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Optimization of Spore and Antifungal Lipopeptide Production During the Solid-state Fermentation of *Bacillus subtilis*

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Abstract *Bacillus subtilis* strain TrigoCor 1448 was grown on wheat middlings in 0.5-l solid-state fermentation (SSF) bioreactors for the production of an antifungal biological control agent. Total antifungal activity was quantified using a 96-well microplate bioassay against the plant pathogen *Fusarium oxysporum* f. sp. *melonis*. The experimental design for process optimization consisted of a 2^{6-1} fractional factorial design followed by a central composite face-centered design. Initial SSF parameters included in the optimization were aeration, fermentation length, pH buffering, peptone addition, nitrate addition, and incubator temperature. Central composite face-centered design parameters included incubator temperature, aeration rate, and initial moisture content (MC). Optimized fermentation conditions were determined with response surface models fitted for both spore concentration and activity of biological control product extracts. Models showed that activity measurements and spore production were most sensitive to substrate MC with highest levels of each response variable occurring at maximum moisture levels. Whereas maximum antifungal activity was seen in a limited area of the design space, spore

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production was fairly robust with near maximum levels occurring over a wider range of fermentation conditions. Optimization resulted in a 55% increase in inhibition and a 40% increase in spore production over nonoptimized conditions.

Keywords Solid-state fermentation · Optimization · *B. subtilis* · Lipopeptides · Spores · Biocontrol

Introduction

The biological control agent (BCA), *Bacillus subtilis*, produces environmentally resistant spores and is known to produce a spectrum of antifungal lipopeptides [1–3], yet it is unclear how each of these properties contributes to the overall efficacy of the final biological control product (BCP). In vitro production of lipopeptides during fermentation could have an immediate effect on the pathogen population postapplication; in vitro production of bacterial spores could allow for potential in situ lipopeptide production; or the combination of both in vitro and in situ lipopeptide production could be necessary for maximum efficacy.

Further complicating the development of an effective *B. subtilis* BCP is the fact that the organism produces a variety of antifungal lipopeptides such as iturin, surfactin, and fengycin [4–6] that are expressed at different levels under different environmental conditions. Published data detailing the effect of fermentation conditions on lipopeptide production come from a combination of both liquid- and solid-state fermentation (SSF) processes [5, 7–9]. These studies have shown maximum iturin A and surfactin production at 25 and 37 °C, respectively, but little data are available for temperature dependency in the production of other compounds [5, 9, 10]. Aeration has been shown to be an important factor in SSF production of iturin A [10], whereas surfactin production is increased under anaerobic, nitrate-limited conditions in liquid fermentation [8]. Other factors such as pH, peptone addition, and shaking rate have been shown to significantly affect *B. subtilis* lipopeptide production in liquid fermentation [9].

Despite the available research on *B. subtilis* lipopeptide production [7, 9, 11–16], little has been done to examine different combinations of factors in SSF and their impact on overall antifungal activity and spore production. Some works have examined production of specific lipopeptides without measuring direct antifungal activity [7, 9]. Given the potential for synergistic or antagonistic relationships between lipopeptide classes in the BCP, antifungal activity may not be a direct function of individual or total lipopeptide concentrations.

A factorial experimental design was chosen to explore the effects of fermentation conditions on BCP antifungal activity and spore concentration. Factors and factor levels for the experimental design were defined based on preliminary experiments and a review of the literature. Production of both iturin A and fengycin has been shown to be highest after exponential growth phase, whereas surfactin production is highest during exponential growth in liquid fermentation [9, 17]. Thus, fermentation time levels of 48 and 72 h were chosen based on preliminary studies evaluating growth profiles. Levels of aeration and nitrate supplementation were based on the work of Davis et al. [8] who reported that surfactin production from a *B. subtilis* culture greatly increased in anaerobic cultures after nitrate depletion. Jacques et al. [9] found that peptone, temperature, and pH all had significant effects on lipopeptide production from *B. subtilis* during liquid fermentation; peptone had the greatest effect of all factors tested with the maximum level of 4 g/l producing the highest levels. Temperature has also been shown to have a significant effect on SSF

production of lipopeptides by *B. subtilis* with maximum production of iturin A and surfactin occurring at 25 and 37 °C, respectively [5]. Preliminary studies indicated that 4.3 g *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES)/l (100 mM) provided the greatest pH buffering without adversely affecting cell growth.

This paper describes the use of a two-stage optimization consisting of a fractional factorial design coupled with a central composite face-centered (CCF) design to model *B. subtilis* SSF. Optimized fermentation conditions were determined with response surface models fitted for both antifungal activity of the BCP and final spore concentration. This approach allowed us to quantitatively assess the effects of multiple fermentation conditions on producing a BCP with a high concentration of antifungal lipopeptides and *B. subtilis* spores to maximize in situ biological control.

Materials and Methods

Experimental Design

First-stage Optimization

A 2^{6-1} fractional factorial experimental design was used to quantify the effects of (A) fermentation time, (B) aeration rate, (C) nitrate level, (D) peptone level, (E) incubator temperature, and (F) pH buffering (see Table 1). Biological control product spore concentrations and extract inhibition measurements were both used as separate response variables. The fractional factorial design was constructed with each factor set at either a high or a low level, coded as +1 and −1, respectively, as shown in Table 1. The design (see Table 2) was created by confounding the sixth design factor (F) with the five-factor interaction effect of the remaining five factors (A–E). Factor effects, defined as the mean difference between treatments at the high and low levels of each factor or interaction, were calculated for each main factor and all two-factor interactions in the experimental design [18]. All three-factor interactions were assumed to be negligible and used to estimate experimental error [18–20].

Second-state Optimization

The second stage of process optimization was composed of a CCF design for three factors. Central composite design is a common follow-up design to a fractional factorial design in optimization studies [18, 19, 21]. It is used to estimate quadratic and interaction effects but requires fewer runs than a full 3^n factorial. The choice of factors included in the second stage design is discussed in the “Results” section.

Table 1 Factor levels in fractional factorial design for BCP optimization.

Level	Time (h)	Airflow (lpm)	Nitrate (g/l)	Peptone (g/l)	Incubator temperature (°C)	pH buffer (g BES/l)
−1	48	0	0	0	22	0
+1	72	0.4	0.8	4	30	4.3

Table 2 First-level fractional factorial design structure for optimization of BCP production.

Treatment	Time A	Airflow B	Nitrate C	Factor peptone D	Incubator temperature E	pH buffer F=ABCDE
1	−1	−1	−1	−1	−1	−1
2	1	−1	−1	−1	−1	1
3	−1	1	−1	−1	−1	1
4	1	1	−1	−1	−1	−1
5	−1	−1	1	−1	−1	1
6	1	−1	1	−1	−1	−1
7	−1	1	1	−1	−1	−1
8	1	1	1	−1	−1	1
9	−1	−1	−1	1	−1	1
10	1	−1	−1	1	−1	−1
11	−1	1	−1	1	−1	−1
12	1	1	−1	1	−1	1
13	−1	−1	1	1	−1	−1
14	1	−1	1	1	−1	1
15	−1	1	1	1	−1	1
16	1	1	1	1	−1	−1
17	−1	−1	−1	−1	1	1
18	1	−1	−1	−1	1	−1
19	−1	1	−1	−1	1	−1
20	1	1	−1	−1	1	1
21	−1	−1	1	−1	1	−1
22	1	−1	1	−1	1	1
23	−1	1	1	−1	1	1
24	1	1	1	−1	1	−1
25	−1	−1	−1	1	1	−1
26	1	−1	−1	1	1	1
27	−1	1	−1	1	1	1
28	1	1	−1	1	1	−1
29	−1	−1	1	1	1	1
30	1	−1	1	1	1	−1
31	−1	1	1	1	1	−1
32	1	1	1	1	1	1

SSF System

Forced aeration batch microreactors used in this study were the same as described previously ([22]. Reactors were constructed of 0.5-l polypropylene bottles (Nalgene, Rochester, NY, USA). Each reactor was fitted with a 3.2-cm-high PVC pipe covered with nylon screen to support the substrate bed. The reactor working volume was approximately 0.4 l. Reactor lids had two pinhole openings with attached rubber septa (Chemglass, Vineland, NJ, USA), allowing thermocouples to be placed in the bed while maintaining a tight air seal. Both thermocouples were inserted into the center of the bed. Inlet and exhaust fittings were glued in the reactor bottom and lid, respectively, for reactor aeration. Each reactor had a 0.2-μm air filter (Pall Corp., East Hills, NY, USA) fitted just before the reactor inlet to eliminate microbial contamination from the incoming air stream.

Substrate for all experiments was prepared separately for each reactor according to experimental design specifications. One hundred twenty-five grams of wheat middlings at a

moisture content (MC) of 8.7% dry basis (d.b.) were added to individual autoclave bags, mixed with 77.5 ml of distilled water, and autoclaved twice at 121 °C for 25 min with 24 h between sterilizations. The remaining water needed to bring the material up to the required initial MC (1–1.7 g/g d.b.) was added directly before reactor loading. A portion of the additional water was mixed with required supplements, as noted in the experimental design, and was sterilized separately before mixing with substrate. The remaining 40 ml of sterile water was mixed with 2.5 ml of inoculum culture for more uniform inoculum distribution throughout the substrate. Inoculum of *B. subtilis* strain TrigoCor 1448, ATCC 202152 [23] was prepared as described previously [22]. Substrate was mixed thoroughly with the diluted inoculum before being loaded into sterilized bioreactors. All mixing of material was done in individual autoclave bags in a sterile laminar flow hood.

Reactors were placed in a refrigerated incubator (Biocold Scientific, Fenton, MO, USA) to control ambient temperatures throughout fermentation. After 48 or 72 h of fermentation, fermentation material was dried to approximately 11–18% (d.b.) by aerating with approximately 4 lpm of dry air for 2–3 days. Reactor temperatures immediately dropped to approximately 15 °C, effectively slowing microbial growth to near minimum. Final dried BCP was homogenized in a Cuisinart Mini-Prep Plus (Stamford, CT, USA) and stored at −20 °C.

Microbial and Biochemical Assays

Final spore concentration for each reactor was calculated by dilution plate count using sterile 0.1% sodium pyrophosphate (SPP) for all dilutions. Approximately 0.5 g of dried, homogenized BCP was placed in a 50-ml vial with 20 ml of sterile SPP, vortexed twice for 1 min to dislodge cells from the solid matrix, and serially diluted in sterile 1.5-ml microfuge tubes. The first dilution was placed in a water bath at 75 °C for 10 min to eliminate viable vegetative cells before plating. Quadruplicate 10-μl spots were placed on nutrient yeast extract agar plates and incubated at 25 °C for 15–20 h. Colonies were counted for all replicate spots and the mean was used to calculate the concentration of *B. subtilis* spores per dry gram of BCP.

Biological control product preparations were extracted with methanol to obtain materials containing antifungal lipopeptides for use in inhibition bioassays. Biological control product samples (5 dry gram) were placed in 40-ml glass vials with 25 ml of methanol and allowed to steep for approximately 16 h. Material was mixed several times by hand, then vacuum-filtered through two 70-mm Whatman no. 1 filter papers; the final volume was brought to 30 ml with methanol. A 15-ml sample of the final volume (equivalent to 2.5 dry gram of BCP) was passed through a 0.2-μm polyethersulfone membrane filter (Whatman, Inc, Clifton, NJ, USA) and then mixed with 15 ml of distilled water in preparation for a solid-phase extraction cleanup step [24].

A 96-well microplate bioassay was used to measure the inhibition of BCP extracts against *Fusarium oxysporum* f. sp. *melonis* [22]. Extract inhibition was tested by adding 100 μl of potato dextrose broth, 10 μl of BCP extract or solvent control, and 100 μl of a *F. oxysporum* spore suspension (10^4 spores/ml) to each well. Fungal cultures were cultivated for 1–2 weeks on quarter strength potato dextrose agar plates. Plates were then flooded with 10–15 ml of sterile distilled water, gently scraped to form a spore suspension, and diluted to 1×10^4 spores/ml for use in bioassays. Biological control product extracts were diluted with methanol to the specified concentration (mg BCP/ml) and all treatments were measured in triplicate. Plates were read using a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT, USA) at an absorbance of 620 nm before and after incubation for 48 h at 25 °C. Percent inhibition was determined by comparing the absorbance increase of the wells containing BCP extract with wells containing control solvent used for extract dilution.

Data Collection

Temperatures were monitored with two type-T thermocouples in the center of each reactor. Ambient and outlet oxygen (O_2) concentrations were measured with two separate O_2 sensors (NeuwGhent Technology, LaGrangeville, NY, USA). One sensor was dedicated to reading ambient O_2 concentrations whereas the second was connected to a multiposition actuator valve (Valco Instruments Co., Inc., Houston, TX, USA) linked to multiple microreactors. The data acquisition system was developed using a Dell OptiPlex GX1p desktop computer. A CIO-DAS08 analog and digital I/O board and two CIO-EXP32 data acquisition board (Measurement Computing- Middleboro, MA, USA) were used to record reactor temperature and outlet O_2 concentration data and to control the multiposition valve.

A LabVIEW (National Instruments, Austin, TX, USA) program was written to monitor and record temperatures from up to 18 microreactors with two separate thermocouples. Mean reactor data were continually uploaded to a real-time chart for process monitoring and a 2-min running average of each thermocouple was written to a file in 5-min intervals. The program also monitored and recorded data from two separate O_2 sensors. The data acquisition program controlled the multiposition valve so that the sensor sampled exhaust gas from a different reactor every 4 min until all reactors were sampled. After a 3-min purge time after each sample, a 1-min running average of O_2 concentration was written to a file for later analysis.

Data Modeling

The CCF design was structured to explore the role of aeration rate, MC, and incubator temperature at three different levels. Main factor effects, quadratic effects, and two-factor interactions were all discernable with this design. Five replicate runs at the design center were completed to measure experimental error in the system. Tables 3 and 4 show the coded factor levels and central composite design table, respectively. The CCF design results were used to develop a multiple linear regression model for the system. For the three-factor design, the regression had ten component parameters (β_{0-9}): one overall mean, three individual main factor effects, three quadratic factor effects, and three two-factor interactions [21]. The model has the form

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1^2 + \beta_5 x_2^2 + \beta_6 x_3^2 + \beta_7 x_1 x_2 + \beta_8 x_1 x_3 + \beta_9 x_2 x_3 \qquad (1)$$

where

y response variable of percent inhibition or spore concentration
x_1 aeration level (lpm)
x_2 substrate initial d.b. MC (g/g)
x_3 incubator temperature (°C)
β_{0-9} model parameters

The method of least squares was used to estimate values for β_{0-9}.

Table 3 Code for factor levels in central composite design for optimization of BCP production.

Level	Aeration (lpm)	Initial moisture content (g/g d.b.)	Incubator temperature (°C)
−1	0.1 lpm	1.00	19
0	0.8 lpm	1.35	23
1	1.5 lpm	1.70	27

Table 4 Central composite face-centered design scheme for optimization of BCP production.

Treatment	Aeration	Moisture	Incubator temperature
1	−1	−1	−1
2	−1	−1	1
3	−1	1	−1
4	−1	1	1
5	1	−1	−1
6	1	−1	1
7	1	1	−1
8	1	1	1
9	−1	0	0
10	1	0	0
11	0	−1	0
12	0	1	0
13	0	0	−1
14	0	0	1
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0

Spore concentrations and bioassay inhibition data were used for response surface modeling. A MATLAB program was written to calculate model parameters for Eq. 1 for each response variable. The MATLAB program was also used to determine theoretical stationary points and local maxima within the design space, create analysis of variance (ANOVA) tables for models, and plot response surface model results.

Model coefficients were tested for significance using SPSS software (SPSS, Inc., Chicago, IL, USA) and parameters whose coefficients had a p value greater than 0.05 were sequentially removed to determine the effect of the parameter on the model R^2 and adjusted R^2 (R^2_{adj}) values. Parameters were permanently removed from the model when it was seen that their inclusion had a negative impact on R^2_{adj} values.

Model coefficients (β_{0-9}) using scaled unit variables were found using the method of least squares and converted for use with standard units for each factor according to Table 3. Coefficients for use with standard unit variables were found by converting each factor level, $x_{1,2,3}$, in Eq. 1 to standard units using the following equations:

$$x_1(\text{lpm}) = 0.7x_1 + 0.8 \tag{2}$$

$$x_2(\text{g/g d.b.}) = 0.35x_2 + 1.35 \tag{3}$$

$$x_3(^\circ\text{C}) = 4x_3 + 23 \tag{4}$$

Results

First Stage of Optimization

Typical reactor temperature profiles are shown in Fig. 1. Temperature profiles were very similar for reactors with the same incubator temperature level and aeration level regardless

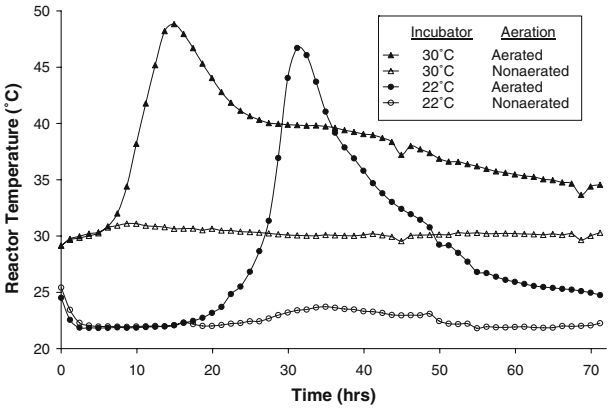

Fig. 1 Typical bioreactor temperature profiles for given incubator temperatures and aeration levels

of all other factor levels. Mean maximum temperatures for all aerated reactors in 30 and 22 °C incubators were 49.8±2.2 and 47.9±2.8 °C, respectively. As expected, the nonaerated reactors did not exhibit significant autoheating whereas aerated reactors showed reactor temperature increases of approximately 20 °C above incubator temperature. Increased convective heat losses were unable to significantly reduce maximum temperatures in aerated reactors in the low-temperature incubator. Incubator temperature did, however, influence lag times as illustrated in Fig. 1. Lag times increased from less than 6 h for the 30-°C incubator reactors to greater than 15 h for the 22-°C incubator reactors. Despite the high temperatures reached in the reactors in the low-temperature incubator, temperatures dropped more rapidly after exponential growth phase.

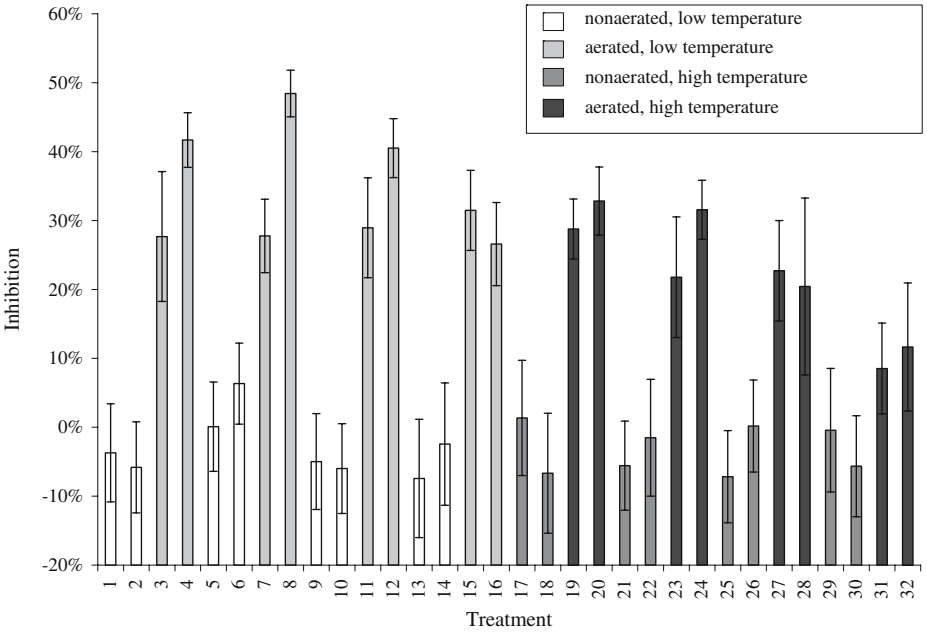

Fig. 2 Inhibition data at 20 mg BCP/ml for fractional factorial design for BCP optimization for treatments listed in Table 2 (*error bars* represent sample standard deviation)

The resultant BCP extracts were tested for growth inhibition of *F. oxysporum* f. sp. *melonis* at concentrations between 2.5 and 160 mg BCP/ml. Aerated reactors had the highest inhibition levels between 20 and 40 mg BCP/ml and declined at higher concentrations (data not shown). Extracts from nonaerated reactors showed little or no inhibition at lower concentrations and less than 25% inhibition even at the highest concentrations tested. Inhibition values at 20 mg BCP/ml were chosen for further statistical analysis; data resolution was greatest at this concentration and lower and higher concentrations had a poor response and were saturated at maximum inhibition, respectively. Inhibition (at 20 mg BCP/ml) and spore concentration results for the fractional factorial design are shown in Figs. 2 and 3, respectively. Inhibition variance is based upon replicate wells from a single sample extraction whereas spore count variance is assumed to be 5% of the measured value based upon preliminary studies of replicate counts from homogenized contents of a single reactor.

Data from Figs. 2 and 3 have been formatted to highlight specific differences between reactors at different aeration levels and incubator temperatures. Although most factor trends are difficult to distinguish from a cursory check of the data, extracts from all aerated reactors showed noticeably higher inhibition levels than those from nonaerated reactors. In addition, extracts from reactors in a lower temperature incubator showed slightly greater inhibition than those in a higher temperature incubator.

Tabulated in Table 5 are factor effects for the first-stage fractional factorial design. Fermentation time and aeration both had significant positive effects on the inhibitory quality of the BCP extract, whereas the addition of nitrate and peptone had significant negative effects. The interaction effects of aeration with both nitrate and peptone (interactions BC and BD, respectively) were both significant and negative. These interactions indicate that the negative effect of each of these factors is greater in magnitude in aerated reactors. As the aeration effect was strong and positive, this provided further evidence that neither nitrate nor peptone should be included in further studies as they negatively impact the production of inhibitory compounds. The strong negative interaction between aeration and temperature also showed that the effect of temperature was

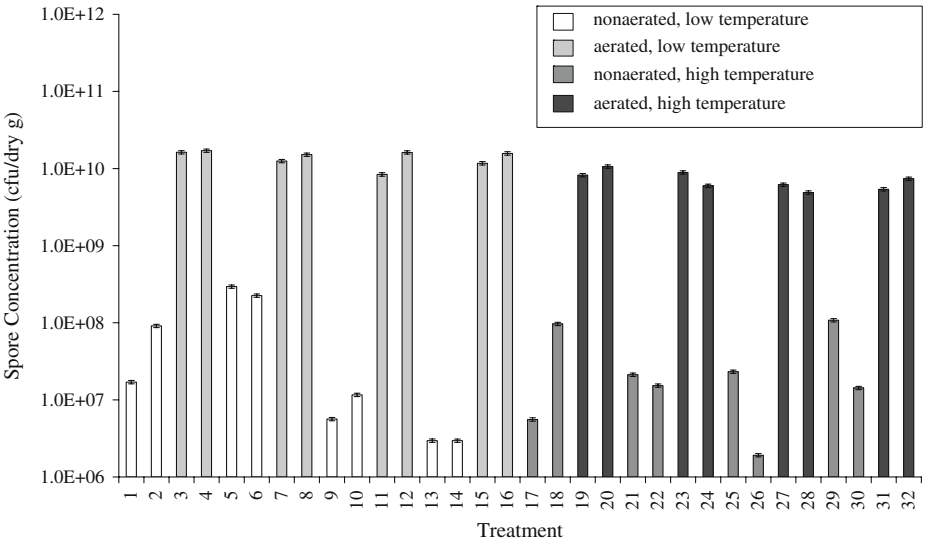

Fig. 3 Spore concentration data for fractional factorial design of BCP optimization for treatments listed in Table 2 (*error bars* represent sample standard deviation)

Table 5 Main factor effects and two-factor interactions from fractional factorial design using microplate inhibition and spore concentration data.

Factor	Effect inhibition at 20 mg BCP/ml	Effect log (spore count)
(Time) A	0.039[a]	0.03
(Airflow) B	0.313[a]	2.69[a]
(Nitrate) C	−0.012	0.12
(Peptone) D	−0.055[a]	−0.41[a]
(Temp) E	−0.060[a]	−0.19
(pH) F	0.028	−0.02
AB	0.031	0.03
AC	0.009	−0.15
AD	−0.022	−0.18
AE	−0.023	−0.15
AF	−0.009	−0.26
BC	−0.032[b]	−0.14
BD	−0.032[b]	0.30[b]
BE	−0.058[a]	−0.11
BF	−0.000	0.11
CD	−0.028	−0.01
CE	−0.028	0.03
CF	0.005	0.16
DE	−0.010	0.29
DF	0.022	−0.00
EF	0.002	−0.11
Standard deviation	0.019	0.18

[a] Indicates significance at the 95% confidence level

[b] Indicates significance at the 90% confidence level

exacerbated at high aeration rates. Incubator temperature had a small negative effect in nonaerated reactors and a sizeable negative effect in aerated reactors, providing further evidence that incubator temperature is an especially important factor in the production of bioactive compounds by *B. subtilis* in small-scale aerobic cultures.

Factor effects for spore production were similar to those for the inhibition data in that aeration and peptone addition showed significant positive and negative effects, respectively. However, unlike the inhibition data, spore counts did not show significant effects from fermentation time or incubator temperature.

Although fermentation time did show a positive effect on inhibition levels, the significant impact of cooler ambient temperature on lag time makes it difficult to compare the reactors on this basis. The 48-h stop time for reactors in the cool and warm incubators occurred at 15 and 22 h after peak temperature, respectively. A follow-up experiment was conducted to explore fermentation time relative to the time of peak temperature. Four reactors were run at 22 and 30 °C and a reactor was killed at 0, 8, 16, and 40 h after peak temperature. Reactor contents were dried and extracted as described earlier.

Results of inhibition assays for those extracts are shown in Fig. 4 and reveal that production of bioactive compounds in reactors with different incubator temperatures followed similar trends. Quadratic equations were fitted through the data for the 22- and 30-°C reactors, as shown in Fig. 4. Maximum inhibition was predicted at 24 h past peak temperature for both data sets. Further studies did not include fermentation time as a design factor; regular aeration was stopped at 24 h past peak temperature for all treatments.

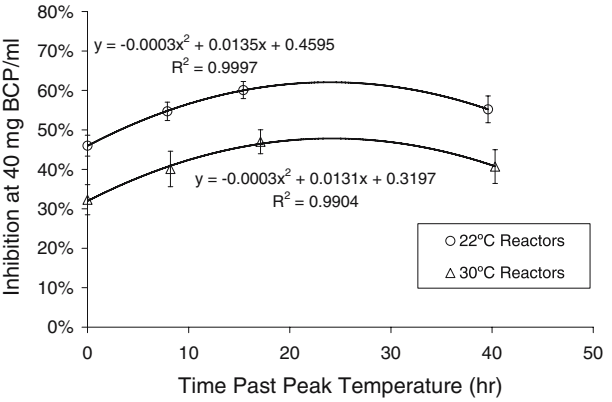

Fig. 4 Inhibition assay results for study of effect of fermentation time on active compound production (*error bars* represent sample standard deviation)

Second Stage of Optimization

Of the factors examined in the first-stage fractional factorial design, only aeration and incubator temperature were chosen for further study in the next stage of optimization. Fermentation time was not included in the design despite its positive effect on inhibition for the reasons described previously. Factors having either a negligible or negative effect on the production of spores and inhibitory compounds were also not included.

Because aerated reactors were shown to have much higher spore and inhibitory compound production, aeration level was included as a factor in the second-stage design. Aeration levels were set at 0.1, 0.8, and 1.5 lpm to provide a range that could include O_2-limiting conditions and significant convective and evaporative heat losses.

Because 30 °C incubator temperatures had a significantly negative impact on BCP extract inhibition, it was hypothesized that further process improvements could be possible through decreasing incubator temperature. The temperature levels chosen for the CCF design were 19, 23, and 27 °C. Temperatures lower than 19 °C were not included because growth was slowed excessively at such extremes and long lag times precluded efficient sample processing.

Although not examined in the first study, substrate MC was included in the second-stage design because MC is linked closely to aeration rates and temperature in many solid-state bioprocessing applications. High aeration rates may help to control reactor temperatures but often lead to drying of the substrate bed. Evaporative and convective heat losses account for approximately 75% of the total heat loss in microreactors at aeration rates of 1.5 lpm with evaporation accounting for more than half of that amount (analysis not shown).

The central composite design results showed much better bioreactor temperature control. Maximum temperatures ranged from 23 to 51 °C whereas the time required to reach maximum temperature ranged from 23 to 72 h (see Table 6). The shape of the temperature profiles were the same for all reactors (as shown in Fig. 1) with the exception of treatment 3 (see Table 4 for treatment conditions). This profile showed a second temperature peak approximately 12 h after the primary peak; this profile was also seen in a preliminary study under the same conditions. Aeration rate affected both reactor peak temperature and minimum outlet O_2 concentrations. Low aeration rates generally led to higher reactor temperatures and outlet O_2 concentrations as low as 2.5%. Reactors with the middle and highest aeration rates showed no evidence of O_2 limitation as minimum outlet concentrations were above 19 and 20%, respectively.

Table 6 Summary of data from CCF design for BCP optimization.

Treatment	Peak temperature (°C)	Peak time (h)	Cumulative oxygen consumption (mol)	Spore concentration (cfu/dry g)	Inhibition at 20 mg BCP/ml (%)
1	37	72	0.47	1.34×10^{10}	28.9
2	47	23	0.70	2.30×10^{10}	32.2
3	40	58	0.62	1.90×10^{10}	53.2
4	49	24	1.00	1.84×10^{10}	13.0
5	23	80	0.35	2.53×10^{10}	5.6
6	36	26	0.72	2.46×10^{10}	20.0
7	26	71	0.88	2.02×10^{10}	55.1
8	48	27	1.19	2.62×10^{10}	54.8
9	51	34	0.50	1.66×10^{10}	38.9
10	32	44	0.64	1.77×10^{10}	59.9
11	31	40	0.44	1.41×10^{10}	45.6
12	37	36	0.76	2.71×10^{10}	58.9
13	31	67	0.57	1.95×10^{10}	60.0
14	49	24	0.88	1.68×10^{10}	50.0
15	41	37	0.63	1.70×10^{10}	44.8
16	40	36	0.70	2.25×10^{10}	55.0
17	39	36	0.72	2.88×10^{10}	51.0
18	37	36	0.66	2.85×10^{10}	59.8
19	40	39	0.64	2.72×10^{10}	59.7

See Table 4 for treatment conditions.

A summary of temperature, oxygen, spore count, and inhibition data for the CCF design is given in Table 6. Extract inhibition was tested at concentrations between 5 and 80 mg BCP/ml, and 20 mg BCP/ml was chosen for further analysis and modeling. Spore and inhibition data were poorly correlated with a correlation coefficient (r) of 0.15. Other variables also had poor correlation with spore and inhibition data; the highest correlation was between cumulative oxygen uptake and spore concentration (r=0.26). The apparent lack of correlation between spore production and sample extract inhibition illustrates the importance of environmental factors in lipopeptide production.

Data Modeling

Both the inhibition data at 20 mg BCP/ml and the log transformation of spore concentration data were used to create response surface models for the SSF process. A significance test on each of the coefficients, β_i, derived for the original form of the model (see Eq. 1) of inhibition data showed that the term associated with the quadratic effect of incubator temperature, β_6, was not significant (p=0.329). This term was dropped from the model and the parameters were recalculated.

The final model for bioassay inhibition at 20 mg BCP/ml is:

$$y = -200 - 62.7x_1 + 324x_2 + 4.37x_3 - 25.0x_1^2 - 76.9x_2^2 + 40.4x_1x_2 + 2.28x_1x_3 - 5.20x_2x_3 \quad (5)$$

The ANOVA table for the model is shown in Table 7. Results of analysis showed that the regression was significant at the 95% confidence level (p=0.001) without significant

Table 7 ANOVA table for inhibition model derived from CCF design.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_0	p value
Regression	4,545.1	8	568.1	9.24	0.001
Residual	614.7	10	61.5	1.88	0.28
Lack of fit	453.9	6	75.7		
Pure error	160.8	4	40.2		
Total	5,159.8	18	286.7		

The R^2 value for the model is 0.881.

The R^2_{adj} value is 0.786.

evidence for lack of fit (p=0.29). The models R^2 and R^2_{adj} were 0.88 and 0.79, respectively. Figure 5 shows the model contour plots at d.b. MCs of 1.0, 1.35, and 1.7 g/g. The model predicted that maximum inhibition (68%) within the design space occurs at a MC of 1.7 g/g (d.b.), incubator temperature of 19 °C, and an aeration rate of 1.0 lpm. The model indicates

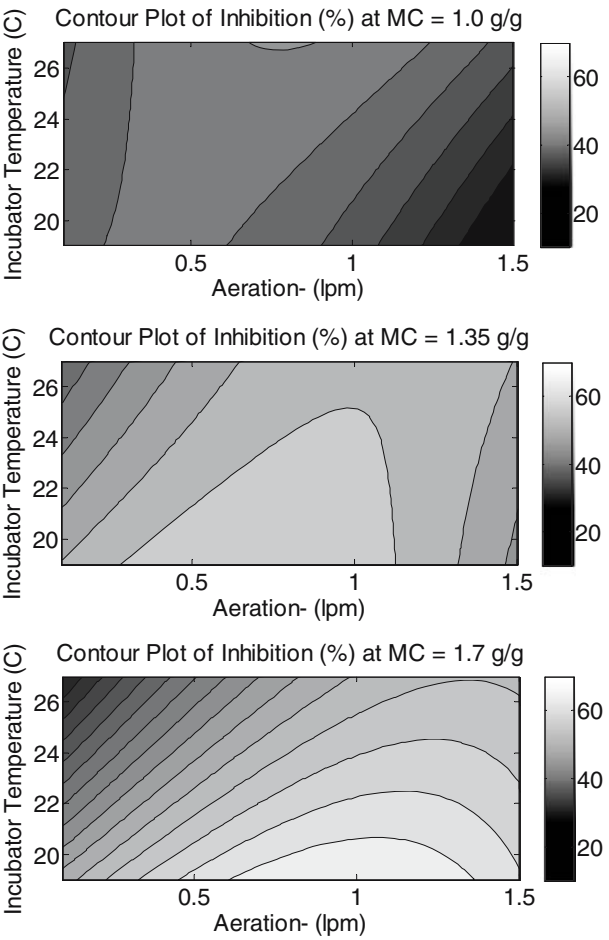

Fig. 5 Contour plots of microplate inhibition at constant MC

Table 8 ANOVA table for model of log (spore concentration) for CCF design.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_0	p value
Regression	0.78	7	0.111	6.40	0.0035
Residual	0.19	11	0.017	2.31	0.22
Lack of fit	0.15	7	0.022		
Pure error	0.04	4	0.009		
Total	0.97	18	0.054		

The R^2 value for the model is 0.803.

The R^2_{adj} value is 0.677.

that inhibition is more sensitive to temperature than to aeration rates in the space immediately surrounding the predicted maximum but falls off more steeply at the lowest aeration rates.

A similar analysis for a model of final BCP spore concentration showed that the quadratic terms for both MC and temperature (β_5 and β_6 from Eq. 1, respectively) were not

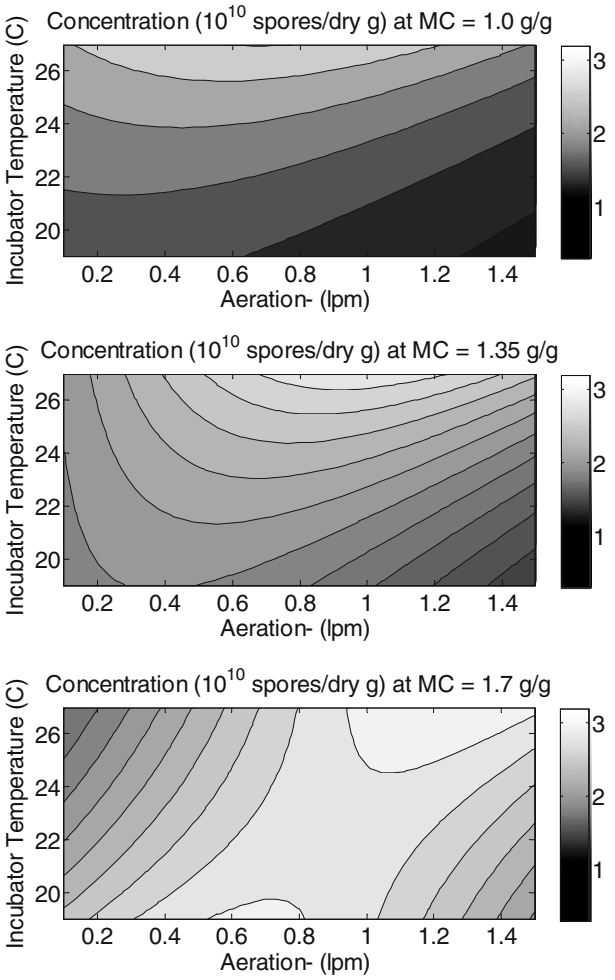

Fig. 6 Contour plots of spore concentrations at constant MC

significant and were thus removed from the final model before recalculating the coefficients. The final model is

$$y = 7.10 - 1.14x_1 + 2.31x_2 + 0.135x_3 - 0.274x_1^2 + 0.448x_1x_2 + 0.039x_1x_3 - 0.100x_2x_3 \quad (6)$$

The ANOVA table for this model is shown in Table 8. Regression was again found to be significant at the 95% confidence level (p=0.004) whereas there was no significant evidence for lack of fit (p=0.22). The R^2 and R^2_{adj} values were 0.80 and 0.68, respectively. Contour plots generated from Eq. 6 are shown in Fig. 6. The model shows that the maximum predicted spore concentration (3.2×10^{10} spores/dry g) within the design space is produced at a MC of 1.7 g/g, incubator temperature of 27 °C, and an aeration rate of 1.2 lpm. The contour plot, however, shows that spore production is fairly robust at high MCs with spore concentration as high as 2.9×10^{10} spores/dry g at an incubator temperature of 19 °C and an aeration rate of 0.4 lpm.

Discussion and Conclusions

This study was undertaken to delineate how environmental and nutritive factors affect overall antifungal activity and spore production of the BCA *B. subtilis*. In the full factorial design analysis, BCP extracts from aerated reactors had significantly more antifungal activity than nonaerated reactors. The significant interaction effect between aeration and nitrate addition showed a small positive effect of nitrate addition in nonaerated reactors and a stronger negative effect in aerated reactors. The presence of nitrate, although beneficial in the absence of aeration, was not able to increase antifungal lipopeptide production beyond levels seen in aerated reactors. Surfactin production has been shown to increase under anaerobic nitrate-limited conditions after nitrate depletion [8]. Although nitrate was not monitored throughout the process or measured after SSF completion, initial nitrate concentrations may have been either not high enough to support sufficient microbial growth or too high so that nitrate did not become limiting during fermentation.

Peptone and pH were previously found to have significant effects on lipopeptide production from *B. subtilis* in liquid culture [9]. In that study, peptone addition had a strong positive effect on lipopeptide production with the highest production levels occurring with the highest peptone concentrations tested. Media pH was also determined to be an important factor and an optimum pH of 7 was reported. In this SSF study, peptone addition had a significantly negative effect on extract antifungal activity whereas the effect of pH buffer addition was not significant. These findings underscore the differences between fermentations using liquid and solid media because each environment has its own growth limitations based on factors such as baseline media nutrient content and buffering capacity. In this study, the addition of such amendments did not significantly increase spore concentration or extract antimicrobial activity of the final product for *B. subtilis* SSF using a wheat middling substrate.

Incubator temperature, initial reactor MC, and aeration rate were all found to have significant effects on both SSF spore production and BCP extract activity. Some similarities were seen between the models quantifying the effects of fermentation conditions on these two response variables. Increasing initial MC had the greatest positive impact on both spore concentration and extract activity with highest values occurring at the highest MC. These results would suggest that increasing MC beyond the maximum level tested (1.7 g/g d.b.) could yield greater process improvements. Preliminary studies (data not shown), however,

showed that 1.7 g/g was near the water-holding capacity of the wheat middlings and increasing initial MC beyond that level led to formation of free water during fermentation.

Whereas spore concentration and extract inhibition models predicted some similarities in optimal conditions, there are certain conditions that favor production of one but not the other. At a MC of 1.0 g/g, an aeration rate of 0.4 lpm, and an incubator temperature of 27 °C, spore concentrations are 92% of the maximum predicted values within the design space (at 1.7 g/g d.b., 1.2 lpm, and 27 °C). Inhibition values at this point, however, are only predicted to be 36% compared to the maximum value of 68%. Generally, spore production was more robust than extract activity across processing conditions. This suggests that although high cell density is necessary for maximum extract activity, specific environmental conditions during fermentation can either enhance or limit production of antifungal compounds despite high spore concentrations.

Based on the data presented here on SSF of *B. subtilis* on wheat middlings, the region of fermentation conditions producing maximum antifungal activity is a subset of those producing near maximum spore concentrations. Therefore, conditions that yield maximum antifungal activity can also produce near maximum spore concentration, whereas the opposite is not always true. Further improvements to BCP product efficacy are likely to be realized by focusing on in vitro extract inhibitory activity as the primary metric for maximizing biological control potential.

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