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Production of antifungal lipopeptide from *Bacillus subtilis* in submerged fermentation using shake flask and fermentor

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Abstract−Optimization of the production of antifungal lipopeptide, iturin A, by *B. subtilis* was carried out in submerged cultural fermentation. In a shake-flask experiment, response surface methodology (RSM) was employed to optimize the cultivation conditions of *Bacillus subtilis* S3 for the enhancement of iturin A production. The optimal conditions for iturin A production obtained from RSM were pH 6.0, 0.93% maltodextrin, 1.11% glucose, 0.72% corn steep powder (C.S.P), 1.5 mM $MgSO_4$, 0.75 mM KH_2PO_4, rotation speed 180 rpm and area of aeration 4.35 cm^2. 57% increase of iturin A productivity (from 47.19 mg/L to 74.22 mg/L) was observed using the one-factor-at -a-time technique; however, 180% increase of iturin A productivity (from 47.19 mg/L to 132.23 mg/L) was observed with RSM. The iturin A production was further studied in a 5-L fermentor with a variation of agitation speed, aeration and baffles. In the 5-L fermentor, it was found that increased agitation speed improved the growth of *B. subtilis* and the production of iturin A. Aeration at 2 vvm gave excellent production of iturin A (175.52 mg/L) at 6 d of fermentation. The addition of baffles in the fermentor has significantly influenced the oxygen mass transfer coefficient (K_La) and iturin A production.

Key words: *Bacillus subtilis*, Submerged Cultural Fermentation, Stirred-tank, K_La, Iturin A, Biomass, Optimization

INTRODUCTION

Synthetic chemical fungicides have long served as power agents for reducing the incidence of plant disease; however, they are costly, can cause environmental pollution, and may induce pathogen resistance. Therefore, the conventional use of chemical pesticides has been seriously questioned [1,2]. To cope with the problem associated with chemical fungicide, biological control agents, which include effective microorganisms and microbial products, have been attracting attention recently as alternatives to chemical pesticides for the management of plant disease. The biological control of plant pathogens, which is without the negative aspects of chemical control, has become an important aspect of sustainable agriculture.

In the past few years, numerous microorganisms with antifungal activities and their antifungal factors have been identified [3-8]; in addition, the mechanisms by which microorganisms inhibit growth of potentially pathogenic fungi have been demonstrated [4,7-13]. *Bacillus subtilis*, a representative gram-positive and spore forming bacterium [11,14-15], shows antagonistic activities against several plant pathogens; thus, it is one potential bio-control agent. Till now, several *Bacillus subtilis* strains and their derivatives were found to have broad suppressive abilities over a variety of plant pathogens *in vitro* [16,17] by producing the lipopeptide antibiotics iturin A and surfactin [14,18].

Iturin A is a cyclic lipopeptide containing a heptapeptide (L-Asn-D-Tyr-D-Asn-L-Gln -L-Pro-D-Asn-L-Ser) cycled with a β-amino fatty acid; it is a small molecule yet displays strong antifungal activity. In contrast, the other lipopeptide, surfactin, which is a biosurfactant constituted by heptapeptide cyclized with a β-hydroxy fatty acid, has weak antibiotic activity. The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides [16,17]. Along with its wide spectrum of antibiotic activity and surface activity, iturin A confers low toxicity, low allergic effect on humans and animals [19], high biodegradability; these characteristics qualify it as a candidate for an environmentally safe biological pesticide [13].

Previously, microbial cultivations of *Bacillus subtilis* for iturin A production have been conducted in submerged fermentation (SMF), in addition to solid-state fermentation (SSF) [3,20-23]. However, they were mostly conducted using a one-factor-at -a-time-technique. Unfortunately, this technique frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account in such a procedure. It was reported that the complexities and uncertainties associated with the large-scale fungi fermentation usually come from a lack of knowledge of the sophisticated interactions among various factors. The response surface methodology (RSM) has been increasingly used for various phases of an optimization process in fermentation [24-28]. It is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such technique [29]. Recently, response surface methodology (RSM) has been adopted to optimize the medium components of *B. subtilis* for cyclic lipopeptide production in shake flask fermentation, which confirmed RSM is qualified to optimize the lipopeptide culture medium [30,31].

In the present study, we adopt RSM to optimize the cultivation conditions of *Bacillus subtilis* S3 for the enhancement of iturin A production in shake flask using SMF. After the optimal conditions

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were sought in the shake-flask fermentation, the iturin A production by *Bacillus subtilis* S3 was further studied in a 5-L fermentor. The effect of agitation speed, aeration rate on the growth of *B. subtilis* and the production of iturin A was studied. Furthermore, the addition of an appropriate baffle in the fermentor that affects the oxygen mass transfer as described and analyzed by means of mass transfer coefficient K_La, which subsequently affect iturin A production, was also investigated.

MATERIALS AND METHODS

1. Chemicals, Microorganism and Seed Culture

Reagents for cultivation such as corn steep powder (CSP) was obtained from Merck & Co., Inc, potato powder and soy bean protein (Zer-Jen Co., Taiwan), peptone was from HIMEDIA laboratory (India), yeast extract was from Taiwan sugar company, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, maltodextrin were obtained from Katamaya Co., Japan. The nutrient agar (NA), Luria Bertani broth and carbon sources, i.e., fructose, glucose, sucrose, maltose, were obtained from DIFCO Laboratories Michigan, USA. All other reagents used were of the highest grade available unless otherwise indicated. *Bacillus subtilis* S3, isolated from soil, was obtained from Taiwan Agricultural Research Institute, Wufeng, Taiwan. The strain was maintained on nutrient agar (NA) slants. Unless otherwise mentioned, the slant was incubated at 30 °C and then stored at 4 °C, which was subcultured every 4 weeks.

To prepare seed culture, a loop of S3 cells from a slant culture of fresh nutrient agar was inoculated into a 500-ml flask containing 100 ml Luria broth. The flask was incubated on a New Brunswick rotary shaker (Model G24) at 30 °C, 200 rpm for 24 h and used as a seed for submerged cultivations.

2. Shake-flask Fermentation

The standard spore suspension inoculums 5% (v/v) were used for shake flask cultures to evaluate the effects of carbon source, nitrogen sources, inorganic salts and plant oil on the production of iturin A from *B. subtilis* S3. Cultures were grown at 30 °C and 200 rpm in a rotary shaker incubator (Yih-Der, LE-539, Taiwan), in 500 mL Erlenmeyer flasks with 100 mL of production media, and no pH control was applied for all cultivations. The production medium for cultivation of *B. subtilis* S3 consisted of 1% of one of the following carbon sources--fructose, glucose, sucrose and maltose, 0.5% of one of the following nitrogen sources--corn steep powder, soy bean protein, yeast extract and peptone, 1-2% of potato powder and maltodextrin, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM KH_2PO_4. In addition, to test the effect of plant oils, 0.5-1% of peanut oil, olive oil, soybean oil and canola oil (president food co., Taiwan) was also added. Submerged cultivations were performed in three replicate experiments, and the analyses were carried out at least in duplicate.

3. Analytical Methods

3-1. Extraction of Iturin A

For the extraction of iturin A, the culture broth was centrifuged at 9,000 g for 20 min, followed by treating the supernatant with 6 M HCl to adjust the pH to 2.0. The treated supernatant was further centrifuged at 9,000 g for 20 min. After the supernatant was discarded, the resulting solid precipitate was added 20 ml of methanol. The mixture was filtered through a Whatman No. 1 filter paper (Whatman, International Ltd, Maidstone, UK) and the solvent was evaporated until no MeOH was left. The resulting concentrate crude extract was re-dissolved in 1 m methanol, centrifuged at 9,000 g for 10 min, the supernatant was filtered through a 0.45 μm - pore-size polytetrafluoroethylene membrane (Millipore, USA), and the filtrate was analyzed by high-performance liquid chromatography (HPLC) as described below.

3-2. Quantization of Iturin A by HPLC

The HPLC system for analysis of iturin A was composed of a JASCO PU-2080 solvent delivery controller, a JASCO PU-2075 UV-Vis detector, and a column (Lichrospher RP-18 EC, 4×250 mm, 5.0 μm; Merck, Germany). The injection volume was 20 μL. The sample was eluted with a mobile phase comprising 10 mM ammonium acetate/acetonitrile (3 : 2, v/v) at a flow rate of 0.8 mL/min. The chromatogram was monitored at 280 nm. Iturin A standard obtained from Sigma Co. (USA) was used to construct a calibration curve from which iturin A concentration in the fermentation media was determined.

4. RSM Experimental Design

In preliminary experiments [32], various nutritional and environmental factors have been investigated by a one-factor-at-a-time fashion for their suitability to sustain good production of iturin A by *Bacillus subtilis* S3. Preliminary data indicated that the major variables affecting the performance of the culture in terms of iturin A yields are the levels of glucose concentration, corn steep powder (CSP), maltodextrin, initial pH, shaking speed and area of aeration. Therefore, these six factors were chosen for further optimization through RSM. Initially, a fractional factorial 2^{6-3} design followed by the method of steepest ascent was carried out to find the general vicinity of optimum conditions for iturin A production.

Table 1. Coded level and real values for of the 2^{6-3} fractional factorial design

Independent variable	Symbol	Level	
		Coded	Real value
		1	1.5
Maltodextrin (%)	X_1	0	1
		−1	0.5
		1	1.5
Glucose (%)	X_2	0	1
		−1	0.5
		1	0.75
CSP (%)	X_3	0	0.5
		−1	0.25
		1	200
Shaking speed (rpm)	X_4	0	150
		−1	100
		1	5.31 (2.6)
Area of aeration (cm^2)[a] (Diameter of rubber eraser, cm)	X_5	0	1.89 (1.55)
		−1	0.20 (0.5)
		1	7.5
Initial pH	X_6	0	6
		−1	4.5

[a]Area of aeration (cm^2) or diameter of rubber eraser (cm) which is shown in parenthesis

4-1. Factorial Design

In the first experiment of this series, the ranges of the variables tested were 0.50-1.5% glucose, 0.25-0.75% CSP, 0.5-1.5% maltodextrin, 4.5-7.5 initial pH, 100-200 rpm shaking speed and 0.20-5.31 cm^2 area of aeration. For the first phase of the optimization process in which the region close to the optimum is to be approached, two-level factorial designs were chosen. In this experimental design, the main effects and interactions of different factors, each at two different levels, can be simultaneously investigated. Table 1 shows the six independent variables and their real values at the different coded levels of the factorial design experiments. The six factors are maltodextrin (X_1), glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5) also expressed as diameter of rubber eraser, initial pH (X_6); their upper and lower levels in this initial design were chosen in reconciliation with the data of our previous work on production of iturin A by *Bacillus subtilis* S3 [32]. For a 2^{6-3} fractional factorial design with six factors at two levels, eight experimental runs are required. The matrix corresponding to 2^{6-3} fractional factorial design, together with the observed experimental data is also shown in Table 2. To avoid bias, a total of 8 runs was performed in a random order (overall randomization).

Table 2. Design and results of 2^{6-3} fractional factorial design

Trial No.	Coded levels of medium composition						Conc. of iturin A (mg/L)
	X_1	X_2	X_3	X_4	X_5	X_6	
1	1	−1	−1	−1	−1	1	26.50
2	−1	1	−1	−1	1	−1	8.67
3	1	1	−1	1	−1	−1	2.12
4	−1	−1	1	1	−1	−1	44.97
5	1	−1	1	−1	1	−1	14.33
6	−1	1	1	−1	−1	1	18.65
7	1	1	1	1	1	1	123.35
8	−1	−1	−1	1	1	1	103.75

X_1: Gmaltodextrin (%); X_2: GGlucose (%); X_3: GC.S.P (%); X_4: G Shaking speed (rpm); X_5: GArea of aeration (cm^2); X_6: Ginitial pH

4-2. Path of Steepest Accent (Descent)

The *method of steepest ascent* (descent) is a procedure for moving sequentially along the path of steepest ascent (descent), that is, in the direction of the maximum increase (decrease) in the response. Based on the results obtained from the factorial design, the fitted first-order model is

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \quad (1)$$

Y is the predicted response; β_0, β_i, are constant coefficients, and x_i is the coded independent variables or factors.

The direction of steepest ascent (descent) is the direction in which Y increases (decreases) most rapidly. This direction is parallel to the normal to the fitted response surface. One usually takes as the path of steepest ascent (descent) the line through the center of the region of interest and normal to the fitted surface. Thus, the steps along the path are proportional to the regression coefficients β_i. The path of steepest ascent (descent) started from the center of the first design. To move away from the first design center along the path of steepest ascent (descent), maltodextrin (X_1), glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5) were moved −0.034, 0.0565, 0.0966, 14.798, 0.398 (expressed as diameter of rubber eraser) units in each step, respectively. These new units were determined from concentration range of unity level from first design and estimated coefficient ratio from Eq. (1). The design and experimental results along the path of steepest ascent are shown on Table 3. It should be noticed that the pH was excluded in path of steepest ascent design because it showed little effect on iturin A production according to the results of fractional factorial experiments which will be

Table 3. Determination of Path of Steepest Ascent according to the results of 2^{6-3} Fractional factorial design (Above) and trials made along Path of Steepest Ascent

	X_1	X_2	X_3	X_4	X_5
(1) Base point	1	1	0.5	150	(1.55)
(2) Unit[a]	0.5	0.5	0.25	50	(1.05)
(3) Slop[b]	−4.59	7.53	25.75	19.73	25.27
(4) Proportion[c] (2×3)	−2.295	3.765	6.4375	986.5	26.53
(5) New unit[d] (4×q) (q=0.015)[e]	−0.034	0.0565	0.0966	14.798	(0.398)
(6) Step[f]					

Trial no.	X_1	X_2	X_3	X_4	X_5	Conc. of iturin A (mg/L)
1	1	1	0.5	150	1.89 (1.55)	89.96
2	0.966	1.057	0.5966	164.8	2.979 (1.948)	119.31
3	0.932	1.113	0.6932	179.6	4.32 (2.346)	123.03
4	0.898	1.169	0.7898	194.4	5.91 (2.744)	119.08
5	0.864	1.226	0.8864	209.2	7.75 (3.142)	76.47

X_1: Gmaltodextrin (%); X_2: GGlucose (%); X_3: GC.S.P (%); X_4: G Shaking speed (rpm); X_5: GArea of aeration (cm^2) or diameter of rubber eraser (cm) which is shown in parenthesis; [a]Unit (Concentration range of unity level); [b]Slope (Estimated coefficient ratio from Eq (1)); [c]Proportion (Corresponding concentration range (2)×(3)); [d]New unit (Actual step size); [e]q is a factor determined by experimenter based on process knowledge and practical consideration, q=0.015 is appropriate in this example; [f]X_1, X_2, X_3, X_4, X_5 were moved −0.034, 0.0565, 0.0966, 14.978, 0.325 units in each step, respectively

Table 4. Process variables and their levels for CCD experiments

Independent symbol variables	Code levels				
	−2	−1	0	+1	+2
Glucose (%) X_1	0.9	1	1.1	1.2	1.3
CSP (%) X_2	0.5	0.6	0.7	0.8	0.9
Shaking speed (rpm) X_3	160	170	180	190	200
Area of aeration (cm^2) X_4	2.69	3.46	4.34	5.31	5.31
(Diameter of rubber eraser, cm)	(1.85)	(2.1)	(2.35)	(2.6)	(2.6)[a]

[a]2.6 cm is the maximum diameter of rubber eraser that was used

described below.

4-3. Central Composite Design

The central composite design (CCD) was conducted in the optimum vicinity to locate the true optimum conditions of glucose (X_1), CSP (X_2), shaking speed (X_3) and area of aeration (X_4) for iturin A production. pH and maltodextrin were excluded in CCD design because it showed little effect on iturin production according to the results of aforementioned experiments. For the 4 factors, this trial was essentially a 2^4 factorial design augmented by eight axial points (or called star points) coded $\pm\alpha$ and two replications of center point (all factors at level 0), resulting in a total number of 26 experiments [29]. The distance of the star points from the center point is given by $\alpha=2^{n/4}$ (for three factors n=4, α=2). The variables were coded according to the Eq. (2):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{2}$$

where x_i is the coded variable of a factor, X_i is natural variable of the factor. X_o is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels for the CCD are shown in the Table 4. The matrix corresponding to the CCD is shown in Table 5, together with the observed experimental data. The experimental results of the CCD were fitted with a second-order polynomial equation by a multiple regression technique.

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii} x_i x_j + \sum\sum_{i<j}\beta_{ij} x_i x_j \tag{3}$$

Y is the predicted response; β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables or factors.

Table 5. Design and results of central composite design

Trial No.	Coded levels of medium composition				Conc. of iturin A (mg/L)
	X_1	X_2	X_3	X_4	
1	−1	−1	−1	−1	59.95
2	−1	−1	−1	1	85.58
3	−1	−1	1	−1	86.11
4	−1	−1	1	1	75.90
5	−1	1	−1	−1	70.59
6	−1	1	−1	1	69.40
7	−1	1	1	−1	82.36
8	−1	1	1	1	74.82
9	1	−1	−1	−1	59.15
10	1	−1	−1	1	61.81
11	1	−1	1	−1	73.84
12	1	−1	1	1	82.50
13	1	1	−1	−1	98.89
14	1	1	−1	1	70.78
15	1	1	1	−1	91.36
16	1	1	1	1	77.11
17	−2	0	0	0	58.32
18	2	0	0	0	84.22
19	0	−2	0	0	58.58
20	0	2	0	0	77.20
21	0	0	−2	0	57.92
22	0	0	2	0	63.45
23	0	0	0	−2	65.13
24	0	0	0	2	59.33
25 (C)	0	0	0	0	130.67
26 (C)	0	0	0	0	126.53

4-4. Software for Experimental Design and Statistical Analysis

The computer software Statistica, version 5.0 (Statsoft, Inc., Tulsa, OK USA) was used for the experimental design and regression analysis of the experimental data obtained. The quality of fit of the model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. The significance of the regression coefficients was tested by a t-test. For analysis of the nature of the fitted response and for prediction of the maximum point, the second-order equation was reduced to its canonical form [33,34], which is one part of the Statistica output.

5. 5-L Stirred Tank Fermentation

Fermentation was carried out in a fully instrumented and computer controlled 5-L jar fermentor (Biotop, BTF-A-5, Taichung Co., Taiwan), equipped with a pH probe (Mettler Toledo 2200) and a dissolved oxygen probe (Mettler Toledo T-96). Agitation was provided by a single four-finned Rushton turbines and aeration was provided by a ring sparger situated below the bottom turbine. Three different baffles (baffle1: 21 cm length, 1.5 cm width; baffle 2: 22 cm length, 3 cm width 0.2 cm pore size; baffle 3: 21 cm length, 3 cm width) were also inserted in for the regulation of oxygen mass transfer. The fermentation medium, which is consisted of the following components: 0.93% maltodextrin, 0.72% corn steep powder (CSP), 1.11% glucose, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM KH_2PO_4, was the optimal medium sought by RSM in the shake-flask fermentation; the medium was inoculated with 10% (v/v) of the inoculum and then cultivated. Unless otherwise specified, fermentation was performed under the following conditions: temperature, 30 °C; aeration rate, 1 vvm; agitation speed, 300 rpm; working volume, 3 L.

In some instances in the course of fermentation, the aeration rate was adjusted in the range of 0.5-2 vvm, and the agitation speed was adjusted in the range 100-300 rpm at 100 rpm interval. The impeller power consumption was calculated by monitoring the amperage of electric current used in impeller motor.

5-1. Determination of the K_La Values

The experimental apparatus consisted of a 5.0 L agitated bioreactor with a working volume of 3.0 L. The temperature was controlled to 30 °C and the experiments were conducted under atmospheric conditions. The dissolved oxygen in the liquid was measured as a fraction of the saturation oxygen concentration by using a submerged polarographic dissolved oxygen probe (Mettler Toledo T-96) fitted with a Teflon membrane. The k_La values for cell free broth in a 5.0 L agitated bioreactor with or without baffle were measured by direct measurement of the rate of increase in dissolved oxygen concentration after it was lowered by passing nitrogen gas (oxygen free), 1.0 L min^{-1}, through the system for 30 min. The nitrogen gas flow was stopped and this was followed by passing the dehumidified air as a source of oxygen through the air sparger at the bottom of the agitated bioreactor. k_La values were obtained from the data by using Eq. (4). The k_La values for cell broth were obtained by using the dynamic gassing-out method. This method is based on the dissolved oxygen concentration in the culture broth. In this method the inlet gas for aeration of actively respiring cell is stopped, and the time course of the consequent decrease in dissolved oxygen due to the oxygen consumption by the microorganisms is monitored. Aeration is resumed when dissolved oxygen drops close to the critical oxygen concentration and the increasing dissolved oxygen concentration follows [35].

$$\frac{dC_L}{dt} = k_La(C^* - C_L) \quad (4)$$

which on integration yields:

$$\ln(C^* - C_L) = -k_La t \quad (5)$$

where k_La is the volumetric mass transfer coefficient (min^{-1}), $(C^* - C_L)$ is the driving force causing the mass transfer, C^* and C_L refer to the liquid phase oxygen concentration at saturation and at any time, respectively. As a result, the k_La value was evaluated from the slope of the straight portion of the curve between $\ln(C^* - C_L)$ vs. time.

RESULTS AND DISCUSSION

1. Shake-flask Fermentation of *Bacillus subtilis S3*

In preliminary experiments, various carbon and nitrogen sources, plant oils were evaluated for their suitability to sustain good production of iturin A by *B. subtilis S3* [32]. The fermentation medium, which consisted of the following components, 2% potato powder, 0.5% corn steep powder (CSP), 2% sucrose, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM KH_2PO_4, was grown at 30 °C and 200 rpm in a rotary shaker incubator. The time course of the cultivation is shown in Fig. 1. This preliminary study [32] showed that when *B. subtilis S3* was cultivated at the aforementioned condition, the highest iturin A production (47.19 mg/L) was obtained after 7 days of cultivation. In addition, only 57% increase of iturin A productivity (from 47.19 mg/L to 74.22 mg/L) was observed using the one-factor-at -a-time technique for optimization; the highest iturin A production (74.22 mg/L) was obtained after 7 days of cultivation when *B. subtilis S3* was cultivated at 30 °C and 200 rpm in a medium consisting of 1% maltodextrin, 0.5% corn steep powder (CSP), 1% glucose, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM KH_2PO_4 (data not shown). The same study also indicated that the major variables affecting the performance of the culture in terms of iturin A yields are the levels of maltodextrin, glucose, CSP, shaking speed, area of aeration and initial pH. Therefore, these six factors were chosen for further optimization through RSM. All plant oils tested showed no effect on the iturin production; thus, they are omitted from the following study.

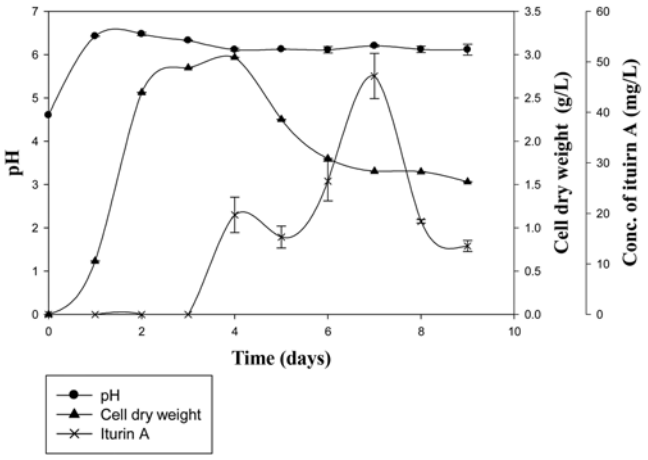

Fig. 1. Time course of *B. subtilis* S3 in submerged culture fermentation.

RSM is a sequential procedure with an initial objective to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. Although two-level (full or fractional) factorial experiments will only yield data to fit a limited model (Eq. (1)), they are the most common initial experiments in the application of RSM, because orthogonality of the design minimizes the variance of the regression coefficients. Besides, any first-order (two-level) orthogonal design is rotatable [34]. Since the location of the optimum is unknown prior to running RSM experiment, it is conceivable to use a design with rotability that ensures equal precision of estimation in all directions. Initially, a 2^{6-3} fractional factorial design with six factors at two levels followed by the method of steepest ascent was carried out to find the general vicinity of optimum conditions for iturin A production.

2. Factorial Design

The experimental results of iturin A productions by 2^{6-3} fractional factorial design experiments are shown in Table 2. To approach the vicinity of the optimum, a first-order model was fitted to the data obtained from the fractional factorial design experiment. From the analysis of the data in Table 2 by least-squares method, a first-order model was best fit to these data. The main effects of the six factors, maltodextrin (X_1), glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5), initial pH (X_6), were calculated to be −4.59, 7.53, 25.75, 19.73, 25.27 and −1.21, respectively. We obtained the following model in the coded variables.

First-order Model Equation

$$Y_{(mg/L)} = 42.8 - 4.59X_1 + 7.53X_2 + 25.75X_3 + 19.73X_4 + 25.27X_5 - 1.21X_6 \quad (6)$$

From the first-order model Eq. (6) and judging from the regression

Table 6. Results of regression analysis and corresponding t and p-value of second-order model for optimization of iturin production of *Bacillus subtilis* S3

Parameter	Parameter estimate	Standard error	T ratio	Probability
Intercept	−7639.9364	1417.8524	−5.3884	0.0002
X_1	2539.3420	834.9107	3.0415	0.0112
X_1^2	−1234.0305	275.4686	−4.4798	0.0009
X_2	2027.0238	733.8269	2.7623	0.0185
X_2^2	−1318.4926	275.4686	−4.7864	0.0006
X_3	56.4510	10.6966	5.2775	0.0003
X_3^2	−0.1499	0.0275	−5.4404	0.0002
X_4	239.3629	74.9861	3.1921	0.0086
X_4^2	−16.7619	3.1990	−5.2397	0.0003
$X_1 \times X_2$	445.0192	288.6003	1.5420	0.1513
$X_1 \times X_3$	0.0320	2.8860	0.0111	0.9914
$X_1 \times X_4$	−26.4718	31.1877	−0.8488	0.4141
$X_2 \times X_3$	−2.2405	2.8860	−0.7763	0.4539
$X_2 \times X_4$	−52.8180	31.1877	−1.6936	0.1184
$X_3 \times X_4$	−0.1458	0.3119	−0.4676	0.6492

Glucose (X_1), CSP (X_2), shaking speed (X_3) and area of aeration (X_4);
[a]Significant at the 5% level;
Coefficient of determination, R-sqr=0.846

coefficients and the corresponding t values (data not shown), it is concluded that linear terms of glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5) had significant positive effect on iturin production, and that the decreasing of the concentrations of maltodextrin (X_1) should enhance iturin production. In contrast, initial pH (X_6) exhibited an insignificant effect on iturin A production; it was then omitted from the experiments of path of steepest ascent and CCD in the study.

Based on the first-order model equation obtained, the path of steepest ascent was determined to find proper direction of changing variables increasing or decreasing the concentration according to the sign of the main effects to improve iturin A production. The path of steepest ascent started from the center of the factorial design and moved along the path in which the concentrations of glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5) were increasing; in contrast, the concentration of maltodextrin (X_1) was decreasing. The design and results of the path of steepest ascent experiments are shown in Table 3 where the highest production response is 123.02 mg/L with the concentrations of maltodextrin (X_1), glucose (X_2), CSP (X_3), shaking speed (X_4) and area of aeration (X_5) are 0.93%, 1.11%, 0.69%, 179 rpm, 4.32 cm^2, respectively. It suggested that this point was near the region of maximum production response.

3. Central Composite Design

To fully explore the subregion of the response surface in the neighborhood of the optimum, an experimental design with more than two levels of each factor is required, so that a second order approximation to the response surface can be developed. A CCD with five coded levels was used for this purpose. The levels of the variables as shown in Table 4 for the CCD experiments were selected according to the results of the previous experiments. The CCD design and the corresponding experimental data are shown in Table 5. By applying multiple regression analysis on the experimental data shown in Table 5, the experimental results of the CCD design were fitted with a second-order polynomial equation (Eq. (3)), and the second-order polynomial equation obtained for iturin A production is shown in Eq. (7).

Second-order Model Equation

$$Y_{(mg/L)} = 128.599 + 5.212X_1 - 25.085X_1^2 + 7.308X_2 - 26.774X_2^2 + 6.577X_3 - 30.377X_3^2 - 2.996X_4 - 29.605X_4^2 + 8.900X_1X_2 + 0.064X_1X_3 - 4.713X_1X_4 - 4.481X_2X_3 - 9.730X_2X_4 - 2.793X_3X_4 \quad (7)$$

The symbols X_1, X_2, X_3, and X_4 in Eq. (7) stands for glucose, CSP, shaking speed and area of aeration, respectively.

Besides the linear effects, the response surface method also gives an insight about the parameters' quadratic and combined effects. The results of the regression analyses are shown in Table 6, which were done by using both Fisher's F-test and Student t-test statistical tools. The student t-test was used to determine the significance of the parameter regression coefficients. The p-values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables [34]. The larger the magnitude of the t-test and smaller the p-value, the more significant is the corresponding coefficient. Judging from the regression coefficients and corresponding t values in Table 6, it was observed that all the linear and square terms displayed a significant effect on the iturin A production at a 5% level ($p<0.05$).

This fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.846, indicating that 84.6% of the variability in the response could be explained by the model. According to the analysis of variance (ANOVA), the test statistics F values for the overall regression are significant at the upper 5% level, which further supported that the second-order model is very adequate in approximating the response surface of the experimental design. After performing the transformation of Eq. (7) to its canonical form, the optimum combination for the iturin production was

found to be the following: glucose (X_1) 1.11%, CSP (X_2) 0.7%, shaking speed (X_3) 180 rpm and area of aeration (X_4) 4.35 cm^2. The model predicted a maximum response of iturin A production at 129.33 mg/L. The calculated maximum was verified with experiments that were performed in the culture media representing the optimum combination found, and the iturin A production of 132.23 mg/L (average of three repeats) was obtained. The excellent correlation between predicted and experimental values justifies the validity of the response model. It is noted that 57% increase of iturin A productivity (from 47.19 mg/L to 74.22 mg/L) was observed using the one-factor-at -a-time technique for optimization as indicated above; however, 180% increase of iturin A productivity (from 47.19 mg/L to 132.23 mg/L) was observed using RSM for the optimization.

Several previous studies used submerged fermentation [11,36,37], in addition to solid-state fermentation [3,20-23], to produce high concentrations of lipopeptide antibiotics from *B. subtilis*. Recently, the iturin A yield has increased significantly through application of the solid-state fermentation and RSM method [31]; the yield of iturin A produced by *B. subtilis* RB14-CS grown on soybean curd residue in SSF under statistically predicted optimum conditions was 5.45 mg/g-wet solid material, a production 2.8 times higher than that observed in solid culture without glucose or soybean meal, and 2.2 times higher than obtained in the basal medium [20]. The application of RSM to optimize iturin A production in SMF is scant; however, Gu and coworkers [30] have successfully demonstrated the optimization of medium constituents for the production lipopeptide antibiotics by *B. subtilis* MO-01 in submerged fermentation (SMF) using response surface methodology (RSM); the predicted lipopeptide yield was 1.712 g/L and the optimum fermentation parameters were 22.432 g/L of sucrose, 2.781 g/L of ammonium chloride, 6.7879 μm of ferrous sulfate and 0.0377 mM of zinc sulfate, respectively.

4. 5-L Stirred Tank Fermentation

For the 5-L fermentor, the culture conditions, such as the agitation speed and aeration rate, are essential factors for batch culture [37]. The effects of the agitation speed (100-300 rpm), aeration rate (0.5-2.0 vvm) and different baffles on the production of iturin A were investigated. The temperature and pH were maintained at 30 °C and pH 6.0, respectively. The effects of agitation speed on the production of cell mass and iturin A are shown in Fig. 2 and Fig. 3, respectively. It is noted that a higher agitation speed resulted in a higher cell

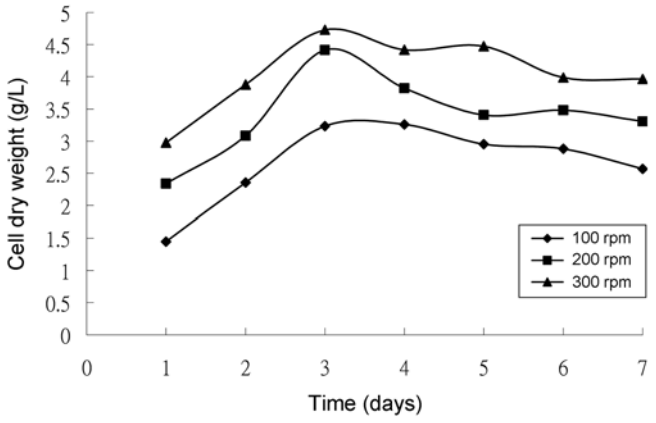

Fig. 2. Effect of agitation speed on the cell dry weight of *B. subtilis* in fermentor.

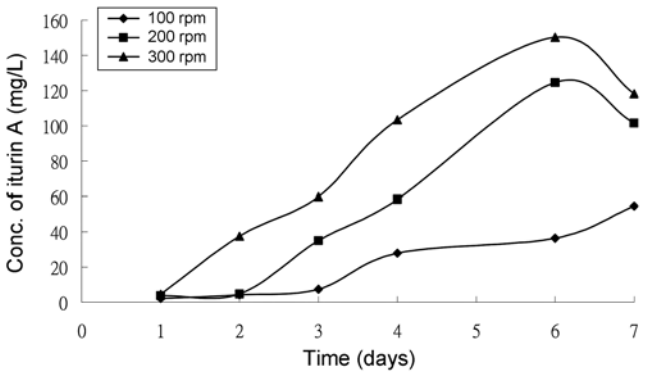

Fig. 3. Effect of agitation speed on the production of iturin A by *B. subtilis* in fermentor.

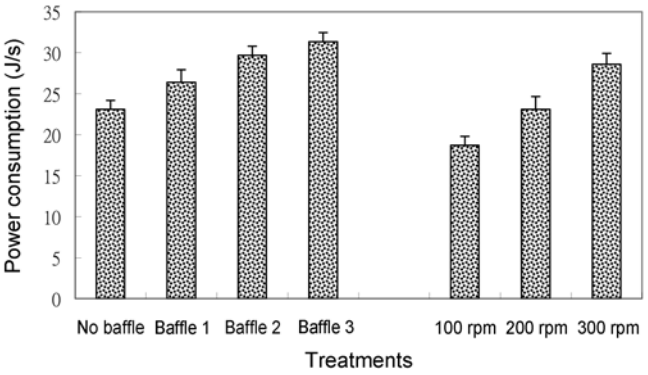

Fig. 4. Effect of power consumption with different agitation speeds and baffles (baffle 1: 21 cm length, 1.5 cm width; baffle 2: 22 cm length, 3 cm width 0.2 cm pore size; baffle 3: 21 cm length, 3 cm width).

mass and iturin A production. The highest cell mass usually appeared at 3 d or 4 d of fermentation for any agitation applied. However, the highest iturin A production appeared after 6 d or 7 d of fermentation. The highest iturin A production for agitation speed at 100, 200 and 300 rpm was 54.72 mg/L (7 d cultivation), 124.6 mg/L (6 d cultivation) and 150.26 mg/L (6 d cultivation), respectively. On the other hand, higher impeller power consumptions were found in higher agitation speed (Fig. 4). The power consumption was found to increase from 18.7 J/s at 100 rpm to 28.6 J/s at 300 rpm. The higher agitation speed might result in higher oxygen transfer rate and higher shear stress, which both influence the cell growth and secondary metabolite production. Thus, the following two experiments were conducted with these two factors on the iturin A production. Increased aeration elevated the oxygen uptake rate (OUR) and the oxygen transfer rate (OTR), showing that a high cell mass is affected by OUR and OTR [38]. The effects of aeration rate on the production of cell mass and iturin A are shown in Fig. 5 and Fig. 6, respectively. It is seen that when the aeration rate was 0.5 vvm, the cell biomass reached the highest (4.2 g/L) at 4 d of fermentation, but less iturin A was produced throughout the 7 d of fermentation. In contrast, when the aeration was increased to 1 vvm or 2 vvm, a little increase of cell biomass was produced (4.4 g/L and 5.01 g/L, respectively), but higher iturin A was produced at the 6 d of fermentation; 150.26 mg/L and 175.52 mg/L of iturin A was produced when the aera-

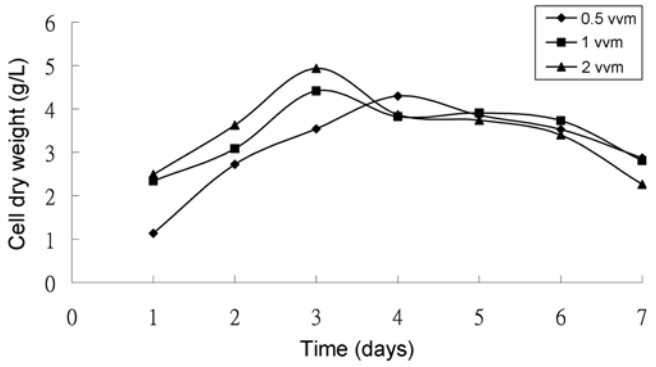

Fig. 5. Effect of aeration rate on the cell dry weight of *B. subtilis* in fermentor.

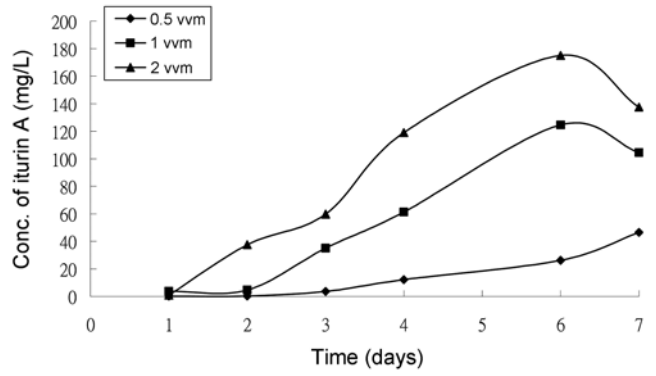

Fig. 6. Effect of aeration rate on the production of iturin A by *B. subtilis* in fermentor.

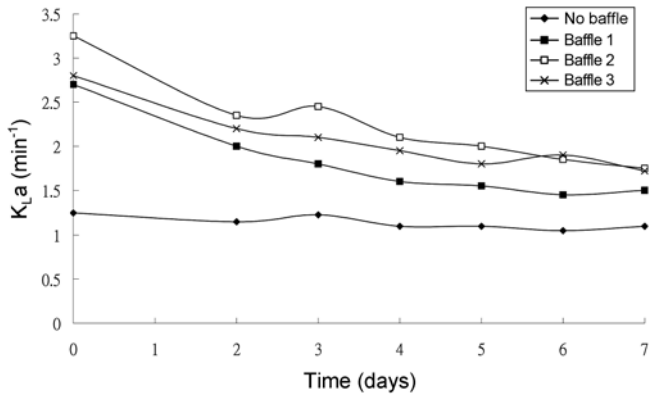

Fig. 7. Effect of different baffles on the oxygen mass transfer coefficient (K_La) in fermentor (baffle 1: 21 cm length, 1.5 cm width; baffle 2: 22 cm length, 3 cm width 0.2 cm pore size; baffle 3: 21 cm length, 3 cm width).

tion rate was at 1 vvm and 2 vvm, respectively. It seems that a low aeration rate such as 0.5 vvm favored the growth of cell biomass but hindered the production of secondary metabolite, the lipopeptide antibiotics. On the other hand, the higher aeration rate resulted in higher oxygen mass transfer (Fig. 7). The oxygen mass transfer can be described and analyzed by means of the mass transfer coefficient, K_La; it represents the most important parameter implied on the design and operation of mixing-sparging equipment of the bioreactors. In a non-baffle fermentor, an aeration increase from 0.5 vvm to 2 vvm has shown increased oxygen mass transfer, but the impeller power consumption was shown to remain at 23.1 J/s.

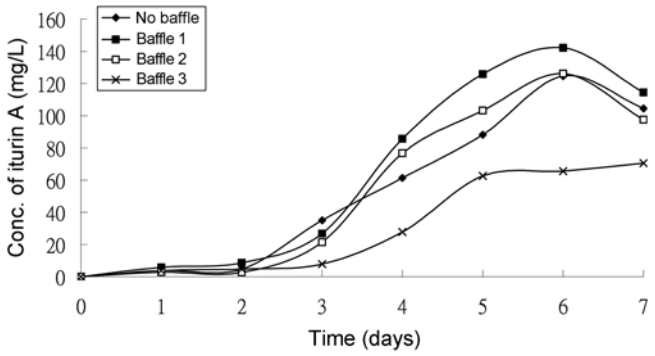

Fig. 8. Effect of different baffles on the production of iturin A by *B. subtilis* in fermentor (baffle 1: 21 cm length, 1.5 cm width; baffle 2: 22 cm length, 3 cm width 0.2 cm pore size; baffle 3: 21 cm length, 3cm width).

The row of baffles inside of a tank directly influences the agitating status of fermentation, which affects the oxygen mass transfer and also the shear stress on the cell. Three different baffles as described above were inserted in the fermentor for the regulation of oxygen mass transfer and shear stress. The effects of different baffles on oxygen mass transfer coefficient (K_La), and production of iturin are shown in Fig. 7 and Fig. 8. It is seen that the addition of baffles in the fermentor has influenced the oxygen mass transfer coefficient (K_La) significantly. Although it appears that the increase of K_La favored the production of cell biomass (data not shown), it did not correlate well with the production of iturin A. The main reason might be that the addition of baffles increases not only the K_La but also the shear stress in the fermentor. The impeller power consumption was found to depend on the agitation speed or the type of baffle used in the fermentor (Fig. 4). The addition of baffle 3 (21 cm length, 3 cm width) gave the highest K_La, but it is the addition of baffle 1 (21 cm length, 1.5 cm width) that gave a significant increase of iturin A production; the highest is 142.13 mg/L at 6 d of fermentation. An interesting result was found that the baffle 1 at 200 rpm and non-baffle 300 rpm have close power consumption and iturin A production. On the other hand, the wide baffle was found to have higher power consumption and lower iturin A production (Fig. 4 & Fig. 8). According this fact of the higher power consumption from baffle 2 and 3, the higher shear stress seems to be an inhibiting factor to iturin A production. This result was consistent with the fact that the higher amount of iturin A was produced with high oxygen mass transfer and non-high shear stress. The oxygen supply into the broths constitutes one of the decisive factors of cultivated microorganisms' growth and can play an important role in the scale-up and economy of aerobic biosynthesis systems. In a baffled bioreactor, the optimal agitating speed of the agitator and aeration must be determined to facilitate cell growth and secondary metabolite production [39].

CONCLUSIONS

Several variables affecting the production of antifungal lipopeptide, iturin A, by *B. subtilis* S3 in SMF were identified; they are the

levels of maltodextrin, glucose, CSP, shaking speed, area of aeration and initial pH. Response surface methodology (RSM) was employed to optimize the cultivation conditions of *Bacillus subtilis* S3 for the enhancement of iturin A production. The optimal conditions for iturin A production obtained from RSM were pH 6.0, 0.93% maltodextrin, 1.11% glucose, 0.72% corn steep powder (C.S.P), 1.5 mM $MgSO_4$, 0.75 mM KH_2PO_4, agitation speed 180 rpm and area of aeration 4.35 cm^2. 180% increase of iturin A productivity (from 47.19 mg/L to 132.23 mg/L) was observed using RSM. This work confirmed that RSM is qualified for the optimization of culture conditions for the production of lipopetide antibiotics. In the 5-L fermentor, it was found that increased agitation speed improved the growth of *B. subtilis* and the production of iturin A. Aeration at 2 vvm gave excellent production of iturin A (175.52 mg/L) after 6 d of cultivation. The addition of baffles in the fermentor significantly influenced the shear stress and oxygen mass transfer coefficient (K_La). Although it appears that the increase of K_La favored the production of cell biomass, it did not correlate well with the production of iturin A. The low iturin A production might due to the effect of shear stress on its production in the baffled fermentor. This is the first report that describes in detail the production lipopeptide antibiotics with a 5-L fermentor. Although much work remains to be done before its application in the field, the results presented here suggest that *B. subtilis* S3 has potential as a bio-control agent.

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NOMENCLATURE

ANOVA: analysis of variance
C^* : the saturated oxygen concentration in liquid phase
C_L : the oxygen concentration in liquid phase
CSP : corn steep powder
CCD : central composite design
D-Asn : D form asparagine
D-Tyr : D form tyrosine
HPLC : high-performance liquid chromatography
K_La : the volumetric mass transfer coefficient [min^{-1}]
L-Asn : L form asparagine
L-Gln : L form glutamine
L-Pro : L form proline
L-Ser : L form serine
NA : nutrient agar
OTR : oxygen uptake rate
OUR : oxygen transfer rate
p : probability
R^2 : amount of reduction in the variability of Y obtained by using the regression variable x_1, x_2 …. in the model
RSM : response surface methodology
SSF : solid-state fermentation
SMF : submerged fermentation
WB : wheat bran
x_i, x_j : coded independent variables
X_i : natural variable of the factor
X_o : value of the natural variable at the center point
ΔX_i : the step change value
Y : predicted response

Greek Letters

α : the distance of the star points from the center point
β_0, β_i : constant coefficients
β_0, β_i, β_{ii}, β_{ij} : constant coefficients

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