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ORIGINAL PAPER

Effect of medium components and culture conditions in *Bacillus subtilis* EA-CB0575 spore production

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Abstract *Bacillus subtilis* spores have important biotechnological applications; however, achieving both, high spore cell densities and sporulation efficiencies in fermentation, is poorly reported. In this study, medium components and culture conditions were optimized with different statistical methods to increase spore production of the plant growth promoting rhizobacteria *B. subtilis* EA-CB0575. Key medium components were determined with Plackett–Burman (PB) design, and the optimum concentration levels of two components (glucose, $MgSO_4.7H_2O$) were optimized with a full factorial and central composite design, achieving 1.37×10^9 CFU/mL of spore cell density and 93.5 % of sporulation efficiency in shake flask. The optimized medium was used to determine the effect of culture conditions on spore production at bioreactor level, finding that maintaining pH control did not affect significantly spore production, while the interaction of agitation and aeration rates had a significant effect on spore cell density. The overall optimization generated a 17.2-fold increase in spore cell density (8.78×10^9 CFU/mL) and 1.9-fold increase in sporulation efficiency (94.2 %) compared to that of PB design. These results indicate the potential of *B. subtilis* EA-CB0575 to produce both, high spore cell densities and sporulation efficiencies, with very low nutrient requirements and short incubation period which can represent savings of process production.

Keywords *Bacillus subtilis* · Bacterial culture optimization · Spore production · Sporulation efficiency

Introduction

Aerobic Endospore-Forming Bacteria (AEFB), especially some species of *Bacillus* spp., have received growing attention because of their industrial potential in the development of biopesticides and biofertilizers. In the agricultural industry, spores are used as alternatives to agrochemicals as Plant Growth Promoting Rhizobacteria (PGPR) or as Biological Control Agents (BCA) [1–4]. In the market economy, *Bacillus*-based products represent about 85 % of the commercially available bacterial BCA [5] but to be able to commercialize these bio-products, industrial exploitation of spores requires high cell density bio-reaction and good sporulation efficiency; for this reason, spore production is a key step in bio-products development when AEFB are the active ingredient [6].

Bacillus spp. uses a system of Quorum-Sensing to initiate its sporulation process that occurs in high cell density populations and only if the cell is in a stressful condition [7]. Furthermore, during the stationary growth phase in a fermentation process, when nutrients are exhausted, the culture initiates sporulation at a cell density of about 10^8 cells/mL [8]. Under ideal conditions, typical cell densities and sporulation efficiencies for *B. subtilis* will be in the range of 1.00×10^8–1.52×10^{10} CFU/mL [9–12] and

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30–80 %, respectively using serial dilution and plate count methods [11–14]. Other studies have reported higher sporulation efficiencies or higher spore cell densities for other *Bacillus* species different from *B. subtilis* [3, 6, 15, 16], but to our knowledge obtaining high values of both variables has not been reported yet.

Different optimum culture media and culture conditions for AEFB sporulation have been reported, where each particular strain has its own requirements and optimum conditions [10, 12, 17, 18]. For example, the presence of glutamate in the medium benefits the growth of *B. cereus* while for other species of *Bacillus* this compound inhibits growth [19]. It has also been reported that adding salts to the media improves sporulation, but high concentrations of heavy metals contained in these salts could affect growth [10]. Elements such as Ca, Mn, Mg, Fe and Zn in appropriate concentrations are essential in sporulation since they are present in the spore layers and enable it to resist high temperatures [11] and the last two elements accelerate the sporulation process [3, 20]. Regarding the culture conditions, several studies have evaluated the effect of different effective volumes (1–70 L), aeration rates (0.5–2.0 vvm), agitation rates (200–500 rpm), pH (5.0–9.0) and inoculum size (1–3 %) in order to obtain large amounts of biomass and secondary metabolites [3, 9, 12, 21, 22], concluding that optimum culture conditions are very specific for each strain. Therefore, optimization of culture medium and culture conditions of new strains can lead to industrially viable processes.

In this study we designed and optimized a culture medium to increase spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575, a PGPR strain, at shake flask level. The optimized medium was used to optimize the culture conditions pH, agitation and aeration rates in a 14 L bioreactor. Finally kinetics of cell growth and substrate uptake are described in optimized conditions.

Materials and methods

Microorganism and culture mediums

The PGPR, *B. subtilis* EA-CB0575, was isolated from the rhizosphere of banana plants in Uraba (Northeast Colombia, 1,352,081 N, 1,044,577 E) in September 2009 and identified by analysis of 16S rDNA gene sequencing (Genbank Accession Number KC170988). The bacteria was stored in TSB (Tripticase Soy Broth, Merck) plus 20 % v/v glycerol at −80 °C (Humboldt Institute Collection No. 191) and activated in TSA (Merck) for 24 h at 30 °C before any experimental use.

Optimized medium SBM (Sporulation *Bacillus* Medium) was composed of: glucose 1.04 g/L, $MgSO_4.7H_2O$ 0.59 g/L, KH_2PO_4 6.0 g/L, meat extract 5.0 g/L, special peptone 3.0 g/L, NaCl 0.01 g/L and stock salt solution (1.136 mL/L of $FeSO_4.7H_2O$ 0.1 M, 300 µL/L of $ZnSO_4.7H_2O$ 0.1 M, 9.9 mL/L of $CaCl_2$ 0.1 M, 30 mL/L of $MnCl_2$ 0.1 M). Modified SBM medium was composed of the same components but we replaced meat extract by yeast extract in the same concentration.

Shake flask culture conditions

For shake flask inoculum preparation, a colony of the strain grown in TSA was transferred to 250 mL Erlenmeyer flaks containing 50 mL of TSB, and incubated at 30 °C and 150 rpm for 24 h. The culture was then centrifuged and the pellet suspended in the respective culture medium with a final optical density (OD_{600}) of 1.0 (equivalent to 1.0×10^8 CFU/mL). Forty mL of each culture medium and 5 mL of a previously filtrated stock salt solution was added to a 250 mL Erlenmeyer flask, inoculated with 5 mL of the inoculum culture, incubated at 30 °C at 150 rpm for 96 h. Total cells and spore cell densities were determined by the spread plate technique.

Bioreactor culture conditions

The inoculum used for the bioreactor fermentations was prepared by transferring two colonies of *B. subtilis* EA-CB0575 grown in TSA to a 2000 mL Erlenmeyer flask containing 240 mL of modified SBM and incubated at 30 °C and 150 rpm for 24 h. Three percent (v/v) of inoculum with a final optical density of 2.0 and 330 mL of sterile stock salts solution was added to 7.43 L of sterile modified SBM into a 14 L Bioreactor (BioFlo110, New Brunswick Scientific Co) equipped with a diffuser ring type, two Rushton turbine, 6 impellers of flat blades and 4 baffles.

Design and formulation of culture medium

Plackett and Burman design $2^{(7 \times 3/32)}$ with 3 central points was used to determine which of eight medium components significantly affected *B. subtilis* EA-CB0575 total cell density, spore cell density and sporulation efficiency in shake flask. The components included glucose, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, KH_2PO_4, yeast extract, meat extract, peptone, and $(NH_4)_2SO_4$ which were evaluated in three concentrations, high (+1), medium (0) and low (−1) in fifteen experimental trials (Table 1). The experiment was independently repeated three times.

Medium optimization

Full factorial and central composite (CC) designs were employed to optimize the component concentrations in

Table 1 PBD design for total cell and spore cell densities of *B. subtilis* EA-CB0575

Factors (g/L)									Response variables		
Treatment	Glucose (X_1)	$MgSO_4.7H_2O$ (X_2)	$MnCl_2.4H_2O$ (X_3)	KH_2PO_4 (X_4)	Yeast extract (X_5)	Meat extract (X_6)	Peptone (X_7)	$(NH_4)_2SO_4$ (X_8)	Total cell density (10^9 CFU/mL)	Spore cell density (10^9 CFU/mL)	Sporulation efficiency (%)
M1	2	0	0	6	5	5	0	4	1.12 ± 1.15	0.61 ± 0.30	54.2 ± 22.0
M2	2	0	0.5	6	5	0	3	4	0.00 ± 0.00	0.00	0.0
M3	20	0	0.5	0	0	0	3	4	0.09 ± 0.00	0.00	0.0
M4	2	0.5	0.5	0	5	0	0	0	0.78 ± 0.23	0.51 ± 0.02	65.5 ± 13.6
M5	20	0	0	0	5	5	3	0	1.55 ± 1.10	0.00	0.0
M6	11	0.25	0.25	3	2.5	2.5	1.5	2	0.29 ± 0.13	0.00	0.0
M7	20	0.5	0	6	5	0	3	0	0.12 ± 0.00	0.00	0.0
M8	11	0.25	0.25	3	2.5	2.5	1.5	2	0.19 ± 0.01	0.00	0.0
M9	20	0.5	0.5	0	5	5	0	4	1.07 ± 0.01	0.00	0.0
M10	11	0.25	0.25	3	2.5	2.5	1.5	2	0.12 ± 0.00	0.00	0.0
M11	2	0.5	0.5	6	0	5	3	0	2.01 ± 0.00	1.87 ± 0.05	93.2 ± 0.3
M12	2	0	0	0	0	0	0	0	0.00 ± 0.02	0.00	0.0
M13	20	0.5	0	6	0	0	0	4	0.08 ± 0.00	0.00	0.0
M14	20	0	0.5	6	0	5	0	0	0.06 ± 0.00	0.00	0.0
M15	2	0.5	0	0	0	5	3	4	1.03 ± 0.01	0.52 ± 0.15	50.7 ± 16.0
Cell density *P* value	0.570	**0.041***	0.301	0.599	0.083	**0.020***	0.954	0.212	Significance $\alpha = 0.05$		
Spore cell density *P* value	**0.001***	0.081	0.991	0.993	0.992	0.964	0.986	0.951			
Sporulation efficiency *P* value	**0.022***	0.096	0.036	0.492	0.989	0.231	0.650	0.231			

Bold values are statistically significant ($P < 0.05$)

order to get optimum spore cell densities and sporulation efficiencies of *B. subtilis* EA-CB0575 in shake flask. Two components (glucose and $MgSO_4.7H_2O$) that significantly affected the response variables were optimized by a 3^2 full factorial design. The concentration levels of glucose were 0.8, 1.4 and 2.0 g/L and the same levels for $MgSO_4.7H_2O$. The other medium components were kept constant at the concentration they had in the best assay of the Plackett and Burman design (peptone 3.0 g/L, meat extract 5.0 g/L, KH_2PO_4 6.0 g/L, $MnCl_2.4H_2O$ 0.5 g/L, and stock salt solution as stated before). A series of nine experiments were carried out in duplicate and the spore cell density and sporulation efficiency were determined 96 h after culture. This experiment was independently repeated three times and the average of the three was statistically analyzed with Design Expert 8.0.7.1. The model employed to correlate the response variable to the independent variables is showed in Eq. (1):

$$y(x_i) = b_0 + \sum_{i=1}^{n} b_i \times x_i + \varepsilon \quad (1)$$

where $y(x_i)$ represents the response variable, x_i is the independent variable (medium components), b_o is the interception coefficient, b_i the coefficient of the linear effect and ε is the random error [23].

Afterwards a CC design 2^{2+star} with 2 center points was employed with glucose and $MgSO_4.7H_2O$ as study factors. A total of 10 experiments were carried out in triplicate and the spore cell density and sporulation efficiency were determined 96 h after culture. The design was statistically analyzed with a second-order polynomial model presented in Eq. (2) through the Response Surface Methodology RSM.

$$y(x_i) = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j + \varepsilon \quad (2)$$

where $y(x_i)$ represents the response variable, x_i is the independent variable (medium component), b_o is the interception coefficient, b_i the coefficient of the linear effect, b_{ii} is the coefficient for the quadratic effect, b_{ij} corresponds to the coefficient for the interaction effect and ε is the random error.

The responses predicted by the second-order models were used to generate a partial desirability function for each response (d_i) that were defined as in Eq. (3).

$$d_{i(y(x_i))} = \begin{cases} 0, & \text{if} \quad y(x_i) < L_i \\ \left(\dfrac{y(x_i) - L_i}{U_i - L_i}\right)^s, & \text{if} \quad L_i \le y(x_i) \le U_i \, l \\ 1, & \text{if} \quad y(x_i) \ge U_i \end{cases} \quad (3)$$

where $y(x_i)$ is the predicted response using the fitted model (Eq. 2), L_i and U_i are the highest and the lowest values obtained for the response i, respectively, and s is the weight of the variable $y(x_i)$ in the multiple optimization. The weights used were equal to 1 for each of the two responses, spore cell density and sporulation efficiency. With the d_i functions defined, they were combined to obtain a global desirability function (D) represented by Eq. (4).

$$D = \left(d_1^{r_1} \times d_2^{r_2} \ldots \times d_n^{r_n}\right)^{\frac{1}{\sum_{i=1}^{n} r_i}} = \left(\prod_{i=1}^{n} d_i^{r_i}\right)^{\frac{1}{\sum_{i=1}^{n} r_i}} \quad (4)$$

where D is the value of the global desirability function, (d_1) and (d_2) are the partial desirability functions computed for the spore cell density and sporulation efficiency, n is the number of variables, and r_i is the relative importance assigned to each response.

This experiment was independently repeated three times. The optimized medium was named SBM medium (Sporulation *Bacillus* Medium).

Validation of the optimum medium and evaluation of modified media at flask level

To validate the prediction of the mathematical optimization, spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575 were determined through an independent trial with SBM medium in Erlenmeyer flasks. Furthermore, the spore production in SBM medium and modified SBM was compared with a unifactorial design with three replicates per treatment. This experiment was independently repeated twice.

Effect of pH on *B. subtilis* EA-CB0575 spore production

The effect of pH on total cell density, spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575 was evaluated using a single factorial design in a 14 L bioreactor. The levels evaluated were pH 6.5, 7.0, and uncontrolled pH (initial pH = 5.5) with two replicates per treatment and the response variables were determinated at 48 h of fermentation. Batch fermentation of *B. subtilis* EA-CB0575 was carried out in a 14 L bioreactor containing 8 L medium, at 30 °C, 300 rpm and 8 L/min of aeration rate.

Effect of agitation and aeration rate on *B. subtilis* EA-CB0575 spore production

The effect of agitation and aeration rates on *B. subtilis* EA-CB0575 spore cell density and sporulation efficiency were evaluated using a CC design 2^{2+star} with two central points in a 14 L bioreactor. A total of ten experiments were carried out in duplicate and the spore cell density and

sporulation efficiency were determined 48 h after culture. The design was statistically analyzed with a second-order polynomial model presented in Eq. (2) through the RSM. Additionally, the responses predicted by the second-order models were used to generate a partial desirability function (d_i) for each response that were defined as in Eq. (3). With the d_i functions defined, they were combined to obtain a global desirability function (D) represented by Eq. (4). The weights used were equal to 1 for each of the two responses, spore cell density and sporulation efficiency.

Kinetics of cell growth and substrate uptake

Kinetics of cell growth and substrate uptake of *B. subtilis* EA-CB0575 were determined in a 14 L bioreactor with 8 L of modified SBM medium at the optimized conditions (432 rpm, 12 L/min, and uncontrolled pH). One milliliter samples were taken every 2 h for the first 30 h and afterwards every 6 h until 50 h for the determination of total cell and spore cell densities (CFU/mL), sporulation efficiency (%), dry weight biomass (g/L), optical density (OD) and sugar uptake (g/L).

Analytical methods

Dry weight biomass (g/L) and glucose concentration (g/L) in the culture medium were determined by the dry weight methodology and DNS methodology [24].

Total cells and spore cell densities were determined by the spread plate technique. Briefly, samples were serially diluted and 100 µL of each dilution was plated in TSA for total cell density determination in CFU/mL. For spore cell density, serially diluted samples were subject to heat shock (80 °C, 20 min) and then plated in TSA. Response variables (total cell and spore cell density) were estimated after 48 h of incubation at 30 °C for those dilutions containing 30–300 CFU/mL. Sporulation efficiency was determined as the ratio between spore cell and total cell densities.

Statistical analysis

The statistical software Design Expert® 8.0.7.1. (Stat-Ease, Inc., Minneapolis, USA) was used to perform the experimental design, the regression and the graphical analysis of data obtained. Analysis of variance (ANOVA) and LSD multiple comparison test were carried out with the data obtained. The confidence level used for ANOVA analyses was 95 % and data of total cell and spore cell density (CFU/mL) were transformed using a logarithmic scale (Log_{10}).

Results and discussion

Selection of medium components for spore production

Among the fifteen media tested by the Plackett and Burman design, only four (M1, M4, M11 and M15) produced spores after 96 h of evaluation (Table 1). Media M1, M4, M11, and M15, all of which had low glucose content in common (2.0 g/L), had spore cell densities and sporulation efficiencies between 0.51×10^9 and 1.87×10^9 CFU/mL, and 50.7 and 93.2 %, respectively. Medium M11 had the highest spore cell density (1.87×10^9) and the highest sporulation efficiency (93.2 %). The media with high quantities of glucose (>11 g/L) did not produce spores and only two of the media with low quantities did not produce them either, such as medium M12 which lacked a source of nitrogen. Media with and without $MnCl_2$ produce spores, but those that have this salt at its highest evaluation level (0.5 g/L) have the highest sporulation efficiencies.

For total cell density, a significant positive effect of $MgSO_4.7H_2O$ (X_2) (P value = 0.041) and meat extract (X_6) (P value = 0.020) factors was seen (Table 1). These results indicate that in order to increase biomass produced by *B. subtilis* EA-CB0575, it is necessary to raise the levels of these two factors. Regarding the production of spores and sporulation efficiencies, glucose (X_1) is the only factor that had a significant negative effect on these variables (P value = 0.001 and 0.022, respectively). Therefore, the concentration of glucose in the culture medium should be reduced to increase the spore cell density and the sporulation efficiency. The negative effect of glucose on sporulation has also been determined by different studies [12, 25] and it suggests that an excess of glucose inhibits sporulation by repressing the transcription of the *spo0A* gen. This gene is responsible of encoding Spo0A, a response regulator activated by phosphorylation in response to several internal and external stimuli and is the master regulator to entry into sporulation [8, 26]. The importance of compounds such as KH_2PO_4, yeast extract, peptone and $(NH4)_2SO_4$ had been shown to significantly stimulate sporulation [6, 27]. Although, in this study these factors didńt affect sporulation possible because the mayor role of glucose on sporulation, which is sensitive to catabolic repression [28].

According to the previous results, the factors that were selected for the next step of optimization, were glucose (range 0.2–1.2 g/L) and $MgSO_4.7H_2O$ (range 0.5–1.5 g/L). Meat extract concentration (5.0 g/L), $MnCl_2.4H_2O$ (0.5 g/L), KH_2PO_4 (6.0 g/L) and peptone (3.0 g/L) were left unchanged. Although, the meat extract had significant

effects on the total cell density, it was left unaltered at the higher level because of its cost and because it had no effect on sporulation. Furthermore, the yeast extract and $(NH_4)_2SO_4$ were eliminated from the culture medium and the concentrations of the previously established salts were left unchanged.

Medium optimization for *B. subtilis* EA-CB0575 spore production

A full factorial design was used to find the optimization area for the two significant components, glucose (X_1) and $MgSO_4.7H_2O$ (X_2). A total of 18 experiments were performed, obtaining spore cell densities between 0.74×10^9 and 1.58×10^9 CFU/mL, and sporulation efficiencies between 50.9 and 95.8 % (Table 2). The ANOVA analysis of the optimization study indicated that among the two significant variables selected by the Plackett and Burman design experiment, $MgSO_4.7H_2O$ (X_2) was found to have a significant effect on spore cell density (P value = 0.001) and sporulation efficiency (P value = 0.022), contrary to glucose concentration. Furthermore, the interaction between ($MgSO_4.7H_2O$) × ($MgSO_4.7H_2O$) ($X_2 \times X_2$) and glucose × glucose ($X_1 \times X_1$) were significant on the spore cell density (P value = 0.002) and sporulation efficiency (P value = 0.032), respectively. Although, glucose did not have a significant effect on the response variables, concentrations between 0.2 and 1.2 g/L were evaluated for the next assay.

The P values and the R^2 for the model of spore cell density (P value = 0.025, $R^2 = 0.820$, R^2 adj = 0.748, Eq. 5) and sporulation efficiency (P value = 0.022, $R^2 = 0.698$, R^2 adj = 0.557, Eq. 6) suggested that the experimental data fit well with the model. These values indicated that less than 18.0 and 30.2 % of the total variation was not explained by the model, respectively. The model predicted a maximum spore cell density and sporulation efficiency of 1.0×10^9 CFU/mL and 133 %, respectively, in the medium containing (g/L): 2.0, glucose; 0.5, $MgSO_4.7H_2O$. A sporulation efficiency of 133 %, which has no biological meaning, can be due to the low determination coefficient of the model.

$$\mathrm{Log}\left(\frac{\mathrm{CFU}}{\mathrm{mL}}\right) = 9.41 + 0.086X_1 - 0.89X_2 - 0.11X_1X_2 + 0.46X_2^2 \quad (5)$$

$$\text{Sporulation efficiency } (\%) = 7.64 + 133.996X_1 + 1.81X_2 - 43.46X_1^2 - 17.58X_1X_2 \quad (6)$$

Different authors have selected factors that have a significant effect by means of factorial designs or fractional factorials [10, 29] but go directly to an optimization design or to a response surface method (RSM). However, in this study, a full factorial design was used to define the optimization zone and, once in this zone, a CC design was used to find the optimum point for each variable.

In the CC design, the spore cell density and sporulation efficiency were between 0.91×10^9 and 1.45×10^9 CFU/mL and 66.3–100.0 %, respectively (Table 3), which represents an increase with respect to the full factorial design. The ANOVA analysis of the CC design indicated that glucose (X_1), $MgSO_4.7H_2O$ (X_2) and $MgSO_4.7H_2O \times MgSO_4.7H_2O$ ($X_2 \times X_2$) had a significant effect on spore cell density (P value = 0.010, 0.052, 0.041, respectively); and glucose (X_1), glucose × $MgSO_4.7H_2O$ ($X_1 \times X_2$), $MgSO_4.7H_2O \times MgSO_4.7H_2O$ ($X_2 \times X_2$) and glucose × glucose ($X_1 \times X_1$) had a significant effect on sporulation efficiency (P value = 0.055, 0.045, 0.024 and 0.011, respectively). The P values for the model and the lack of fit test of spore cell density (0.027, 0.343, Eq. 7) and sporulation efficiency (0.022, 0.133, Eq. 8), indicated

Table 2 Full factorial design for glucose and $MgSO_4$ factors respect to spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575

RUN	Glucose (X_1) (g/L)	$MgSO_4.7H_2O$ (X_2) (g/L)	Spore cell density (10^9 CFU/mL)		Sporulation efficiency (%)	
			Observed	Predicted	Observed	Predicted
1	0.8	0.5	1.30 ± 0.05[a]	1.26	85.1 ± 5.8	80.9
2	1.4	0.5	1.16 ± 0.04	1.31	72.0 ± 2.3	98.7
3	2	0.5	1.58 ± 0.06	1.36	95.8 ± 4.4	85.2
4	0.8	1	0.88 ± 0.11	0.89	72.5 ± 8.7	74.8
5	1.4	1	0.96 ± 0.09	0.86	88.8 ± 0.5	87.3
6	2	1	0.74 ± 0.13	0.82	64.9 ± 1.8	68.5
7	0.8	1.5	0.99 ± 0.03	1.06	66.7 ± 1.3	68.6
8	1.4	1.5	1.06 ± 0.03	0.96	80.0 ± 8.6	75.9
9	2	1.5	0.83 ± 0.02	0.85	50.9 ± 0.4	51.8

[a] Intervals represent standard errors of the mean

Table 3 Central composite design for glucose and $MgSO_4$ factors respect to spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575

RUN	Glucose (X_1) (g/L)	$MgSO_4.7H_2O$ (X_2) (g/L)	Spore cell density (10^9 CFU/mL)		Sporulation efficiency (%)	
			Observed	Predicted	Observed	Predicted
1	0.7	0.7	1.15 ± 0.04[a]	1.30	100.0 ± 7.4	99.4
2	0.2	0.2	0.91 ± 0.04	1.03	66.3 ± 1.7	70.2
3	1.2	0.2	1.10 ± 0.10	1.35	96.4 ± 0.8	97.2
4	0.2	1.2	1.02 ± 0.05	1.04	89.5 ± 10.2	87.1
5	1.2	1.2	1.35 ± 0.02	1.2	76.6 ± 6.8	81.2
6	0	0.7	1.15 ± 0.04	1.05	84.6 ± 3.5	78.6
7	1.41	0.7	1.45 ± 0.05	1.38	95.1 ± 2.7	93.0
8	0.7	0	0.97 ± 0.05	1.15	83.5 ± 5.7	82.3
9	0.7	1.41	1.07 ± 0.13	1.05	89.3 ± 1.4	83.4
10	0.7	0.7	1.20 ± 0.08	1.30	98.7 ± 4.1	99.4

[a] Intervals represent standard errors of the mean

that the model fit appropriately with the experimental data. The second-order polynomial equations (Eqs. 7, 8), obtained from the ANOVA analysis, showed that the value of R^2 was 0.917 (R^2 adj = 0.815) for spore cell density and 0.927 (R^2 adj = 0.835) for sporulation efficiency, indicating that less than 8.3 and 7.3 % of the total variation was not explained by the model, respectively.

$$\mathrm{Log}\left(\frac{\mathrm{CFU}}{\mathrm{mL}}\right) = 8.94 + 0.23X_1 + 0.22X_2 - 0.07X_1^2 - 0.06X_1X_2 - 0.15X_2^2 \quad (7)$$

$$\text{Sporulation efficiency } (\%) = 45.31 + 71.95X_1 + 71.56X_2 - 27.38X_1^2 - 33.0X_1X_2 - 34.32X_2^2 \quad (8)$$

Multiple response regression was evaluated using spore cells density and sporulation efficiency responses and glucose and $MgSO_4.7H_2O$ were used as factors. Desirability was the dependent variable for this regression and the optimum value for both responses was determined. Desirability measures the overlap for the optimum value of the response variables. In this case, the desirability value for multiple response regression was 0.875 with 1.2 g/L of glucose and 0.66 g/L of $MgSO_4.7H_2O$, which would reach 1.33×10^9 CFU/mL of spore cell density and 97.7 % of sporulation efficiency. Supplementary material Figure S1 presents the multivariable optimization and the stated values of those compounds that make it possible to get the highest desirable function.

The optimization of the medium by the CC design resulted in a 1.5-fold increase in spore cell density compared to that of the full factorial design prediction (1.0×10^9 CFU/mL). These values do not surpass the spore cell density reached by Chen et al. [9], who reported a concentration of 1.52×10^{10} CFU/mL for the *B. subtilis* WHK-Z12 strain, but to our knowledge, the sporulation efficiency reached in this work (93.5 ± 4.0 %) is the highest reported to date for *B. subtilis* strains that are in the range of 30–80 % [11, 12, 14].

Model verification and evaluation of modified medium

In order to verify the optimization results, an experiment was performed with the optimized levels of nutrients predicted by the model. Spore cell density and sporulation efficiencies of $(1.37 \pm 0.15) \times 10^9$ CFU/mL and 93.5 ± 4.0 %, respectively, were obtained, suggesting that experimental and predicted values were in good agreement with percentage errors of 6.8 and 2.8 %, respectively, validating the model. Additionally, the medium optimization resulted in a 2.7- and 1.9-fold increase in spore cell density and sporulation efficiency, respectively, as compared to that of the Plackett and Burman treatment that showed lower spore cell density (5.10×10^8 CFU/mL). In this optimization process glucose concentration was also reduced form 2.0 g/L used in the Plackett and Burman design to 1.2 g/L used in the SBM medium, representing savings of 40 %.

The effect of changing the nutrient meat extract by a nutrient of lower cost (yeast extract) in the optimized SBM medium on the production of spores was also determined. Spore cell density and sporulation efficiency had no significant differences between the SBM and modified SBM medium (P value = 0.232 and 0.249, respectively), suggesting that spore production is not affected by changing the complex nitrogen source.

Influence of pH, agitation and aeration rate on *B. subtilis* EA-CB0575 spore production

To determine the effect of pH on *B. subtilis* EA-CB00575 spore production at bioreactor level a single factorial design was conducted. The ANOVA analysis indicated that maintaining the pH at a constant value (6.5 or 7.0) or under noncontrolled pH bioreaction did not affect significantly total cell density (P value = 0.209), spore cell density (P value = 0.285) and sporulation efficiency (P value = 0.895). Under noncontrolled conditions, pH variation was between 5.5 and 7.0 during 48 h of fermentation, and spore cell densities and sporulation efficiencies of $(2.27 \pm 0.56) \times 10^9$ CFU/mL and 92.9 ± 5.1 % were achieved. Contrary to our results, Monteiro et al. [12], reported that maintaining the pH at a constant value during the fermentation increases spore production, although in the ranges of 6–9 the sporulation efficiency did not depend on the pH value (11). Therefore our results could indicate that the low variation on pH during the fermentation process did not affect the sporulation achieved.

To determine the effect of the agitation and aeration rate on *B. subtilis* EA-CB0575 spore production under noncontrolled pH bioreaction, operating conditions between 300 and 500 rpm of agitation rate and 8–16 L/min of aeration rate were evaluated. In the CC design, the spore cell density and sporulation efficiency were between 1.24×10^9 and 9.33×10^9 CFU/mL and 78.9–96.2 %, respectively (Table 4), which represents a 6.8 increase in spore cell density with respect to the shake flask conditions. The ANOVA analysis of the CC design indicated that agitation rate (X_1), agitation × agitation ($X_1 \times X_1$) and agitation × aeration ($X_1 \times X_2$) had a significant effect on spore cell density (P value = 0.053, 0.021, 0.024, respectively), while none of the two variables had significant effects on sporulation efficiency. The P values for the model and the lack of fit test of spore cell density (0.027, 0.339, Eq. 9) indicated that the model fit appropriately with the experimental data. The second-order polynomial equation (Eq. 9), obtained from the ANOVA analysis, showed that the value of R^2 was 0.900 (R^2 adj = 0.754), indicating that less than 10 % of the total variation was not explained by the model.

$$\mathrm{Log}\left(\frac{\mathrm{CFU}}{\mathrm{mL}}\right) = 2.21 + 0.02X_1 + 4.1X_2 - 2.5 \times 10^{-5}X_1^2 - 2.9 \times 10^{-5}X_1X_2 - 1.32X_2^2 \quad (9)$$

CC design showed higher total cell and spore cell densities (CFU/mL) around the central points of agitation and aeration ranges (400 rpm and 12 L/min). The highest value for spore cell density was 9.33×10^9 CFU/mL, reporting a sporulation efficiency of 91.2 % at 400 rpm and 12 L/min of air (Table 4). The response surface curve for simple and multivariate optimization shown in Supplementary material Figure S2 shows an optimum value for both response variables at 432 rpm and 12 L/min, with a total cell and spore cell densities prediction of 1.02×10^{10} CFU/mL and 9.59×10^9 CFU/mL, respectively, and 94.0 % of spore efficiency. These results are in accordance with several reports were an increase on agitation and aeration rates generate higher biomass and metabolites production on different *Bacillus* species [22, 30–32].

Table 4 Central composite design for agitation and aeration factors respect to spore cell density and sporulation efficiency *of B. subtilis* EA-CB0575 at bioreactor level

RUN	Agitation (X_1) (rpm)	Aeration (X_2) L/min (vvm)	Spore cell density (10^9 CFU/mL)		Sporulation efficiency (%)
			Observed	Predicted	Observed
1	400	12 (1.5)	9.33 ± 0.0[a]	8.81	91.2 ± 1.1
2	300	8 (1.0)	1.56 ± 0.2	1.59	96.2 ± 3.8
3	500	8 (1.0)	5.20 ± 1.0	3.55	83.9 ± 4.8
4	300	16 (2.0)	1.24 ± 0.0	1.56	91.9 ± 7.8
5	500	16 (2.0)	3.60 ± 0.7	3.18	81.8 ± 9.0
6	259	12 (1.5)	2.14 ± 0.3	1.70	79.8 ± 6.0
7	541	12 (1.5)	3.30 ± 0.3	4.70	97.1 ± 1.4
8	400	6.5 (0.8)	1.53 ± 0.5	1.96	78.9 ± 1.3
9	400	18 (2.2)	2.17 ± 0.2	1.89	95.2 ± 2.6
10	400	12 (1.5)	8.72 ± 0.1	8.81	95.5 ± 4.3

[a] Intervals represent standard errors of the mean

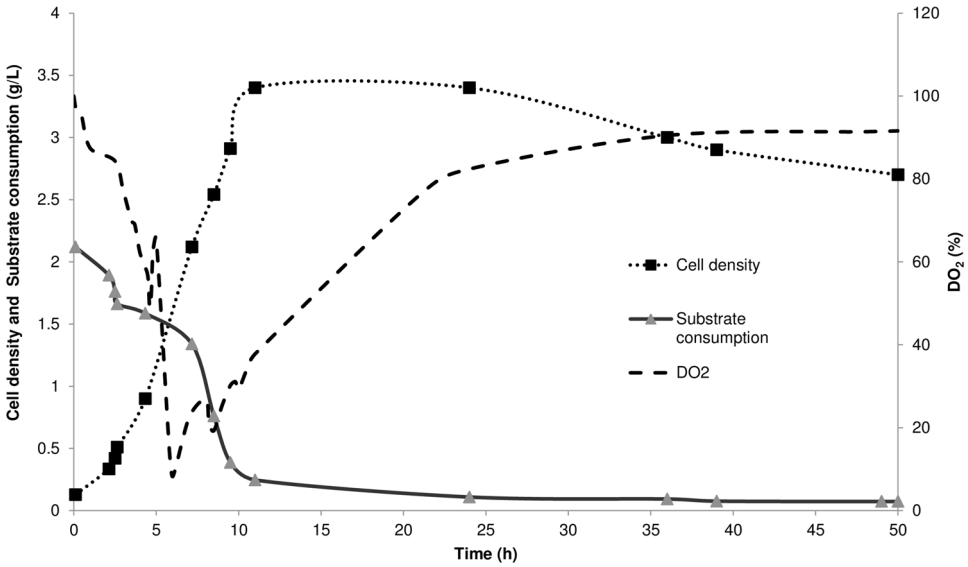

Fig. 1 Growth, substrate and oxygen uptake of *B. subtilis* EA-CB0575 in optimized conditions at bioreactor level

Kinetics of cell growth and substrate uptake of *B. subtilis* EA-CB0575

The kinetics of cell growth and substrate uptake of *B. subtilis* EA-CB0575 in modified SBM medium were determined at the optimal conditions in a batch culture at bioreactor level (Fig. 1). Biomass concentration (g/L) increased during 10 h in the exponential phase reaching 3.5 g/L, consuming 96.9 % of reducing sugars and 92.0 % of dissolve oxygen. After 10 h of growth, cells entered a stationary phase and after 40 h of culture total biomass decline and dissolve oxygen reached 91.6 % suggesting the formation of spores [12, 15].

Additionally in order to verify the optimization results at bioreactor level, spore cell density and sporulation efficiencies were determined at 50 h of culture obtaining 8.78×10^9 CFU/mL and 94.2 %, respectively. These results suggest that experimental and predicted values were in good agreement with percentage errors of 7.9 and 1.1 %, respectively, validating the model. The optimization of the culture conditions resulted in a 6.4-fold increase in spore cell density compared to that obtained at shake flask (1.37×10^9 CFU/mL) and the overall optimization generated a 17.2-fold increase in spore cell density and 1.9-fold increase in sporulation efficiency compared to that of PB treatment that showed the lower values (5.10×10^8 CFU/mL and 50.7 %). Our results did not surpass the spore cell density (1.56×10^{10} spores/mL) reported previously for *B. subtilis* WHK-Z12 in a 30 L bioreactor with 16.18 g/L of corn steep liquor, 17.53 g/L of soybean flour and 8.14 g/L of yeast extract, although we achieved a high spore cell density and sporulation efficiency with very low carbon and nitrogen requirements (1.04 g/L glucose and 5 g/L of yeast extract), with no lag phase and a short exponential phase (10 h), which can represent savings of culture medium.

Conclusions

High cell density and sporulation efficiency are important issues for the profitability of the production of *Bacillus* used as bioproducts. In literature, spore cell densities and sporulation efficiencies have been reported up to 1.52×10^{10} CFU/mL (Chen et al. [9]) and 80 % (Monteiro et al. [12]), respectively, although obtaining high values of both variables is poorly reported. In the present study, the improvement of both spore cell density and sporulation efficiency of *B. subtilis* EA-CB0575 was achieved by the optimization of medium components and culture conditions. In general, spore production of *B. subtilis* EA-CB0575 was affected by the medium components glucose and $MgSO_4.7H_2O$ and by the culture conditions agitation and aeration rate. The culture conditions stablished in this study, permitted to reach a high final spore cell density of 8.78×10^9 CFU/mL and a high sporulation efficiency of 94.2 % at bioreactor level, being the sporulation efficiency the highest value reported to date for *B. subtilis* in batch culture. Finally, with this culture approach the quantity of nutrients needed for the fermentation were reduced, the costs of the reagents used for pH control were avoid, and the high spore production was obtained in a short incubation period, which could represent savings on bioprocess costs.

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