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# Extractive fermentation for enhanced production of thailandepsin A from *Burkholderia thailandensis* E264 using polyaromatic adsorbent resin Diaion HP-20

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**Abstract** Thailandepsin A is natural product of *Burkholderia thailandensis* E264 with potent histone deacetylase inhibitory activities and promising anticancer activities. The titer of thailandepsin A is very low (less than 10 mg/l) from limited empirical fermentation. To facilitate preclinical evaluations and potentially clinical development of thailandepsin A, systematic optimization and extractive fermentation of thailandepsin A from *B. thailandensis* E264 culture in flasks were investigated in this pilot study. The main fermentation parameters—28°C, pH 7.0, inoculum ratio 1% (v/v), incubation duration 60 h, medium volume 26%, shaking speed 170 rpm, and chloroform as extracting solvent—were determined by single factor experiments. Polyaromatic adsorbent resin Diaion HP-20, when added at a concentration of 4% (w/v), was most effective to reduce feedback inhibition of thailandepsin A and to significantly increase the titer of target product. Central composite design was used to further optimize the fermentation medium for *B. thailandensis* E264. The optimized medium contains glucose 17.89 g/l, tryptone 34.98 g/l, potassium phosphate 24.84 g/l, and sodium citrate 0.01 g/l, which resulted in a large increase of the titer of thailandepsin A to 236.7 mg/l. Finally kinetic models based on the modified logistic and Luedeking–Piret equations were developed, delivering a good description of temporal variations of biomass, product, and substrate in the fermentation process, which could be used as references for developing large-scale fermentation.

**Keywords** *Burkholderia thailandensis* E264 · Central composite design · Extractive fermentation · Thailandepsin A

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## Introduction

Histone deacetylase (HDAC) inhibitors have emerged as a new class of cancer drugs, with one synthetic compound SAHA and one natural product FK228 approved by the US Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma [14, 24]. Thailandepsin A from *Burkholderia thailandensis* E264 (Fig. 1) is a natural FK228 analog which possesses potent HDAC inhibitory activities and broad-spectrum antiproliferation activities against NCI-60 human cancer cell lines at low nanomolar concentrations [2, 28]. However, the titer of thailandepsin A is very low (less than 10 mg/l) from our limited empirical fermentation, which hinders preclinical evaluations and potentially clinical development. The objective of this work was thus to significantly increase the

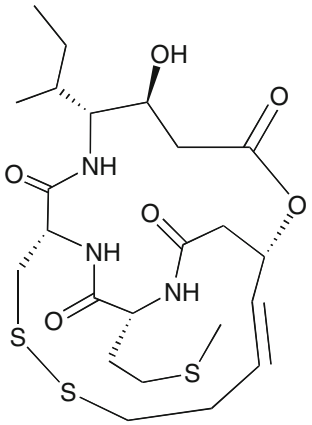


**Fig. 1** Chemical structure of thailandepsin A

concentration of thailandepsin A production through systematic optimization of fermentation.

Feedback inhibition is a common problem during fermentation processes when the concentration of the end product reaches a certain level [20]. Inclusion of an adsorbent resin in the production medium in combination with extractive fermentation is an effective approach to reduce feedback inhibition and to increase the product titer. Successes have been made using the polyaromatic resin Diaion HP-20 in actinomycete fermentation to improve the concentration of important antibiotics [8, 10] or to reduce the self-toxicity of the fungal metabolite [23], but such an approach has never been applied to improve metabolite production by *Burkholderia* fermentation.

Optimization of fermentation conditions, such as medium components and physicochemical parameters, is often critical and attainable. Single factor experiments can identify individual parameters that are most favorable to the product titer but cannot assess the interactions among factors [11]. Response surface methodology (RSM) allows the responses to be varied simultaneously with a limited number of experiments [17, 22], which is time-saving and labor-saving. RSM facilitates model construction for the evaluation of variables and their interactions, and subsequently allows for predicting optimal production conditions.

Here we report the significant improvement of thailandepsin A production by *B. thailandensis* E264 through systematic optimization of fermentation conditions and inclusion of Diaion HP-20 resin to reduce product feedback inhibition. Based on the optimization, RSM was employed to build models to assess the interaction between two critical factors, and to guide the selection of optimal fermentation conditions. Kinetic models for thailandepsin A fermentation by *B. thailandensis* E264 were also constructed to describe temporal variations of cell growth, product concentration, and substrate consumption; these models could serve as references for developing large-scale fermentation.

## Materials and methods

### Strain and cultivation conditions

*B. thailandensis* E264 (ATCC 700388) was purchased from the American Type Culture Collection (Manassas, VA, USA) for the discovery of thailandepsin A [2, 28]; it is a Gram-negative *β-proteobacterium* strain originally isolated from a rice field in central Thailand [1]. This strain was routinely plated on Luria–Bertani (LB) agar and grew at 25–37°C. Aggregated bacteria were suspended in 20% glycerol (v/v) for long-term preservation at −80°C. For seed culture, colonies from plates were transferred into 50 ml of LB medium with 50 μg/ml of apramycin in a 250-ml Erlenmeyer flask and grew at 37°C for 24 h in a shaker at 180 rpm. For fermentation, seed culture was added aseptically at varying ratios to each 250-ml flask containing 50 ml or 65 ml of fermentation medium. Inoculated flasks were incubated at varying temperatures on a rotary shaker at varying speeds. After 12 h of initial cultivation, varying amounts of activated Diaion HP-20 resin (see next section for description) were added to each flask and fermentation was allowed to continue to varying lengths of time.

### Activation of adsorbent resin Diaion HP-20

Diaion HP-20 resin was manufactured by Mitsubishi Chemical Industries Ltd, Tokyo, Japan, and was purchased through a vendor. Prior to use, the resin was soaked in 50% (v/v) ethanol for 24 h and then rinsed thoroughly with distilled water. Wet resin was weighed and sterilized in separate packages at 121°C for 20 min and then added to each flask containing fermentation medium at the correct time point.

### Extraction and analysis of thailandepsin A

After fermentation, the residue (resin and precipitate) was separated from each 50 ml of culture with a 100-micron nylon mesh plankton net and air-dried on a filter paper for 24 h. The dried residue was extracted with 4 ml of chloroform for 1 h and then the organic solvent was evaporated under reduced pressure to give a crude extract. The amount of thailandepsin A in the extract was quantified by HPLC (KNAUER, Smartline Pump 1000, Smartline UV Detector 2500) under the following operating conditions: C18 reversed-phase column (Agilent Eclipse, XDB-C18, 5 μm, 4.6 × 150 mm) eluted with 40% acetonitrile–water (v/v), UV monitored at 200 nm, flow rate 1.0 ml/min, and temperature 25°C. Thailandepsin A has a retention time of 10.5 min. A standard curve was generated with purified compound.

For thailandepsin A in culture without resin addition, 600 µl of chloroform was added to 200 µl of culture, and the mixture was agitated intensely for 60 min and centrifuged at 10,000×*g* for 10 min. The lower layer (organic phase) was collected for quantitative HPLC analysis to determine the titer of target compound.

### Analyses of glucose concentration and biomass

For glucose concentration estimation, 0.2 ml of 3,5-dinitrosalicylic acid reagent [16] was added to each 0.5 ml of culture and the mixture was heated in boiling water for 5 min, and then further diluted with 4 ml of water. Optical density was measured with a UV/Vis spectrometer (model UV-2000; UNICO, Dayton, NJ) at 540 nm, and the glucose concentration was calculated as follow:

$$C_{\text{glucose}} = 1.706\,\text{OD}_{540} - 0.013 \quad (1)$$

where $C_{\text{glucose}}$ is the glucose concentration (g/l) and $\text{OD}_{540}$ is the optical density value at 540 nm.

For biomass quantification, 250 µl of fermentation broth was centrifuged at 10,000×*g* for 10 min and the cell pellet was then suspended in 3 ml distilled water. Optical density was measured with a UV/Vis spectrometer at 600 nm. Meanwhile 20 ml of the fermentation broth was centrifuged at 10,000×*g* for 10 min; the resulting pellet was dried in an oven at 60°C for 24 h and weighed accurately to determine the dry cell weight (DCW). The relationship of DCW to optical density value was determined by a linear regression equation as follow:

$$\text{DCW} = 0.276\,\text{OD}_{600} - 0.006 \quad (2)$$

where DCW stands for dry cell weight (g/l) and $\text{OD}_{600}$ is the optical density value at 600 nm. All experiments were carried out in triplicate, and the results were averaged.

### Identification of fermentation parameters and medium components by single factor experiments

Our initial identification of fermentation parameters (incubation temperature, pH, inoculum ratio, medium volume, shaking speed, volume of Diaion HP-20 resin, and extracting solvent) and optimal medium components (carbon sources, nitrogen sources, inorganic salts) required for the enhanced production of thailandepsin A by *B. thailandensis* E264 was carried out in 250-ml Erlenmeyer flasks. Experiments were performed in triplicate and the mean values were used to minimize variations.

### Statistical experimental design and RSM

Central composite design (CCD) was employed to optimize the most significant factors (glucose, tryptone, potassium phosphate, and sodium citrate) identified by single factor experiments. These four independent factors were studied at five coded levels (−2, −1, 0, +1, +2) as shown in Table 1. The actual values of variables in the CCD design were chosen according to information obtained from the single factor experiments. CCD with a total of 30 experimental trials included 16 one-factorial designs, 8 axial point trials, and 6 replications at the center points to minimize the experimental uncertainty (Table 2). The axial distance was chosen as 2 to make the design rotatable. The factors were coded on the basis of Eq. 3:

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ i = 1, 2, \ldots, k \quad (3)$$

where $X_i$ and $x_i$ are the actual value and coded value of variables, respectively. $X_0$ is $X_i$ value at the center point, and $\Delta X_i$ is the step change value. The second-order model was used to fit the response surface to the independent variables in Eq. 4:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, \quad i = 1, 2, \ldots, k \quad (4)$$

where $Y$ is the predicted response, $x_i$ and $x_j$ are the coded independent variables that influence the response variable $Y$, $\beta_0$ is the intercept, $\beta_i$ represents the linear effect of $x_i$, $\beta_{ij}$ represents the interaction between $x_i$ and $x_j$, and $\beta_{ii}$ represents the quadratic effect of $x_i$. All experiments were conducted in triplicate.

Design-Expert version 7.1.3 statistical software (Stat-Ease, Minneapolis, MN) was used for experimental design, regression analysis of data, and creation of response surface plots. The quality of quadratic model equations was checked by the determination of $R^2$. The statistical significance of models was determined by Fischer's test and the regression coefficients were determined by Student's test. The optimum concentrations of the variables were calculated by differentiation of the quadratic model.

**Table 1** Coded and real values of variables in CCD

| Variables | Level of variables | | | | |
|---|---|---|---|---|---|
| | −2 | −1 | 0 | 1 | 2 |
| $X_1$: glucose (g/l) | 12.8 | 17.6 | 22.4 | 27.5 | 32.0 |
| $X_2$: tryptone (g/l) | 12.0 | 18.0 | 24.0 | 30.0 | 36.0 |
| $X_3$: potassium phosphate (g/l)[a] | 12.9 | 15.9 | 18.9 | 21.9 | 24.9 |
| $X_4$: sodium citrate (g/l) | 0.006 | 0.155 | 0.304 | 0.453 | 0.602 |

[a] Potassium phosphate consists of dibasic potassium phosphate trihydrate and monobasic potassium phosphate in 9.17:2 ratio, which generates a buffered pH of 7.0

**Table 2** Experimental design and results of CCD

| Run | Factor variables | | | | Experimental results | |
|---|---|---|---|---|---|---|
| | Coded level | | | | Thailandepsin A production (mg/l) | |
| | $x_1$ | $x_2$ | $x_3$ | $x_4$ | Actual | Predicted |
| 1 | −1 | −1 | −1 | −1 | 135.01 | 127.70 |
| 2 | +1 | −1 | +1 | −1 | 110.97 | 113.31 |
| 3 | −1 | +1 | −1 | −1 | 136.58 | 138.75 |
| 4 | +1 | +1 | −1 | −1 | 86.55 | 85.05 |
| 5 | −1 | −1 | +1 | −1 | 177.99 | 167.65 |
| 6 | +1 | −1 | +1 | −1 | 124.04 | 132.45 |
| 7 | −1 | +1 | +1 | −1 | 193.52 | 199.52 |
| 8 | +1 | +1 | +1 | −1 | 137.10 | 125.01 |
| 9 | −1 | −1 | −1 | +1 | 130.05 | 135.77 |
| 10 | +1 | −1 | −1 | +1 | 101.01 | 104.53 |
| 11 | −1 | +1 | −1 | +1 | 125.54 | 126.65 |
| 12 | +1 | +1 | −1 | +1 | 52.14 | 56.10 |
| 13 | −1 | −1 | +1 | +1 | 144.00 | 155.01 |
| 14 | +1 | −1 | +1 | +1 | 111.48 | 102.95 |
| 15 | −1 | +1 | +1 | +1 | 175.43 | 166.72 |
| 16 | +1 | +1 | +1 | +1 | 58.52 | 75.35 |
| 17 | −2 | 0 | 0 | 0 | 111.77 | 113.54 |
| 18 | +2 | 0 | 0 | 0 | 12.67 | 7.78 |
| 19 | 0 | −2 | 0 | 0 | 140.01 | 139.19 |
| 20 | 0 | +2 | 0 | 0 | 124.94 | 122.63 |
| 21 | 0 | 0 | −2 | 0 | 134.50 | 131.08 |
| 22 | 0 | 0 | +2 | 0 | 189.97 | 190.27 |
| 23 | 0 | 0 | 0 | −2 | 163.95 | 171.69 |
| 24 | 0 | 0 | 0 | +2 | 140.96 | 130.09 |
| 25 | 0 | 0 | 0 | 0 | 157.80 | 159.04 |
| 26 | 0 | 0 | 0 | 0 | 167.57 | 159.04 |
| 27 | 0 | 0 | 0 | 0 | 162.45 | 159.04 |
| 28 | 0 | 0 | 0 | 0 | 154.31 | 159.04 |
| 29 | 0 | 0 | 0 | 0 | 150.155 | 159.04 |
| 30 | 0 | 0 | 0 | 0 | 161.92 | 159.04 |

## Kinetic modeling of thailandepsin A fermentation

Kinetic models for thailandepsin A fermentation by *B. thailandensis* E264 including temporal variations of biomass ($X$, dry cell weight, g/l), product ($P$, thailandepsin A titer, mg/l), and substrate ($S$, glucose, g/l) were built. Certain properties of thailandepsin A fermentation process were deduced from our previous work [2, 28].

First of all, similar biomass values were found in the stationary phase of batch fermentation with different initial glucose concentrations (15, 20, and 25 g/l tested), indicating that thailandepsin A fermentation follows the classical kinetic model of substrate-independent logistic equation. Consequently the following logistic equation (Eq. 5) was employed to describe the microbial growth:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \tag{5}$$

where $\mu_m$ is the maximum specific growth rate ($h^{-1}$) and $X_m$ is the maximum attainable biomass concentration (g/l).

Secondly, the following Luedeking–Piret's equation [13] was introduced to describe the kinetics of thailandepsin A formation:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \tag{6}$$

where $\alpha$ and $\beta$ ($h^{-1}$) are the product formation constants. The larger the $\alpha$ value, the more growth-associated the fermentation process. The $\beta$ value indicates a non-growth-associated process.

Finally, the following classical kinetic model originally proposed by Luedeking and Piret [13] was used to describe the consumption of substrate glucose:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + mX + \frac{1}{Y_{P/S}}\frac{dP}{dt} \quad (7)$$

where $Y_{X/S}$ is the cell yield coefficient for glucose (g/g), $Y_{P/S}$ is the product yield coefficient for glucose (g/g), and $m$ is the maintenance coefficient ($h^{-1}$). To explain more conveniently, it is desirable to symbolize these parameters as $K_1$ and $K_2$:

$$\frac{dS}{dt} = K_1\frac{dX}{dt} + K_2X, \quad -K_1 = \frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}}, \quad -K_2 = m + \frac{\beta}{Y_{P/S}}. \quad (8)$$

## Results and discussion

### Identification of fermentation parameters and medium components

Previously we identified by a reverse transcription polymerase chain reaction (RT-PCR) method several common bacterial cultivation media in which two critical genes involved in thailandepsin A biosynthesis were found to be expressed at adequate levels; furthermore we found by HPLC that thailandepsin A is produced in a notable level (less than 10 mg/l) in one of the media (modified minimal broth—MMB; composition per liter: glucose 10.0 g, dibasic potassium phosphate trihydrate 7.0 g, monobasic potassium phosphate 2.0 g, ammonium sulfate 1.0 g, sodium citrate 0.5 g, magnesium sulfate heptahydrate 0.1 g) [2, 28]. This level of production is too low for the drug development process; thus MMB was selected as the starting point for medium optimization. A suite of medium components and fermentation parameters, including different carbon sources (sucrose, lactose, glycerol, soluble starch, or glucose) added at 10 g/l and different nitrogen sources (ammonium sulfate, soybean meal, corn steep liquor, yeast extract, tryptone, ammonium chloride, or potassium nitrate) at 1 g/l, were tested to determine the suitable carbon and nitrogen sources; different inorganic salts (dibasic potassium phosphate trihydrate, monobasic potassium phosphate, sodium citrate, or magnesium sulfate), incubation temperature (25–37°C), pH (2.5–9.0), inoculum ratio (0.5–10%, v/v) of seed culture, medium volume (20–80 ml) in 250-ml Erlenmeyer flasks, shaking speed (150–190 rpm), and extracting solvent (dichloromethane, chloroform, tetrachloride, or ethyl acetate) were tested by single factor experiments. As a result, glucose as carbon source and tryptone as nitrogen source were found to be the most conductive to thailandepsin A production, and the favorable fermentation parameters were identified as follows: 28°C, pH 7.0 (before autoclaving), 1% inoculum ratio, incubation duration 60 h, medium volume 65 ml (26% by v/v), shaking speed 170 rpm, and chloroform as extracting solvent. Representative fermentation data are shown in Fig. 2.

Furthermore, concentration optimizations of the key medium components (data not shown) and Diaion HP-20 adsorbent resin were accomplished by further single factor experiments; 158 mg/l of thailandepsin A was obtained from a medium consisting of glucose 22.4 g/l, tryptone 24 g/l, potassium phosphate 18.9 g/l, sodium citrate 0.304 g/l, and 4% (w/v, wet weight) of HP-20 resin (Fig. 3). Potassium phosphate consists of dibasic potassium phosphate trihydrate and monobasic potassium phosphate in 9.17:2 ratio, which generates a buffered pH of 7.0.

The effect of HP-20 resin is significant. Addition of 4% HP-20 resin resulted in a 91% increase in thailandepsin A

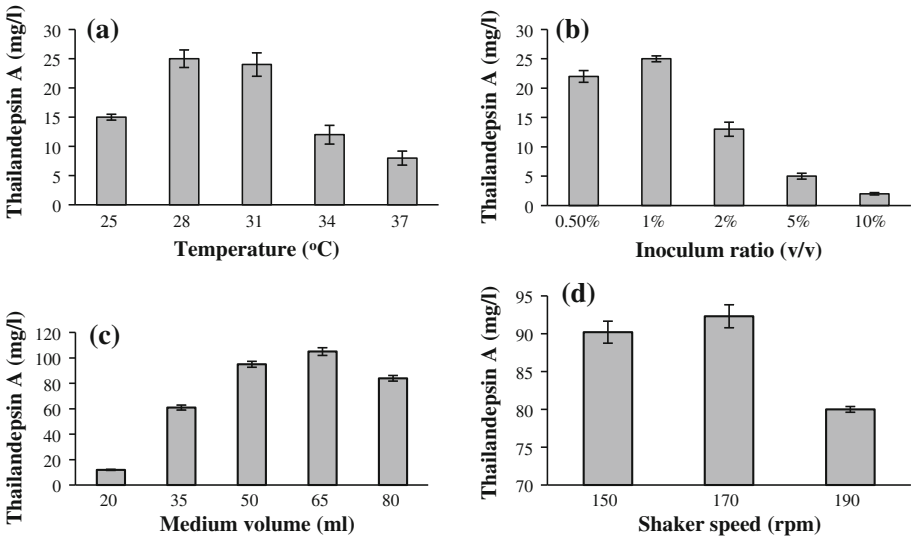


**Fig. 2** Effects of **a** fermentation temperature, **b** inoculum ratio, **c** medium volume, and **d** shaker speed on thailandepsin A production. The data shown are the averages of triplicate experiments with standard deviations

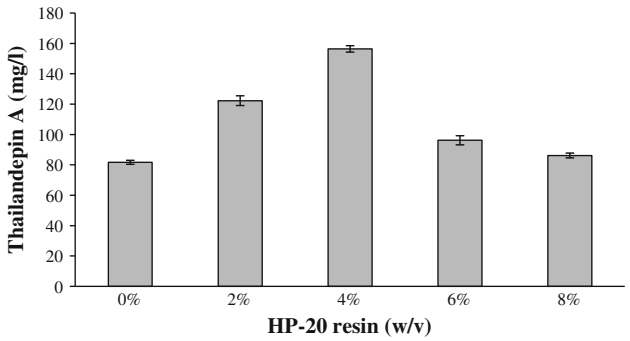


**Fig. 3** Thailandespin A production (mg/l) in fermentation medium containing varying amounts of HP-20 resin

production compared to a control fermentation without HP-20 (Fig. 3). Under this circumstance, most of the thailandepsin A was found adsorbed to the resin in the medium. It is believed that HP-20 reduced the feedback inhibition of thailandepsin A during fermentation by sequestering the target product. However, more than 4% of HP-20 is inhibitory to thailandepsin A production, probably due to a negative impact on the availability of nutrients, space, or dissolved oxygen for bacterial growth. For example, under such conditions, the resin may interfere with the bacteria's nutrient absorption process; too much resin may be attached to the bacteria and nutrients. Also, addition of an excess of resin may give a poor environment for the production of thailandepsin A in fermentation, e.g., a larger shear force due to limited medium space. Excess resin may also lead to higher dissolved oxygen. Therefore, 4% of Diaion HP-20 was identified as optimal for the production and recovery of thailandepsin A. For teicoplanin fermentation in the presence of HP-20 resin, 5% (w/v) gave the highest titer with the benefit of relieving product inhibition, offering better environment conditions such as agitation, nutrition, and pH [9].

In comparison with the traditional methods of metabolite separation from culture broth, e.g., organic extraction or membrane separation, which are often laborious and time-consuming, resin adsorption and extractive fermentation carries many advantages and therefore has been used widely in recent years. Resin sequesters product and thus lowers feedback inhibition and improves concentration; resin increases product stability; resin collection is easy and does not require sophisticated instrumentation; elution of metabolites from resin consumes much less organic solvent; resin is reusable and thus is environmentally friendly; and resin reduces the length and scale of exposure to organic solvent. Examples of successful usage of HP-20 resin include 100-fold increase of the titer of a fungal self-toxic metabolite [23], significant titer improvement of metabolites produced by *Streptomyces* [5, 7, 15, 19], and significant increase of product stability during fermentation [7, 9], to list only a few.

## Optimization of culture medium by RSM

Four nutrient variables—glucose, tryptone, potassium phosphate, and sodium citrate—were subjected to further studies by RSM. CCD was employed to determine the optimal level of those variables. The respective low and high levels of each variable in five coded levels are given in Table 1 and the CCD design as a function of thailandepsin A concentration is shown in Table 2. Furthermore, the predicted responses were deduced from statistical analysis. Multiple regression analysis was used to analyze the data and deduced the following polynomial equation:

$$\begin{aligned} Y = {} & 159.04 - 26.44\,x_1 - 4.14x_2 \\ & + 14.80x_3 - 10.40x_4 - 9.83x_1x_2 - 5.20x_1x_3 - 4.21x_1x_4 \\ & + 5.21x_2x_3 - 5.08x_2x_4 - 5.18x_3x_4 - 24.59x_1^2 - 7.03x_2^2 \end{aligned} \quad (9)$$

where $Y$ represents the response (predicted thailandepsin A concentration), $x_1$ is glucose, $x_2$ is tryptone, $x_3$ is potassium phosphate, and $x_4$ is sodium citrate. This 12-term quadratic equation includes four linear terms, six two-factorial interactions, and two quadratic terms. $P$ values less than 0.10 indicate factors which are significant at the probability level of 90%. The non-significant terms, i.e., $x_3^2$ and $x_4^2$, were excluded from the model.

Potassium phosphate exhibited a positive effect on the response, but glucose, tryptone, and sodium citrate exhibited a negative effect. The negative effect implied that the titer of thailandepsin A initially rose to the maximum and then began to decline with higher variable values. On the other hand, glucose, potassium phosphate, and sodium citrate expressed strong linear effects on the response; glucose and tryptone also expressed strong quadric effects on the thailandepsin A titer ($P < 0.005$). Remarkable interactions were detected between glucose as carbon source and tryptone as nitrogen source, whereas interactions among other components were relatively less significant.

The fitness of the model was verified by analysis of variance (ANOVA) which was tested by Fisher's statistical analysis as shown in Table 3. This $F$ test with an extremely low probability value [$(P_{\text{model}} > F) < 0.0001$] and a large model $F$ value (33.87) suggests there is less than a 0.01% chance that the model $F$ value could occur due to noise. An $R^2$ value (correlation coefficient) closer to 1 implies very good correlation between predicted and observed values. $R^2 = 0.9693$ denotes that more than 96% of the sample variation was attributed to the variables and only less than 4% of the total variance could not be account for in the

**Table 3** ANOVA for the full second-order polynomial quadratic model

| Source | Sum of squares | Mean squares | *F* value | *P* value |
|---|---|---|---|---|
| Model | 46,109.04 | 3,293.50 | 33.87 | <0.0001 |
| $X_1$ | 16,776.15 | 16,776.15 | 172.50 | <0.0001 |
| $X_2$ | 410.98 | 410.98 | 4.23 | 0.0576 |
| $X_3$ | 5,256.41 | 5,256.41 | 54.05 | <0.0001 |
| $X_4$ | 2,595.12 | 2,595.12 | 26.68 | 0.0001 |
| $X_1$ $X_2$ | 1,599.44 | 1,599.44 | 15.89 | 0.0012 |
| $X_1$ $X_3$ | 433.46 | 433.46 | 4.46 | 0.0519 |
| $X_1$ $X_4$ | 284.19 | 284.19 | 2.92 | 0.1080 |
| $X_2$ $X_3$ | 433.53 | 433.53 | 4.46 | 0.0519 |
| $X_2X_4$ | 406.58 | 406.58 | 4.18 | 0.0588 |
| $X_3$ $X_4$ | 429.16 | 429.16 | 4.41 | 0.0530 |
| $X_1^2$ | 16,591.44 | 16,591.44 | 170.61 | <0.0001 |
| $X_2^2$ | 1,356.32 | 1,356.32 | 13.95 | 0.0020 |
| $X_3^2$ | 4.46 | 4.46 | 0.047 | 0.8307 |
| $X_4^2$ | 113.81 | 113.81 | 1.17 | 0.2964 |

$R^2 = 0.9693$, adj $R^2 = 0.9407$, predicted $R^2 = 0.8411$, CV = 7.45

model. Additionally, the predicted determination coefficient (predicted $R^2 = 0.8411$) and adjusted determination coefficient (adjusted $R^2 = 0.9407$) validated the significance of the model. The low coefficient value of variation (CV = 7.45) in the current case denotes that the experiments performed were reliable and precise.

The optimal levels of the variables for the maximal production of thailandepsin A by *B. thailandensis* E264 and the interactive effects were determined by plotting the 2D contour plot and 3D response surface curve [4, 25] as shown in Fig. 4. The response surface plot depicts the interaction between two variables by keeping other variables unchanged in their central zero levels. A response surface with an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables, whereas a circular contour plot indicates negligible interaction [18]. It is often straightforward to locate the optimum levels from elliptical contour plots on account of the remarkable convex appearance of the 3D response surface curve.

Apparently there is a significant interaction between glucose and tryptone for thailandepsin A production (Fig. 4). As glucose concentration increases, the maximal response appeared near the middle of tryptone concentrations. C/N ratio is well known to be critical for thailandepsin A production, because the carbon and nitrogen proportion directly affects the rate of microbial growth and the titer of product biosynthesis [21]. The optimized C/N ratio for *B. thailandensis* E264 growth and for thailandepsin A accumulation was identified here as 0.51. Tryptone appeared to be a significant factor beneficial to

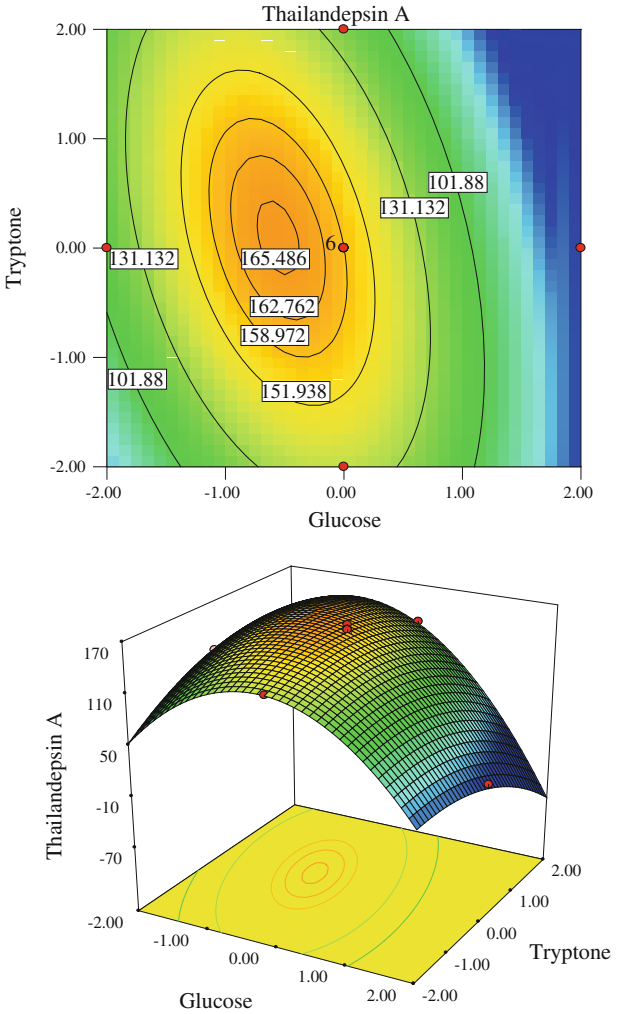


**Fig. 4** Response surface curve and its corresponding 2D contour plot fitted to the full quadratic model: interaction of glucose and tryptone

thailandepsin A production, for tryptone is rich in amino acids and inorganic salts that influence the growth of microorganism and the production of thailandepsin A. Considering that thailandepsin A is biosynthesized by a hybrid polyketide synthase–nonribosomal peptide synthetase pathway which polymerizes building blocks of short carboxylic acids, amino acids, or amino acid derivatives [28], the production process of thailandepsin A needs abundant nitrogen sources.

In combination with the statistically based experimental designs and the optimized nutrient solution obtained by analyzing the response surface contour plots, the optimal values of each nutrient component were determined as follows: glucose 17.89 g/l, tryptone 34.98 g/l, potassium phosphate 24.84 g/l, and sodium citrate 0.01 g/l. The predicted production of thailandepsin A is 251.9 mg/l by using the optimized concentrations of the variables. Experimental validation of the predicted model was conducted in

triplicate and the concentration of target product reached 236.7 mg/l. The prominent correlation between the actual and predicted values justifies the validity of the response model and indicates the successful optimized production of thailandepsin A in flask fermentation. It is obvious that RSM has effectively identified the optimal medium composition and made a remarkable titer increase.

## Kinetic models for thailandepsin A production

*B. thailandensis* E264 culture fermentation typically experiences a 12-h lag period when initially exposed to nutrient rich medium, then grows rapidly up to its maximum in 25 h of cultivation; after this point the vitality of cells starts to decline (Fig. 5). Rapid increase of cell mass during fermentation proceeded with rapid consumption of glucose (and presumably other nutrients as well) whose concentration is almost exhausted to less than 2 g/l after 36 h of cultivation. Thailandepsin A production commenced at 20 h during the exponential phase of cell growth (12–30 h), and continued to accumulate along with biomass increase at the end of log phase until 60 h when the culture reached the stationary phase. After this point, thailandepsin A production starts to fall into recession with the degeneration of cells and deficiency of medium nutrient.

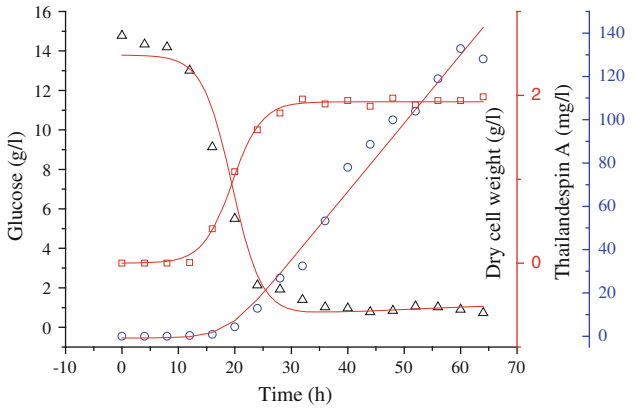


**Fig. 5** Typical time course batch fermentation of *B. thailandensis* E264 for the production of thailandepsin A. *Squares*, dry cell weight; *circles*, thailandepsin A; *triangles*, glucose. The *solid lines* represent model prediction

Fermentation kinetic models contribute to describe the fermentation process and predict the suitable cultivation conditions based on glucose as substrate. Consequently, kinetic models featuring the fermentation process of *B. thailandensis* E264 were established consisting of the characteristics of thailandepsin A production and substrate glucose consumption under the early conditions with 15.0 g/l of glucose in batch culture. Coefficient determination ($R^2$) of simulation and kinetic parameters including $X_m$, $\mu_m$, $\alpha$, $\beta$, $K_1$, $K_2$, $S_0$, $X_0$, $P_0$ are listed in Table 4. Also $S_0$, $X_0$, and $P_0$ as the calculated parameters based on the initial experimental values of $S$, $X$, and $P$ from the built model could be used to validate this model; here the difference between real and calculated values is acceptable. If we want to predict the time course of fermentation at a different initial glucose concentration ($S_{0exp}$), $S_0$ could be changed to that initial glucose concentration ($S_{0exp}$) with some modification of known deviation ($S_0 - S_{0exp}$) which was estimated on the basis of the regression with approximate initial glucose concentration.

In thailandepsin A batch fermentation, our kinetic models based on the logistic and Luedeking–Piret equations described cell growth, thailandepsin A formation, and substrate consumption well. The correlation coefficients of cell growth, thailandepsin A formation, and substrate consumption were up to 0.9974, 0.9873, and 0.9835, respectively. This shows a good correspondence between the model predictions and experimental data. These correlations among cell growth, thailandepsin A formation, and glucose uptake will be useful in further investigations of the fermentation process.

The Luedeking–Piret equations provide a good description of *B. thailandensis* E264 growth behavior. The growth-associated constant $\alpha$ (0.542 $h^{-1}$) and the non-growth-associated constant $\beta$ (1.64 $h^{-1}$) are within the same magnitude, indicating that thailandepsin A formation is a partial growth-associated fermentation process. Thailandepsin A produced by *B. thailandensis* E264 under laboratory conditions showed a typical partial cell density-dependent response, which is consistent with its "assembly-line" mechanism of biosynthesis that utilizes intermediates from the primary metabolism [2, 28]. The kinetic model is further verified and consistent with biosynthetic analysis. Biosynthesis does not initiate fully simultaneously with cell growth, whereas primary metabolite

**Table 4** Coefficients of kinetic model and calculated kinetic parameters

| $X_m$(g/l) | $\mu_m$ ($h^{-1}$) | $\alpha$ ($h^{-1}$) | $\beta$ ($h^{-1}$) | $K_1$ | $K_2$ | $S_0$ (g/l) | $X_0$ (g/l) | $P_0$ (mg/l) |
|---|---|---|---|---|---|---|---|---|
| 1.92 ± 0.02 | 0.382 ± 0.03 | 0.542 ± 1.16 | 1.64 ± 0.08 | −2.63 ± 0.13 | 0.006 ± 0.01 | 13.79 ± 0.35 | 0.00112 ± 0.0006 | −0.80 ± 3.09 |

$R^2$ biomass = 0.9974, $R^2$ product = 0.9873, $R^2$ glucose = 0.9835

(short carboxylic acid, amino acid, or amino acid derivative) was produced within the logarithmic phase. Thailandepsin A does not accumulate until when cell density and building blocks reach a certain level.

Similar to thailandepsin A fermentation, many microbial metabolite productions could be optimized under the guidance of fermentation kinetics. For example, production of leukotoxin, a lead drug for respiratory disease obtained from *Mannheimia haemolytica*, is regarded as a partial growth-associated model [3]. In contrast, the vast majority of secondary metabolites produced by fungi and bacteria such as *Trametes versicolor*, *E. coli*, and *Pseudomonas* are considered as growth-associated models and the desired product formation rate is found to be highly correlated with the cell growth rate [12, 26, 27]. Furthermore, the formation of metabolite phenazine-1-carboxylic acid (PCA) in *Pseudomonas* sp. M18G is found to be a weak growth-associated fermentation process [6]. Therefore, kinetic models integrating cell growth, glucose substrates consumption, and partially growth-associated thailandepsin A formation by *B. thailandensis* E264 introduced in this paper can be used as a reference for improving the fermentation process for other similar types of bacterial products. In addition, these results provide useful information for future commercial application of large-scale industrial fermentation facilities for thailandepsin A production. Tests on thailandepsin A production in a 5-l fermentor are being conducted under the guidance of these kinetic models.

In conclusion, this study provides much needed guidance for the enhanced production of thailandepsin A, an HDAC inhibitor anticancer drug candidate produced by *B. thailandensis* E264. Growth parameter optimization through single factor experiments, inclusion of HP-20 adsorbent resin in the fermentation process, and central composite design all contributed to the significant increase of the thailandepsin A titer. Kinetic models constructed in this work will serve as a reference for further process optimization for large-scale production of thailandepsin A in fermentor systems.

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