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Optimisation of Hydrocortisone Production by *Curvularia lunata*

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Abstract A new method for breeding the hydrocortisone overproducing strain *Curvularia lunata* by screening ketoconazole-resistance mutant was developed. A hydrocortisone overproducing mutant *C. lunata* KA-91 with ketoconazole-resistance marker was obtained from protoplasts treated with ultraviolet radiation. The hydrocortisone conversion rate of *C. lunata* KA-91 was increased by 42.1% compared to the original strain CL-114 at the substrate 17α-hydroxypregn-4-en-3, 20-dione-21-acetate addition concentration of 1.0 g/L. The by-products produced by KA-91 were fewer than those of the original strain. It was assumed that the higher cytochrome P450 content of ketoconazole-resistance mutant resulted in the increase of 11β-hydroxylation capacity. The culture conditions for biotransformation of 17α-hydroxypregn-4-en-3, 20-dione-21-acetate to hydrocortisone were optimized by response surface methodology. Plackett–Burman design was applied to elucidate the key factors affecting the hydrocortisone production, and the results indicated that glucose, initial pH, and glucose to total nitrogen sources ratio (ω) had significant effects on hydrocortisone production. Box–Behnken design was employed to search for the optimal parameters of those three key factors. According to the model, the trial checking at the optimal conditions showed a high hydrocortisone conversion rate of 82.67%.

Keywords *Curvularia lunata* · Cytochrome P450 · Hydrocortisone
Ketoconazole-resistance screening · Optimization · Response surface methodology

Nomenclature

HCCR	hydrocortisone conversion rate (%)
RT	retain time (min)

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Y experimental response of Plackett–Burman design (HCCR, %)
Z experimental response of Box–Behnken design (HCCR, %)
x_i coded value of the variable X_i
X_i variable
X_0 value of X_i at the center point
X step change

Greek Symbols

α_i linear coefficient
α_0 intercept of α_i
β_i linear coefficients
β_{ii} squared coefficients
β_{ij} interaction coefficients
β_0 intercept of β_i
ω glucose to total nitrogen sources ratio

Introduction

Among the microbiological transformations of steroids, the 11β-hydroxylation of 17α-hydroxypregn-4-en-3, 20-dione (Reichstein's substance S, RS) or 17α-hydroxypregn-4-en-3, 20-dione-21-acetate (RSA) to hydrocortisone is one of the most important reactions. It is a direct approach to produce hydrocortisone (11β-OH-S, HC), which is not only a matured medical steroid but also the starting substrate for the manufacturing of several other potential steroids, such as prednisolone (i.e., 1-dehydrocortisol) [1–3]. Among the strains used for 11β-hydroxylation, a fungus *Curvularia lunata* is a very important strain [4, 5].

During an 11β-hydroxylation reaction, there are many undesirable by-products which are similar to the main product hydrocortisone, such as 11α-OH-S, 14α-OH-S, 7α-OH-S, 20β-OH-S, and 6β-OH-S, etc., which greatly decrease the economic efficiency of the production. To increase the hydrocortisone conversion rate (HCCR), elimination of by-products using *C. lunata* clone regenerated from protoplasts mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was proved efficiently [3].

Classical physical and chemical mutation methods in strain improvement for steroid biotransformations were proved to be effective approaches. However, the classical mutation without the help of resistant mutation selection is random and with no direction, which decreases the screening efficiency. Although there are several successful examples of combing classical mutation and resistant selection method in *Streptomyces* species improvement [6, 7], reports about the resistant mutation selection for steroid biotransformation are rarely seen in literatures.

Cytochrome P450 is the key component of steroid 11β-hydroxylation systems, which is responsible for the oxygen insertion into the steroid substrate molecule [8, 9]. Ketoconazole, which belongs to the azole fungicides, is a well-known cytochrome P450 inhibitor. With the wide use of azoles, there are increasing reports which show that some strains are resistant to ketoconazole and other azoles. One of the reasons for azole-resistance occurrence is the cytochrome P450 gene point mutation, which decreases the affinity between cytochrome P450 and azoles [10–12]. Another reason is the over-expression of cytochrome P450 [13, 14]. Based on the later reason, it might be possible to increase the hydrocortisone production by means of screening the cytochrome P450 overexpression mutant with the property of azole resistance.

Being an experimental strategy for seeking the optimal conditions for a multivariable system, response surface methodology (RSM) is a very efficient tool compared to the one-factor-at-a-time approach [15]. RSM has been successfully applied to optimize the culture conditions in several fermentation systems [16–19].

In this study, the ketoconazole-resistance screening method was adopted to improve the hydrocortisone biotransformation ability of *C. lunata* for the first time. Comparison of cytochrome P450 content between the mutants and the parent strain was investigated. In our previous paper [20], we reported the effects of carbon sources, nitrogen sources, initial pH, and metal ions on hydrocortisone production from *C. lunata* using the one-factor-at-a-time methodology. To further investigate the effects of culture conditions on the improvement of HCCR by the hydrocortisone high-producing strain, Plackett–Burman design and Box–Behnken design were employed in this paper.

Materials and Methods

Microorganism and Medium

C. lunata CL-114 without ketoconazole-resistance marker was isolated from soil and maintained on potato-dextrose-agar slant: glucose (4 g) and agar (4 g), dissolved in 200 ml 20% (*w*/*v*) potato decoction, with an initial pH 6.8. The fermentation medium for hydrocortisone production was as follows: glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L, and soybean powder 10 g/L, with initial pH 6.5. The protoplast regeneration medium was as follows: glucose 10 g/L, yeast extract 4 g/L, agar 15 g/L, and KCl 0.6 mol/L, dissolved in 6° Bx malt extract juice, with initial pH 6.5.

Protoplast Preparation

An improved protoplast preparation method was adopted [21].

Minimum Inhibition Concentration of Ketoconazole

0.1 ml protoplast suspension (1×10^6 spores/ml) was transferred to a petri dish with protoplast regeneration medium containing different dosages of ketoconazole. After 5 days of incubation at 28 °C, the hyphae and colonies of fungi could be observed. The minimum concentration at which the fungi could not grow was the minimum inhibition concentration (MIC) of ketoconazole.

Ultraviolet Radiation Treatment

A 10-ml protoplast suspension (1×10^6 spores/ml) was added into a petri dish (ϕ75 mm I.D.) and irradiated by ultraviolet (UV) (wavelength 253.7 nm, power 15 W, and radiation length 30 cm) for 15 to 115 s.

Protoplast Regeneration and Ketoconazole-Resistance Mutant Strain Isolation

The irradiated protoplast suspension was centrifuged, washed with 0.6 mol/L KCl, and then diluted in 0.6 mol/L KCl. A 0.1-ml protoplast suspension was transferred to a petri dish with regeneration medium containing different dosages of ketoconazole. After 5 days of

incubation at 28 °C under darkness condition, the spores or hyphae separated from the single colony were inoculated into the slants for conservation. These colonies tested the capacity of hydrocortisone conversion, and the nonirradiation parent strain was used as the control culture at the same conditions. Finally, the mutants whose hydrocortisone conversion potential was beyond their parent strain were defined as the positive mutant. The positive mutation rate for each irradiation condition was calculated as the number of the positive mutant strains divided by the number of colonies on the regeneration medium plate.

Cultivation Conditions

A 1-ml spore suspension with the concentration of 3×10^6 spores/ml was inoculated into a 250-ml Erlenmeyer flask containing 30 ml fermentation medium and incubated at 28 °C and 180 rpm. The pH of broth was adjusted to 6.5 with 10% NaOH or 6 mol/L HCl at 24 h, and then 1.0 g/L RSA dissolved in 80% (*v*/*v*) ethanol was fed in.

Cytochrome P450 Content Determination

The cytochrome P450 content was determined by the reduced carbon monoxide difference spectrum method [22] with a modification [8]. A 1-g wet mycelium was used to prepare protoplasts. Protoplasts, suspended in 5 ml buffer containing 0.1 mol/L Tris–HCl (pH 7.5), 0.25 mol/L glucose, 1.0 mmol/L KCl, 10% (*v*/*v*) glycerol, and 5 mmol/L DTT were counted. A 0.2-ml antimycin A solution containing 100 μg/ml antimycin, 1.0 mmol/L KCl, and 0.1 mol/L Tris–HCl (pH 7.5) was added into the protoplast suspension and was mixed. The protoplast suspension was poured averagely into two 2-ml vessels and then incubated on ice for 15 min. One of the vessels, with carbon monoxide bubbling for 5 min, was considered as the sample, whereas the other one without carbon monoxide bubbling was regarded as the control. The UV spectra of two suspensions were recorded by a 756 MC spectrophotometer (optical length of 1 cm) with the scan wavelength from 400 to 500 nm. The carbon-monoxide difference spectra was obtained, and the cytochrome P450 content was calculated by Eq. 1:

$$\mathrm{P450(mmol/L)} = \frac{A_{450} - A_{490}}{91} \tag{1}$$

where A_{450} and A_{490} are the absorbances at 450 and 490 nm, respectively.

Product Analytical Process

An improved high-performance liquid chromatography (HPLC) analysis method was used to determine the products [23]. The HCCR was the molar ratio of the product hydrocortisone to the substrate RSA.

Experimental Design and Statistical Analysis

Plackett–Burman Design

Plackett–Burman design was employed to determine the key factors affecting hydrocortisone production, and it was used based on the following first-order model:

$$Y = \alpha_0 + \sum \alpha_i X_{i\ (i=1,2,3,\ldots,)} \tag{2}$$

where Y is the experimental response (HCCR, %), α_0 is the intercept, α_i is the linear coefficient, and X_i is the variable. This model does not consider the interaction effects among the variables. The design is shown in Table 1. Seven variables were investigated in 12 experiments with one dummy variable. Each variable was represented in two levels, namely, a high level designated by (+) and a low level designated by (−). All experiments were performed three times, and the average of HCCR was taken as the Y. The variables whose confidence levels were greater than 90% were considered to have significant influence on hydrocortisone production.

Box–Behnken Design

Box–Behnken design was used to optimize the value of the key factors obtained from the above Plackett–Burman design. The independent variables were coded according to Eq. 3:

$$x_i = \frac{(X_i - X_0)}{X} (i = 1, 2, 3, \ldots j) \tag{3}$$

where x_i is the coded value of the variable X_i, X_0 is the value of X_i at the center point, and ΔX is the step change. Each variable was studied at three different levels (−1, 0, and +1). In the experiments, the HCCR was taken as the experimental response denoted as Z. The mathematical relationship of Z and the coded value X_i of the independent variable X_i were denoted by the quadratic model equation Eq. 4:

$$Z = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 \sum \beta_{ij} x_i x_j \tag{4}$$

where Z is the experimental response (HCCR, %), β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the squared coefficient, and β_{ij} is the interaction coefficient. The RSM was developed using the Design Expert System (version 5.0.3, Stat-Ease Inc., Minneapolis, MN

Table 1 Plackett–Burman design matrix and experimental results for hydrocortisone production by *Curvularia lunata* KA-91.

Run no.	Variables/levels[a]								HCCR (%)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	
1	1	−1	1	−1	−1	−1	1	1	58.5±0.02
2	1	1	−1	1	−1	−1	−1	1	68.2±0.01
3	−1	1	1	−1	1	−1	−1	−1	59.4±0.02
4	1	−1	1	1	−1	1	−1	−1	61.3±0.01
5	1	1	−1	1	1	−1	1	−1	62.0±0.02
6	1	1	1	−1	1	1	−1	1	64.4±0.01
7	−1	1	1	1	−1	1	1	−1	60.9±0.02
8	−1	−1	1	1	1	−1	1	1	54.4±0.02
9	−1	−1	−1	1	1	1	−1	1	53.5±0.01
10	1	−1	−1	−1	1	1	1	−1	58.6±0.02
11	−1	1	−1	−1	−1	1	1	1	50.3±0.01
12	−1	−1	−1	−1	−1	−1	−1	−1	48.7±0.03

[a] X_1, Glucose at a high level of 25 g/L and at a low level of 20 g/L; X_2, initial pH at a high level of 7.5 and at a low level of 6.5; X_3, inoculum size at a high level of 12.5% (*v*/*v*) and at a low level of 10% (*v*/*v*); X_4, ω at a high level of 1.25:1 and at a low level of 1:1; X_5, dummy variable; X_6, yeast extract at a high level of 6.25 g/L and at a low level of 5 g/L; X_7, peptone at a high level of 6.25 g/L and at a low level of 5 g/L; X_8, soybean meal at a high level of 12.5 g/L and at a low level of 10 g/L

55413, USA) to obtain optimal working parameters and generate response surface graphs. The data were analyzed by SAS software [24].

Statistics

All experiments were repeated three times. The data shown in the corresponding tables and figures of the "Results and Discussion" section were the mean values of the experiments, and it was indicated that the relative standard deviations of HCCR and P450 contents in protoplasts were all within ±3%.

Results and Discussion

The Screening of Ketoconazole-Resistance Mutant Strain

It was found that the ketoconazole MIC to *C. lunata* CL-114 was 0.03 mmol/L. The positive mutation rate reached to the highest 18.8% level when UV radiation time was 75 s. Thus, 75 s was selected as the optimal radiation time. One-hundred thirty-six isolates of ketoconazole-resistance mutant strains were screened out on the plate containing 0.03 mmol/L ketoconazole. Fourteen strains, which have relative higher hydrocortisone production ability, are shown in Table 2. As shown in Table 2, among these mutant strains, strain KA-91 was the most effective with an HCCR of 68.05% without optimizing the culture conditions. These positive mutants were continuously transferred through 10 generations from one nutrient agar slant to the next, and the strains of each generation were tested for HCCR, colony characteristics, and cell conformation in the same culture. The results showed that KA-91 was stable in continuous transfers (dry cell concentration of 12.01–12.24 g/L, HCCR of 67.37–68.73%). The genetic stability experiments of these 14 mutants indicated that KA-91 showed higher genetic stability than other mutants. Thus, KA-91 was selected as the optimal strain for further experiments. It was interesting that the

Table 2 The results of mutation experiments.

Strains	HCCR (%)	P450 content in protoplasts (10^{-3} mmol·L^{-1}/10^{8} protoplasts)	MIC of ketoconazole (10^{-3} mmol/L)
CL-114	47.89±0.01	7.39±0.00	30±0.00
KA-05	54.60±0.02	16.29±0.02	82±0.01
KA-08	66.09±0.01	32.21±0.01	118±0.00
KA-18	51.72±0.03	13.89±0.03	80±0.01
KA-26	56.03±0.01	18.22±0.02	78±0.01
KA-29	57.47±0.03	20.01±0.03	102±0.01
KA-35	62.74±0.02	30.02±0.02	126±0.01
KA-44	60.83±0.01	27.22±0.02	76±0.01
KA-49	56.99±0.03	16.33±0.03	78±0.00
KA-57	58.90±0.01	19.89±0.02	116±0.00
KA-64	66.57±0.02	34.44±0.01	122±0.01
KA-73	59.86±0.01	21.23±0.02	96±0.01
KA-80	59.38±0.01	21.01±0.02	126±0.00
KA-91	68.05±0.01	36.67±0.01	144±0.01
KA-112	60.34±0.01	26.17±0.02	122±0.01

ketoconazole MIC of mutant strain with higher HCCR was higher than for the original strain CL-114.

It was found from the screening experiments that the 11β-hydroxylation ability of the mutant strains was related to several factors, such as clone morpha, spore color, spore amount, and mycelium growth speed. The experiments showed that the strains with higher HCCR have similar clone characteristics as follows. Firstly, clone morpha was surface flat, floss-shaped, and spread around. Secondly, the mycelium color varied from white to dark green during growth. Thirdly, the spores were in puce and abundance. Moreover, finally,

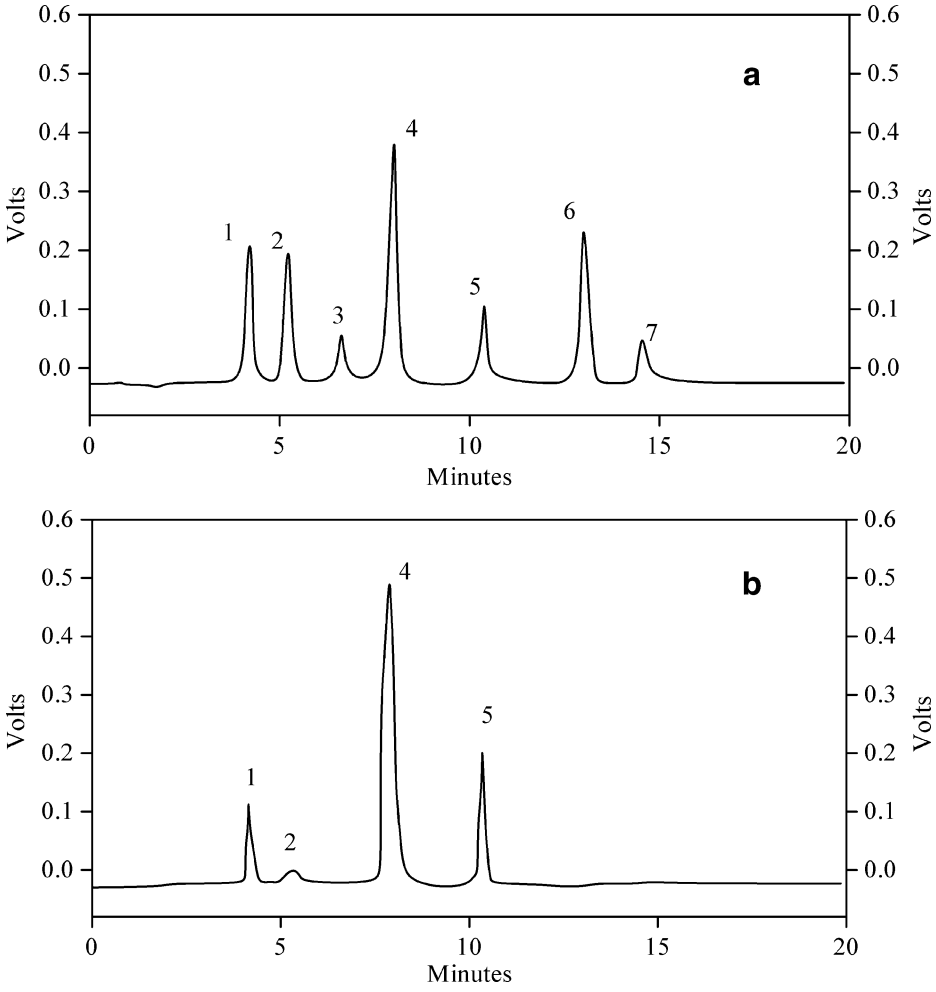

Fig. 1 HPLC chromatography of steroids produced by the parent strain **(a)** and the mutant strain **(b)** of *Curvularia lunata*. *1* RSA. *2* RS. *3* Not identified. *4* 11β-OH-S. *5* 6β-OH-S. *6* 11α-OH-S. *7* 14α-OH-S. The retention time (minute) of the peaks for chromatography **(a)** and **(b)** was as follows [the data in parentheses for chromatography **(b)**]: *1*, 4.213 (4.205); *2*, 5.405 (5.437); *3*, 6.822; *4*, 8.056 (7.998); *5*, 10.734 (10.772); *6*, 13.015; *7*, 14.568. The peak integral area for chromatography **(a)** and **(b)** was as follows [the data in parentheses for chromatography **(b)**]: *1*, 11.86 (9.87); *2*, 10.11 (5.89); *3*, 5.03; *4*, 35.97 (70.12); *5*, 11.44 (13.69); *6*, 20.01; *7*, 4.88

the mycelium growth speed was slow. This result was beneficial for screening the excellent mutant strain apparently.

The mutation of *C. lunata* CL-114 conidia and hyphal fragments by UV radiation was proved ineffective in our research. The possible reason might be that the wall of conidia was thick and insensitive to mutagenic treatment. Furthermore, the multinucleate conidia and hyphal fragments could result in mixed cultures, which were unsuitable for stable mutant strain selection, as well as in the loss of the potential mutant strains by the "rescue" repair of the DNA from undamaged nuclei [3]. By treatment of the uninucleate fractions of *C. lunata* protoplasts with UV, the desired mutant strains could be obtained with a relative high frequency.

The 11β-Hydroxylation of Mutant Strain and Parent Strain

For the parent strain CL-114, besides the 11β-OH-S, several other biotransformation products were formed, among which RS, 11α-OH-S, 14α-OH-S, and 6 β-OH-S have been recognized. Figure 1 shows the HPLC chromatograms of the steroids produced by the parent strain and the mutant strain KA-91. It was found that the original strain transformed RSA multidirectionally compared to the mutant strains. It was showed that when KA-91 was used, some of the by-products, such as 11α-OH-S and 14α-OH-S, disappeared. It is interesting that the HPLC profile pattern in other resistant strains is similar with that of KA-91.

Cytochrome P450 Content of Parent Strain and Mutant Strain

It is well known that cytochrome P450 acts as the key component in the 11β-hydroxylation of steroid. Our previous experiments have also proved that the substrate RSA could induce the expression of endoenzyme cytochrome P450, and the cytochrome P450 content has positive relativity to HCCR [25].

The cytochrome P450 content of ketoconazole-resistance mutant strains including KA-91 were detected, as shown in Table 2. It was shown that the mutant strains with higher HCCR had higher cytochrome P450 content. The data suggested that the increase in 11β-hydroxylation ability of mutant strains might be the result of the overexpression of cytochrome P450. Paraszkiewicz and Długonski [8] found that the ketoconazole-resistance strain with higher hydrocortisone conversion ability had higher cytochrome P450 content than the wild strain, and they believed that the higher amount of cytochrome P450 and higher steroid biotransformation ability of the mutant strain resulted from mutation(s) of genes involved in steroid 11β-hydroxylase synthesis regulation. In our experiments, a more efficient approach to screen the mutant strain with high 11β-hydroxylation ability by means of ketoconazole-resistance mutation was developed.

Screening of Culture Conditions for Hydrocortisone Production by Plackett–Burman Design

Based on our previous research [20], the relative important factors including $X_{1(\text{glucose})}$, $X_{2(\text{initial pH})}$, $X_{3(\text{inoculum size})}$, $X_{4(\omega)}$, $X_{6(\text{yeast extract})}$, $X_{7(\text{peptone})}$, and $X_{8(\text{soybean meal})}$ were investigated by means of the Plackett–Burman design. The design and the experimental results of the Plackett–Burman design are illustrated in Table 1. The analysis of variables and their effect estimates are shown in Table 3. Three conclusions could be drawn as follows: (1) variables including $X_{6(\text{yeast extract})}$, $X_{7(\text{peptone})}$, and $X_{8(\text{soybean meal})}$, as well as $X_{3(\text{inoculum size})}$ did not affect hydrocortisone production obviously, suggesting that their low settings (−)

Table 3 Analysis of variance from the Plackett–Burman design experiments for hydrocortisone production by the mutant strain KA-91.

Variable	Estimate	t	$P > \|t\|$	Confidence level (%)[a]
X_1	6.27	4.7732	0.0175	*
X_2	4.73	3.6052	0.0366	*
X_3	2.17	1.6503	0.1975	
X_4	4.57	3.4783	0.0401	*
X_6	−0.17	−0.1270	0.9070	
X_7	−0.5	−0.3808	0.7287	
X_8	0.17	0.5840	0.6003	

[a] Significant levels of regression coefficient are denoted by (*) 90% by t test.

were appropriate; (2) the confidence levels of variables $X_{1(\text{glucose})}$, $X_{2(\text{initial pH})}$, and $X_{4(\omega)}$ were above 90%, thus they were considered to have a significant influence on hydrocortisone production; and (3) the estimate of glucose, initial pH, and ω were all positive, which suggested that the increase of their value would result in the promotion of hydrocortisone production. Glucose was found to be the optimal carbon source for hydrocortisone production in our research, which was consistent with the studies of Wilmanska et al. [3] and Clark et al. [26].

Optimization of the Important Parameters Using Box–Behnken Design

The three variables $X_{1(\text{glucose})}$, $X_{2(\text{initial pH})}$, and $X_{4(\omega)}$ will be further investigated with the Box–Behnken design. The levels for X_1 were 20, 25, and 30 g/L; X_2 were 5.5, 6.5, and 7.5 for X_4; were 1:1, 2:1, and 3:1 respectively. The total number of trials needed for this design was 15, which was fewer than those of the central composite design or the 33 factorial designs. Among the 15 experiments, three variables were at their central coded values [27].

Table 4 Box–Behnken design matrix and experimental results of three independent variables for hydrocortisone production by the mutant strain KA-91.

Run no.	Glucose		Initial pH				*HCCR* (%)
	X_1	Code x_1	X_2	Code x_2	X_4	Code x_4	
1	20.0	−1	5.5	−1	1	0	52.6±0.01
2	20.0	−1	7.5	1	3	0	50.7±0.02
3	30.0	1	5.5	−1	3	0	56.6±0.01
4	30.0	1	7.5	1	3	0	68.9±0.00
5	25.0	0	5.5	−1	1	−1	70.1±0.01
6	25.0	0	5.5	−1	1	1	72.4±0.01
7	25.0	0	7.5	1	3	−1	75.6±0.02
8	25.0	0	7.5	1	3	1	78.4±0.03
9	20.0	−1	6.5	0	1	−1	70.3±0.02
10	30.0	1	6.5	0	2	−1	78.7±0.02
11	20.0	−1	6.5	0	2	1	68.5±0.01
12	30.0	1	6.5	0	2	1	74.4±0.02
13	25.0	0	6.5	0	2	0	83.2±0.00
14	25.0	0	6.5	0	2	0	81.9±0.01
15	25.0	0	6.5	0	2	0	82.8±0.01

Table 5 Regression coefficients, standard error, t values, and parametric estimation of a full second-order polynomial model for hydrocortisone production by the mutant strain KA-91[a].

Term	Regression coefficient	Standard error	t value	Probability ($P > \|t\|$)	Parametric estimation
β_0	82.63	0.783			
β_1	4.56	0.783	5.827	0.0021	4.563
β_2	2.74	0.783	3.496	0.0174	2.738
β_3	−0.13	1.152	−0.16	0.8794	−0.125
β_{11}	−13.30	1.107	−11.534	0.0001	−13.292
β_{12}	3.55	1.107	3.206	0.0238	3.550
β_{13}	−0.63	1.152	−0.564	0.5968	−0.625
β_{22}	−12.14	1.107	−10.536	0.0001	−12.142
β_{23}	0.13	0.125	0.113	0.9145	0.125
β_{33}	3.63	1.152	3.153	0.0253	3.633

[a] The determination coefficient R^2 value is 0.9839.

The results of the Box–Behnken design, along with the mean experimental response, are presented in Table 4. The regression coefficients, standard error, parameter estimation, and t values for the second-order model are shown in Table 5. The R^2 value was 0.9839, which means that a 98.39% variation could be explained by this model, indicating that this regression model was suitable to represent the hydrocortisone production. The F value of the regression model was 34.01, which was greater than the tabular $F_{0.99}$ (9.5) value (10.16), confirming that the linearity between all the independent variable and the attributive variable was significant. Judging by the regression coefficients and t values, it might be concluded that glucose and initial pH had more significant effects on hydrocortisone production than ω, and also the interaction term had no significant effects on hydrocortisone production.

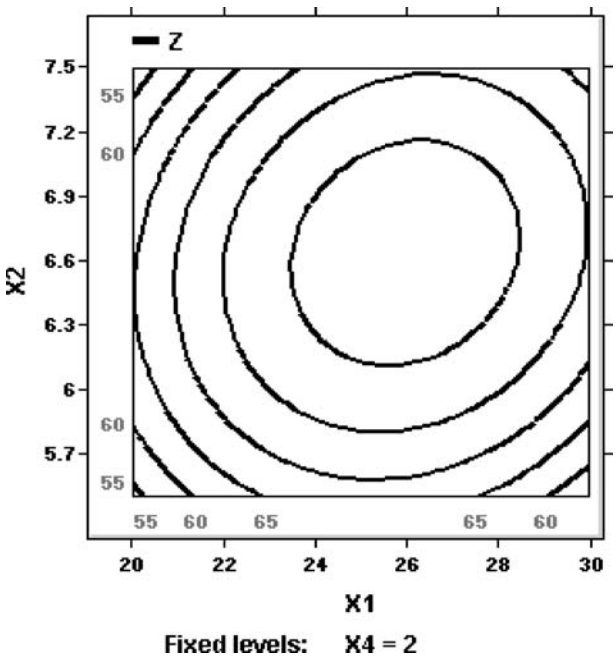

Fig. 2 Isoresponese contour plot of hydrocortisone production for X_1 (glucose) and X_2 (initial pH) when X_4 (ω) is at 2:1

The regression Eq. 5 obtained by applying multiple regression analysis on the experimental data was applied to describe the hydrocortisone production:

$$Z = 82.63 + 4.56x_1 + 2.74x_2 - 0.13x_4 - 13.3(x_1)^2 + 3.55x_1x_2 - 0.63x_1x_4 - 12.14(x_2)^2 + 0.13x_2x_4 + 3.63(x_4)^2 \quad (5)$$

where Z is the response (HCCR, %), whereas x_1, x_2, and x_4 are the coded values of $X_{1(\text{glucose})}$, $X_{2(\text{initial pH})}$, and $X_{4(\omega)}$, respectively.

The canonical analysis was a mathematical procedure used to simplify a second-order polynomial model and to decide the stationary point to be a maximum, minimum, or saddle point. One of the response surface graphs was plotted as Fig. 2. The plots indicated that linear effects were more predominant over interactive effects. During the three factors, glucose showed the highest significant effect on hydrocortisone production. The stationary points were determined to be the maximum points by canonical analysis for hydrocortisone production.

The regression Eq. 5 was solved by a design expert. The optimal values of test variables in the coded units were x_1=0.1897, x_2=−0.1406, and x_4=0.0311. At these values, the $X_{1(\text{glucose})}$, $X_{2(\text{initial pH})}$, and $X_{4(\omega)}$ were 25.95 g/L, 6.6, and 2.03, respectively. Based on the optimal ω and the ratios between nitrogen sources in initial fermentation medium, the optimal medium was obtained as follows: glucose 25.95 g/L, peptone 3.20 g/L, yeast extract 3.20 g/L, and soybean powder 6.39 g/L. This combination made an HCCR of 82.67% by repeating the experiments three times, which was less than the predicted value of 83.26% obtained from the above equation, which may due to the slight variation in experimental conditions.

Conclusions

A hydrocortisone high-producing mutant strain *C. lunata* KA-91 was obtained by means of ketoconazole-resistance screening method through protoplast treatment with UV radiation. The HCCR was increased by 42.01% compared with the parent strain CL-114. Additionally, some of the undesirable by-products disappeared when this mutant strain was used to produce hydrocortisone. It was assumed that the higher cytochrome P450 content of ketoconazole-resistance mutant strain resulted in the increase in 11β-hydroxylation capacity.

Based on the Plackett–Burman design and the Box–Behnken design of RSM, the optimal culture conditions for hydrocortisone production were obtained as follows: glucose 25.95 g/L, peptone 3.20 g/L, yeast extract 3.20 g/L, and soybean powder 6.39 g/L, with initial pH 6.6.

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