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Optimization of *Bacillus subtilis* cell growth effecting jiean-peptide production in fed batch fermentation using central composite design

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ABSTRACT

Background: Optimization of nutrient feeding was developed to improve the growth of *Bacillus subtilis* in fed batch fermentation to increase the production of jiean-peptide (JAA). A central composite design (CCD) was used to obtain a model describing the relationship between glucose, total nitrogen, and the maximum cell dry weight in the culture broth with fed batch fermentation in a 5 L fermentor.
Results: The results were analyzed using response surface methodology (RSM), and the optimized values of glucose and total nitrogen concentration were 30.70 g/L and 1.68 g/L in the culture, respectively. The highest cell dry weight was improved to 77.50 g/L in fed batch fermentation, which is 280% higher than the batch fermentation concentration (20.37 g/L). This led to a 44% increase of JAA production in fed batch fermentation as compared to the production of batch fermentation.
Conclusion: The results of this work improve the present production of JAA and may be adopted for other objective products' production.

1. Introduction

Different strains of *Bacillus subtilis* can produce a variety of antimicrobial cyclic lipopeptides, including iturin A, fengycins, and surfactins [1]. In 2001, our laboratory in Chengdu, China isolated a new strain of *B. subtilis* that was able to produce an agricultural antibiotic jiean-peptide (JAA), which was believed to belong to the iturin family [2]. Members of this family are cyclic lipoheptapeptides linked by a β-amino acid residue; and have strong antibiotic activity and moderate surfactant activity [3]. Previous studies have shown that JAA could be used as a fungicide against various crop diseases, including cotton fusarium wilt, tomato rhizoctonia rot, and wheat powdery mildew, all of which can decrease significantly the growth and productivity of these crops [4]. JAA poses little risk to the environment because of its low levels of toxicity and lack of allergic effects on the host [5]. Therefore, JAA has great potential for use against crop fungus diseases. Despite these decisive benefits, the commercial production of JAA has been limited, resulting from the low cell density of *B. subtilis* cultures and low production in fermentation.

Fed batch fermentation techniques have been applied extensively in industrial fermentation processes for enhancing cell density and the generation of products of interest. The major advantage of fed batch fermentation is the ability to adjust the substrate concentration in the culture broth to a value suitable for cell growth and production [6,7,8]. However, optimization of fed batch processes is challenging. The challenge of dynamic fed batch optimization often involves the resolution of high-order, nonlinear and multimodal systems [9]. Empirical feeding policies have been developed to achieve high cell density cultures. The simplest technique is a constant feeding rate. These methods, however, do not consider cell dynamics. An exponential feed rate has been used by taking into consideration the cell growth dynamics [10]. In recent efforts, both stochastic and deterministic search techniques have been applied to maximize cell concentration and productivity in the process of fed batch fermentation. Evolutionary algorithms, such as genetic algorithms mimicking the principles of natural biological evolution [11,12], or an ant colony algorithm mimicking the cooperative search behavior of ants in real life [13], have also been applied to solve for optimal feed-rate profiles. In addition to these population-based search

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techniques, many point-based search techniques have been applied to determine optimal feed-rate profiles [14]. For instance, Lee et al. [10] successfully applied the conjugate gradient algorithm for optimizing fed batch fermentations for poly-β-hydroxybutyric acid production. Cuthrell and Biegler employed an orthogonal collocation-based sequential quadratic programming to optimize fed batch culture for penicillin production [15]. In all of these cases, a mathematical model was used to describe the relationship between biomass and the concentration of the limiting substrate.

Previous studies on JAA fermentation have found that the limiting substrates including glucose and total nitrogen are the main influencing factors for cell growth and JAA accumulation [16] and that the response surface methodology (RSM) is an effective strategy for optimizing JAA production by *B. subtilis* ZK8 in a shake flask culture [2]. Compared with conventional methods for optimization, RSM is a time- and labor-saving method [17,18], which consists mainly of the central composite design (CCD), the box-behnken design (BBD), the one factor design, the D-optimal design, the user-defined design, and the historical data design. The CCD and BBD are the most commonly used response surface design methods, and have 5 levels and 3 levels, respectively, for one numeric factor. RSM has been used successfully for production optimization of many products, including enzyme [19,20], antibiotics [21,22] and biofuel [23,24].

In this study, we have used RSM with a CCD for optimization of cell growth in fed batch fermentation by *B. subtilis* ZK8 in order to improve JAA production.

2. Materials and methods

2.1. Microorganism

B. subtilis ZK8, which produces large amounts of JAA, is a mutant of the original *B. subtilis* ZK.

2.2. Culture media

The seed medium was composed of (in g/L) peptone, 30; glucose, 25; $MgSO_4 \times 7H_2O$, 3; KH_2PO_4, 4.

The fermentation medium was composed of (in g/L) glucose, 37; total nitrogen of soybean meal hydrolysate (TNSMH), 2.4; yeast extract, 0.8; $MgSO_4 \times 7H_2O$, 3.5; KH_2PO_4, 1.1.

The initial pH values of the two media were 7.0.

The fed concentrated carbon source was composed of (in g/L) glucose, 377.

The fed concentrated nitrogen source was composed of (in g/L) TNSMH, 9.0.

2.3. Culture conditions

A seed culture was grown in a 500 mL flask (containing 120 mL of seed medium) on a shaker at 30°C for 20 h. An inoculum of 200 mL was introduced into a 5 L stirred bioreactor (BG-5, Baoxing Biotech Co., Shanghai, China) containing 2 L of fermentation medium. The bioreactor was equipped with pH, temperature and agitation speed controls and a dissolved oxygen display. The temperature and the initial stirring speed were maintained at 30°C and 300 rpm, respectively. The aeration rate was fixed at 1.0 vvm. The dissolved oxygen concentration (DOC) was maintained at 30% ± 5% saturation by dynamic linkage 30% DOC and stirring speed during the fastigium of oxygen consumption.

The fed batch culture was started as a batch culture. A concentrated carbon source and nitrogen source were fed into the bioreactor from the 10th h to the 15th h of cultivation to maintain the concentration of glucose and total nitrogen at an appropriate value. The two peristaltic pumps were calibrated before starting the fed batch fermentation.

2.4. Design of the optimal concentration of glucose and total nitrogen

A CCD with five coded levels (-1.41, -1, 0, +1 and +1.41) was used to elucidate the influence of the glucose concentration and total nitrogen concentration on cell dry weight, from the 11th h to the 15th h of cultivation. According to this design, the total number of treatment combinations was $2^k + 2\,k + n_0$, where k is the number of independent variables and n_0 is the number of repetitions of experiments at the center point [25]. The treatments were carried out in duplicate as independent experiments to take into account the non-adjustable data and the analysis of variance (ANOVA). The results of the CCD were fit with a second-order polynomial equation using a multiple regression technique in [Equation 1]:

$$Y = \beta_0 + \sum_{i}^{k} \beta_i x_i + \sum_{ii}^{k} \beta_{ii} x_i^{\,2} + \sum_{i<j} \beta_{ij} x_i x_j \qquad \text{[Equation 1]}$$

where Y is the predicted response, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

All experiments were carried out in duplicate, and the results were averaged. The CCD and statistical analysis of the data were performed with the Design Expert software package (version 7.1.5, State-Ease Inc., Minneapolis, MN, USA). The models were analyzed statistically by using the analysis of variance (ANOVA). The quality of the polynomial model equations was judged statistically by the coefficient of determination, R^2, and its statistical significance was determined by the F-test. The significance of the regression coefficients was tested by Student's t-test [26].

2.5. Cell concentration

Cell mass was monitored intermittently by measuring the cell density of the culture broth with a blood cell count board. The dry cell weight was determined from a calibration curve between the dry cell mass and the cell density. For accurate cell mass measurements at a high concentration range, the dry cell mass was measured by centrifuging 10 mL of culture broth, washing with distilled water, and drying in the oven at 80°C for 12 h.

2.6. Glucose and total nitrogen concentration

The cell cultures taken from the fermentor were centrifuged and the glucose concentration was determined by the dinitrosalicylic acid (DNS) method [27]. The total nitrogen concentration was determined by a modified Kjeldahl method [28].

2.7. Extraction and quantification of JAA

A 10 mL sample of *B. subtilis* ZK8 culture was centrifuged at 12,000 × *g* for 10 m. The supernatant was recovered, adjusted to pH 2.0 with 15% (v/v) HCl, and then centrifuged at 12,000 × *g* for 10 min. The precipitate was recovered and mixed with 10 mL of methanol/50 mM ammonium acetate (8:2, v/v). After extraction for 30 min, the mixture was centrifuged at 12,000 × *g* for 10 min and the supernatant was recovered. Samples of the supernatant were analyzed by reverse-phase, high-performance liquid chromatography (RP-HPLC) using an HC-C_{18} column (4.6 mm × 150 mm, Agilent, USA) in an LC-10AT HPLC system (SHIMADZU, Kyoto, Japan) operated at a flow rate of 0.8 mL/min. A mixture of methanol and 50 mM ammonium acetate (8:2, v/v) was used as the eluent, and the outflow of the column was monitored by measuring the absorbance at 214 nm.

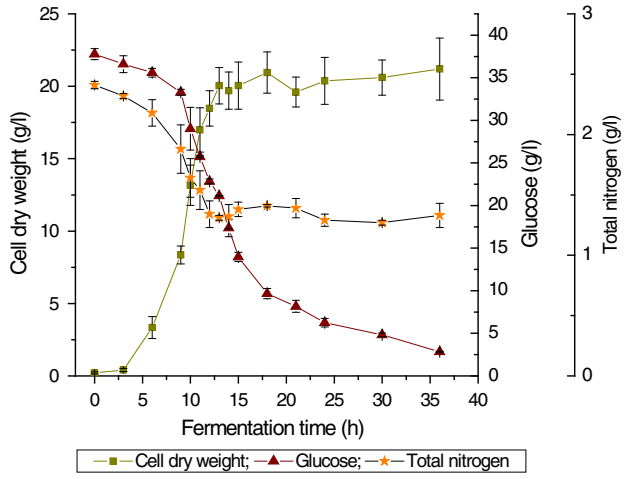

Fig. 1. The development of *Bacillus subtilis* cell growth, glucose and total nitrogen consumption with time in batch fermentation.

3. Results and discussion

3.1. Cell growth and substrates consumption in batch fermentation

Prior to the fed batch study, batch fermentation was carried out to study the cell growth and substrate consumption. Fig. 1 shows the development of *B. subtilis* cell growth, glucose and total nitrogen consumptions with time in batch fermentation. Cell growth started immediately after inoculation of the fermentor. Exponential growth was observed for a short period (6–15 h), in which glucose and total nitrogen consumptions were very fast. According to the parameters of cell dry weight, glucose and total nitrogen concentrations in the course of exponential growth, the specific rates of glucose and total nitrogen consumptions were calculated as 0.2863 and 0.0132 h^{-1}, respectively. Although the maximum cell growth was obtained at 18 h, the fermentation time of 10 h, having the highest specific rates of glucose and total nitrogen consumption was considered as the optimal time to start feeding both substrates. At this time, glucose and total nitrogen concentrations were 30.0 g/L and 1.74 g/L, respectively.

3.2. Optimal feed rate profiles by a nonsingular control algorithm

According to the above results of the specific rates of glucose and total nitrogen consumptions, the feed rate equations for both substrates in the 10th h–11th h of the fermentation process can be written as follows:

$$V_{C\text{-}10} = 0.2863 \text{ x } X_{10} + (x_1\text{-}30.0) \quad \text{[Equation 2]}$$

$$V_{N\text{-}10} = 0.0132 \text{ x } X_{10} + (x_2\text{-}1.74) \quad \text{[Equation 3]}$$

Where $V_{C\text{-}10}$ (g/L/h) and $V_{N\text{-}10}$ (g/L/h) are the feed rates of glucose and total nitrogen in the 10th h–11th h of the fermentation process. X_{10} (g/L) is the concentration of cells at the 10th h of cultivation, and x_1 and x_2 are the designated concentrations of glucose and total nitrogen in CCD.

The feed rate equations for both substrates in the 11th h–15th h of the fermentation process can be written as follows:

$$V_{C\text{-}S} = 0.2863 \times X_S \quad \text{[Equation 4]}$$

$$V_{N\text{-}S} = 0.0132 \times X_S \quad \text{[Equation 5]}$$

where $V_{C\text{-}S}$ (g/L/h) and $V_{N\text{-}S}$ (g/L/h) are the feed rates of glucose and total nitrogen, respectively, at the Sth h of cultivation. X_S (g/L) is the concentration of cells at the Sth h of cultivation.

The key feature of the above approach was the formulation of the feeding sequence, and a feedback law expressed in terms of state variables and a few parameters for singular feed rate calculation as decision variables. Depending on the process kinetics, the feedback law maintains the substrate concentration constant, or allows its variation in a predetermined manner in the singular interval [29].

3.3. Optimized control of the concentrations of glucose and total nitrogen in fed batch fermentation

The optimal control of the concentration of glucose and total nitrogen from the 11th h to 15th h of cultivation versus the maximum cell dry weight was conducted by CCD. The design matrix and the corresponding experimental data are given in Table 1. The experimental results of the CCD were fit to a second-order polynomial in [Equation 6]:

$$Y = 74.5 + 7.42X_1 + 5.58X_2\text{-}X_1X_2\text{-}9.31{X_1}^2\text{-}9.06{X_2}^2 \quad \text{[Equation 6]}$$

The fit of the model Y was evaluated by the coefficient of determination, R^2, which was 0.9531, indicating that 95.31% of the variability in the response could be explained by the model (Table 2). The statistical significance of the model equation was evaluated by an F-test ANOVA, which revealed that this regression was statistically significant (P =0.0092) at the 99% confidence level. Table 2 shows the significance of the regression coefficient of the model, indicating that the glucose (X_1) (P = 0.0057) and the total nitrogen (X_2) (P = 0.0156) had highly significant effects on the maximum cell dry weight. The effect of the interaction of glucose (X_1) and total nitrogen (X_2) was not significant (P = 0.6221) at the 90% confidence level. The contour plot described by the model Y is represented in Fig. 2, which shows that the maximum cell dry weight was approximately 75 g/L. The optimal concentrations for glucose (X_1) and total nitrogen (X_2) obtained from the maximum point of the model were 30.70 g/L for X_1 and 1.68 g/L for X_2. The model predicted a maximum cell dry weight of 76.79 g/L for this point.

Many authors have studied and continue to study the application of advanced controls to fermentative processes. The advanced controls

Table 1
Design and responses of the central composite design (CCD).

Run	Coded values and real values		Maximum cell dry weight (g/L)	
	X_1	X_2	Experimental	Predicted
1	-1 (28.0)	-1 (1.40)	46.01	42.20
2	-1 (28.0)	1 (1.80)	59.21	55.46
3	1 (32.0)	-1 (1.40)	60.19	59.18
4	1 (32.0)	1 (1.80)	69.22	68.27
5	-1.41 (27.2)	0 (1.60)	41.11	45.61
6	1.41 (32.8)	0 (1.60)	66.13	66.61
7	0 (30.0)	-1.41 (1.32)	46.23	48.78
8	0 (30.0)	1.41 (1.88)	62.12	64.54
9	0 (30.0)	0 (1.60)	75.03	74.74
10	0 (30.0)	0 (1.60)	74.45	74.74

X_1: Glucose, g/L; X_2: Total nitrogen, g/L.

Table 2
Analysis of variance for the experimental results of the central composite design (CCD).

Source	DF	Sum of squares	Mean square	F value	Prob > F
X_1[a]	1	443.63	443.63	29.06	0.0057**
X_2	1	249.78	249.78	16.34	0.0156*
X_1^2	1	401.36	401.36	26.25	0.0069**
X_2^2	1	377.94	377.94	24.72	0.0076**
X_1X_2	1	4.35	4.35	0.28	0.6221
Model	5	1243.45	248.69	16.27	0.0092**
Error	4	61.069	15.29		
Total	9	1304.60			
$R^2 = 0.9531$		Adj-$R^2 = 0.8945$			

[a] The symbols are the same as those in Table 1.
* Statistically significant at a probability level of 90%.
** Statistically significant at a probability level of 99%.

have been utilized mostly with respect to a particular substrate and the substrate feed rate [30,31]. However, San and Stephanopoulos [32] hypothesized that controlling the feed rate of a substrate could lead to a suboptimal reactor performance. The fermentor performance might depend heavily on the biomass and the reactor substrate concentration, especially when the proportion of carbon and nitrogen sources utilized by the organisms is variable in different fermentation processes. Therefore, San and Stephanopoulos [32] proposed another optimal strategy: controlling the concentration of different substrates by controlling the feed rate of the substrates. In this study, feeding both substrates and optimizing their concentration at the same time were carried out by CCD. The results showed that it was a successful and effective optimal feeding strategy for improving biomass production.

3.4. Validation of the optimized fed batch fermentation

To verify the modeling results, experiments were done in triplicate, using the optimized conditions to represent the maximum point of the cell dry weight in the course of fed batch fermentation. The predicted maximum dry cell weight was 76.79 g/L, and the average value obtained in the experiments was 77.50 g/L. This is an improvement of concentration by about 280% relative to that in batch fermentation (20.37 g/L). The good correlation between the predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point. At this point, the maximum concentration of JAA was 0.574 g/L, which is an improvement of the concentration by about 44% relative to that in batch fermentation. The dry cell weight value of 77.50 g/L was higher than those reported for fed batch fermentation by some *B. subtilis* variants using an optimized feeding strategy for the substrate [9,33].

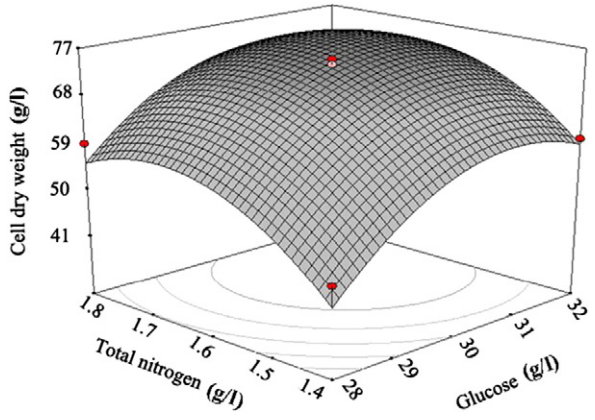

Fig. 2. Response surface plot, described by the model Y fitted from the experimental results of the central composite design (CCD) represents the effect of total nitrogen and glucose on cell dry weight.

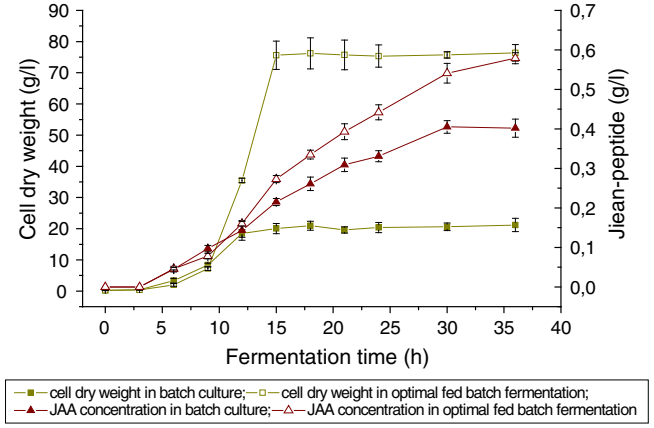

Fig. 3. Comparison of the development of *Bacillus subtilis* cell growth and JAA production with time in batch and optimal fed batch fermentation.

3.5. Comparison of JAA production in batch and optimal fed batch fermentation

To enable comparison with batch fermentation, the values of the parameters and the initial conditions were kept the same; the final volume of fed batch fermentation should not be higher than 10% that of batch fermentation. The superiority of optimal fed batch operation is evident from Fig. 3. The dry cell weight and JAA production are consistently higher than batch fermentation with the same starting condition after 10 h of cultivation. At the end of 36 h period, the optimal fed batch fermentation produced 280% more cell dry weight than batch fermentation, but only 44% more JAA production than batch fermentation. We observed that JAA production through optimal fed batch fermentation continued to rise after 36 h of cultivation, although the rate of increase slowed down. While in batch fermentation, JAA production declined after 30 h of cultivation. It might therefore be possible to improve this performance by allowing the duration of fermentation to exceed 36 h so as to trade off between improved JAA production and the time allowed. In addition, Fig. 3 shows that there were two phases of JAA accumulation in batch and fed batch fermentation: the first phase was associated with cell growth, and the second phase was not associated with cell growth. These results concur with a previous report stating that the relation between the cell growth and JAA formation was a relation combining growth-associated and nongrowth-associated processes [2]. In this study, the cell dry weight was improved more than JAA production in fed batch fermentation as compared to the production of batch fermentation. Future studies will focus on further optimization, in which the cell dry weight and JAA production will be considered at the same time.

4. Concluding remarks

The RSM with a CCD was used to optimize the growth of *B. subtilis* to increase the production of JAA in fed batch fermentation using a 5 L fermentor. The optimized values of glucose and total nitrogen concentrations in the culture were identified as 30.70 g/L and 1.68 g/L, respectively. Operated under the optimized conditions, the highest cell dry weight was improved to 77.50 g/L in fed batch fermentation. This value was 280% higher than the batch fermentation concentration (20.37 g/L), leading to a 44% increase of JAA production in fed batch fermentation as compared to the production of batch fermentation.

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Author contribution

Proposed theoretical frame: HT, JZ, XZ; Conceived and designed the experiments: JZ, XZ; Contributed reagents/materials/analysis tools: JZ, XZ, YR; Wrote the paper: JZ, XZ; Performed the experiments: JZ, XZ, YR, JY; Analyzed the data: JZ, XZ, HT, JZ.

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