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ORIGINAL ARTICLE

Environmental dynamics of *Bacillus amyloliquefaciens* CCMI 1051 antifungal activity under different nitrogen patterns

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antifungal activity, *Bacillus amyloliquefaciens,* phytopathogenic fungi, spore formation, statistical design.

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Abstract

Aims: The aim of this study was to evaluate the influence of environmental conditions on the antifungal activity of the *Bacillus* sp. CCMI 1053 cultures.
Methods and Results: The electrospray ionization mass spectra (ESI-MS) analysis was used to detect the active peptides produced by *Bacillus amyloliquefaciens* CCMI 1051 cultures in a glucose-containing medium to which four different nitrogen sources were added. The cultures produced different patterns of *Bacillus* sporulation and distinct antifungal activity of the cell-free culture broths.
Conclusions: The highest sporulation obtained corresponds to higher antifungal activity when it is formed after 3 days of microbial growth. The antifungal activity against *Trichoderma harzianum* CCMI 783 is more influenced by the concentration on the nitrogen source than the culture time of incubation. The association of nitrogen concentration and the time of incubation is particularly relevant in the expression of the antifungal activity.
Significance and Impact of the Study: The present findings allow the reduction of the use of chemical pesticides and to limit some plant diseases. The association of the nitrogen source and the time of incubation is a novelty, which would improve the production of secondary metabolites. Both economical and environmental benefits arise from the study.

Introduction

In the search of alternative biological means to act against phytopathogenic fungi in the field, the knowledge about the factors that trigger the production of antifungal compounds by bacteria is as important as understanding the ecology of naturally occurring strains that harbour specific biocontrol traits (Raaijmakers *et al.* 2002).

Bacillus species produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities (Eppelmann *et al.* 2001; Wright *et al.* 2001; Yazgan *et al.* 2001; Hindré *et al.* 2003) being often used as biocontrol agents (El-Ghaouth 1997; Tsuge *et al.* 2001; Reva *et al.* 2004; Romero *et al.* 2004).

In response to nutritional stress conditions, *Bacillus* activates a variety of defence processes, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production (Dieckmann *et al.* 2001). Many strains are known to suppress fungal growth *in vitro* by the production of antifungal antibiotics (Seifert *et al.* 1987; Leifert *et al.* 1995; Hiradate *et al.* 2002; Yu *et al.* 2002); however, *Bacillus* spp. produce a range of other metabolites, like biosurfactants (Yakimov *et al.* 1999) and enzymes capable of wall-degrading the fungal cells (Seifert *et al.* 1987).

The antimicrobial activity in *Bacillus* is highly dependent on the culture-medium composition (Benko and Highley 1990). In fact, different nitrogen and other nutrient sources have evidenced different antibiotic production (Katz and Demain 1977; Chevanet *et al.* 1986; Besson *et al.* 1987; Davis *et al.* 1999; Volpon *et al.* 2000). The utilization of glutamic acid as nitrogen source seems to be advantageous for the bacilomicina L production (Chevanet *et al.* 1986), and aspartic acid is a more favourable

nitrogen source for iturin A production in *Bacillus subtilis* (Besson *et al.* 1987). The incubation time is another factor to be considered on antibiotic production, as the response to adverse environmental conditions could lead to the activation of different mechanisms for the production of antibiotics giving a competitive advantage to the producer micro-organism (Dieckmann *et al.* 2001).

The purpose of this study was to evaluate the influence of environmental conditions on the antifungal activity of the *Bacillus amyloliquefaciens* CCMI 1051 cultures. The nitrogen source is a key factor as excess carbon induces product formation, and the time of incubation plays a role in secondary metabolites to be produced. The association between sporulation and antifungal activity was also assessed.

Materials and methods

Micro-organisms

Bacillus amyloliquefaciens CCMI 1051 was isolated from *Quercus suber*. The strain was characterized by the morphological, physiological and biochemical characteristics based on the Bergey's manual of systematic bacteriology and the partial sequence of 16S rDNA. The 16S rDNA partial sequence was registered with the GenBank accession number AY785773. *Bacillus amyloliquefaciens* CCMI 1051 was maintained on nutrient agar slants and stored at 4°C. *Trichoderma pseudokoningii* CCMI 304, *Aspergillus niger* CCMI 296, *Rhizopus* sp. L-122, *Penicillium expansum* CCMI 625, *Trichoderma harzianum* CCMI 783, *Trichoderma koningii* CCMI 868, *T. harzianum* CCMI 822, *Cladosporium resinae* CCMI 262, *Fusarium oxysporum* CCMI 898, *Fusarium solani* F4 and *Cephalosporium* sp. F25 were used as test micro-organisms and were obtained from the Culture Collection of Industrial Micro-organisms (Lisbon). The cultures of the test micro-organisms were maintained on malt extract agar slants.

Batch conditions

Growth took place in 3-l shake flasks, each containing 500 ml of the defined medium with (g l^{-1}): KH_2PO_4, 1·7; $Na_2HPO_4.2H_2O$, 1·7; $MgSO_4.7H_2O$, 0·2; yeast extract, 0·1; glucose 10·0, 2 ml of Vishniac solution sterilized separately (Vishniac and Santer 1957) and 23 mmol l^{-1} of nitrogen made available from ammonium sulfate, aspartic acid, glutamic acid or glycine.

The medium components were dissolved in 80% of the total distilled water volume. Glucose (10·0 g l^{-1}) was added to the remaining 20% of the final volume of distilled water and sterilized separately. The solutions were sterilized in an autoclave at 121°C and 1 bar for 20 min. Amino acids were filter sterilized using 0·22-μm membranes (Gelman Sciences, Ann Arbor, MI, USA) and added to the medium after autoclaving.

The shake flasks containing 500 ml of the medium described were inoculated with 50 ml stationary-phase culture. The cultures were incubated for 10 days at 30°C in an orbital shaker at 200 rev min^{-1} (Heidolph unimax 1010; Heidolph, Kelheim, Germany).

The specific growth rate values were determined from experimental data in exponential phase, using the equation $\ln x = \ln x_0 + \mu t$, where x is Abs_{620nm}, t is time (h^{-1}) and μ is specific growth rate.

For the experimental design cultures, the cells of a new slant were used to inoculate each 500-ml shake flask containing 100 ml of medium, on the conditions corresponding to the model. The cultures were incubated at 30°C in an orbital shaker at 200 rev min^{-1} (Heidolph unimax 1010).

To characterize the antimicrobial activity, batch assays were performed in a bench scale CH-4103 bioreactor (Infors HT, Bottmingen, Switzerland) with 1-l working volume. The defined medium is described earlier containing 2·0 g l^{-1} of glucose. The culture pH was maintained at 7 ± 0·2 by the automatic addition of a base (NaOH, 2 mol l^{-1}) and an acid (H_2SO_4 1 mol l^{-1}) solutions. The temperature was controlled at 30°C. The dissolved oxygen level was measured by a polarographic electrode (Ingold, Leicester, UK). The stirring speed was set to 700 rev min^{-1} and the aeration rate was 2 vvm.

Sporulation

To measure *Bacillus* sporulation, 4 ml of the culture was heated at 77°C for 12 min in a thermostat bath. The number of *Bacillus* spores (CFU ml^{-1}) was determined by plating the sampled medium on nutrient agar in a serial dilution procedure. The number of colonies was counted after 24-h incubation at 30°C.

Antifungal assays

Ten millilitres of *Bacillus* sp. CCMI 1051 cell-free supernatant was centrifuged and filtered through a sterile 0·22-μm membrane (Gelman Sciences, Ann Arbor, MI, USA). The resulting filtrate was used as the source of the antifungal activity.

A fungal spore suspension (from the test organisms described earlier) was prepared suspending loopfuls of hyphae and spores from a malt extract agar slant (7 day cultures at 25°C) in 5 ml of 0·85% NaCl solution. The suspension was filtered through sterilized cotton. A 10^8 CFU ml^{-1} spore suspension was obtained through dilutions and adjusted to 10^5 CFU ml^{-1} using a serial

dilution operation. Malt extract (2·5 ml), 0·25 ml of 10^5 CFU ml^{-1} of fungi and 2·5 ml of the *Bacillus* culture supernatant were added to a test tube and incubated at 25°C for 24 h. A control was made only with the malt extract and the fungal spore suspension. One millilitre of each tube was transferred to Petri dishes, incorporated in 20 ml of Cooke Rose Bengal agar (Difco, Detroit, MI, USA) and incubated at 25°C for 24–48 h. After this period, the colonies were counted. Inhibition was determined as per cent inhibition = (1 − CFU of test/CFU of control) − 100 (Moita *et al.* 2005).

Mass spectrometric analysis

A 100-ml culture sample was spun down (16 000 ***g*** for 20 min at 6°C) and lipopeptides were recovered as escribed by McKeen *et al.* (1986). The dried material was dissolved in a minimum volume of methanol–water (1 : 1). The electrospray mass spectra were recorded on a Bruker, Esquire 3000 plus ion trap mass spectrometer in the positive polarity mode (Bruker Daltonics, Billerica, MA).

The samples were diluted 50-fold in acetonitrile–water (1 : 1) with formic acid (0·1%) and injected at a rate of 100 μl^{-1} h^{-1} into the electrospray ionization (ESI) probe. The capillary temperature and voltage were set to 300°C and 166 V, respectively. All spectra acquisitions were done using Esquire Control and analysed with the data analysis software.

Experimental design

The experimental distribution for two factors, according to the Doehlert uniform design (Doehlert 1970) was used to produce response surfaces. To study the effect of two independent variables, the experimental distribution is based on seven combinations of the variables to which three experiments at the centre of the experimental domain were added. The experimental domain was defined by the time of incubation (X_1) between 1 and 9 days and aspartic acid concentration (X_2) varying between 0 and 6 g l^{-1}. The concentration of the aspartic acid and sampling days were selected according to preliminary studies. The coded and real values are shown below

Coded values	0	1	−1	0·5	−0·5
Time of incubation (days)	5	9	1	7	3
Coded values	0	0·866	0·866		
Aspartic acid (g l^{-1})	3	5·598	0·402		

The responses studied in this design were: final sporulation (Y_1), antifungal activity (Y_2) and final biomass concentration (Y_3). The model used to express the responses was a second-order polynomial model: $Y_i = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_j + \Sigma\beta_{ii} X_i^2$, where Y_i is response from experiment 1, β refers to the parameters of the polynomial model and X is experimental factor level (coded units).

Results

The effect of nitrogen compounds on the antifungal activity of *Bacillus* sp. cultures

The batch cultures of *B. amyloliquefaciens* CCMI 1051 using nitrogen, made available from different sources, revealed similar kinetic features. The cultures showed 0·487 h^{-1} specific growth rate when growing with ammonium sulfate, 0·485 h^{-1} for aspartic acid cultures, 0·451 h^{-1} for glutamic acid and 0·489 h^{-1} for glycine cultures. The final biomass concentration (Ln Abs = 620 nm) was virtually the same in all the cases studied. However, *B. amyloliquefaciens* CCMI 1051 sporulation was distinct when the cultures were grown in different nitrogen sources. Cultures with glutamic acid and glycine attained a maximum between 2×10^8 and 3×10^8 CFU ml^{-1} after 140 h of growth, remaining steady thereafter. Cultures containing aspartic acid developed further and attained a maximum between 5×10^8 and 6×10^8 CFU ml^{-1} after 220 h of growth (Fig. 1). Ammonium sulfate induced a much smaller sporulation than any other culture conditions tested showing $1{\cdot}0 \times 10^8$ CFU after 100 h, decaying after.

Supernatants from all the cultures of *B. amyloliquefaciens* CCMI 1051, developed in the presence of different

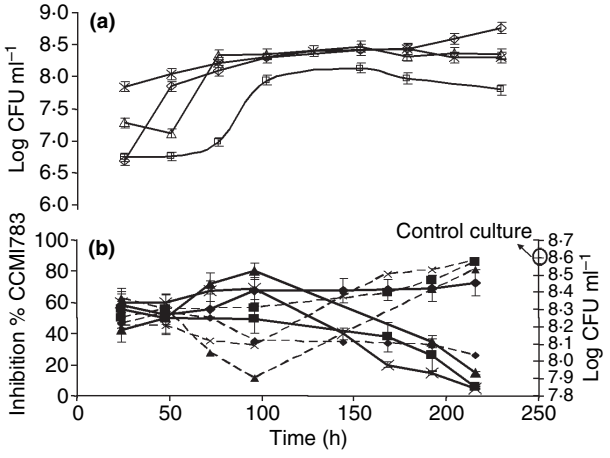

Figure 1 Time course profiles of sporulation (a) and antifungal activity (b) of *Bacillus amyloliquefaciens* CCMI 1051 grown on $(NH_4)_2SO_4$ (MD), aspartic acid (Asp), glycin (Gly) or glutamic acid (Glu) as nitrogen source. Inhibition data are presented as percentage against a control culture in the absence of the *B. amyloliquefaciens* CCMI 1051 supernatant. All data was determined in triplicate. –□–, MD; –◇–, Asp; –△–, Glu; –×–, Gly; (_ _ _ _) log CFU ml^{-1}; (_____) inhibition %.

nitrogen sources, induced the inhibition of *T. harzianum* CCMI783. This effect increased during the first 100 h, displaying inhibition values above 50%. After this period, the percentage of fungal inhibition decreased. In the culture developed with aspartic acid, the inhibition increased up to 100 h, thus remaining at high values (Fig. 1).

To characterize the antimicrobial activity of *B. amyloliquefaciens* CCMI 1051, the bacteria were tested against a set of superficial contamination fungi, blue-strain fungi and phytopathogenic fungi as shown in Table 1. The inhibition data is presented as log (colony counting units) against a control culture of the same fungus in the absence of the *B. amyloliquefaciens* CCMI 1051 supernatant. A particularly high activity (above 90% inhibition) was observed against the superficial contamination fungus *Rhizopus* sp., *A. níger* and *T. harzianum*. The same standard was not observed against blue-strain fungus and phytopathogenic fungus, although one of the *C. resinae* strains showed an 84% inhibition. Fungi from all groups show an acceptable level of sensitivity (around 50%) of activity (*T. harzianum*, *T. koningii* and *F. oxysporum*).

The supernatant extracts from the cultures developed in the presence of four different nitrogen sources were characterized by ESI mass spectroscopy (Fig. 2). Some preliminary tests [ninhydrin and 4,4′-tetramethyldiaminodiphenylmethane (TDM) (von Arx *et al.* 1976)] performed on these extracts revealed the possible presence of peptides. Mass spectrometer acquisition parameters were optimized using commercial iturin A and surfactin. The mass spectra of the broth extracts from cultures grown with different nitrogen sources have been acquired in the *m/z* range 200–3000. We report the results for *m/z* peaks higher than 1000. The ammonium sulfate cultures show a major peak at *m/z* 1058 and several smaller peaks (size smaller than 20% relative size) (Fig. 2a). The cultures grown with aspartic acid, glutamic acid and glycine showed major peaks at *m/z* 1044 and 1058 (Figs 2b–d). These *m/z* peaks correspond to peptides expelled by *Bacillus* into the broth during the development of the culture; in the spectra acquired from the culture medium, those peaks are not observed.

A zoom of the full ESI mass spectra present several well-separated groups of mass peaks, detected in the *m/z* range between 1000 and 1100. Different isoforms exist for each lipopeptide, which vary in the chain length of their fatty acid components and amino acid residues in their peptide rings. Accordingly, some of the corresponding *m/z* values differ by a value of 14, which corresponds to the molecular weight of one CH_2 group (e.g. 1031, 1045 and 1059 *m/z*). The presence of sodium and potassium adducts also favour differences of 22 Da (1031 *m/z* and 1053 *m/z*; 1045 *m/z* and 1067 *m/z*) or 38 Da (1045 *m/z* and 1083 *m/z*) in the peaks.

The peak mass exhibited on those experimental conditions was compared with commercial sample of iturin A and surfactin, but the samples contained peaks which did not correspond to the peaks exhibited by the commercial samples subjected to the same conditions. Nevertheless, the commercial iturin A and surfactin samples show the same peak profile and the presence of adducts (data not shown).

Table 1 Antifungal activity of *Bacillus amyloliquefaciens* CCMI 1051 cell-free supernatants against superficial contamination fungus – blue-strain fungus and phytopathogenic fungus

Antifungal activity against superficial contamination fungus		
Micro-organism	Log CFU of control	Log CFU of test
Trichoderma pseudokoningii CCMI 304	8·14	7·57
Aspergillus niger CCMI 296	8·30	7·29
Rhizopus sp. L-122	8·51	6·18
Penicillium expansum CCMI 625	8·45	8·45
Trichoderma harzianum CCMI 783	7·90	7·31
Trichoderma koningii CCMI 868	7·37	7·02
T. harzianum CCMI 822	7·91	5·70
Antifungal activity against blue-strain fungus		
Cladosporium resinae CCMI 262	7·28	6·48
C. resinae CCMI 667	6·98	6·85
Antifungal activity against phytopathogenic fungus		
Fusarium oxysporum CCMI 898	8·81	8·50
Fusaruim solani F4	7·08	6·98
Cephalosporium sp. F25	8·14	8·14

Dynamics of antifungal activity in *Bacillus* sp. cultures

Table 2 shows the results of the experiments of *B. amyloliquefaciens* CCMI 1051 growth in the presence of different concentrations of aspartic acid for distinct periods of time. The experiments were conducted according to a Dohlert experimental design. The data of final sporulation (Y_1), antifungal activity against *T. harzianum* CCMI 783 (Y_2) and final biomass concentration (Y_3) for each experiment are presented separately (tests 1–7) being complemented by experiments at the centre of the experimental domain (tests 8–10). The analysis of data in Table 2 allows identifying the input given by the different culture variables on the level of the antifungal activity. Higher aspartic acid concentrations produce biomass above 2 g l^{-1} for incubation below 3 days (tests 3 and 7). Extending the incubation does not produce more biomass (tests 2 and 4). A low concentration of aspartic acid produced the lowest biomass concentration (tests 5 and 6). Conversely, the sporulation is highly induced by the *B. amyloliquefaciens* CCMI 1051 time of incubation showing a direct relationship between sporulation and culture age

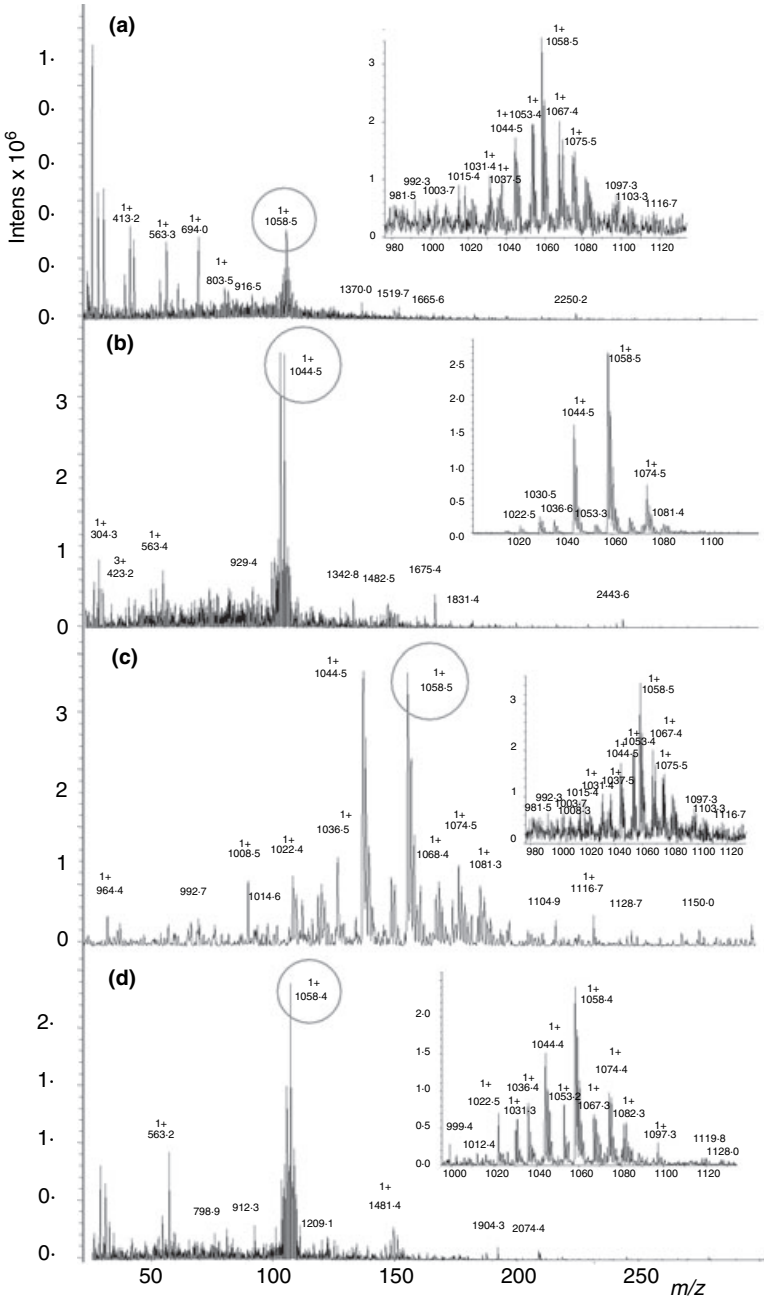

Figure 2 Electrospray mass spectra of antifungal compounds produced by *Bacillus amyloliquefaciens* CCMI 1051 grown on $(NH_4)_2SO_4$ (a); aspartic acid (b), glutamic acid (c) or glycin (d) as nitrogen source. Zoom scan of the full electrospray ionization mass spectra.

(tests 1–3). A limited incubation and low aspartic acid concentration induces a poor spore formation (tests 3 and 5). In addition, the role of aspartic acid on sporulation is shown by the data from tests 5 and 7. The *B. amyloliquefaciens* CCMI 1051 cultures were grown for 3 days, but the aspartic acid concentration was 14-fold higher in test 7 (0·40 g l^{-1}) than in test 5 (5·59 g l^{-1}). The high nitrogen source concentration increased the sporulation twofold. Even with this level of sporulation, the antifungal activity remained the same in both tests showing that there is no relevant association between sporulation and antifungal activity.

The profiles shown by the isoresponse contours of sporulation (Y_1), antifungal activity (Y_2) and final biomass concentration (Y_3) with growing concentrations of aspartic acid and different incubation periods are shown in Fig. 3. The response surfaces show maximal and minimal values in each sector. The response surfaces concerning the sporulation (Fig. 3a) show that spores are formed after longer periods of incubation. The graphs also reveal

Table 2 Comparison between measured and evaluated responses by using polynomial models for each fermentation of the Doehlert design

	Factors		Y_1 (sporulation) ($\times 10^8$ CFU ml^{-1})		Y_2 (antifungal activity) (inhibition % of CCMI 738)		Y_3 (biomass concentration) (Abs $_{620nm}$)	
Test	Time (day)	Aspartic acid (g l^{-1})	Measured (Y)	Evaluated (Y')	Measured (Y)	Evaluated (Y')	Measured (Y)	Evaluated (Y')
1	5	3·00	3·27 (±0·04)	3·32	41·28 (±1·00)	39·41	1·60 (±0·04)	1·56
2	9	3·00	5·68 (±0·14)	5·37	73·19 (±0·577)	71·14	1·34 (±0·03)	1·49
3	1	3·00	1·20 (±0·11)	1·51	58·02 (±0·577)	60·07	2·23 (±0.05)	2·08
4	7	5·59	5·40 (±0·14)	5·71	69·08 (±0·500)	71·13	1·93 (±0·04)	1·78
5	3	0.40	1·85 (±0·07)	1·54	52·62 (±2·31)	50·57	1·21 (±0·02)	1·37
6	7	0·40	3·02 (±0·11)	3·33	41·28 (±4·36)	43·33	1·59 (±0·02)	1·44
7	3	5·59	3·94 (±0·08)	3·63	54·89 (±3·00)	52·84	2·29 (±0·04)	2·44
8	5	3·00	3·00 (±0·14)	3·32	38·72 (±2·64)	39·41	1·45 (±0·04)	1·56
9	5	3·00	3·72 (±0·17)	3·32	39·57 (±1·00)	39·41	1·76 (±0·02)	1·56
10	5	3·00	3·28 (±0·11)	3·32	38·08 (±0·50)	39·41	1·44 (±0·06)	1·56

CFU, colony forming units; Y_1, final sporulation; Y_2, antifungal activity; Y_3, final biomass concentration; Y, measured responses; Y', estimated responses.

an important contribution of aspartic acid towards sporulation. Isoresponse contours for antifungal activity against CCMI 783 (Fig. 3b) show a complex dependence upon the time of incubation and aspartic acid concentration. A maximal antifungal activity is observed in the same region of spore formation. On the whole, the time of incubation has a positive effect on the antifungal activity almost independent of the aspartic acid concentration present as nitrogen source. Biomass formation is highly dependent on the aspartic acid concentration and takes place in the initial days of fermentation (Fig. 3c).

The data obtained from the experimental design were further used for regression analysis, and polynomial model-derived parameters (β_0 to β_{22}) were obtained (Table 2). These parameters describe the relative influence of both factors individually on the responses and how they interact within the experimental domain. β_0 represents the analysed response at the centre of the experimental domain. The magnitude of β_1 and β_2 indicates the importance of each factor (time of incubation and aspartic acid concentration as nitrogen source) on the responses. β_{12} is an interaction parameter and express how the effect of one factor depends on the level of the other factor β_{11}, and β_{22} determines how the response surface folds downward or upward quadratically more or less rapidly depending on the magnitude of the absolute value.

The values of β_1 and β_2 parameters show a much more limited influence of time of incubation than to aspartic acid concentration on the antifungal activity (Table 3). However, the interaction factor (β_{12}) of both factors is highly important for this response. The β_1 and β_2 parameters to final biomass concentration show a negative effect on time of incubation and a positive influence on the aspartic acid concentration as nitrogen source. The interaction of both factors also has a negative influence on this response as shown by the negative value of β_{12}.

The strong dependence of biomass on aspartic acid concentration can be observed in Fig. 3, where the biomass concentration increases along with aspartic acid concentrations. However, within the experimental domain, biomass drops for extended incubation times.

Analysis of experimental data

Fischer-statistical tests (F-tests) were applied to evaluate the significance of the variances owing to the effectiveness of the model fitting and the experimental error. The adequacy of the models was performed using the F-test for the effectiveness of the factors, which detects whether the source of variance is to the result of the models for the reproduction of the experimental data. For responses Y_1, Y_2 and Y_3, the levels of confidence at which the null hypothesis can be rejected are 99·01, 99·82 and 99·72, respectively.

Model analysis by the coefficient of multiple determination (R^2) was also performed. The coefficients of multiple determination of 0·951 for Y_1, 0·980 for Y_2 and 0·974 for Y_3 were found. These coefficients showed the amount of the sum of squares corrected for the mean that is accounted for by the residuals (4·9% for sporulation, 2·0% for antifungal activity and 2·6% for biomass).

Discussion

The *Bacillus* species are well-known producers of metabolites with antimicrobial properties. Usually, three different classes of bioactive peptides can be distinguished: antifungal peptides, such as bacilysin and rhizocticin; antifungal lipopeptides, such as surfactins, iturins and fengycins; and

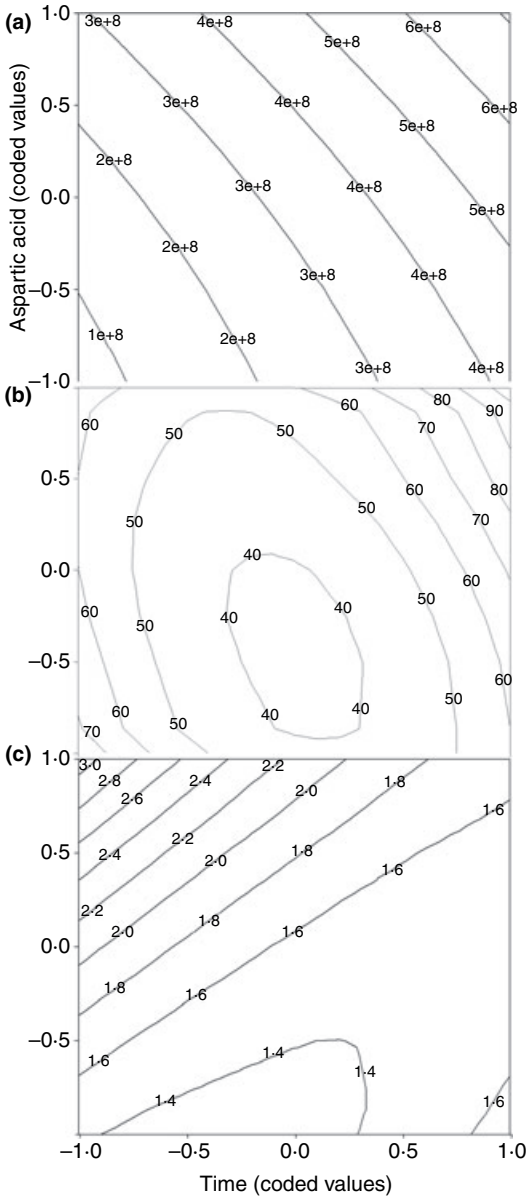

Figure 3 (a) Isoresponse contours for sporulation (Y_1) showing its dependence upon the time of incubation (factor 1) and aspartic acid concentration (factor 2). (b) Isoresponse contours for antifungal activity against CCMI 783 (Y_2) showing its dependence upon the time of incubation (factor 1) and aspartic acid concentration (factor 2). (c) Isoresponse contours for biomass (Y_3) showing its dependence upon the time of incubation (factor 1) and aspartic acid concentration (factor 2).

antimicrobial polypeptides, such as subtilin (Pabel *et al.* 2003).

The species *B. amyloliquefaciens* has been reported to produce lipopeptides with antifungal proprieties. Yu *et al.* (2002) demonstrated that the antifungal compounds produced by the strain B94 of *B. amyloliquefaciens* (*m/z* values 1044·3, 1047·9 and 1069·5) are isomers of iturin A produced by *B. subtilis*. Hiradate *et al.* (2002), using the strain RC-2 of *B. amyloliquefaciens*, had attributed the compounds' bioactivity to the production of iturin A2–A8 (*m/z* values of 1043 for iturin A2; 1057 for iturin A3–A5; 1071 for iturin A6 and A7; 1085 for iturin A8). The iturinic composition seems to depend on the strain of *B. amyloliquefaciens* used.

Antifungal peptides are usually less than 2000 Da in size and ESI full scan mass spectra help elucidate the composition of the mixture directly. The results presented in this work show that several compounds of masses between 1000 and 1100 Da, comparable with that of iturin and surfactin compounds, are produced by *B. amyloliquefaciens* CCMI 1051, suggesting the production of different antifungal compounds, or different proportion of them, depending on the nitrogen source used in the culture medium. The presence of these compounds and/or hit conjugation conferred antifungal activity to the cultures.

The association between the antibiotic production and *Bacillus* sporulation are not fully understood. Some authors refer antifungal activity and sporulation as associated phenomena (Dieckmann *et al.* 2001; Yazgan *et al.* 2001). However, some studies with mutant strains describe sporulation without antibiotic formation (Leifert *et al.* 1995), and some modifications in the culture conditions resulted in the increase of sporulation and a decrease in the antibiotic production (Chevanet *et al.* 1986).

González-Pastor *et al.* (2003) described that a system of programmed death in *B. subtilis* is crucial for sporulation. In this process, named 'cannibalism' by the authors, some cells produce and export a killing factor and a signalling protein that acts cooperatively to block sister cells from sporulating and causes endogenous metabolism. In this mechanism, the cultures seem to work as a multicellular organism capable of 'self-digestion' (Engelberg-Kulka and Hazan 2003) in unfavourable nutritional conditions to maintain the culture alive. Some genes of the sporulating killing factor operon seem to present great similarity to genes involved in the production of peptide antibiotics (González-Pastor *et al.* 2003).

The production of antifungal compounds seems to be related to the sporulation of the *Bacillus* sp. once the largest inhibition observed corresponded to more CFU ml^{-1} formed. In *Bacillus* sp., when the nutritional conditions are adverse, the vegetative growth makes way for bacterial spore formation (Liu and Tzeng 2000) and to the synthesis of some enzymes associated with the cessation of vegetative growth and other activities (Piggot and Hilbert 2004). During sporulation, there is little net synthesis of protein and the amino acid biosynthesis pathways are less

Table 3 β Parameters of the polynomial model used to represent the three responses analysed

	Responses					
Parameters	Sporulation (Y_1)	*P*-value	Antifungal activity (Y_2)	*P*-value	Biomass (Y_3)	*P*-value
β_0	$3{\cdot}32 \times 10^8$	0·000	39·095	0·000	1·564	0·000
β_1	$1{\cdot}93 \times 10^8$	0·002	0·557	0·026	−0·292	0·091
β_2	$1{\cdot}29 \times 10^8$	0·008	8·701	0·006	0·409	0·036
β_{12}	$1{\cdot}67 \times 10^7$	0·767	14·781	0·010	−0·425	0·182
β_{11}	$1{\cdot}22 \times 10^7$	0·772	26·189	0·000	0·222	0·324
β_{22}	$2{\cdot}725 \times 10^7$	0·529	11·342	0·009	0·186	0·400

Y_1, final sporulation; Y_2, antifungal activity; Y_3, final biomass concentration; β_0, response in the centre of the experimental domain; β_1 and β_2, parameters of the factors 1 and 2; β_{12}, interaction parameter of the factors 1 and 2; β_{11} and β_{22}, self-interaction parameters of the factors 1 and 2.

active as compared with vegetative growth. The aspartate pathway plays a critical role in sporulation because two products of this pathway are required by the sporulating cells: dipicolinate, which makes up to 10% of the dry weight of the spore and diaminopimelate, a component of the spore cortex peptidoglycan (Paulus 1993; Setlow 2003).

Aspartic acid induces a higher level of sporulation than the other nitrogen sources in this work and corresponds to a higher antifungal activity. No correlation between biomass and sporulation was observed. Furthermore, in agreement with our results, the incubation time influences the sporulation of the *Bacillus* strain more markedly than the use of aspartic acid as nitrogen source. However, the antifungal activity against *T. harzianum* CCMI 783 is more influenced by the concentration of the nitrogen source than the culture time of incubation, although the association of these two factors is particularly relevant in the expression of the antifungal activity. The most advantageous conditions for antifungal compound production lay between the seventh and the ninth day of incubation (0·5–1 coded units) and aspartic acid concentration between 3·0 and 6·0 g l^{-1} (0–1 coded units). In these conditions, the fungal growth inhibition of *T. harzianum* CCMI 783 was above 70%. The data from the experimental design shed light on the relationship between the sporulation and the expression of antifungal activity. In fact, the highest sporulation corresponds to higher antifungal activity (tests 2 and 4). A 14-fold increase of aspartic acid in 7-day cultures (tests 4 and 6) resulted in a 41·6% increase of sporulation that produced a 40% increment of the fungal inhibition, revealing a strong contribution of the medium composition towards the production of the bioactive compounds. The similar reduction of both sporulation and activity suggests a close correlation between the two. Exactly the same effect was observed when aspartic acid was increased by 40% in 3-day cultures (tests 5 and 7). During this period, spore formation increased by 57·6%. At this stage, the antifungal activity only increased 4% indicating that a longer period is needed for bioactive compounds to be formed.

Feio *et al.* (2004) showed that *B. subtilis* CCMI 355 reached the highest values of sporulation between 7 and 11 days of growth. Similar observations showed that medium changes, including the nitrogen source and culture conditions, influenced *B. subtilis* strain AU195 sporulation and affected the antifungal activity of the culture broth. The antifungal activity increased with the incubation time and a maximum was observed after 6 days of culture (Moyne *et al.* 2001).

References

von Arx, E., Faupel, M. and Brugger, M. (1976) Das 4,4′-Tetramethyldiamino-diphenylmethan reagens (TDM) eine modifikation der Chlor-o-tolidin Farbereaktion für die Dünnschichtchromatographie. *J Chromatogr* **120**, 224–228.

Benko, R. and Highley, T. (1990) Selection of media for screening interaction of wood-attacking fungi and antagonistic bacteria. *Mater Org* **25**, 173–180.

Besson, F., Chevanet, C. and Michel, G. (1987) Influence of the culture medium on the production of iturin A by *Bacillus subtilis*. *J Gen Microbiol* **3**, 767–772.

Chevanet, C., Besson, F. and Michel, G. (1986) Effect of various growth conditions on spore formation and bacillomycin L production in *Bacillus subtilis*. *Can J Microbiol* **32**, 254–258.

Davis, D.A., Lynch, H.C. and Varley, J. (1999) The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. *Enzyme Microb Technol* **25**, 322–329.

Dieckmann, R., pavela-Vrancic, M. and Döhren, H. (2001) Synthesis of (di)adenosine polyphosphates by non-ribosomal peptide synthetases (NRPS). *Biochem Biophys Acta* **1546**, 234–241.

Doehlert, D.H. (1970) Uniform shell designs. *Appl Stat* **19**, 231–239.

El-Ghaouth, A. (1997) Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. *J Ind Microbiol Biotechnol* **19**, 160–162.

Engelberg-Kulka, H. and Hazan, R. (2003) Cannibals defy starvation and avoid sporulation. *Science* **301**, 467–468.

Eppelmann, K., Doekel, S. and Marahiel, M.A. (2001) Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host *Bacillus subtilis*. *J Biol Chem* **14**, 34824–34831.

Feio, S., Barbosa, A., Cabrita, M., Nunes, L., Esteves, A., Roseiro, J.C. and Marcelo Curto, M. (2004) Antifungal activity of *Bacillus subtilis* 355 against wood-surface contaminant fungi. *J Ind Microbiol Biotechnol* **31**, 199–203.

González-Pastor, J., Hobbs, E. and Losick, R. (2003) Cannibalism by sporulating bacteria. *Science* **301**, 510–513.

Hindré, T., Didelot, S., Pennec, J., Haras, D., Dufour, A. and Vallée-Réhel, K. (2003) Bacteriocin detection from whole bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* **69**, 1051–1058.

Hiradate, S., Yoshida, S., Sugie, H., Yada, H. and Fujii, Y. (2002) Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. *Phytochemistry* **61**, 693–698.

Katz, E. and Demain, A.L. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. *Bacteriol Rev* **41**, 449–474.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H. and Harbour, A. (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. *J Appl Bacteriol* **78**, 97–108.

Liu, B. and Tzeng, Y. (2000) Characterization study of the sporulation kinetics of *Bacillus thuringiensis*. *Biotechnol Bioeng* **68**, 11–17.

McKeen, C., Reilly, C. and Pusey, P. (1986) Production and partial characterization of antifungal substances antagonistic to *Monilinia fruticola* from *Bacillus subtilis*. *Phytopathology* **76**, 136–139.

Moita, C., Feio, S.S., Nunes, L., Marcelo Curto, M.J. and Roseiro, J.C. (2005) Optimisation of physical factors on the production of active metabolites by *Bacillus subtilis* 355 against wood surface contaminant fungi. *Int Biodet Biodegrad* **55**, 261–269.

Moyne, A., Shelby, R., Cleveland, T. and Tuzun, S. (2001) Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J Appl Microbiol* **90**, 622–629.

Pabel, C., Vater, J., Wilde, C., Franke, P., Hofemeisrer, J., Adler, B., Bringmann, G., Hacker, J. *et al.* (2003) Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of *Bacillus* isolates from the marine sponge *Aplysina aerophoba*. *Mar Biotechnol* **5**, 424–434.

Paulus, H. (1993) Biosynthesis of the aspartate family of amino acids. In *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics* ed. Sonenshein, A., Hoch, J. and Losick, R. pp. 237–267 Washington: American Society of Microbiology.

Piggot, P.J. and Hilbert, D.W. (2004) Sporulation of *Bacillus subtilis*. *Curr Opin Biotechnol* **7**, 579–586.

Raaijmakers, J., Vlami, M. and Souza, J. (2002) Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* **81**, 537–547.

Reva, O.N., Dixelius, C., Meijer, J. and Priest, F.G. (2004) Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Microbiol Ecol* **48**, 249–259.

Romero, D., Pérez-García Rivera, M.E., Cazoela, F.M. and Vicente, A. (2004) Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery milder fungus *Podospheara fusca*. *Appl Microbiol Biotechnol* **64**, 263–269.

Seifert, K.A., Hamilton, W.E., Breuil, C. and Best, M. (1987) Evaluation of *Bacillus subtilis* C186 as a potential biological control of sapstain and mould on unseasoned lumber. *Can J Microbiol* **33**, 1102–1107.

Setlow, P. (2003) Spore germination. *Curr Opin Microbiol* **6**, 550–556.

Tsuge, K., Akiyama, T. and Shoda, M. (2001) Cloning, sequencing, and characterization of the iturin A operon. *J Bacteriol* **183**, 6265–6273.

Vishniac, W. and Santer, M. (1957) The Thiobacilli. *Bacteriol Rev* **21**, 195–213.

Volpon, L., Besson, F. and Lancelin, J.M. (2000) NMR structure of antibiotics plistatins A and B from *Bacillus subtilis* inhibitors of phospholipase A2. *FEBS Lett* **485**, 76–80.

Wright, S., Zumoff, C., Schneider, L. and Beer, S. (2001) *Pantoea agglomerans* strain EH318 produces two antibiotics that inibit *Erwinia amylovora* in vitro. *Appl Environ Microbiol* **67**, 284–292.

Yakimov, M.M., Abraham, W.R., Meyer, H., Giuliano, L. and Golyshin, P.N. (1999) Structural characterization of lichenysin A components by fast atom bombardment tandem mass spectrometry. *Biochim Biophys Acta* **1438**, 273–280.

Yazgan, A., Özcengiz, G., Özcengiz, E., Kilinç, K., Marahiel, M.A. and Alaeddinoglu, N.G. (2001) Bacolysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis*. *Enzyme Microb Technol* **29**, 400–406.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturine A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biol Biochem* **34**, 955–963.