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ORIGINAL RESEARCH ARTICLE

Optimization of the Biosynthesis Conditions of Daptomycin by the Biostatistical Methodology

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Abstract Response surface methodology (RSM) was employed to optimize medium components including oxygen vector of *n*-dodecane of a mutant strain GC-63 of *Streptomyces roseosporus* NRRL 11379. The two-level Plackett–Burman design (PB factorial design) with fourteen variables including oxygen vector was used to screen the most significant factors affecting antibiotic production. Then, the RSM based on center composite design was used to identify the optimum levels of the significant variables to generate optimal response. Glucose, soybean meal, asparagine and *n*-dodecane were screened to significantly influence the daptomycin production. The medium composition optimized with response surface methodology was (g/L): glucose, 9.46; soluble starch, 25; dextrin, 12.5; yeast extract, 12.5; soybean meal, 21.34; peptone, 25; casein, 5; asparagine, 2.68; K_2SO_4, 6; $(NH_4)_2Fe(SO_4)_2$, 2; $MgSO_4$, 1; $CaCO_3$, 5; $MnCl_2$, 0.5; *n*-dodecane, 7.47 % (v/v). The maximum daptomycin concentration reached 979.36 mg/L which was nearly 2.2-fold higher compared to that in the basal medium, with predicted optimal concentrations in a 7.5-L fermentor.

Keywords Daptomycin · *Streptomyces roseosporus* · Biostatistical analysis · Response surface methodology · Optimization · Oxygen vector · *n*-Dodecane

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1 Introduction

Daptomycin is a member of the A21978C family of the cyclic anionic 13-amino acid lipopeptide, produced by a non-ribosomal peptide synthetase (NRPS) mechanism in *Streptomyces roseosporus* against a broad range of gram-positive pathogens, including methicillin- and vancomycin-resistant *Staphylococcus aureus* [1]. However, the yield needs to be further improved and cost of industrial production needs to be further reduced. Yet, there was little information available regarding enhancement of daptomycin productivity by medium optimization.

RSM was a collection of statistical techniques for evaluating the effective factors, building models to study the interaction between the variables, and selecting the optimum conditions of variables or desirable responses [2]. Using this technique, a few experimental trials were needed and significant interactions between the factors could be identified and quantified. Thus, RSM has been widely proved to be very effective for the applications on the optimization of medium compositions for fermentation processes [3, 4].

One of the major problems in industrial production of antibiotics was the inadequate supply of dissolved oxygen, which does not meet the demand of the cultures by only increasing the agitation speed. The addition of non-aqueous solvent called oxygen vector was one of the most effective approaches to improve oxygen transfer rate in aerobic fermentation [5].

In summary, the present investigation was aimed to enhance daptomycin production of strain GC-63 of *S. roseosporus* by optimizing medium components including oxygen vector of *n*-dodecane using the RSM method. The factorial design of Plackett–Burman (PB) was used to screen the most significant factors affecting antibiotic production. Center composite design (CCD) was used to

identify the optimum levels of the significant variables to generate optimal response.

2 Materials and Methods

2.1 Microorganism

The mutant strain GC-63 (He–Ne Laser and NTG induced mutants) of *S. roseosporus* NRRL 11379 used in the study was stored in 20 % glycerol at −80 °C.

2.2 Medium and Culture Conditions

Seed medium, fermentation medium and culture conditions both in flasks and in a 7.5-L bioreactor were same as previous research [6].

2.3 Analytical Methods

The analysis methods of the daptomycin, dry cell weight (DWC) and reducing sugar concentrations were same as previous research [6]. The pH and dissolved oxygen values were monitored by the digital electrode on-line.

2.4 Experimental Design and Optimization by RSM

2.4.1 Screening of Factors Affecting Daptomycin Production

The PB factorial design was employed for selecting factors that significantly influenced daptomycin production. In the present study, fourteen assigned variables were screened in twenty experimental runs (Tables 1, 2).

2.4.2 Path of Steepest Accent (Descent) Experiment Design

According to the first-order model equation obtained and the three important effect factors above, we moved in the three most important effect factors directions, respectively. The path of steepest ascent (descent) was determined to find proper direction of changing variables increasing or decreasing the concentration according to the sign of the main effects to improve daptomycin production. The design of the path of steepest ascent experiments is shown in Table 3.

2.4.3 Response Surface Methodology and Central Composite Design

RSM was used to optimize the screened variables for enhanced daptomycin production based on CCD. Glucose, soybean meal, asparagine and *n*-dodecane were major variables for daptomycin production using CCD and RSM. The four-factor, five-level CCD was used to optimize the response of four variables. The range and levels of experimental variables investigated in this study are listed in Table 4.

The analysis of all the data was conducted by Design Expert software package 7.0.

3 Results and Discussion

3.1 PB Method for Selection of Significant Factors Affecting Daptomycin Production

To evaluate which factors play significant effects on the daptomycin production, the PB design was employed. The previous studies indicated that the factors such as K^+, Mg^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+} exerted important effects on the production of daptomycin through fermentation by *S. roseosporus* (data no shown). In addition to these factors, others including yeast extract, peptone, casein, soybean meal, glucose, soluble starch and dextrin were also investigated in our experiment. Furthermore, *n*-dodecane was used in this investigation as a dissolved oxygen vector in the media. Based on the above results, the upper and lower limits of the variables for each factor were then chosen as shown in Table 1. The PB design for 20 trials with two levels for each variable and corresponding daptomycin production is presented in Table 2. To approach the neighborhood of the optimum response, a fitted first-order model for daptomycin production was obtained from the PB design as follows:

$$\begin{aligned} Y = {} & 532.63 - 52.55X_1 - 2.07X_2 + 4.33X_3 + 65.55X_4 \\ & + 31X_5 + 43.41X_6 + 21.33X_7 + 45.4X_8 + 9.13X_9 \\ & + 18.84X_{10} + 20.7X_{11} - 5.18X_{12} + 12.3X_{13} \\ & - 24.45X_{14} \end{aligned} \quad (1)$$

The coefficient of each variable in Eq. (1) represents the effect extent of this variable on the daptomycin yield. The linear regression coefficient R^2 was 0.9411 and the adjusted determination coefficient (Adj R^2) was 0.7763 for the model, which indicated that the model was reasonable for the PB design. And glucose (X_1), soybean meal (X_4), asparagine (X_6) and *n*-dodecane (X_8) were found to be the most significant variables affecting daptomycin production.

According to reports, the synthesis of many antibiotics and other secondary metabolites was often affected by carbon catabolic inhibition [7]. Daptomycin production could suffer from carbon catabolic repression which explained the negative effect of glucose. And the soybean

Table 1 Placket–Burman design for screening variables in daptomycin production

Term	Code	Low level (−1)	High level (+1)	Effect	Coefficient	*t* value	*P* value
Constant					532.63	40.12	0
Glucose	X_1	12	18	−105.1	−52.55	−3.96	0.011*
Soluble starch	X_2	20	30	−4.14	−2.07	−0.16	0.882
Dextrin	X_3	10	15	8.65	4.33	0.33	0.758
Soybean meal	X_4	12	18	131.1	65.55	4.94	0.004*
Peptone	X_5	20	30	62.01	31	2.34	0.067
Asparagine	X_6	1	2	86.82	43.41	3.27	0.022*
Casein	X_7	5	7.5	42.67	21.33	1.61	0.169
n-Dodecane	X_8	4	6	90.81	45.4	3.42	0.019*
$(NH_4)_2Fe(SO_4)_2$	X_9	2	4	18.27	9.13	0.69	0.522
Yeast extract	X_{10}	10	15	37.69	18.84	1.42	0.215
$MgSO_4$	X_{11}	1	2	41.41	20.7	1.56	0.18
$CaCO_3$	X_{12}	5	7.5	−10.36	−5.18	−0.39	0.712
$MnCl_2$	X_{13}	0.5	1	24.59	12.3	0.93	0.397
K_2SO_4	X_{14}	6	9	−48.91	−24.45	−1.84	0.125

$R^2 = 0.9411$; R^2 (Adj) = 0.7763

* Significant at 95 % confidence degree ($P < 0.05$)

Table 2 Placket–Burman design variables (in coded levels) with daptomycin as response

Run	Coded values														Daptomycin (mg/L)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	Observed	Predicted
1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	495.99	512.87
2	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	393.30	414.89
3	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	548.04	510.73
4	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	693.12	718.12
5	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	569.70	590.95
6	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	324.89	303.29
7	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	728.52	724.63
8	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	589.22	564.22
9	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	428.91	429.73
10	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	494.53	536.56
11	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	419.97	444.63
12	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	578.60	598.55
13	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	699.16	682.62
14	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	588.78	587.96
15	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	380.41	443.20
16	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	452.59	407.16
17	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	706.30	702.07
18	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	345.36	344.88
19	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	643.44	580.65
20	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	571.73	554.85

meal was also known to play a major role by supplying various amino acids and cofactors. As one of amino acids, addition of asparagine could increase the precursors for daptomycin production, which may account for the positive effect. For the oxygen vector *n*-dodecane, the results showed that dissolved oxygen plays an important role for the synthesis of daptomycin, as the research results of Wang [8].

Table 3 Design and results of the steepest ascent experiment

Run	Variables				Daptomycin (g/L)
	Glucose	Soybean meal	Asparagine	*n*-Dodecane	
Center point	15	15	1.5	5	669.41
1	13.5	16.2	1.75	5.5	735.33
2	12	17.4	2	6	789.06
3	10.5	18.6	2.25	6.5	873.15
4	9	19.8	2.5	7	946.68
5	7.5	21	2.75	7.5	881.47
6	6	22.2	3	8	731.37
7	4.5	23.4	3.25	8.5	623.89

Table 4 Central composite design with four independent variables for the experimental and predicted results

Run	Coded level				Real level				Daptomycin (mg/L)	
	X_1	X_2	X_3	X_4	x_1	x_2	x_3	x_4	Observed	Predicted
1	−1	−1	−1	−1	7	18	1.5	6	765.51	765.91
2	1	−1	−1	−1	11	18	1.5	6	865.92	871.64
3	−1	1	−1	−1	7	22	1.5	6	817.20	812.46
4	1	1	−1	−1	11	22	1.5	6	936.05	938.58
5	−1	−1	1	−1	7	18	3.5	6	769.09	787.58
6	1	−1	1	−1	11	18	3.5	6	793.55	815.39
7	−1	1	1	−1	7	22	3.5	6	825.68	834.64
8	1	1	1	−1	11	22	3.5	6	898.71	882.84
9	−1	−1	−1	1	7	18	1.5	8	753.24	769.07
10	1	−1	−1	1	11	18	1.5	8	803.75	814.39
11	−1	1	−1	1	7	22	1.5	8	851.03	848.78
12	1	1	−1	1	11	22	1.5	8	933.01	914.49
13	−1	−1	1	1	7	18	3.5	8	849.94	867.01
14	1	−1	1	1	11	18	3.5	8	829.71	834.41
15	−1	1	1	1	7	22	3.5	8	953.00	947.24
16	1	1	1	1	11	22	3.5	8	915.83	935.03
17	−2	0	0	0	5	20	2.5	7	806.52	792.31
18	2	0	0	0	13	20	2.5	7	891.18	885.83
19	0	−2	0	0	9	16	2.5	7	821.51	783.94
20	0	2	0	0	9	24	2.5	7	913.10	931.11
21	0	0	−2	0	9	20	0.5	7	788.19	793.17
22	0	0	2	0	9	20	4.5	7	859.91	835.38
23	0	0	0	−2	9	20	2.5	5	880.21	871.32
24	0	0	0	2	9	20	2.5	9	937.34	926.67
25	0	0	0	0	9	20	2.5	7	967.73	982.04
26	0	0	0	0	9	20	2.5	7	989.61	982.04
27	0	0	0	0	9	20	2.5	7	980.00	982.04
28	0	0	0	0	9	20	2.5	7	992.31	982.04
29	0	0	0	0	9	20	2.5	7	965.19	982.04
30	0	0	0	0	9	20	2.5	7	997.39	982.04

3.2 The Path of Steepest Ascent

The path of steepest ascent was determined by Eq. (1). The coefficients of the X_4, X_6 and X_8 in the refined model were positive, which indicated a positive impact on daptomycin production. The X_1 was negative and generated an opposite effect. Table 3 exhibits the results of the experiment and the directions which the variables

changed. The concentrations of the other factors were fixed at the center of the fractional factorial design, because they were not significant at the probability level of 95 %. Regarding the results from the path of steepest ascent, it was clearly seen that the daptomycin concentration profile showed a maximum for the run four (Table 3). Consequently, this point was chosen for further optimization.

3.3 CCD and RSM

The optimum levels of significant independent variables (glucose, soybean meal, asparagine and *n*-dodecane) were determined based on the above results by CCD. The CCD with four-factor and five-level, including six replicates at the center point, was used for fitting a second-order response surface. The design matrix and the corresponding experimental data are presented in Table 4. The results were analyzed by standard analysis of variance (ANOVA), and the following quadratic regression equations were obtained in terms of daptomycin production (Table 5). Using the designed experimental data, the polynomial model for daptomycin yield Y was regressed by only considering the significant terms and is shown below:

$$\begin{aligned} Y = {} & 982.03 + 23.38A + 36.79B + 10.55C + 13.84D \\ & + 5.10AB - 19.48AC - 15.10AD + 0.13BC \\ & + 8.29BD + 19.07CD - 35.74A^2 - 31.13B^2 \\ & - 41.94C^2 - 20.76D^2 \end{aligned} \quad (2)$$

where Y was the predicted response, A, B, C, D were coded values of glucose, soybean meal, asparagine and *n*-dodecane, respectively.

As shown in Table 5, the model F value was 26.82 and the F value for lack of fit was 3.04. The F value and nonsignificant lack of fit indicated that the model was a good fit. The P values of the model (<0.0001) and the lack of fit (0.1155) also suggested that the obtained experimental data were good fit with the model. The fit of the model was also checked by determination of coefficient (R^2). The value of determination coefficient $R^2 = 0.9616$ for daptomycin yield, indicating that about 96.16 % of the total variations could be explained by the model. This value indicated that the accuracy and general ability of the polynomial model was good. The adjusted determination coefficient (Adj $R^2 = 0.9257$) could be used to verify the significance of the model. Analysis of the response trends using the model was considered to be reasonable.

Table 5 Results of regression analysis and analysis of variance (ANOVA) for optimization of daptomycin in the central composite design

Variable	Sum of squares	Df	Mean square	F value	P value Prob >F
Model	158,167.5	14	11,297.68	26.82468	<0.0001*
A-Glucose	13,119.36	1	13,119.36	31.15	<0.0001*
B-Soybean meal	32,485.13	1	32,485.13	77.13119	<0.0001*
C-Asparagine	2671.851	1	2671.851	6.34392	0.0236**
D-*n*-Dodecane	4594.05	1	4594.05	10.9079	0.0048*
AB	415.6502	1	415.6502	0.986901	0.3363
AC	6070.825	1	6070.825	14.41429	0.0018*
AD	3649.972	1	3649.972	8.666326	0.0101**
BC	0.267289	1	0.267289	0.000635	0.9802
BD	1100.017	1	1100.017	2.611829	0.1269
CD	5817.647	1	5817.647	13.81315	0.0021*
A^2	35,039.41	1	35,039.41	83.19597	<0.0001*
B^2	26,576.7	1	26,576.7	63.10248	<0.0001*
C^2	48,247.49	1	48,247.49	114.5566	<0.0001*
D^2	11,821.91	1	11,821.91	28.06939	<0.0001*
Residual	6317.508	15	421.1672		
Lack of fit	5426.245	10	542.6245	3.044134	0.1155
Pure error	891.2625	5	178.2525		
Cor total	164,485	29			

$R^2 = 0.9616$; R^2 (Adj) = 0.9257

* Significant at 99 % confidence degree ($P < 0.01$)

** Significant at 95 % confidence degree ($P < 0.05$)

The model coefficients calculated by regression analysis for each variable are also given in Table 5. The P value <0.05 indicates that the model terms were significant. In this case, the A, B, C, D, AC, AD, CD, A^2, B^2, C^2, D^2 were significant model terms. They were proved to have important effects for daptomycin production.

The three-dimensional response surfaces plot is shown in Fig. 1. There was a clear elongated hill running along the concentration axis on the plot of the three-dimensional response surfaces of the quadratic model (Fig. 1b, c, e, f), which indicated a significant interactive effect on daptomycin between the two independent variables. On the other hand, three-dimensional response surface of glucose and soybean meal, soybean and asparagine had a slightly circular nature, suggesting that their interactive effects were not significant (Fig. 1a, d) [4, 9, 10].

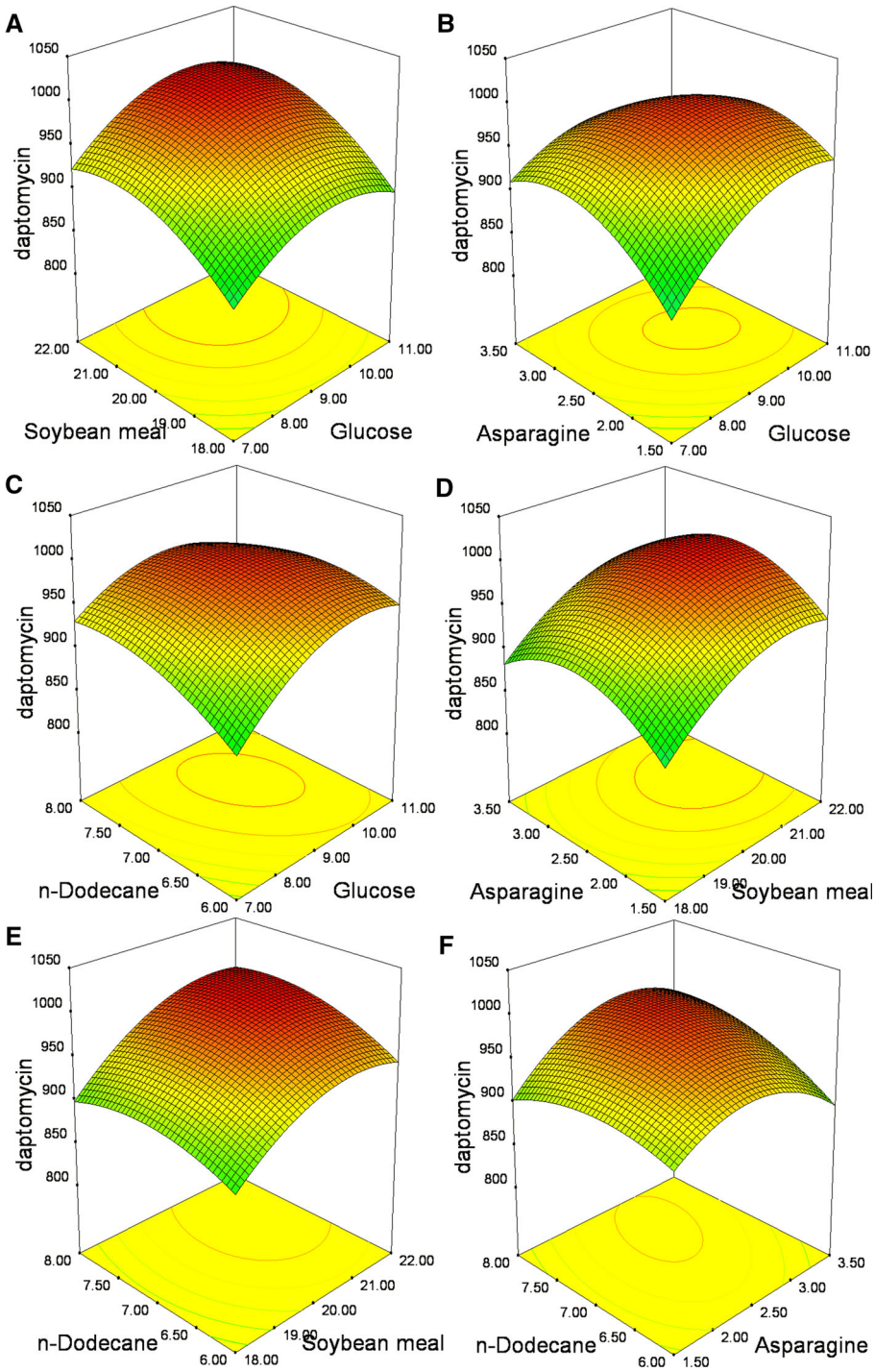

Fig. 1 Response surface plot for daptomycin production by *S. roseosporus*. **a** Effects of glucose and soybean meal, **b** effects of glucose and asparagine, **c** effects of glucose and *n*-dodecane, **d** effects of soybean meal and asparagine, **e** effects of soybean meal and *n*-dodecane, **f** effects of asparagine and *n*-dodecane. Each figure shows the effect of two variables on the production of daptomycin, while other two variables were held at zero level

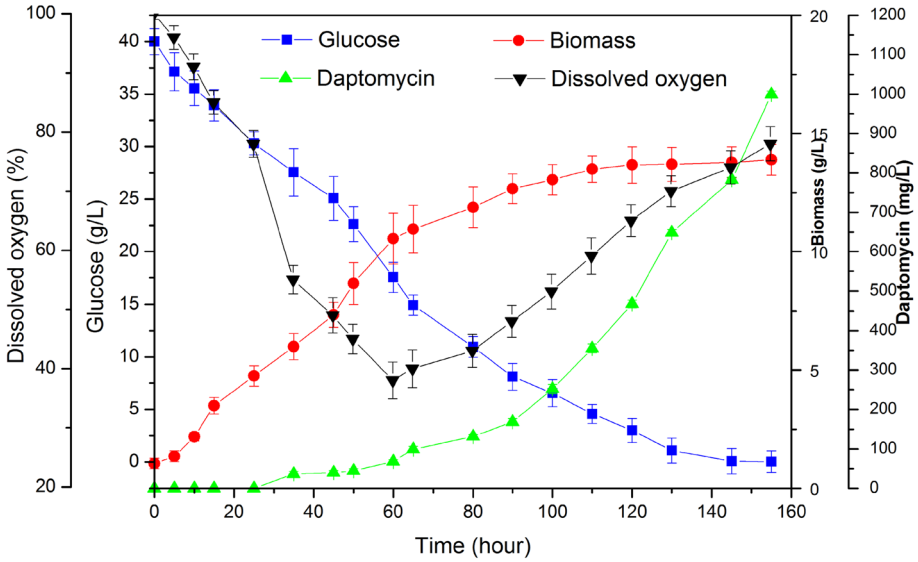

Fig. 2 Time course of batch fermentation in a 7.5-L bioreactor for daptomycin production by *S. roseosporus* GC-63 with the optimized medium

According to the canonical analysis, the results predicted by the model showed that the maximum daptomycin production could be achieved when the glucose, soybean meal, asparagine and *n*-dodecane were set at 9.46, 21.34, 2.68 g/L and 7.47 % (v/v), respectively. The maximum predicted value of daptomycin yield obtained was 1001.24 mg/L.

3.4 Experimental Validation of the Optimized Condition

In order to verify the model adequacy for predicting the maximum daptomycin production, three verification experiments in shake flasks under this optimum medium composition were performed. The mean value of daptomycin concentration was 918.87 mg/L, which was coincident with the predicted value (1001.24 mg/L), indicating that the model was proved to be adequate.

After optimization of medium components, scale-up studies were carried out in a 7.5-L laboratory bioreactor. The batch-fermentation profile of daptomycin production, including reducing sugar, DWC and dissolved oxygen concentration, with the optimized medium is shown in Fig. 2. And the daptomycin yield reached 979.36 mg/L. The ranges of DWC were from 1.35 g/L at the lag phase to 13.89 g/L at the end of the exponential phase. The reducing sugar decreased to 0.1–0.2 % when the cells reached the stationary phase. The results indicated that the optimum medium components obtained by statistical experimental design in shake flasks were equally effective to the overproduction of daptomycin by bioreactor fermentation. The addition of *n*-dodecane enhanced the dissolved oxygen with the constant agitation speed, which enhanced the cell growth and daptomycin biosynthesis.

4 Conclusions

Response surface methodology (RSM) was employed to enhance daptomycin production by a mutant strain GC-63 of *S. roseosporus* NRRL 11379. The glucose, soybean meal, asparagine and *n*-dodecane were screened to significantly influence the daptomycin production. The predicted optimized concentration of glucose, soybean meal, asparagine and *n*-dodecane were 9.46, 21.34, 2.68 g/L and 7.47 % (v/v), respectively, by CCD. Furthermore, the validation experiments were carried out to prove the adequacy and the accuracy of the model, and the maximum daptomycin concentration reached 979.36 mg/L which was nearly 2.2-fold higher compared to that in the basal medium, with predicted optimal medium concentrations in a 7.5-L fermentor. The study had certain guiding significance for improvement of antibiotic production in other *Streptomyces*.

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Compliance with Ethical Standards

Conflict of interest The author declared that no potential conflict of interest existed in this study.

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