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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.

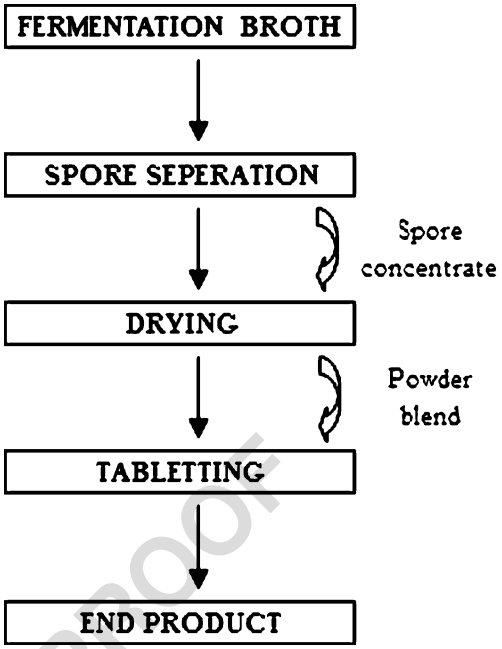

Fig. 1 Conceptual downstream process flow sheet for production of a tablet product

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 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.

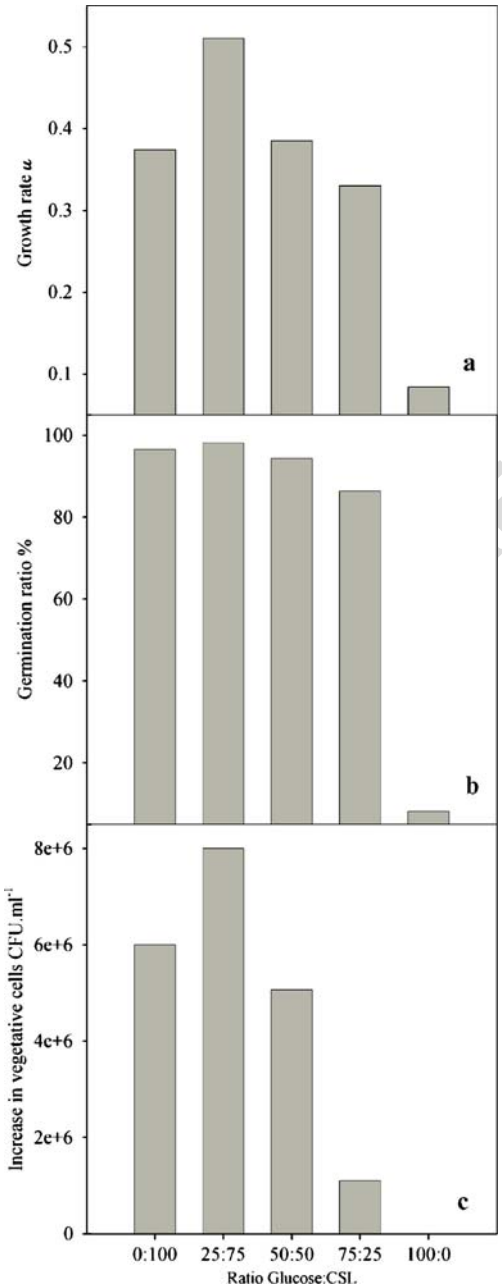

Fig. 2 a–c Influence of glucose to CSL ratio in supporting germination and growth

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.

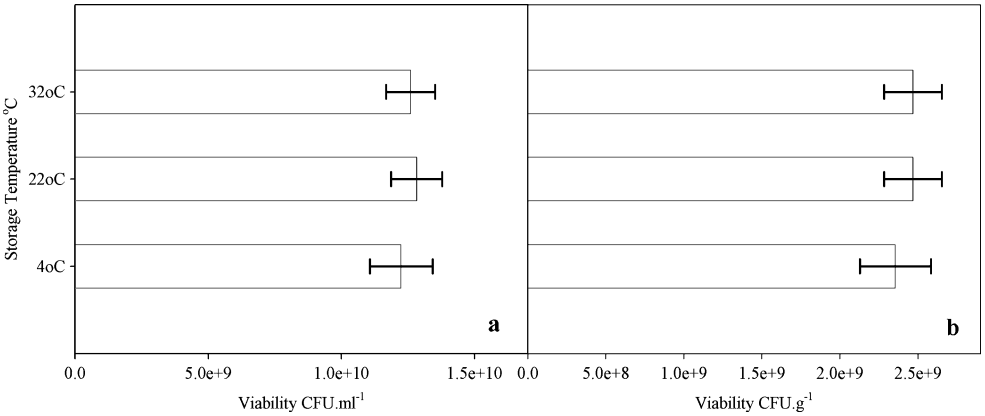

Fig. 3 Viability of product intermediates **a** spore concentrate and **b** powder blend at different temperatures

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.*

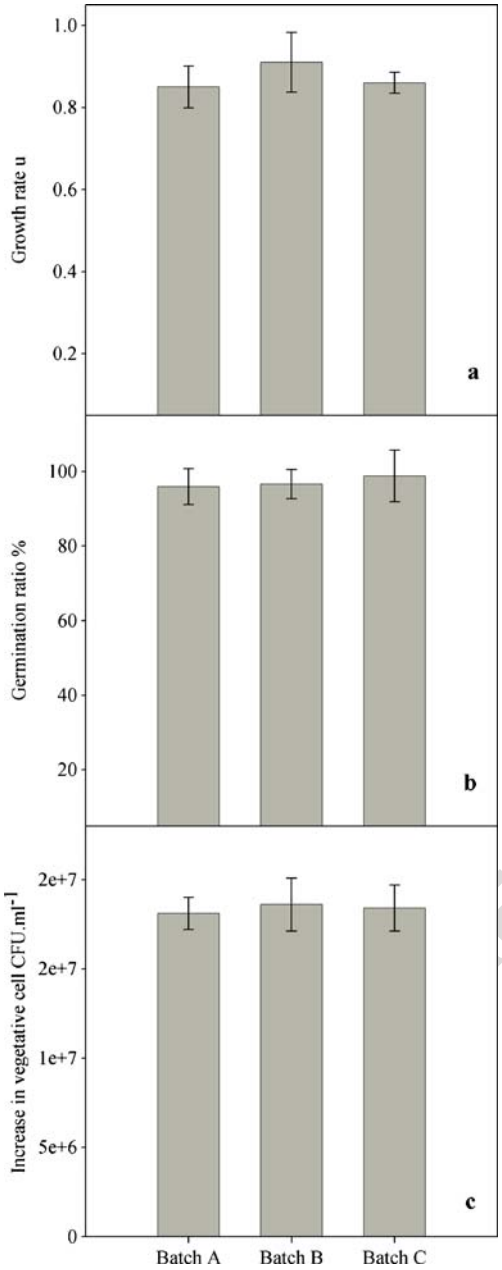

Fig. 4 **a**–**c** Germination and growth of *B. cereus* in the tablet end product (CV<10% across batches *A*, *B* and *C*)

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

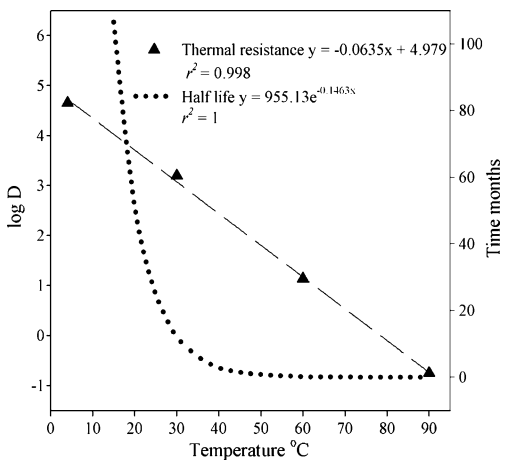

Fig. 5 Thermal resistance and half-life plots showing end product stability

t2.1 **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 gh^{-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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