 89:840–846

Frances J, Nowak BF, Allan GL (2000) Effects of ammonia on juvenile silver perch (*Bidyanus bidyanus*). Aquaculture 183:95–103

Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas flourescens* AH2, a possible probiotic treatment of fish. Appl Environ Microbiol 65:969–973

Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270

Gross A, Nemirovsky A, Zilberg D, Khaimov A, Brenner A, Snir E, Ronen Z, Nejidat A (2003) Soil nitrifying enrichments as biofilter starters in intensive re-circulating saline water aquaculture. Aquaculture 223:51–62

Guetsky R, Shtienberg Y, Elad Y, Fischer E, Dinoor A (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 92:976–985

Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29:813–835

Irianto A, Austin B (2002) Probiotics in Aquaculture. J Fish Dis 25:633–642

Irie T, Watarai S, Iwasaki T, Kodama H (2005) Protection against experimental *Aeromonas salmonicida* infection in carp by oral immunisation with bacterial antigen entrapped liposomes. Fish Shellfish Immunol 18:235–242

Jana BB, Jana S (2003) The potential and sustainability of aquaculture in India. J Appl Aquac 13:283–316

Jeney Z, Jeney G (1995) Recent achievements in studies on disease of common carp (*Cyprinus carpio* L.). Aquaculture 129:397–420

Jobin MP, Clavel T, Carlin F, Schmitt P (2002) Acid tolerance response is low-pH and late stationary growth phase inducible in *Bacillus cereus* TZ415. Int J Food Microbiol 79:65–73

Kielwein G, Gerlach RU, Johne H (1969) Untersuchungen über das Vorkommen von *Aeromonas hydrophila* in Rohmilch. Arch F Lebensmittelhyg 20:34–38

Kielwein G (1971) Die Isolierung und Differenzierung von Pseudomonaden aus Lebensmitteln. Arch F Lebensmittelhyg 22:29–37

Kim JD, Kim KS, Song JS, Lee JY, Jeong KS (1998) Optimum level of dietary monocalcium phosphate based on growth and phosphorous excretion of mirror carp, *Cyprinus carpio*. Aquaculture 161:337–344

Kim JK, Park KJ, Cho KS, Nam S, Park T, Bajpai R (2005) Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. Bioresour Technol 96:1897–1906

Lalloo R, Ramchuran S, Ramduth D, Gorgens J, Gardiner N (2007) Isolation and selection of *Bacillus spp*. as potential biological agents for enhancement of water quality in culture of ornamental fish. J Appl Microbiol 103:1471–1479

Lammens M (2004) The Koi Doctor. A Publishing/Kindai bvba, Belgium

Larmoyeux JD, Piper RG (1973) Effects of water reuse on rainbow trout in hatchery. Prog Fish-Cult 35:2–8

Leguerinel I, Couvert O, Mafart P (2000) Relationship between the apparent heat resistance of *Bacillus cereus* spores and the pH and NaCl concentration of the recovery medium. Int J Food Microbiol 55:223–227

Liao PB, Mayo RD (1974) Intensified fish culture combining water reconditioning with pollution abatement. Aquaculture 3:61–85

Lin Y, Tanaka S, Kong H (2006) Characterization of a newly isolated heterotrophic nitrifying bacterium. Water Practice & Technology 1:3

Martienssen M, Schöps R (1999) Population dynamics of denitrifying bacteria in a model biocommunity. Water Res 33:639–646

Metz JR, van den Burg E, Wendelaar Bonga SE, Flik G (2003) Regulation of branchial N^+/K^+-ATPase in common *Cyprinus carpio* L. acclimated to different temperatures. J Exp Biol 206:2273–2280

Meza RA, Monroy AF, Mercado M, Poutou RA, Rodriguez P, Pedroza AP (2004) Study of the stability in real time of cryopreserved strain banks. Universitas Scientiarum 9:35–42

Monteiro SM, Clemente JJ, Henriques AO, Gomes RJ, Carrondo MJ, Cunha AE (2005) A procedure for high-yield spore production by *Bacillus subtilis*. Biotechnol Progr 21:1026–1031

Morales JA, de Graterol LS, Mesa J (2000) Determination of chloride, sulfate and nitrate in groundwater samples by ion chromatography. J Chromatogr A 884:185–190

Moriarity DJW (1999) Disease Control in Shrimp Aquaculture with Probiotic Bacteria. Microbial Interactions in Aquaculture. In: Bell CR, Brylinsky M (eds) Proceedings of the 8th International Symposium on Microbial Ecology, Canada

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64:548–572

Paperna I (1991) Disease caused by parasites in the aquaculture of warm water fish. Ann Rev Fish Dis 1:155–194

Ratkowsky DA, Lowry RK, McMeekin TA, Stokes AN, Chandler RE (1983) Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J Bacteriol 154:1222–1226

Rengipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasaveta P (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191:271–288

Robertson LA, Kuenen JG (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiospaera pantotropha* and other bacteria. Antonie van Leeuwenhoek 57:139–152

Sakai K, Ikehata Y, Ikenaga Y, Wakayama M (1996) Nitrite oxidation by heterotrophic bacteria under various nutritional and aerobic conditions. J Ferment Bioeng 82:613–617

Sakai K, Nakamura K, Wakayama M, Moriguchi M (1997) Change in nitrite conversion direction from oxidation to reduction in heterotrophic bacteria depending on the aeration conditions. J Ferment Bioeng 86:47–52

Sanders ME, Morelli L, Tompkins TA (2003) Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus* and *Brevibacillus*. Comprehensive Reviews in Food Science and Food Safety 2:101–110

Shimeno S, Shikata T, Hosokawa H, Masumoto T, Kheyyali D (1997) Metabolic response to feeding rates in common carp, *Cyprinus carpio*. Aquaculture 151:371–377

Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177:333–343

Sze CP (2000) Antibiotics use in aquaculture. Infofish Int 19:24–28

Su JJ, Liu BY, Lin J, Yang CP (2001) Isolation of an aerobic denitrifying bacteria strain NS-2 from the activated sludge of piggery wastewater treatment systems in Taiwan processing denitrification under 92% oxygen atmosphere. J Appl Microbiol 91:853–860

Ugoji EO, Laing MD, Hunter CH (2006) An Investigation of the shelf-life (storage) of *Bacillus* isolates on seeds. J Bot 72:28–33

Vanbelle M, Teller E, Focant M (1990) Probiotics in animal nutrition. Archives of Animal Nutrition 40:543–567

Vaseeharan B, Ramamsamy P (2003) Control of pathogenic *Vibrio spp*. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Peneaus monodon*. Lett Appl Microbiol 36:83–87

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671

Wolken WAM, Tramper J, van der Werf MJ (2003) What can spores do for us. Trends Biotechnol 21:338–345