Appl Microbiol Biotechnol (2009) 83:59–66
DOI 10.1007/s00253-008-1845-z

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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

Rajesh Lalloo · Dheepak Maharajh · Johann Görgens · Neil Gardiner · J. F. Görgens

Received: 27 August 2008 / Revised: 26 December 2008 / Accepted: 26 December 2008 / Published online: 16 January 2009
© Springer-Verlag 2009

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

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

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

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 ultra-filtered 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 $\sim 1\times10^7$ 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}

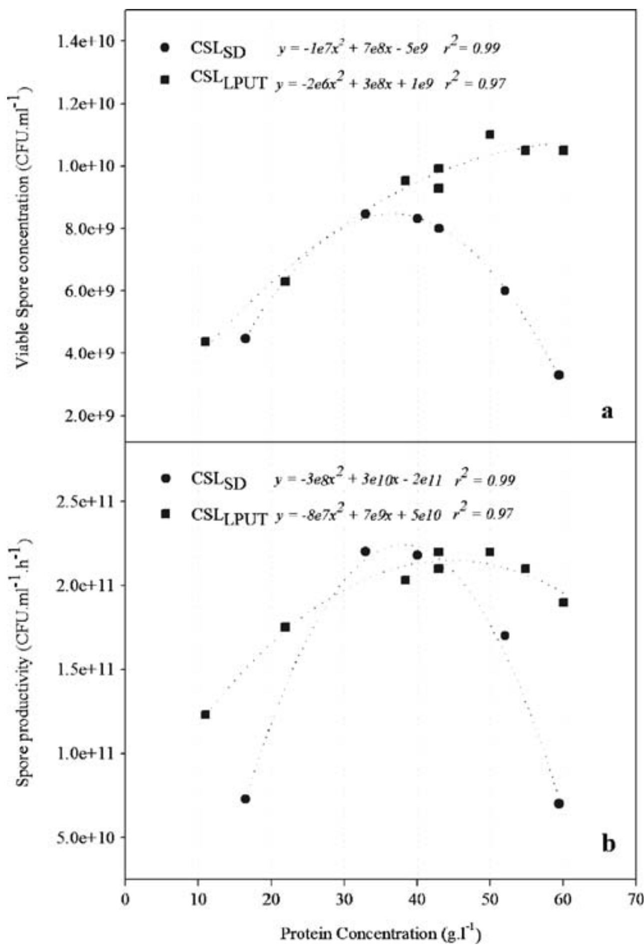

Fig. 1 *B. cereus* viable spore concentrations (**a**) and spore productivity (**b**) when different concentrations of CSL_{SD} or CSL_{LPUT} based on total protein were tested

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

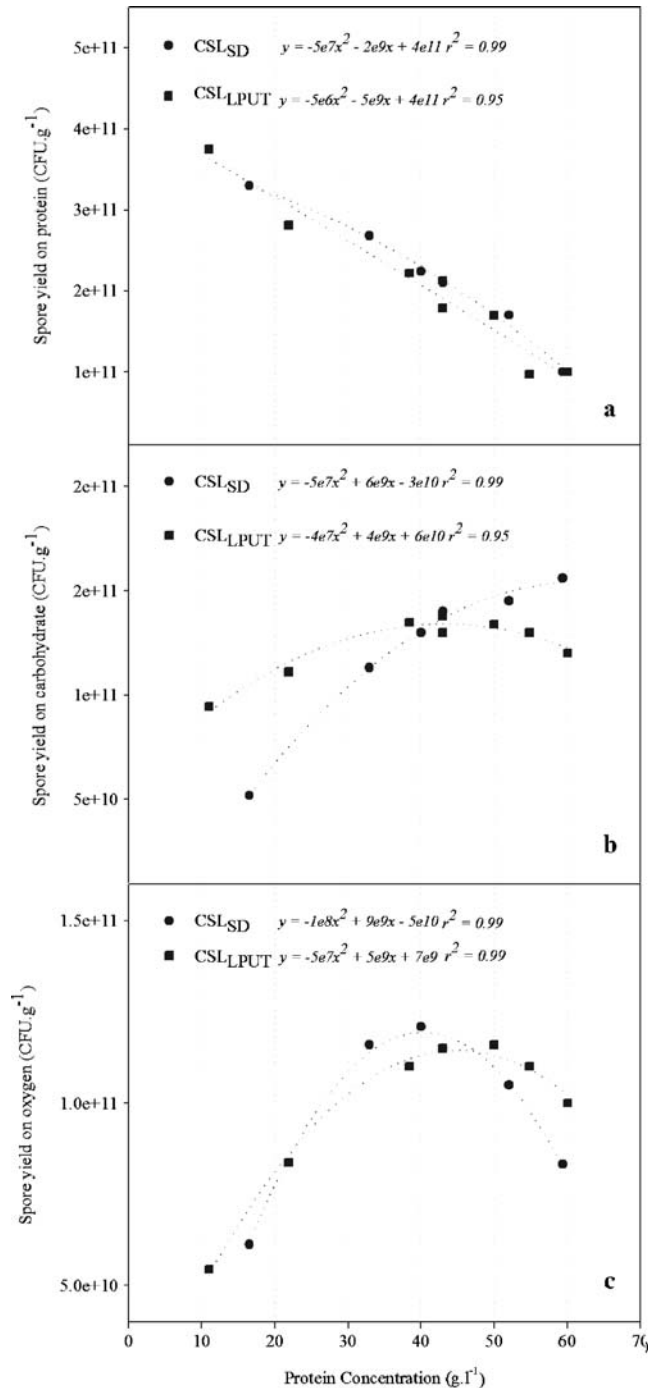

Fig. 2 *B. cereus* yield on protein (**a**), yield on carbohydrate (**b**), and yield on oxygen (**c**) when different concentrations of CSL_{SD} or CSL_{LPUT} based on total protein were tested

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.

Acknowledgements This work was supported by BioPAD Biotechnology Regional Innovation Center.

References

Avignonne-Rossa C, Arcas J, Mignone C (1992) *Bacillus thuringiensis* growth, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures. World J Microbiol Biotechnol 8:301–304

Chang M, Zhou S, Lu N, Ni J (2008) Starch processing wastewater as a new medium production of *Bacillus thuringiensis*. World J Microbiol Biotechnol 24:441–447

Dingman DW, Stahly DP (1983) Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl Environ Microbiol 46:860–869

Errington J (2003) Regulation of endospore formation in *Bacillus subtilis*. Nat Rev Microbiol 1:117–126

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

Gouda MK, Swellam AE, Omar SH (2001) Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol Res 156:201–207

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

He N, Li YL, Chen J (2004) Production of a novel polygalacturonic acid bioflocculant REA-11 by *Corynebacterium glutamicum*. Bioresource Technol 94:99–105

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

Kuppusamy M, Balaraman K (1991) Effect of corn-steep liquor on growth and mosquito larvicidal activity of *Bacillus thuringiensis var irealensis* de Barjac and *B. sphaericus* Neide 1904. Indian J Exp Biol 29:87–189

Lalloo R, Ramchuran S, Ramduth D, Görgens 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

Lalloo R, Maharajh D, Görgens J, Gardiner N (2008) Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in aquaculture. Appl Microbiol Biotechnol 79:111–118

Liu WM, Bajpai R, Bihari V (1994) High-density cultivation of spore formers. Ann N Y Acad Sci 721:310–325

Lopez JM, Dromenck A, Freese E (1981) Response of guanosine 5′-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J Bacteriol 146:605–613

Mao W, Pan R, Freedman D (1992) High production alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using synthetic medium. J Ind Microbiol 11:1–6

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

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

Msadek T (1999) When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. Trends Microbiol 7:201–207

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

Nickerson K, Bulla LA Jr (1974) Physiology of spore forming bacteria associated with insects: minimal nutritional requirements for growth, sporulation and parasporal crystal formation of *Bacillus thuringiensis*. Appl Microbiol 28:124–128

Nohata Y, Kurane R (1997) Complete defined medium for large-scale production of polysaccharide bioabsorbent from *Alcaligenes latus* B-16. J Ferment BioEng 83:116–117

Nori H, Yamane T, Kobayashi T, Shimizu S (1983) Comparison of cell productivity among fed-batch, repeat fed-batch and continuous cultures at high cell concentration. J Ferment Technol 61:391–401

Ochi K, Kandala JC, Freese E (1981) Initiation of *Bacillus subtilis* sporulation by the stringent responses to partial amino acid deprivation. J Biol Chem 256:6866–6875

Papanikolaou S, Aggelis G (2002) Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresource Technol 82:43–49

Payot T, Chemaly Z, Fick M (1998) Lactic acid production by *Bacillus coagulans*—kinetic studies and optimization of culture medium for batch and continuous fermentations. Enzyme Microbial Technol 24:191–199

Prabakaran G, Balaram K, Hoti SL, Manonmani AM (2007) A cost-effective medium for the large-scale production of *Bacillus sphaericus* H5a5b (VCRC B42) for mosquito control. Biol Cont 41:379–383

Preeta R, Jayaprakash NS, Philip R, Singh ISB (2006) Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic (*Pseudomonas* MCCB 103) using response surface methodology. J Appl Microbiol 102:1043–1051

Purushothaman M, Anderson RKI, Narayana S, Jayaraman VK (2001) Industrial byproducts as cheaper medium components influencing the production of polyhydroxyalkanoates (PHA)-biodegradable plastics. Bioprocess Biosyst Eng 24:131–136

Rättö M, Poutanen K, Viikari L (1992) Production of xylanolytic enzymes by an alkali tolerant *Bacillus circulans* strain. Appl Microbiol Biotechnol 37:470–473

Reisenberg D, Guthke R (1999) High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 51:422–430

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

Sanders ME, Morelli L, Tompkins TA (2003) Spore formers as human probiotics: *Bacillus*, *Sporolactobacillus* and *Brevibacillus*. Comp Rev Food Sci Safety 2:101–110

Schilling O, Frick O, Herzberg C, Ehrenreich A, Heinzle E, Wittman C, Stulke J (2007) Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: transcription regulation is important but not sufficient to account for metabolic adaptation. Appl Environ Microbiol 73:499–507

Sharpe ES, Bulla LA Jr (1978) Characteristics of the constituent substrains of *Bacillus popillae* growing in batch and continuous cultures. Appl Environ Microbiol 35:601–609

Shi F, Zhu Y (2007) Applications of statistically-based experimental designs in medium optimization for spore production of *Bacillus subtilis* from distillery effluent. BioControl 52:845–853

Shikata S, Saeki K, Okoshi H, Yoshimatsu T, Ozaki K, Kawai S, Ito S (1990) Alkaline cellulases for laundry detergents: production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. Agric Biol Chem 54:91–96

Silveira MM, Wisbeck E, Hoch I, Jonas R (2001) Production of glucose-fructose oxidoreductase and ethanol by *Zymomonas mobilis* ATCC 29191 in medium containing corn steep liquor as a source of vitamins. Appl Microbiol Biotechnol 55:442–445

Sonenshein AL (2000) Control of sporulation initiation in *Bacillus subtilis*. Curr Opin Microbiol 3:561–566

Srivasta RAK, Baruah JN (1986) Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. Appl Env Microbiol 52:179–184

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

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

Vuolanto A, von Weymarn N, Kerovuo J, Ojamo H, Leisola M (2001) Phytase production by high cell density culture or recombinant *Bacillus subtilis*. Biotechnol Lett 23:761–766

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

Zhang J, Greasham R (1999) Chemically defined media for commercial fermentations. Appl Microbiol Biotechnol 51:407–421