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Optimizing bioconversion of ferulic acid to vanillin by *Bacillus subtilis* in the stirred packed reactor using Box-Behnken design and desirability function

Peng Chen, Lei Yan[1,*], Shuang Zhang[1], Zhengrong Wu, Suyue Li[2], Xiaojuan Yan[2], Ningbo Wang[2], Ning Liang[2], and Hongyu Li

School of Pharmacy, Lanzhou University, Lanzhou 730020, China
[1]*College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, China*
[2]*Gansu Institute of Business and Technology, Lanzhou 730010, China*

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*Corresponding Author
Tel/Fax: +86-459-6819299
E-mail: 15045908934@163.com;
hekouyanlei@gmail.com

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Abstract A stirring bioreactor packed with a carbon fiber textiles (FT) biofilm formed by *Bacillus subtilis* was used to produce vanillin from ferulic acid. Biofilm formation was characterized by scanning electron microscopy. The interactive effects of three variables on vanillin molar yield (M) and conversion efficiency of ferulic acid (E) were evaluated by response surface methodology (RSM) with a Box-Behnken design (BBD). The optimal conversion conditions with a maximum overall desirability D of 0.983 were obtained by a desirability function. Considering the actual operation, the confirmation tests were performed using the slightly modified optimal conditions (initial ferulic acid concentration 1.55 g/L, temperature 35°C, stirring speed 220 rpm). The results showed that M and E were 57.42 and 93.53%, respectively. This was only 1.03% and 1.87%, respectively, different from the predicted values, confirming the validity of the predicted models. These revealed that the stirred packed reactor could be successfully used in vanillin bioconversion from ferulic acid.

Keywords: vanillin, stirred packed reactor, *Bacillus subtilis*, desirability function, response surface methodology

Introduction

Vanillin, one of the most important aromatic compounds, is widely used in foods, cosmetics, fragrances, beverages, and pharmaceutical industries (1,2). There are two types of commercial vanillin, namely, synthetic vanillin produced by chemical synthesis and natural vanillin obtained by extraction and bioconversion (3). The price of synthetic vanillin is 100 to 330 times lower than that of natural vanillin (4). It is well known that synthetic vanillin cannot be considered as natural owing to the recent US and European legislation (5). Natural vanillin produced by extraction cannot fulfill the increasing demand for natural aroma chemicals because direct extraction has some drawbacks such as low concentration, high extracting cost, and low hydrolysis rate (2). The high price of natural vanillin and the increasing customer-led demand for natural and healthy flavors have resulted in growing interest to produce natural vanillin via bioconversion (1). This is because the vanillin obtained by bioconversion can be regarded as safe and considered as natural according to the international standards (6).

Many natural substrates including phenolic stilbenes, lignin, eugenol, isoeugenol, ferulic acid, aromatic amino acids, and glucose can be used for the production of vanillin via bioconversion (7). Among them, ferulic acid is an attractive inexpensive substrate for biotechnological production of vanillin (8). This hydroxycinnamic acid is abundant substance since it is a constituent of cell walls of all families of the comme-linoid group of monocotyledons, such as in graminaceous plants, including wheat straw, maize stems, and grass (8). Various microorganisms including *Pseudomonas* ssp., *Escherichia coli*, *Rhodococcus* ssp., *Aspergillus niger*, *Bacillus subtilis*, *Amycolatopsis* ssp., *Pycnoporus cinnabarinus*, and *Streptomyces setonii* have been reported to produce vanillin from ferulic acid (7). However, in most cases, the yield of vanillin is low and the bioconversion reaction is slow. These may be attributed to the inhibitory effects of vanillin on growth and metabolic activity of free cells during the bioconversion (6). Microbial cell immobilization has been used to eliminate inhibition caused by high concentration of product and substrate as well as to enhance productivity, avoid wash-out of cells, and protect cells against shear forces in the stirred reactor (9). Although the immobilized cells using polyurethane, synthetic sponge, and porous glass as supports have been evaluated to produce vanillin from

ferulate (10), the vanillin yield is still low. This may be ascribed to the low biomass retention of these supports and the mass transfer limitation in the fixed bed reactor (11). Recently, carbon fiber textiles, an immobilization support has been given more attention as it has the advantages of possessing a high surface area for cell adhesion and excellent biocompatibility (12). It is well known that stirring plays a significant role in mass transfer performance during fermentation. Indeed, to our knowledge, thus far, there has no report on the bioconversion of ferulic acid to vanillin in the stirring reactor packed with CFT carrier, which immobilizes microbial cells.

The production of vanillin via biotransformation depends on several working parameters such as culture time, incubation temperature, oxygen concentration, types of species, pH, and initial substrate concentration (1,3,5,13). To explore the optimum combination of working parameters, most of the experiments were designed based on the one-factor-at-a-time approach (OFAT) in previous studies (1,13). However, this OFAT approach is inefficient and ineffective in the detection of possible interactions between parameters that are often significant (14). In addition, the working parameters not only affect the vanillin production but also influence the conversion efficiency of the substrate. It has been reported that vanillin and other products such as vanillin acid, protocatechulic acid, and vanillyl alcohol are produced during microbial bioconversion of ferulic acid (15). In the actual production, the yield of vanillin and the conversion efficiency of ferulic acid are both important, but the parameters, which are individually optimize with each response variable, are contradictory. Thus, dealing with several parameters at a time with multiple responses is a great challenge. This challenge can be handled first by building appropriate response surface methodology (RSM) for each response and later applying a desirability function to find a set of conditions that produce adequately balanced optima for all responses involved. Until recently, no attempt had been made to simultaneously consider the vanillin yield and the ferulic acid conversion efficiency during bioconversion.

In this study, the combined effects of initial ferulic acid concentration, temperature, and stirring speed on vanillin molar yield (M) and ferulic acid conversion efficacy (E) in the stirring reactor packed with CFT carrier, which immobilizes *B subtilis*, were investigated using Box–Behnken design (BBD) in RSM. The optimum conditions for bioconversion were determined by the desirability function. In addition, validation experiments were performed on a random set of experiments under optimal conditions.

Materials and Methods

Microorganism and media *B. subtilis* BS-7 (CCTCC-M-2011162), capable of converting ferulic acid to vanillin, was used (16). The seed medium included 5 g/L glucose, 10 g/L peptone, 3 g/L yeast extract, and 5 g/L NaCl, and the conditions were 25°C for 24 h. Cell immobilization and biofilm formation were performed with the same medium but replacing glucose by 0.5 g/L analytical grade ferulic acid (Aladdin, Shanghai, China).

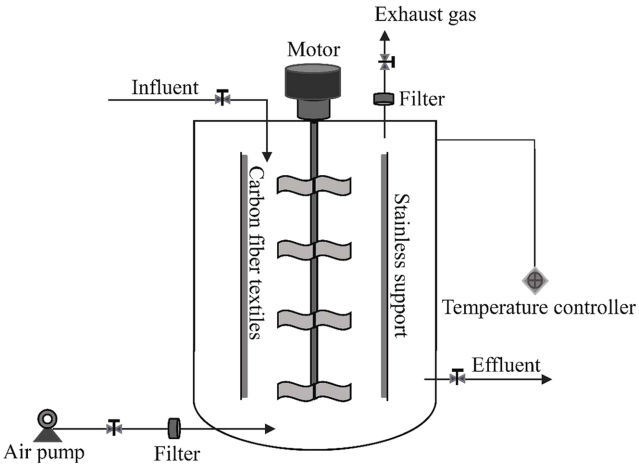

Fig. 1. Schematic of the stirred packed reactor used for vanillin production (Fermentation was performed in bioconversion medium containing 10 g/L peptone, 3 g/L yeast extract, 5 g/L NaCl, and 1.0–2.0 g/L ferulic acid. Fermentation conditions: pH 8.5, hydraulic retention time 20 h, temperature were at 30, 35, and 40°C, respectively, string speed were at 150, 200, and 250 rpm, respectively. Optimized fermentation conditions: pH 8.5, HRT 20 h, initial ferulic acid 1.55 g/L, temperature 35°C, and stirring speed 220 rpm).

Bioreactor setup and biofilm formation The stirred stainless steel fermenters with a working volume of 10 L were applied in this study (Fig. 1). Carbon fiber textiles (Xintong Activated Carbon Fiber Co., Ltd., Nantong, China) were used as the biomass carrier and bound to a conical frustum stainless support, which was fixed inside the fermenter. The conical frustum has dimensions of 12 cm in top diameter, 16 cm in bottom diameter, and 25 cm in height.

The whole setup was steam sterilized at 120°C for 45 min. Then, 8.0 L of broth including the sterilized seed medium without glucose and the filtered ferulic acid solution (dissolved in 1 M NaOH solution) was added aseptically to the reactor. For cell immobilization and biofilm formation, a 24-h grown culture of *B. subtilis* BS-7 (5.0%, v/v) was inoculated. The system operated for approximately 40 h at conditions with no pH control, stirring speed of 150 rpm, and temperature of 35°C.

Batch bioconversion After the formation of biofilm on CFT carrier, the batch bioconversion experiments were performed without inoculation of fresh culture at pH 8.5 and hydraulic retention time (HRT) 20 h. The initial ferulic acid concentration, temperature, and stirring speed were adjusted to the desired values, respectively. The other conditions were the same as the conditions for biofilm formation described above. The bioconversion started as soon as the fresh medium was transferred to the reactor.

Experimental design and validation In this study, the Box-Behnken

design approach of RSM was used to determine the relationship between the variables and response functions M and E. Our previous single-factor experiments conducted with five parameters (HRT, temperature, stirring speed, initial ferulic acid concentration, and pH) showed that only three factors including initial ferulic acid concentration, temperature, and stirring speed significantly affected the production of vanillin (17). Accordingly, three important parameters, i.e., initial ferulic acid concentration (X_1), temperature (X_2), and stirring speed (X_3) and their three levels for each were selected. The range and levels of the variables investigated are shown in Table 1. To define the relationship among the responses of interest and the independent variables, a mathematical model in the form of a second order polynomial was applied, as expressed in Eq. 1:

$$Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum\sum_{i<j}^{3}\beta_{ij}X_iX_j \quad (1)$$

where Y is the response (M or E), β_0 is a constant term, and β_i, β_{ii}, and β_{ij} are the coefficients of parameters for linear, squared, and interaction effects, respectively. The experimental design and optimization were applied using Design Expert 8.0.6.1 software (Stat-Ease Inc., Minneapolis, MN, USA). The analysis of variance (ANOVA) for each response was performed using Minitab.16 (Minitab Inc., State College, PA, USA). The adequacy of the model was assessed by R^2, adjusted-R^2 (adj-R^2), lack-of-fit, CV, and p-values.

To obtain the best compromise between M and E based on RSM mathematical models that might combine multiple responses into a single response, the desirability method described by Derringer and Suich (18) was applied. In general, each estimated response (Y_i) is converted to a desirability function (d_i) as expressed in Eq. 2:

$$d_i=\begin{cases}0 & Y_i\le Y_{i-\min}\\ \left[\dfrac{Y_i-Y_{i-\min}}{Y_{i-\min}-Y_{i-\max}}\right]^r & Y_{i-\min}<Y_i<Y_{i-\max}\\ 1 & Y_i\ge Y_{i-\max}\end{cases} \quad (2)$$

where $Y_{i\text{-min}}$ and $Y_{i\text{-max}}$ are the minimum and the maximum values of the response Y_i, respectively. Also, r is a weight used to determine scale of desirability and equals to 1 in this study. The individual desirability of multiple responses were then combined into an overall desirability function D by using the geometric mean according to the following equation:

$$D=(d_1\times 1_2\times \ldots d_n)^{1/n} \quad (3)$$

where n is the number of responses being optimized. The Design-Expert software was used to determine the maximum desirability value by an extensive grid search over the domain.

To verify the predicted results, a validation experiment was performed in triplicate by employing the recommended optimum conditions of initial ferulic acid concentration, temperature, and stirring speed. During bioconversion, approximately 20 mL fermentation liquid and 4 cm^2 CFT biofilm carrier were withdrawn every 5 h. The ferulic acid concentration, vanillin titer, and ATP content in the fermentation liquid were analyzed. Mean while, the ATP content on CFT carrier was also determined.

Analysis Ferulic acid and vanillin were determined by HPLC (LC-2010A; Shimadzu, Kyoto, Japan) with a C18 reverse-phase column (4.6×250 mm, Elite, Dalian, China) maintained at 25°C using a UV–Vis detector (280 nm). Elution was performed with a 40:60 (v/v) mixture of methanol and water. The injection volume was 20 μL and the flow rate was maintained at 0.8 mL/min. Ferulic acid and vanillin used for HPLC analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). The surface morphologies of the CFT biofilm carriers before and after immobilization were examined by a field-emission scanning electron microscope (S4800; Hitachi, Tokyo, Japan). The cell growth was evaluated using the ATP assay. The ATP content was analyzed by an ATP assay kit (Beyotime, Nanjing, China) according to the manufacturer's instructions.

Vanillin molar yield (M) was calculated as the ratio between the produced vanillin (mM) and the initial ferulic acid (mM) (3). The conversion efficacy (E) of ferulic acid was as the ratio between the concentrations (g/L) of converted and initial ferulic acid.

Results and Discussion

Immobilization To verify bacterial immobilization onto CFT carrier, SEM observations were performed. The surface morphologies of CFT carriers before and after immobilization are shown in Fig. 2. It can be observed that there were many cracks and grooves on the surface of original CFT carrier (Fig. 2A). This rough surface was favorable to the bacterial adhesion, which would lead a well immobilization of bacteria on CFT carrier. The image of bacteria immobilized on CFT carrier is shown in Fig. 2B. It can be seen that the bacterial cells were immobilized on CFT carrier and the cells were dispersed in the multilayer. Some holes and channels on CFT carrier surface indicated biofilm formation and extracellular polymeric substances (EPS) production (19).

Vanillin molar yield in batch bioconversion RSM with a BBD was used to statistically and mathematically determine the optimal conditions of initial ferulic acid concentration, temperature, and stirring speed in this study. The various combinations of experimental conditions with their response values are given in Table 1. The experimental data were subjected to an ANOVA and were fitted by Eq. 1 to evaluate these parameters on M (Y_1). A quadratic polynomial regression equation was then obtained as

$$Y_1=-624.586+182.550X_1+25.593X_2+0.831X_3-56.538X_1^2-0.351X_2^2-0.002X_3^2-0.348X_1X_2+0.009X_1X_3-0.0007X_2X_3 \quad (4)$$

The adequacy of the model was evaluated by several indicators viz.

Table 1. The experimental conditions and results of RSM Box-Behnken design for M, E, and D

Run	Ferullic acid concentration (g/L)	Temperature (°C)	Stirring speed (rpm)	M (%)	E (%)	D
1	1.0	30	200	30.29	52.55	0
2	2.0	30	200	33.44	71.83	0.228
3	1.0	40	200	35.57	61.15	0.197
4	2.0	40	200	35.24	70.78	0.278
5	1.0	35	150	33.12	55.93	0.091
6	2.0	35	150	36.44	65.68	0.263
7	1.0	35	250	38.34	62.61	0.263
8	2.0	35	250	42.58	79.43	0.531
9	1.5	30	150	38.73	68.34	0.338
10	1.5	40	150	42.47	79.62	0.531
11	1.5	30	250	43.86	94.12	0.695
12	1.5	40	250	46.85	83.44	0.661
13	1.5	35	200	55.87	92.56	0.936
14	1.5	35	200	56.68	93.67	0.964
15	1.5	35	200	57.09	92.92	0.961

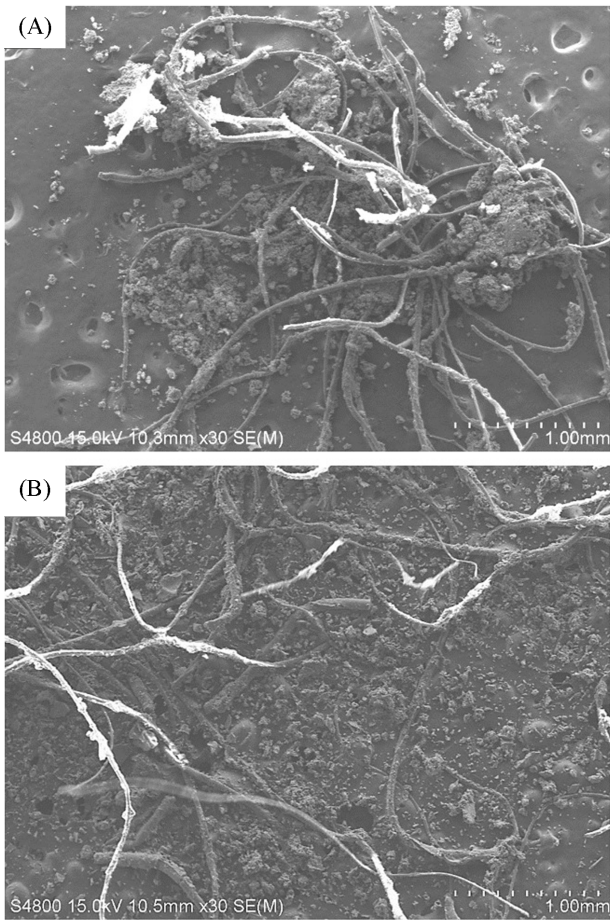

Fig. 2. SEM of carbon fiber textile before (A) and after (B) immobilizing.

model significance (*F*-value), correlation coefficient (R^2), coefficient of variation (CV), adequate precision etc. (20,21), as depicted in Table 2. The *F*-value, i.e., ratio of the noise to response, for the regression model was 150.49; this implied that the model was highly significant (p<0.0001). There was only a 0.01% chance that for a "model *F*-value," such magnitude could occur owing to noise. R^2 is the proportion of variation in the response that is explained by the model. It falls between 0 and 1, and a value above 0.90 suggests a good fit for a model (20). A higher value of R^2 (0.996) indicated that the second order polynomial could be used for prediction with reasonable precision, and only 0.4% of the total variation was not explained by the model. Adjusted R^2 (adj-R^2), derived from the sample size and from the number of terms in the model, is also a measure of goodness of fit (22). In fact, the adj-R^2 is more suitable than R^2 for assessing the fit of a multiple regression model (20). If the sample size is not very large and there are many terms in the model, adj-R^2 may be noticeably smaller than R^2 (22). In the present case, the high adj-R^2 value (0.989) demonstrated a high significance of the model. In general, predicted R^2 (pred-R^2) can be used to evaluate how well the model predicts responses for new observations (20). The value of pred-R^2 (0.951) was also high and advocated a high correlation between the predicted and observed values. In addition, the difference between values of adj-R^2 and pred-R^2 was smaller than 0.2, which indicated no large block effect or no possible problem with the model and/or data (23). The CV is the ratio of the standard error of estimated data to the average value of the observed response. Usually, the CV value should be less than 10% and the lower value indicates higher reliability of the experiment (24). The low CV value of 2.15% showed that the response of the developed model was dependable. The lack of fit, the variation of the data around the fitted model, is a special evaluation for the adequacy of a model (18). Here, the lack of fit value of 2.81 was non-significant relative to the pure error, implying good response to the model. Adequate precision is a measure of the range in predicted response relative to its associated error (25). Its value of 36.60, greater than 4, implied an adequate signal and thus, the predicted model could be used for navigating the design space. Furthermore, a standard deviation (SD) of 0.9 indicated

that the model was compliant with the predicted response.

It can be seen from Table 2 and Eq. 4 that linear terms X_1, X_2, and X_3 and quadratic terms X_1^2, X_2^2, and X_3^2 had significant positive and negative effect on M, respectively. The initial ferulic acid concentration (X_1) had the highest positive and significant impact on M followed by the positive linear impact of temperature (X_2) and stirring speed (X_3). This indicated that M increased with increase in levels of the variables. The significant negative effect of these quadratic terms implied that they tended to decrease vanillin yield markedly at high levels. Thus, the positive linear impact and negative quadratic effect led to an overall curvilinear effect on M. From Table 2, it can be observed that the interaction terms X_1X_2, X_1X_3, and X_2X_3 were non-significant (p>0.05), which indicated that they had little influence on vanillin yield. However, these terms were still considered in the equation owing to the hierarchy of the model (26). In addition, Eq. 4 shows that temperature interacts negatively with all process variables. This means that effect of initial ferulic acid concentration (X_1) and stirring speed (X_3) on M depended on the level of temperature used.

To illuminate the interactive effect of the variables followed by optimization of each variable for maximization of M, contour plots of the RSM were drawn as a function of two variables at a time, holding the remaining variable at the zero level. A total of three response plots and three corresponding contour plots were produced for the responses (Fig. 3). Figure 3A shows the interactive effect of initial ferulic acid concentration and temperature on M at a fixed stirring speed of 200 rpm. The yield of vanillin was relatively low at low initial ferulic acid concentration (1.0 g/L) and temperature (30°C) but increasing substrate concentration at 1.5 g/L and temperature 35°C led to increase in the response. After vanillin yield reached its highest level, a further increase of these two independent variables resulted in a decrease of dependent variable (Fig. 3A). It has been demonstrated that high concentrations of ferulic acid have adverse effect on microbial cells and could inhibit their growth and metabolism (3). Here, in the CFT packed stirring bioreactor, *B. subtilis* used ferulic acid as a sole carbon source for growth; thus, increasing substrate concentration in an appropriate range could increase the activity of microbes to produce vanillin. However, substrate concentrations at much higher levels could affect the biofilm formation (27), and reduce the volumetric oxygen mass transfer (28), which in turn would negatively impact bioconversion.

Figure 3A and 3C illustrate the interaction effects of temperature with initial ferulic acid concentration and stirring speed, respectively on vanillin yield. It can be observed that vanillin yield significantly increased with an increase in temperature from 30 to 35°C; however, a further increase in temperature resulted in decrease of vanillin yield. Temperature is an imperative factor that affects the bacterial growth and the product generation (29,30). The optimal fermentation temperature range for *Bacillus* species is 30 to 40°C (30); increasing the temperature appropriately would promote the microbial growth and biofilm formation, but a higher temperature could reduce the EPS production and biofilm formation (31). The interaction effects of stirring speed with initial ferulic acid concentration and temperature on vanillin yield are depicted in Fig. 3B and 3C. It can be observed that the vanillin yield increased with stirring speed over the range of 150 to 200 rpm. Over 200 rpm, vanillin yield decreased, possibly owing to the reduction in biofilm thickness and cell density, and the change of the hydrodynamic condition at higher stirring speed during fermentation (28,32).

As shown in the 3D response surface (Fig. 3), each response surface had a clear peak and the corresponding contour plot had a clear highest peak, implying that the optimal condition was inside the experimental region. Based on Eq. 4, a maximum vanillin molar yield of 57.01% was predicted at the following optimized conditions: an initial ferulic acid concentration of 1.52 g/L, a temperature of 35.47°C, and a stirring speed of 213.59 rpm.

Table 2. ANOVA results for dependent variable: M

Source	Sum of squares	df	Mean square	*F*-value	Significant
Model	1089.62	9	121.07	150.49	<0.0001
X_1	13.47	1	13.47	16.74	0.0094
X_2	23.84	1	23.84	29.63	0.0028
X_3	54.44	1	54.44	67.68	0.0004
X_1X_2	3.03	1	3.03	3.76	0.1101
X_1X_3	0.21	1	0.21	0.26	0.6299
X_2X_3	0.14	1	0.14	0.17	0.6932
X_1^2	737.67	1	737.67	916.95	<0.0001
X_2^2	284.45	1	284.45	353.57	<0.0001
X_3^2	84.79	1	84.79	105.4	0.0002
Residual	4.02	5	0.8		
Lack of fit	3.25	3	1.08	2.81	0.2732
Pure Error	0.77	2	0.39	pred-R^2	0.951
Cor Total	1093.64	14		Adeq Precision	36.60
SD	0.9	R^2	0.996		
CV	0.0215	adj-R^2	0.989		

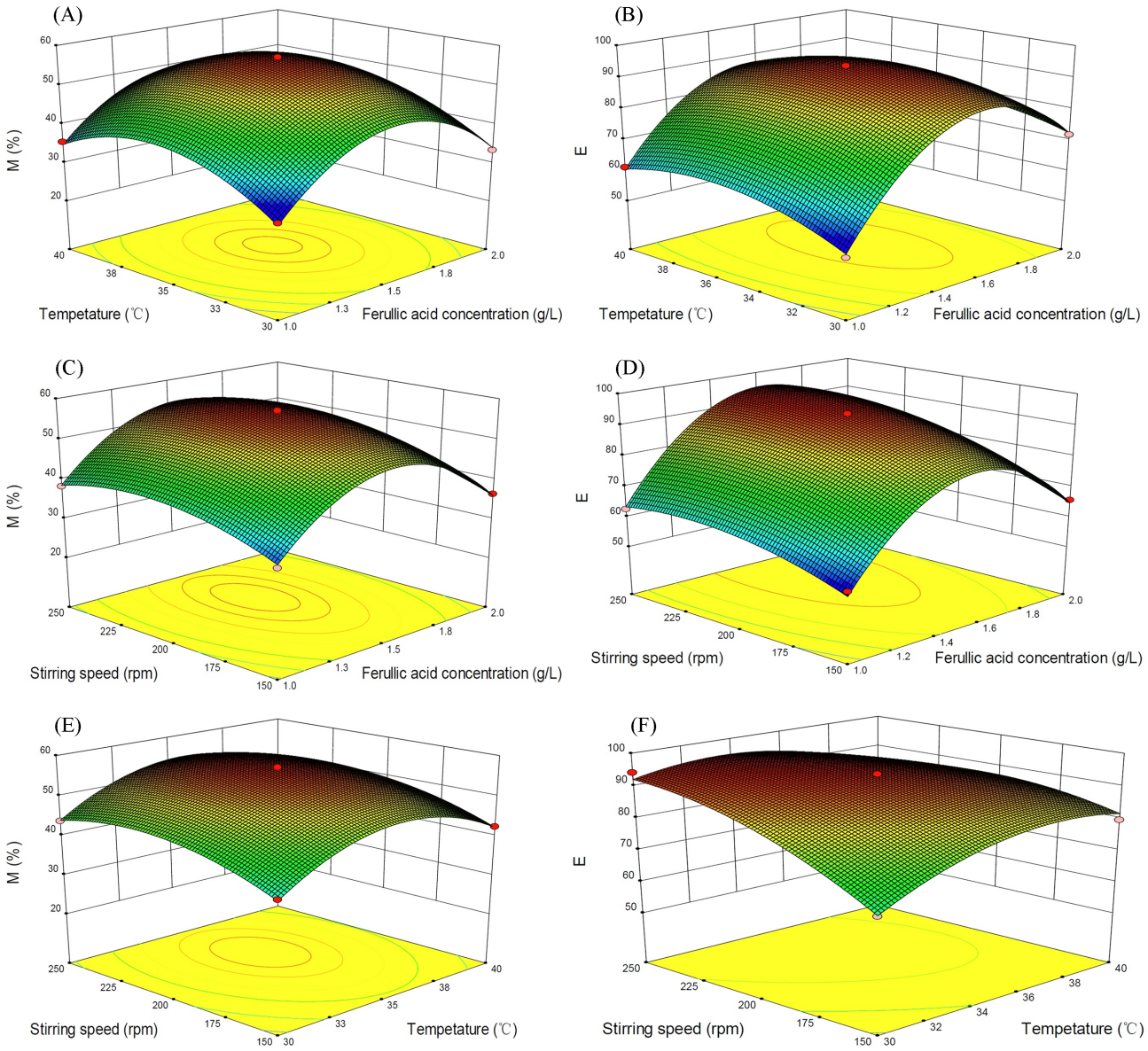

Fig. 3. Two-dimensional contour plots and three-dimensional graphs of the effects of initial ferulic acid concentration, temperature, and stirring speed on M (A, B, C) and E (D, E, F): (A, D) fixed stirring speed 200 rpm; (B, E) fixed temperature 35°C; and (C, F) fixed initial ferulic acid concentration 1.5 g/L.

Conversion efficacy of ferulic acid in batch bioconversion The initial ferulic acid concentration (X_1), temperature (X_2), and stirring speed (X_3) were found to be the important factors that influenced the conversion efficacy of ferulic acid during bioconversion. To evaluate the effects of these parameters, experiments designed by RSM with a BBD were performed. The experiment design and response values are presented in Table 1. The response data were subjected to regression analysis, and the following equation was obtained:

$$Y_2=752.606+300.145X_1+24.950X_2+1.574X_3-88.880X_1^2-0.370X_2^2 -0.002X_3^2-0.965X_1X_2+0.070X_1X_3-0.022X_2X_3 \quad (5)$$

The sufficiency of the fitted model was examined by ANOVA and the results are depicted in Table 3. The calculated model *F*-value was found to be high (87.34) with low probability value ($p<0.0001$), indicating that the model was significant. The high R^2 value of 0.994 implied that only 0.6% of the total variation was not explained and the fitted model could be used for prediction with reasonable precision. The pred-R^2 of 0.902 was found in reasonable agreement with the adj-R^2 of 0.982 followed by a high adequate precision value of 25.48 indicating the fitted model equation adequately. In addition, the low CV of 2.52% and a non-significant lack of fit value showed that the model adequately described the response surface of E.

It can be observed from Table 3 that the main effect of X_1 and X_3, the interactive effect of X_2X_3, and the quadratic effect of X_1^2, X_2^2, and X_3^2 were the significant model terms ($p<0.05$). Other model terms

were insignificant (p>0.05) and still included in Eq. 5 because it was a hierarchical model. As shown in Eq. 5, the positive coefficient for the main effect showed a linear effect to increase the conversion efficiency of ferulic acid, whereas the negative coefficient of the quadratic effect implied a strong effect to decrease ferulic acid conversion at very high levels of variables.

The response surface and contour plots of ferulic acid conversion are presented in Fig. 3. Figure 3D and 3E show the effects of initial ferulic acid concentration with temperature and stirring speed, respectively, on the conversion efficiency of ferulic acid. It can be observed that the E was very low at low initial ferulic acid concentration and increased with increase in initial substrate concentration till it attained a peak value. However, further increasing the initial ferulic acid concentrations resulted in a decreased E. The microbial growth inhibition may occur at high initial ferulic acid concentration, which in turn leads to decreased substrate utilization (3). From the response surface plots (Fig. 3D and 3F), it can be observed that E increased with an increase in temperature, the highest value appeared in the middle of the temperature range, in the vicinity of 35°C. Further increase in temperature led to decrease in E. Although elevating the temperature could increase substrate solubility and biofilm thickness (30), a higher temperature would result in the growth inhibition and the reduction in enzyme involved in ferulic acid degradation (15,30). A similar trend was observed for effects of stirring speed and initial ferulic acid concentration (Fig. 3E) as well as the effects of stirring speed and temperature (Fig. 3F). It has been reported that stirring speed was an important parameter for *B. subtilis* and its products formation in the bioreactor (33). Increasing stirring speed in the appropriate range (150–200 rpm) led to an increase in E because elevating stirring speed could improve the oxygen and mass transfer (34). However, a higher stirring speed would lead to the increase in shear stress and foam which in turn affected the utilization of ferulic acid (28,33). Therefore, with the help of this model, the maximum E of 96.24% was obtained at initial ferulic acid concentration 1.60 g/L, temperature 33.48°C, and stirring speed 242.05 rpm.

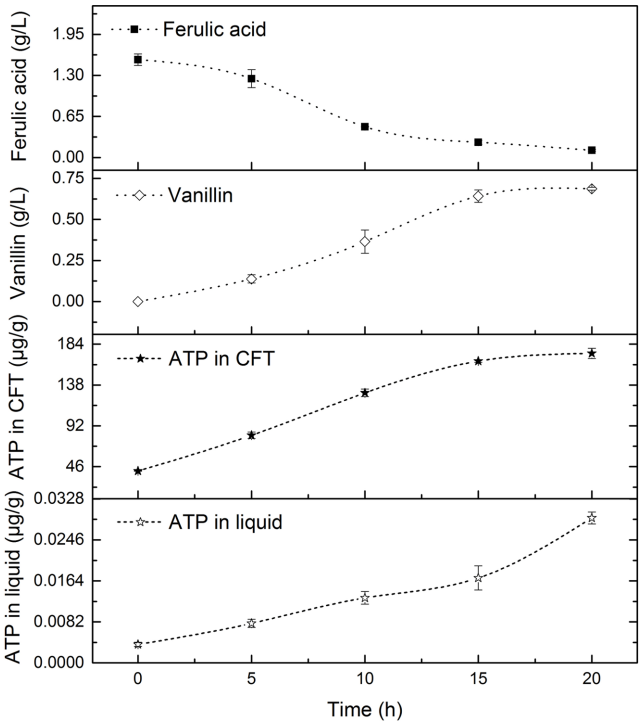

Fig. 4. Time profiles of ferulic acid concentration, vanillin titer, ATP content in CFT, and fermentation liquid during bioconversion.

Optimization using desirability function For a bioconversion system, both products yield and substrate utilization were important aims to be maximized. However, according to the above results, the optimal conditions for vanillin yield and ferulic acid conversion were not same. Thus, the desirability function approach was used to obtain the maximum M and E simultaneously. Based on RSM data for M, the minimum and maximum acceptable values were 30.29% (the

Table 3. ANOVA results for dependent variable: E

Source	Sum of squares	df	Mean square	*F*-value	Significant
Model	2810.02	9	312.22	87.34	<0.0001
X_1	384.75	1	384.75	107.62	0.0001
X_2	8.30	1	8.30	2.32	0.1880
X_3	312.88	1	312.88	87.52	0.0002
X_1X_2	23.28	1	23.28	6.51	0.0511
X_1X_3	12.50	1	12.50	3.50	0.1205
X_2X_3	120.56	1	120.56	33.72	0.0021
X_1^2	1823.00	1	1823.00	509.94	<0.0001
X_2^2	168.36	1	168.36	47.09	0.0010
X_3^2	89.29	1	89.29	24.98	0.0041
Residual	17.87	5	3.57		
Lack of fit	17.23	3	5.74	17.91	0.0533
Pure Error	0.64	2	0.32	pred-R^2	0.902
Cor Total	2827.89	14		Adeq Precision	25.48
SD	1.89	R^2	0.994		
CV	0.0252	adj-R^2	0.982		

minimum experimental value) and 57.01% (the maximum predicted value), respectively. Therefore, according to Eq. 2, the following one-sided transform of M (d_1) was obtained:

$$d_1=\begin{cases}0 & Y_1\leq 30.29\\ \left[\dfrac{Y_1-30.29}{57.01-30.29}\right]^r & 30.29<Y_i<57.01\\ 1 & Y_1\geq 57.01\end{cases} \quad (6)$$

Similarly, a one-sided transform of E (d_2) was achieved as

$$d_2=\begin{cases}0 & Y_2\leq 52.55\\ \left[\dfrac{Y_2-52.55}{96.24-52.55}\right]^r & 52.55<Y_i<96.24\\ 1 & Y_2\geq 96.24\end{cases} \quad (7)$$

With the help of Eq. 3, the overall desirability D was calculated as

$$D=\sqrt{d_1 d_2} \quad (8)$$

Thus, regression analysis of the new response D indicated that the optimized conditions for bioconversion (with a maximum D of 0.983) were initial ferulic acid concentration 1.55 g/L, temperature 35.16°C, and stirring speed 220.73 rpm. Correspondingly, the vanillin molar yield (M) and conversion efficiency of ferulic acid (E) were 56.83% and 95.31%, respectively.

Validation test Based on the optimized conditions acquired by RSM and the desirability function, verification experiments were performed in triplicate at 1.55 g/L initial ferulic acid concentration, 35°C temperature, and 220 rpm stirring speed for practical purposes. The average vanillin molar yield (M) and conversion efficiency of ferulic acid (E) were observed to be 57.42 and 93.53%, respectively. The difference between estimated and actual values for M and E was less than 1.87%, implying the optimal conditions could be practically used for bioconversion of ferulic acid to vanillin.

Time courses of ferulic acid concentration and vanillin production during bioconversion under the optimal condition mentioned above are shown in Fig. 4. It can be observed that there was approximately a 5-h lag phase and the maximum titer of vanillin was obtained after 20 h bioconversion. With the proceeding of fermentation, a continuous increase in vanillin titer was found from the hour 0 to hour 20, whereas a contrary trend was observed on ferulic acid concentration (Fig. 4). The rapid assimilation of ferulic acid indicated that the immobilized cells could efficiently convert ferulic acid. It also can be observed that the minimum titer of ferulic acid in fermentation broth was 0.11 g/L at 20 h, which means that the conversion efficiency of ferulic acid was high. However, the corresponding vanillin molar yield was only 56.59%. This is because that vanillin converted from ferulic acid was unstable and transformed to vanillic acid and/or vanillyl alcohol during fermentation (15).

The ATP assay has proven to be applicable for measuring active biomass in various aquatic environments (35). To determine whether the immobilized cells were separated from CFT carrier during bioconversion, the cell growth on CFT carrier and in liquid was monitored using ATP assay (Fig. 4). It can be observed that the ATP content on CFT carrier increased during bioconversion and reached the maximum value at the end of fermentation. A similar profile was observed on the ATP content of fermentation liquid. The ATP content on CFT carrier varied in a range of 41.01–173.69 μg/g, while the ATP titer in fermentation liquid ranged from 0.0037 to 0.029 μg/g (Fig. 4). These indicated that very little amount of cells separated from the CFT carrier (approximately 10^4–10^3 fold less than the values of cells immobilized on CFT carrier) and their contribute to bioconversion was minimal.

Table 4 summarizes some reported vanillin bioconversion from ferulic acid using different system and conditions. The vanillin molar yield in this study was markedly higher than those with other fermentation system and conditions (2,7,10,36-38). The difference in the yield of vanillin was attributed primarily to the different system and conditions applied. For large-scale production, the repeatability

Table 4. Production of vanillin from ferulic acid by various system and conditions

System and conditions	Microorganisms	Substrate (g/L)	Time (h)	Yield (%)	References
Fed batch (150 rpm, 30°C and 120 mL in 250 mL flask)	*Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725	0.3	60	14.9	(38)
Fed batch	*Enterobacter* sp. Px6-4	1.0	108	1.76	(37)
Fed batch	*Amycolatopsis* HR167	5.1	6.5	50	(2)
Resting cells	*Streptomyces halstedii*	1	8	8	(2)
Fed batch	*Streptomyces setonii*	8	26	47	(2)
Resting cells, 25 mL in 125 mL flask at 37°C, 200 rpm	*Bacillus licheniformis* SHL1	1	39	0.05	(36)
Resting cell	*Halomonas* sp. B15	0.5	48	49	(2)
Fed batch (30°C and 100 mL in 250 mL flask)	*Escherichia coli* JM109/pBB1 immobilized on reticulated polyurethane	0.3	144	27.5	(7)
Fed batch (30°C and 50 mL in 250 mL flask)	*Escherichia coli* JM109/pBB1 immobilized on synthetic sponge	0.2	72	51.05	(10)
10 L stirring packed reactor (220.73 rpm, 35.16°C)	*B. subtilis* BS-7 immobilized on CFT	1.55	20	57.42	This study

and continuity of the fermentation system played an important role in reducing costs. Therefore, the stirred reactor packed with CFT carrier biofilm might offer potential advantages over fed batch and resting cells for vanillin bioconversion from ferulic acid. In addition, the vanillin molar yield could be possibly elevated by optimizing conditions with RSM-BBD design and desirability function approach. In conclusion, bioconversion of vanillin from ferulic acid was performed in the stirring bioreactor packed with CFT biofilm formed by *B. subtilis*. SEM was used to characterize the formation of biofilm. The bioconversion condition was optimized with RSM and desirability function. ANOVA results demonstrated that all parameters significantly (p<0.05) affected the vanillin yield. The maximum D value of 0.983, along with the maximum M (56.83%) and E (95.31%), were obtained at the optimal conditions optimized by desirability function. The confirmation tests revealed that M and E were 57.42% and 93.53%, respectively. The average absolute error between experimental and predicted values was less than 1.87%, implying the high predictive power of model.

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