Biocatalysis and Agricultural Biotechnology 3 (2014) 378–385

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Original Research Paper

Multiple response optimization of *Bacillus subtilis* EA-CB0015 culture and identification of antifungal metabolites

Sandra Mosquera [a,c], Lina María González-Jaramillo [a], Sergio Orduz [b], Valeska Villegas-Escobar [a,*]

[a] Grupo CIBIOP, Departamento Ingeniería de Procesos, Universidad EAFIT, Carrera 49 No 7 Sur 50, Medellín, Colombia
[b] Escuela de Biociencias, Facultad de Ciencias, Universidad Nacional de Colombia, Medellín, Colombia
[c] Centro de Investigaciones del Banano - Augura, Carepa, Colombia

ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form
29 August 2014
Accepted 3 September 2014
Available online 16 September 2014

Keywords:
Bacillus subtilis
Medium optimization
Lipopeptides
Mycosphaerella fijiensis
Multiple response optimization

ABSTRACT

The low yields of biomass and antimicrobial metabolites obtained in fermentation processes are limiting factors for implementing biological control agents in the field. In this context, optimization of the culture medium for the biological control agent *Bacillus subtilis* EA-CB0015 was conducted in submerged culture to maximize the biomass production and antifungal activity. Additionally, the active metabolites against the phytopathogen *Mycosphaerella fijiensis* produced under optimized conditions were identified. Carbon and nitrogen constituents of the culture medium were optimized using a 2^2 factorial design with central point followed by a multiple response optimization coupled to the desirability function approach. The optimized medium (33.4 g/L of glucose and 32.5 g/L of yeast extract), showed significant increases in both, cell density by 3.6 folds (13.2 ± 1.0 g/L) and antifungal activity by 1.2 folds ($77.7 \pm 0.3\%$ inhibition). To gain insight into the type of active compounds, they were purified and identified by mass spectrometry (MS). The MS analysis revealed the presence of three families of lipopeptides: surfactin, fengycin and iturin. The high antifungal activity was associated with the novel fengycin C and with iturin A which were partially growth-associated with a maximum concentration of 781.4 and 355.4 mg/L at 36 h of growth, respectively. These results indicate the potential of *B. subtilis* EA-CB0015 to produce high concentrations of biomass and lipopeptides that can be exploited for biotechnological application.

1. Introduction

Black Sigatoka, caused by the fungus *Mycosphaerella fijiensis* Morelet, is the most serious and destructive disease of banana crops (Marín et al., 2003) generating losses in production and reductions in fruit weight when not treated (Castelan et al., 2012). The increasing use of synthetic fungicides to control this disease has brought problems related to economic and environmental aspects and to the emergence of resistant strains of the pathogen to the traditional fungicides (Marín et al., 2003). Therefore, alternative control approaches that are sustainable, such as biological control, have gained interest. The implementation of biological control agents has become an important strategy for the integrated management of diseases in commercial crops (Bailey et al., 2010) and different bacteria, especially from the *Bacillus* genus, have been selected as potential biological control agents (Raaijmakers et al., 2010; Heins et al., 2002).

Contrary to the high number of potential biological control agents of plant diseases, the number of biological products commercially available for their control is reduced (Hynes and Boyetchko, 2006). These products represent less than 5% of the pesticides used in agriculture (Bailey et al., 2010), probably due to difficulties in the fermentation and formulation processes of the biological control agent (Hynes and Boyetchko, 2006). A limiting factor for development, commercialization and implementation of *Bacillus* spp.-based products for the control of plant diseases at commercial levels is the low yields obtained during the fermentation process and high production costs (Schisler et al., 2004). Hence, the development of a culture medium that optimizes production of the microbial strain is crucial (Hynes and Boyetchko, 2006).

Culture medium optimization, enhancing microbial growth and production of metabolites using different strategies, has been studied for different applications (Masurekar, 2008; Parekh et al., 2000). Between these strategies, the Response Surface Methodology (RSM) has been increasingly used and successfully applied to optimize the medium components of *Bacillus* spp. for lipopeptide (Shih et al., 2009; Gu et al., 2005) and spore (Rao et al., 2007)

* Corresponding author. Tel.: +57 4 2619500x9823.
E-mail address: vvilleg2@eafit.edu.co (V. Villegas-Escobar).

http://dx.doi.org/10.1016/j.bcab.2014.09.004
1878-8181/

production in shake flask fermentation. Additionally, this technique allows the optimization of several factors simultaneously using few treatments (Shih et al., 2009). During culture media optimization process, there are more than one response of interest and therefore, the multiple response optimization coupled to the desirability function approach is a useful technique for the analysis of experiments in which several responses have to be optimized simultaneously (Del Castillo et al., 1996). This methodology has been effectively applied to optimize the culture medium composition of *Cordyceps jiangxiensis* (Xiao et al., 2006) and *Bacillus thuringiensis* (Moreira et al., 2007).

A new strain of *Bacillus subtilis* EA-CB0015, isolated from the phyllosphere of banana plants from Urabá (Colombia), has been identified as a producer of antimicrobial compounds active against *M. fijiensis* (Villegas-Escobar et al., 2013; Ceballos et al., 2012). When used as the active ingredient of a liquid formulation, the strain and its metabolites have shown the ability to reduce black Sigatoka severity, with reductions comparable to that obtained with mancozeb and chlorothalonil chemical controls (unpublished data). More recently, part of the active compounds produced by *B. subtilis* EA-CB0015 in shake flask fermentation were purified and identified (Villegas-Escobar et al., 2013). They were associated with the surfactin and fengycin lipopeptide families and they include a novel fengycin isoform, fengycin C (Villegas-Escobar et al., 2013). In the present study, RSM coupled to the desirability function approach was applied to optimize the levels of glucose and yeast extract of a culture medium for *B. subtilis* EA-CB0015 biomass and metabolites production in order to simultaneously maximize cell density and Cell-Free Supernatant (CFS) activity. Additionally, the compounds produced in the new medium responsible for inhibition of *M. fijiensis* were isolated and identified in order to determine the production time course of the active metabolites.

2. Materials and methods

2.1. Microorganism

B. subtilis EA-CB0015 strain (GenBank accession number KC006063) was isolated from the phyllosphere of a banana plant in Urabá, Colombia (Northeast Colombia, 1352081N, 1044577 E) in September 2008 and identified by sequencing of the 16S rDNA gene. The strain was maintained in TSB (Tripticase Soy Broth, Merck) with 20% glycerol at −80 °C (Humboldt Institute Collection no 191) and activated in TSA (Tripticase Soy Agar, Merck) for 48 h at 30 °C before any experimental use. The fungal strain *M. fijiensis* EASGK15 was used for the evaluation of the antimicrobial compounds produced by *B. subtilis* EA-CB0015. This fungal strain was obtained from diseased banana leaves from Urabá, Colombia using the DuPont methodology (Dupont, 1982). Twenty days old monosporic cultures obtained in PDA (Potato Dextrose Agar, Merck) were fragmented and stored (Ceballos et al., 2012) and the activation was performed by placing mycelia fragments into the liquid cultures with Sabouraud (Merck) for 10 days at 150 rpm and 30 °C. Identification of this strain was carried out by analysis of the Internal Transcribed Spacers (ITS) regions using the oligonucleotide primers PN3 F (5′ CCGTTGGTGAACCAGCGGGATC 3′) and PN16 R (5′ TCCCTTTCAACAATTTCACG 3′). Each reaction contained 25 μL 1 × colorless GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 200 nM PCR Nucleotide Mix, 625 nM GoTaq® DNA Polymerase, 600 nM of each primer and 200 nM of probe. Sterilized, deionized water was added to give a total reaction volume of 25 μL. Cycling conditions were: 5 min at 95 °C; and 35 cycles of 1 min at 95 °C followed by 1 min at 55 °C and 1.5 min at 72 °C, the final extension was carried out for 10 min at 72 °C.

2.2. Culture medium and growth conditions

B. subtilis EA-CB0015 seed culture was prepared by transferring one colony of *B. subtilis* EA-CB0015 to a 100 mL Erlenmeyer flask containing 20 mL of the basal medium (CIB medium) composed of $MnSO_4 \cdot 4H_2O$ (Carlo Erba), 0.042 g/L; $CaCl_2$ (Carlo Erba), 0.031 g/L; KH_2PO_4 (Carlo Erba), 0.5 g/L; K_2HPO_4 (Carlo Erba), 0.5 g/L; $(NH_4)_2SO_4$ (Carlo Erba), 1.0 g/L; yeast extract (Oxoid), 8.0 g/L; $MgSO_4 \cdot 7H_2O$ (Carlo Erba), 4.0 g/L and glucose (Merck), 8.0 g/L (Atehortúa et al., 2007) and cultured for 12 h at 30 °C and 200 rpm in an orbital shaker. The pH was adjusted to 7 before the sterilization. Then, 10% (v/v) of the seed culture was added to a 100 mL Erlenmeyer flask containing 10 mL of the experimental medium consisting of the basal medium with concentrations of glucose and yeast extract determined by the experimental design. The cultures were incubated for 48 h at 30 °C and 200 rpm in an orbital shaker.

2.3. Experimental design

In order to optimize glucose and yeast extract concentrations in the culture medium, a 2^2 factorial design with a central point and three replicates was build and consisted of 15 runs. The lowest and the highest levels of the variables were: glucose, 20 and 40 g/L and yeast extract 25 and 45 g/L which were coded as −1 and +1 while the central point was coded as 0 (Table 1). The cell density and the activity of the CFS obtained after 48 h culture were individually fitted to the second-order polynomial model presented in Eq. (1) through the RSM

$$Y_i = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X^2{}_i + \sum_{i,j=1}^{2} \beta_{ij} X_i X_j \quad (1)$$

where Y_i is the predicted response, X_i and X_j are the input variables, β_0 is the intercept term, β_i are the linear effects, β_{ii} are the squared effects and β_{ij} are the interaction effects. The responses predicted by the second-order models were used to generate a partial desirability function for each response i (d_i) that

Table 1
Bacillus subtilis EA-CB0015 cell density and inhibition percentage of the cell-free supernatant over *Mycosphaerella fijiensis* for the 2^2 factorial design.

Run	Glucose (g/L)	Yeast extract (g/L)	Cell density (g/L)		Inhibition %	
			Observed	Predicted (Y_1)	Observed	Predicted (Y_2) (%)
1	−1	−1	11.0 ± 0.5 (a)	10.98	69.6 ± 0.6% (b)	68.6
2	−1	1	12.5 ± 0.3 (ab)	12.49	62.9 ± 0.5% (a)	64.4
3	1	−1	12.7 ± 0.1 (b)	12.72	71.6 ± 0.4% (b)	73.6
4	1	1	15.3 ± 0.4 (c)	14.93	70.3 ± 1.1% (b)	69.4
5	0	0	12.8 ± 0.4 (b)	12.77	75.7 ± 1.9% (c)	75.9

Intervals represent standard errors of the mean (n=3); different letters indicate that the mean is significantly different ($P < 0.05$) based on Tukey multiple range tests.

were defined as (Moreira et al., 2007)

$$d_i = \left[\frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}}\right]^{w_i}, \quad Y_{\min} < Y_i < Y_{\max} \tag{2}$$

where Y_i is the predicted response using the fitted model (Eq. (1)), $Y_{\max}$ and $Y_{\min}$ are the highest and the lowest values obtained for the response i respectively and w_i is the weight. The weights used in this work were equal to 1 for each of the two responses. With the d_i functions defined, they were combined to obtain a global desirability function (D) represented by (Moreira et al., 2007)

$$D = \left(d_1^{r1} \times d_2^{r2}\right)^{1/\sum_{i=1}^{3} r_i} \tag{3}$$

where D is the value of the global desirability function, (d_1) and (d_2) are the partial desirability functions computed for the cell density and the activity of the CFS and (r_i) is the relative importance assigned to each response. The relative importance used in this work was equal to 1 for each of the two responses. Then, the levels of glucose and yeast extract concentrations that predict the highest value of D were selected.

2.4. Kinetic analysis

Kinetic analysis of *B. subtilis* EA-CB0015 growth, antifungal activity of CFS and lipopeptide production were performed by inoculating 20 mL of an overnight culture into 1000 mL Erlenmeyer flasks containing 180 mL of the optimized medium and incubated at 30 °C and 200 rpm with three replicates. At regular time intervals, 2 mL of samples was removed from each Erlenmeyer flask to determine the cell density, antifungal activity of CFS and lipopeptide concentration, as described in Section 2.6.

2.5. Lipopeptide purification, quantification and identification

The recovery of antimicrobial compounds was carried out by the modified methodology previously described (Villegas-Escobar et al., 2013). Briefly, one colony of *B. subtilis* EA-CB0015 was added to 1000 mL Erlenmeyer flasks containing 100 mL of the optimized medium and incubated for 120 h at 30 °C and 200 rpm. After 24 h of incubation, 4% of Amberlite XAD16® was added to the fermentation. The adsorbent resin was recovered from the culture broth, and washed with 100 mL of distilled water three times. Adsorbed products from two fermentations were subsequently eluted with 100 mL of 100% methanol, then mixed to a final volume of 200 mL and solvent was removed by evaporation at reduced pressure (−50 psig, 50 °C). After evaporation, the solid residue was suspended in 10 mL of distilled water and injected into a solid phase extraction (SPE) C18 cartridge (Agilent®, 10 g) that was rinsed successively with 80 mL of water, 80 mL of 20% methanol, 80 mL of 40% methanol, 80 mL of 70% and 80 mL of 100% methanol. The eluates were evaporated at reduced pressure (−50 psig, 50 °C) and the residues were weighed and dissolved in methanol to a final concentration of 50 mg/mL. The fractions were evaluated against *M. fijiensis* by dual plate cultures and the active fractions analyzed by HPLC, as described below.

The active fractions obtained with 100% methanol and 70% methanol from the SPE were purified by reversed-phase high pressure liquid chromatography (RP-HPLC) using a Zorbax Eclipse XDB C18 Column (250 by 4.6 mm, 5 μm, Agilent) connected to an Agilent G1311A quaternary pump. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). 40 μL of the samples (50 mg/mL) was injected into the column and the compounds were eluted by a gradient program developed from 30–70% B in 60 min, 70–100% B in 5 min and 100% B for 10 min at a flow rate of 1 mL/min and UV detection at 214 nm. Peaks with different retention times were collected, evaporated with gaseous N_2 and stored at 4 °C.

For the structural identification of active compounds, mass spectrometry analyses were carried out on an Agilent 6300 Series Ion Trap LC/MS instrument equipped with ESI source or on an Applied Biosystems Q-Star XL quadrapole-TOF tandem mass spectrometer, as previously described (Villegas-Escobar et al., 2013).

2.6. Analytical methods

2.6.1. Cell density

Cell density in the culture medium was determined by dry weight and optical density methodology. To determine cell density, 2 mL of the samples obtained in different time intervals was centrifuged at 10000*g* for 10 min. The pellet was washed with distilled water, centrifuged at the same conditions stated above and then dried at 50 °C for 48 h to determine dry weight or suspended in 2 mL of distilled water to determine optical density at 600 nm.

2.6.2. Antifungal activity

The activity of the CFS and the fractions collected from SPE and HPLC against *M. fijiensis* EASGK15 were performed by determining the mycelial growth inhibition in dual plate cultures (Ceballos et al., 2012). To obtain the CFS, samples of *B. subtilis* EA-CB0015 culture were centrifuged (10,000 × *g*, 10 min) and filtered (cellulose acetate membrane filter, 0.45 μm, Sartorious Biolab). Fractions collected from SPE and HPLC were evaporated and suspended in 1 mL or 100 μL of methanol respectively, in order to determine their activity. The inoculum of *M. fijiensis* EASTK15 was obtained by the fragmentation method described previously. Dual plate culture was carried out by plating 70 μL of the sample (CFS, SPE or HPLC fraction) and 70 μL of *M. fijiensis* inoculum in PDA supplemented with 200 ppm chloramphenicol ($n=2$). Two negative controls were used by plating 70 μL of water (or 70 μL of methanol) with 70 μL of *M. fijiensis* in PDA. Fungal colony diameters were measured after 10 days of incubation (30 °C, darkness) and the inhibition percentage for each treatment was calculated considering fungal growth in the absolute control as 100%.

2.6.3. Lipopeptide quantification

The CFS obtained in different time intervals of the fermentations was injected into a SPE C18 cartridge (Agilent®, 500 mg) which was rinsed successively with 4 mL of water, 4 mL of 20% methanol, 4 mL of 40% methanol, 4 mL of 70% methanol and 4 mL of 100% methanol. The eluates (70% methanol and 100% methanol) were evaporated at reduced pressure (−50 psig, 50 °C) and the residues were suspended in 1 mL of methanol to be purified by RP-HPLC using a Zorbax Eclipse C18 (250 mm by 4.5 mm, 5μm, Agilent) column connected to an Agilent G1311A quaternary pump. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). 40 μL of the samples were injected into the column with a gradient program developed from 30–100% B in 25 min, 100% B for 10 min at a flow rate of 1 mL/min and UV detection at 214 nm. Calibration curves were determined for surfactins ($A = 9.75 * [S] - 543.36$; $R^2 = 0.97$), iturins ($A = 9.39 * [I] + 1.54$; $R^2 = 0.99$) and fengycins ($A = 11.99 * [S] - 862.79$; $R^2 = 0.98$) where [*S*], [*I*] and [*F*] represent the concentration (mg/L) of surfactin, iturin and fengycin; and *A* represents the area of each metabolite in m AU min.

2.7. Statistical analysis

The statistical software Statgraphics plus 5.1 was used to perform the experimental design, the regression and the graphical analysis of data obtained. Analysis of variance (ANOVA) and Tukey

multiple comparison test were carried out with the data obtained and the confidence level used for ANOVA analyses was 95%.

3. Results and discussion

3.1. Response surface model

To optimize glucose and yeast extract concentrations a 2^2 factorial design with central point was constructed. Table 1 shows the experimental design matrix, the cell density and activity of the CFS of each culture. The highest cell density (15.3 ± 0.4 g/L) of *B. subtilis* EA-CB0015 was obtained with the highest glucose (40 g/L) and yeast extract (45 g/L) levels (run 4); however, the highest activity of the CFS ($75.7 \pm 1.9\%$) was obtained with the central point for glucose and yeast extract levels (run 5) and concentrations above the central point generated lower activities. From Table 1 it can be suggested that higher levels of glucose and yeast extract will produce higher cell densities; however, it negatively affected the activity of the CFS of *B. subtilis* EA-CB0015.

Regression analyses were performed through the RSM to fit the experimental data for each response through the second-order polynomial presented in Eq. (1). The obtained models presented in Eq. (4) and Eq. (5) showed that the cell density and the activity of the CFS were empirical functions of the factors evaluated. The statistical parameters of the models are presented in Table 2. Both empirical models were significant (P-Model < 0.05) and the lack of fit was not significant relative to the pure error (P-Lack of fit > 0.05). The goodness of fit of the models was examined by determination coefficients ($R^2 > 0.8$), which implied that more than 80% of the total variance in cell density and activity of the CFS could be explained by the models. The adjusted determination coefficients (adjusted $R^2 > 0.755$) were also satisfactory to confirm the significance of the models

$$Y_1 = 8.228 + 0.0433X_1 + 0.0404X_2 + 0.00175X_1X_2 \quad (4)$$

$$Y_2 = 0.136 + 0.0439X_1 - 0.0021X_2 - 0.00069X_1^2 \quad (5)$$

where Y_1 is the predicted cell density, Y_2 the activity of the CFS, X_1 and X_2 the glucose and yeast extract concentrations, respectively.

Once the independent models were validated, the multiple response optimization by means of the desirability function (D) was performed in order to find the glucose and yeast extract optimum concentrations. The goal was to establish the levels that produce the highest values of both cell density and activity of the CFS. Fig. 1 shows the three-dimensional surface plot for D as function of the glucose and yeast extract concentrations. The analysis predicted a maximum D value (D=0.775) that corresponded to 33.4 g/L of glucose and 32.5 g/L of yeast extract. The corresponding predicted response values were 13.0 g/L for cell density and 76.0% for CFS inhibition percentage of *M. fijiensis* growth.

To confirm the analysis adequacy for predicting the maximum values, three additional experiments using the optimum medium

Table 2
Statistical results obtained for the models and lack of fit test obtained with 2^2 factorial design.

Statistical parameter	Cell density	Inhibition %
F-Model	21.3	15.4
P-Model	0.0001	0.0003
F-Lack of fit test	0	4.49
P-Lack of fit test	0.9529	0.0602
R^2	0.853	0.808
Adjusted R^2	0.813	0.755

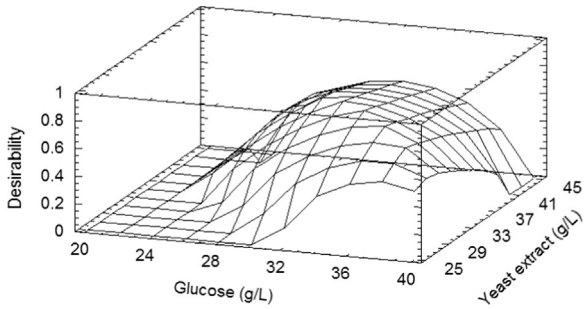

Fig. 1. Response surface plot obtained for the global desirability function for the cell density of *Bacillus subtilis* EA-CB0015 and the activity of the cell-free supernatant against *Mycosphaerella fijiensis* as a function of glucose (g/L) and yeast extract (g/L). Culture conditions: all cultures were incubated 48 h at 30 °C and 200 rpm in an orbital shaker using the basal medium with the different glucose and yeast extract concentrations given by the statistical design.

Table 3
Cell density of *Bacillus subtilis* EA-CB0015 and cell-free supernatants inhibition percentage over the growth of *Mycosphaerella fijiensis* for the optimal and the basal media.

Culture media	Cell density (g/L)	Inhibition (%)
Basal medium	3.7 ± 0.2 (a)	66.5 ± 1.6 (a)
Optimun medium	13.2 ± 1.0 (b)	77.7 ± 0.3 (b)

Intervals represent standard errors of the mean (n=3); different letters indicate that the mean is significantly different ($P < 0.05$) based on Tukey multiple range tests.

composition were performed. Table 3 shows that subsequent cultures with the optimized medium yielded an average maximum concentration of 13.2 ± 1.0 g/L and 77.7 ± 0.3 inhibition percentage. The good agreement between the predicted and experimental results, with errors less than 2.4%, verifies the validity of the models constructed. To further determine the effect of glucose and yeast extract constituents on biomass production and antifungal activity, the basal and optimized medium were compared (Table 3). The yields obtained for the optimized medium were significantly higher than those obtained with the basal medium. The cell density (13.2 ± 1.0 g/L) was increased 3.6 folds and the activity of CFS ($77.7 \pm 0.3\%$) was increased 1.2 folds. The multiple response optimization with the desirability function approach has been successfully used for the optimization of the culture media (Moreira et al., 2007; Xiao et al., 2006). This work confirmed that this methodology is appropriate to optimize culture media when more than one variable is involved.

This is the first time that medium constituents have been optimized for biomass and antifungal activity of *B. subtilis* EA-CB0015 against *M. fijiensis* using RSM coupled to multiple response optimization, and as far as it is known, this is the highest cell density reported for *B. subtilis* in shake flask fermentation and represents a 1.4 fold increase compared with the previous highest documented value obtained after 60 h (Tang et al., 2004), and a 2.6 fold increase compared to the values of *B. subtilis* biomass obtained after 48 h (Tang et al., 2004; Wei et al., 2003). The increase in cell density can be due to the nature of carbon and nitrogen sources, glucose and yeast extract used. Although other carbon sources, such as sucrose, lactose and maltose, allowed the appropriate growth of *B. subtilis* (Fu et al., 2010; Gu et al., 2005; Tang et al., 2004), this bacterium uses glucose as the most preferred source of carbon and energy (Stulke and Hillen, 2000). On the other hand, yeast extract, which can be used as a carbon and nitrogen source, contains all the precursors needed for microbial biomass synthesis, such as amino acids and nucleotides, and they are incorporated by cells without previous chemical

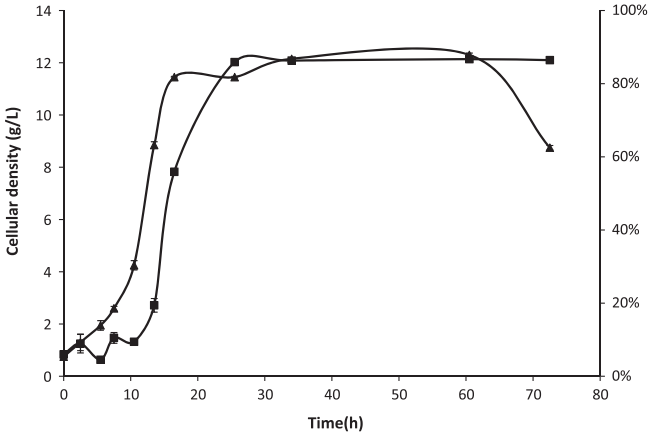

Fig. 2. Cell growth and antifungal activity kinetics of *Bacillus subtilis* EA-CB0015 under optimized culture conditions. (▲) Cell density of *B. subtilis* EA-CB0015; (■) Activity of the cell-free supernatants against *M. fijiensis*. Intervals represent standard errors of the mean ($n=3$). Culture conditions: the culture was incubated at 30 °C and 200 rpm in an orbital shaker with the optimized medium.

modifications and are preferentially used for biomass production (Berbert-Molina et al., 2008; Mignone and Avignone-Rossa, 1996). The high levels of carbon and nitrogen sources are also associated with high biomass production; cultures of *B. subtilis* using glucose and yeast extract at lower levels cause low values of cell densities (Tang et al., 2004). Although the nutrient concentrations used in this work were inside the range of values normally used for *B. subtilis* culture media (Ye et al., 2012; Fu et al., 2010; Wei et al., 2003, 2007), the cell density and the biomass to glucose yield (0.4 g/g) were significantly higher.

3.2. Growth and antifungal activity kinetics

The kinetics of cell growth and antifungal activity of the CFS under optimized culture conditions can be observed in Fig. 2. At the beginning of the fermentation process a rapid adaptation period of the cells to the culture medium can be seen, resulting in a short lag phase. When the fermentation process reached 14 h, it entered into a stationary phase which was characterized by a stable cell density and lasted until the 60 h of culture when the growth curve started to present a decreasing profile. This cell density reduction is probably related to the loss of cellular mass that is associated with the sporulation process common in the species from the genus *Bacillus* (Berbert-Molina et al., 2008).

The antifungal activity of the CFS started increasing at the end of the exponential phase and continued increasing during the stationary phase until it reaches its highest value around 30 h of culture (Fig. 2). The increase in the antifungal activity of the CFS can be due to the accumulation of lipopeptides, especially those from the fengycin and iturin families which are known for their high antifungal activity (Raaijmakers et al., 2010) and are mostly synthesized by *B. subtilis* once the culture has entered into the stationary phase (Jacques et al., 1999). It has been reported that *B. subtilis* EA-CB0015 produces surfactin and the new isoform fengycin C when grown in a medium optimized for lipopeptide production (MOLP) (Villegas-Escobar et al., 2013), although production of iturin has not been reported. To determine if the accumulation of the antifungal activity is associated with the lipopeptides suggested, the active compounds were purified and identified.

3.3. Identification of lipopeptides produced in the optimized medium

To identify the compounds produced in the optimum medium responsible for the inhibition of *M. fijiensis*, metabolites produced by *B. subtilis* EA-CB0015 were obtained by eluting the compounds adsorbed by the resin Amberlite XAD16® with methanol. The methanol extract obtained was active against *M. fijiensis* and after evaporation approximately 473 mg of solid residue was obtained from 200 mL of the culture. The solid residue was suspended in 10 mL of distilled water and was further separated using reverse SPE, as described Section 2. The active fractions obtained by SPE, eluting with 70% and 100% methanol, were then purified on reverse-phase HPLC.

Two differential HPLC groups of peaks from the fraction 100% methanol were detected. The first group (G1) between retention times of 29.3 and 48.2 min (eluting with 48–60% of acetonitrile–0.1% TFA, solvent B) was highly active against *M. fijiensis* ($65.7 \pm 3.0\%$ inhibition) and was identified as fengycins C (Supp. Fig. S1a), while the second group (G2) obtained between retentions times of 67 and 75 min (eluting with 100% of acetonitrile–0.1% TFA, solvent B) was not active against *M. fijiensis* and was identified as surfactins (Supp. Fig. S1a). The first group of peaks exhibited signals at *m/z* 1399.5, 1413.6, 1429.6, 1443.6 and 1457.6, corresponding to the protonated molecules $[M+H]^+$ (Supp. Fig. S1b). These peaks revealed differences of 14 Da, suggesting a series of homologous molecules differing by one $-CH_2$ and with molecular weights of 1399, 1413, 1429, 1443 and 1457 Da, respectively, as previously reported for fengycin C (Villegas-Escobar et al., 2013). The second group showed the main protonated signals at *m/z* $[M+H]^+$ 1008.6, 1022.6, 1036.7 and 1050.7 and sodiated signals $[M+Na]^+$ at *m/z* 1044.6, 1058.6 and 1072.6 (Supp. Fig. S1c) corresponding to C13, C14, C15 and C16 surfactins. Interestingly, C16 surfactin is produced in this optimized medium but not in MOLP medium, as previously reported (Villegas-Escobar et al., 2013).

From the 70% methanol fraction, one group of peaks was collected between retention times 15 and 20.5 min (Fig. 3a). This fraction was active against *M. fijiensis* with a growth inhibition of $42.7 \pm 1.2\%$. When analyzed by MALDI-TOF-MS, two main signals were detected at *m/z* 1065.5 and 1079.5 corresponding to the sodiated molecules $[M+Na]^+$ and one signal at *m/z* 1095.5 corresponding to potassium molecule $[M+K]^+$ (Fig. 3b). Therefore, the molecular weights of the two molecules were respectively 1043 and 1057 Da. The two sodiated molecules were used as precursor ions for further MS/MS analysis (Fig. 3c), and the results showed that the product ions had regularities and corresponded to C14 and C15 iturin A. Product ions, obtained from the precursor ion at *m/z* 1065.5, are shown in Fig. 3b. The y ion sequence was 1065.5→854.2 (y_6)→767.2 (y_5)→542.2 (y_4)→428.2 (y_3)→265.1 (y_2) corresponding respectively to the losses of Pro-(Asn-Ser)-βAA-Asn-Tyr-Asn. The b ion series contained the ions at *m/z* 1065.5→937.3 (b_7)→823.1 (b_6)→660.2 (b_5)→546.1 (b_4)→321.1 (b_3)→234.1 (b_2) which correspond to the neutral losses of Gln-Asn-Tyr-Asn-βAA-Ser-Asn. The connection of the two series suggests that the ion at 1065.5 has the sequence Pro-(Asn-Ser)-βAA-Asn-Tyr-Asn-Gln, where βAA is the β fatty acid chain of 14 carbons. The other precursor ions at *m/z* 1079.5 had the same y ions and differ in the b ions by 14 Da between them (data not shown). Production of iturin A by different *B. subtilis* strains and its high antifungal activity have been previously reported (Shih et al., 2009; Touré et al., 2004; Ye et al., 2012), but it is the first time described in *B. subtilis* EA-CB0015. In this work, iturin A was significantly less active against *M. fijiensis* when compared to fengycin C. This low activity can be related to the amount of iturin A that is being produced in the optimized media compared to the amount of fengycin C produced by this strain or to the specificity of fengycin C towards *M. fijiensis*.

3.4. Lipopeptide production time course

In order to determine the lipopeptide production time course, *B. subtilis* EA-CB0015 was grown on the optimized media and lipopeptide was purified and quantified by HPLC. Fig. 4 illustrates that surfactins are growth-associated metabolites produced during

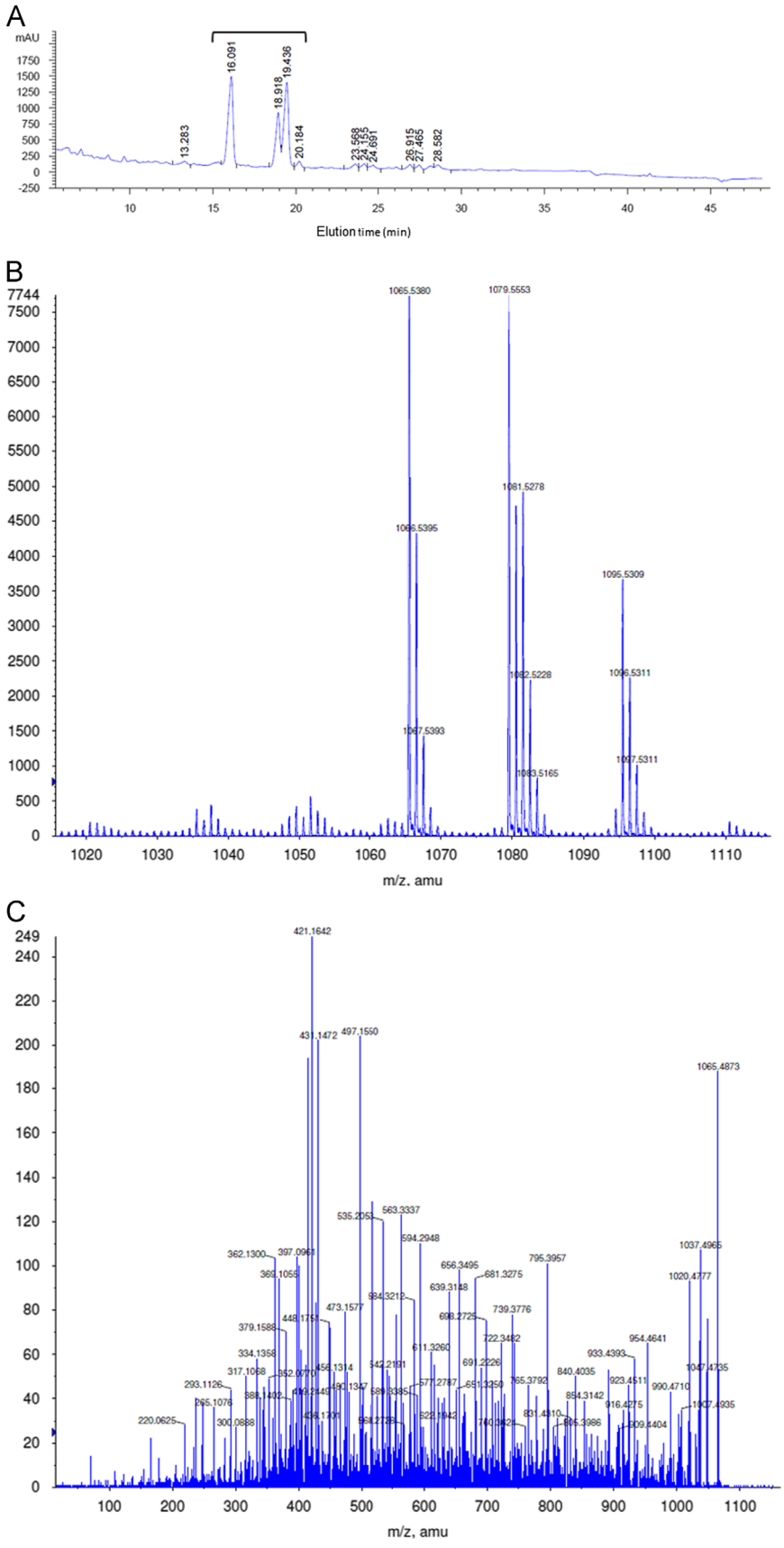

Fig. 3. HPLC chromatogram of 70% methanol-eluted fraction from a C18 SPE cartridge (A) MALDI-TOF-MS (B) and MS/MS (C) spectrum of HPLC fraction F3 collected from 70% methanol-injected sample. The square bracket indicates the fraction collected for MS analysis.

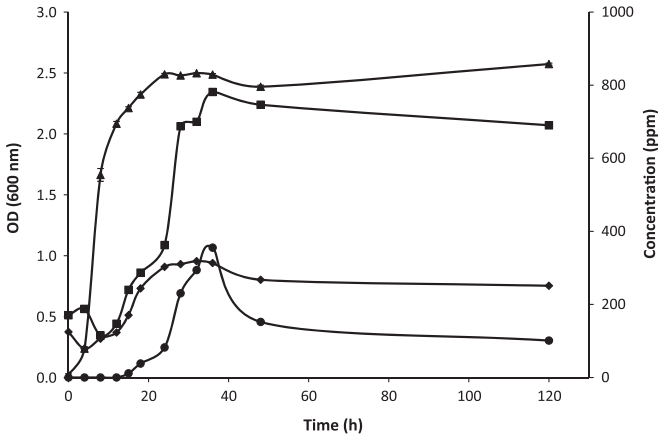

Fig. 4. Cell growth and lipopeptide production kinetics of *Bacillus subtilis* EA-CB0015 under optimized culture conditions. (▲) Optical density at 600 nm, (■) Fengycin C (C14–C18), (◆) Surfactin (C13–C16), (●) iturin A (C14 and C15). Culture conditions: the culture was incubated at 30 °C and 200 rpm in an orbital shaker with the optimized medium.

exponential phase with a maximum production of 302.7 mg/L after 24 h of growth, while fengycin C and iturin A are partially growth associated metabolites produced during late exponential and stationary phase with a maximum production of 781.4 and 355.4 mg/L at 36 h of growth, respectively. Interestingly, iturin A concentration is reduced after 36 h, dropping to 101.1 mg/L in 120 min. These results are in accordance with previous reports for lipopeptide production kinetics in terms of surfactins being growth associated and iturins and fengycins are mainly produced in stationary phase (Jacques et al., 1999; Shih et al., 2009; Wei et al., 2007). Under optimal conditions evaluated, the amount of fengycin C produced was higher than the amount of surfactin and iturin A, contrary to other lipopeptide production profile reported for another *B. subtilis* strain (Jacques et al., 1999). The high antifungal activity observed for *B. subtilis* EA-CB0015 could be explained by the high amount of fengycin and iturin produced; these lipopeptides have been associated with disruption of fungal cell membrane and growth inhibition (Ceballos et al., 2012; Romero et al., 2007).

4. Conclusion

This study shows that the RSM, the multiple response optimization and the desirability function are useful methodologies that can be used when there are two or more variables of interest that need to be optimized. This is the case of many fermentation processes that involve secondary metabolite production; in this type of process, the final concentrations of cells and secondary metabolites are equally important and normally do not have matching optimal conditions. Using this methodology, it is possible to find the conditions which simultaneously maximize both variables. Additionally, it was possible to describe the time course production of surfactins, fengycin C and iturin A, the latter two being responsible for the antifungal activity of *B. subtilis* EA-CB0015, and to recognize the importance of determining the optimum time to harvest the culture for a future product formulation.

Acknowledgments

The authors are thankful to the Universidad EAFIT and the Association of Banana Producers of Colombia (Asociación de Bananeros de Colombia, AUGURA) for the financial support of this project (Grant number 261-000027) and for the support of Universidad Nacional de Colombia. This research was made possible by the Subscribe Contract number 89 from December 20 of 2013 with the Ministerio de Medio Ambiente y Desarrollo Territorial in the category “Contrato de Acceso a Recursos Genéticos para la Investigación Científica sin Interés Comercial”.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcab.2014.09.004.

References

Atehortúa, P., Álvarez, H., Orduz, S., 2007. Modeling of growth and sporulation of *Bacillus thuringiensis* in an intermittent fed batch culture with total cell retention. Bioprocess Biosyst. Eng. 30 (6), 447–456.
Bailey, K.L., Boyetchko, S.M., Längle, T., 2010. Social and economic drivers shaping the future of biological control: a Canadian perspective on the factors affecting the development and use of microbial biopesticides. Biol. Control 52 (3), 221–229.
Berbert-Molina, M.A., Prata, A.M.R., Pessanha, L.G., Silveira, M.M., 2008. Kinetics of *Bacillus thuringiensis* var. *israelensis* growth on high glucose concentrations. J. Ind. Microbiol. Biotechnol. 35 (11), 1397–1404.
Castelan, F.P., Saraiva, L.A., Lange, F., de Lapeyre de Bellaire, L., Cordenunsi, B.R., Chillet, M., 2012. Effects of black leaf streak disease and Sigatoka disease on fruit quality and maturation process of bananas produced in the subtropical conditions of southern Brazil. Crop Prot. 35, 127–131.
Ceballos, I., Mosquera, S., Angulo, M., Mira, J., Argel, L., Uribe-Velez, D., Romero-Tabarez, M., Orduz-Peralta, S., Villegas, V., 2012. Cultivable bacteria populations associated with leaves of banana and plantain plants and their antagonistic activity against *Mycosphaerella fijiensis*. Microb. Ecol. 64 (3), 641–653.
Del Castillo, E., Montgomery, D.C., McCarville, D.R., 1996. Modified desirability functions for multiple response optimization. J. Qual. Technol. 28 (3), 337–345.
Dupont, 1982. Black and Yellow Sigatoka: Improved Identification and Management Techniques. Du Pont Latin America, Coral Gables, USA p. 17.
Fu, G., Huang, S., Ye, Y., Wu, Y., Cen, Z., Lin, S., 2010. Characterization of a bacterial biocontrol strain B106 and its efficacies on controlling banana leaf spot and post-harvest anthracnose diseases. Biol. Control 55 (1), 1–10.
Gu, X.-B., Zheng, Z.-M., Yu, H.-Q., Wang, J., Liang, F.-L., Liu, R.-L., 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem. 40 (10), 3196–3201.
Heins, S.D., Manker, D.C., Jimenez, D.R., McCoy, R.J., Marrone, P.G., Orjala, J.E. 2002. Compositions and methods for controlling plant pests. US patent 6417163 B1.
Hynes, R.K., Boyetchko, S.M., 2006. Research initiatives in the art and science of biopesticide formulations. Soil Biol. Biochem. 38 (4), 845–849.
Jacques, P., Hbid, C., Destain, J., Razafindralambo, H., Paquot, M., De Pauw, E., Thonart, P., 1999. Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett–Burman design. Appl. Biochem. Bitechnol. 77 (1–3), 223–233.
Marín, D.H., Romero, R.A., Guzmán, M., Sutton, T.B., 2003. Black Sigatoka: an increasing threat to banana cultivation. Plant Dis. 87 (3), 208–222.
Masurekar, P., 2008. Nutritional and engineering aspects of microbial process development (65). In: Petersen, F., Amstutz, R. (Eds.), Natural Compounds as Drugs Volume I. Birkhäuser, Basel, Switzerland, pp. 291–328.
Mignone, C.F., Avignone-Rossa, C., 1996. Analysis of glucose carbon fluxes in continuous cultures of *Bacillus thuringiensis*. Appl. Microbiol. Biotechnol. 46 (1), 78–84.
Moreira, G.A., Micheloud, G.A., Beccaria, A.J., Goicoechea, H.C., 2007. Optimization of the *Bacillus thuringiensis* var. *kurstaki* HD-1 δ-endotoxins production by using experimental mixture design and artificial neural networks. Biochem. Eng. J. 35 (1), 48–55.
Parekh, S., Vinci, V.A., Strobel, R.J., 2000. Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol. 54 (3), 287–301.
Raaijmakers, J.M., De Bruijn, I., Nybroe, O., Ongena, M., 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol. Rev. 34 (6), 1037–1062.
Rao, Y.K., Tsay, K.-J., Wu, W.-S., Tzeng, Y.-M., 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem. 42 (4), 535–541.
Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.-W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M., Pérez-García, A., 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant Microbe 20 (4), 430–440.
Schisler, D.A., Slininger, P.J., Behle, R.W., Jackson, M.A., 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. Phytopathology 94 (11), 1267–1271.
Shih, I.-L., Lin, C.-Y., Wu, J.-Y., Hsieh, C., 2009. Production of antifungal lipopeptide from *Bacillus subtilis* in submerged fermentation using shake flask and fermentor. Korean J. Chem. Eng. 26 (6), 1652–1661.
Stulke, J., Hillen, W., 2000. Regulation of carbon catabolism in *Bacillus* species. Annu. Rev. Microbiol. 54, 849–880.

Tang, X.-J., He, G.-Q., Chen, Q.-H., Zhang, X.-Y., Ali, M.A.M., 2004. Medium optimization for the production of thermal stable β-glucanase by *Bacillus subtilis* ZJF-1A5 using response surface methodology. Bioresour. Technol. 93 (2), 175–181.

Touré, Y., Ongena, M., Jacques, P., Guiro, A., Thonart, P., 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J. Appl. Microbiol. 96 (5), 1151–1160.

Villegas-Escobar, V., Ceballos, I., Mira, J.J., Argel, L.E., Orduz Peralta, S., Romero-Tabarez, M., 2013. Fengycin C produced by *Bacillus subtilis* EA-CB0015. J. Nat. Prod. 76 (4), 503–509.

Wei, Y.-H., Lai, C.-C., Chang, J.-S., 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. Process Biochem. 42 (1), 40–45.

Wei, Y.-H., Wang, L.-F., Changy, J.-S., Kung, S.-S., 2003. Identification of induced acidification in iron-enriched cultures of *Bacillus subtilis* during biosurfactant fermentation. J. Biosci. Bioeng. 96 (2), 174–178.

Xiao, J.-H., Chen, D.-X., Wan, W.-H., Hu, X.-J., Qi, Y., Liang, Z.-Q., 2006. Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions. Process Biochem. 41 (8), 1887–1893.

Ye, Y.-F., Li, Q.-Q., Fu, G., Yuan, G.-Q., Miao, J.-H., Lin, W., 2012. Identification of antifungal substance (iturin A2) produced by *Bacillus subtilis* B47 and Its effect on southern corn leaf blight. J. Integr. Agric. 11 (1), 90–99.