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Screening of Dhase/Dcase-producing Strain and its Optimal Fermentation Process for the Production of D-Hydroxyphenylglycine

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In the present study, 33 soil samples were collected from Longyan City, Fujian Province, and 23 strains with the capability to produce Dhase (D-hydantoinase) and Dcase (D-decarbamoylase) were screened. In addition, one strain with excellent growth status and high substrate-converting efficiency was selected by morphological observation, and physiological and biochemical identification to explore the optimal fermentation process of D-hydroxyphenyl glycine (D-HPG) as the original strain. Experimental results showed that industrial glucose and corn syrup are the most suitable carbon and nitrogen sources of the starting strain fermentation, respectively. Plackett-Burman design in response surface methodology was used to explore the effects of eight factors on fermentation efficiency. On the basis of Box-Behnken design, the optimal fermentation process of the strain include inoculum size of 3%(v/v), industrial glucose concentration of 30 g/L, corn syrup concentration of 10 g/L, inducer (DL-p-hydroxyphenylhydantoin, DL-HPH) amount of 3.0 g/L, liquid volume of 50 mL/250 mL, pH 6.0, fermentation temperature of 32.8 °C and fermentation time of 60 h. Among these factors, induction agent, fermentation pH and fermentation temperature revealed the most important impact on D-HPG fermentation, and significant effect on fermentation efficiency. Under optimal fermentation conditions, the yield of D-HPG could reach up to 0.262 g/L, which is better than or close to the strains reported at home and abroad for the production of D-HPG through fermentation. In our study, the fermentation medium is relatively cheap, and the operation is relatively simple and easy to control, which will provide the reference or theoretical basis for industrial production of D-HPG.

Key words: D-acetic acid urease, D-off carbamoyl hydrolase, D-hydroxyphenyl glycine; Aeruginosa, Response surface methodology, Optimization.

D-hydroxyphenyl glycine (D-HPG), as a white flaky solid, is an intermediate for the synthesis of beta-lactam antibiotics such as amoxicillin and cephalosporins, pesticides and peptide hormones in pharmaceutical, chemical, and chemical engineering fields[1]. Meanwhile, it also can be used as analytical reagent for silicon, phosphorus and iron as well as the application in the photosensitive field[2].

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Currently, the production methods of D-HPG at home and abroad are chemical method, enzymatic assay method and enzymatic conversion method[3-5]. Chemical method is characteristics of high reaction temperature, low yield, large energy consumption, highly toxic raw materials, and difficult post-processing and high pollution, so that it is gradually being replaced by both enzymatic methods. However, enzymatic synthesis of D-HPG has the advantages of mild reaction conditions, simple process, high specificity, high yield, low energy consumption and low pollution, which is suitable for industrial production. Therefore, in 1993, the International Biological Symposium formally proposed that

enzymatic synthesis is the most economical and effective method for the large-scale industrial production of D-HPG[6].

Enzymatic production of D-HPG is normally completed by DL-p-hydroxyphenylhydantoin (DL-HPH). DL-HPH can be converted into N-carbamoylmethyl-D-hydroxyphenylglycine (CpHPG) in the presence of D-hydantoinase (Dhase), and then hydrolyzed as D-HPG in the presence of D-decarbamoylase (Dcase). According to the types of bacteria for enzyme sources, enzymatic methods for the production of D-HPG include single bacterium and single enzyme method, dual bacteria and dual enzyme method, and single bacterium and dual enzyme method. Currently, single bacterium and dual enzyme method reveals the best fermentation efficiency, which is the hot topic in previous reports[7]. In 1979, one strain of *Agrobacterium radiobacter* is Dhase/Dcase-producing strain has been reported[8], and in 1981, this strain has been successfully used for the synthesis of D-amino acids[9].

In 2001, DL-HPH was also successfully converted into D-HPG by this strain with the theoretical yield of 100% [10]. In 2007, a strain with Dhase/Dcase was extracted from *Burkholderia cepacia* JS-02 to prepare D-HPG with theoretical yield of 94%[11]. In addition, Dhase/Dcase-producing *Agrobacterium SP.* CPU1295 and *Pseudomonas SP.* 2262 with higher enzyme activity were screened in China Pharmaceutical University and Shanxi Institute of Biology[12]. Besides above strains, Dhase/Dcase-producing *Microbacterium oxydans, Sinorhizobium morelens, Arthrobacter SP., Blastobacter SP. and halophilic Ochrobactrum SP and Delftia SP*[13-14] belong to *Agrobacterium, Pseudomonas, Arthrobacter sp, Sprouting bacilli and Colorless spp. Agrobacterium* is a genus with the most popular study[15-16].

Currently, the screening methods for strains with common and quick production of Dhase at large scale are flask-shaking cell culture method, solid plate method, microporous rapid screening method, selective medium culture method using DL-HPH as single nitrogen source, and substrate analogue resistance screening method[17-18]. Dcase-producing bacterial screening methods include ninhydrin colorimetric method, paper chromatography, and selective medium culture method using CpHPG as single nitrogen source[19]. In the present study, taking DL-HPH as the sole nitrogen source, the designed methods were used to screen positive strains with the capability to produce Dhase/Dcase from 33 soil samples. The isolated strains were identified on the basis of physiological and biochemical properties. Meanwhile, response surface methodology was used explore the optimal fermentation process conditions to prepare D-HPG using DL-HPH as the substrate, which will provide the theoretical basis for industrial production of D-HPG.

MATERIALS AND METHODS

Soil samples

Totally 33 soil samples were collected from Longyan City, Fujian Province, China.

Media

Enrichment medium (1000 mL): p-hydroxybenzoate (DL-HPH) 2 g, yeast extract 5 g, K_2HPO_4 5 g, $MgSO_4 \cdot 7H_2O$ 2 g, trisodium citrate 0.1g, pH 7.0.

Selective medium with DL-HPH as the sole nitrogen source (1000 mL): Hydroxyphenylglycine (DL-HPH) 2 g, glycerol 5 g, K_2HPO_4 3g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 1.0 g, $MnSO_4 \cdot H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.02 g, $FeSO_4 \cdot 5H_2O$ 0.02 g, $CaC1_2$ 0.02 g, actinomycetes cycloheximide 50 mg, agar 20 g, pH 7.0.

Selective medium with CpHPG as the sole nitrogen source (1000 mL): N-carbamoylmethyl-D-hydroxyphenylglycine (CpHPG) as the sole nitrogen source 2 g, the remaining ingredients similar as DL-HPH selective medium.

Common bacterial culture medium

beef extract peptone medium.

Induction medium

common bacterial culture medium with the addition of 2 g/L DL-HPH as the inducer.

Seed culture medium

glucose 20 g, corn syrup 25 g, sodium chloride 3 g, ammonium sulfate 1 g, magnesium sulfate 0.55 g, pH 7.0.

Fermentation medium (1000 mL)

DL-HPH 2.5 g, glucose 20 g, corn syrup 15 g, $(NH_4)_2SO_2$ 1 g, sodium chloride 5 g, $CoCl_2$ 0.1 g, pH 7.0.

Reagents

D-HPG and CpHPG were synthesized in our laboratory, and the rest reagents were of analytical grade or chemically pure. Biochemical identification kits were purchased from Tianhe Microbiological Reagent Co., Ltd. in Hangzhou, China.

Major instruments

SW-CJ-ID clean bench was purchased from Suzhou Purification Equipment Co., Ltd., China. ZHWY-2102 shaker was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd. China. UV-2000 UV-visible spectrophotometer was purchased from UNICO Shanghai Instrument Co., Ltd. China.

Enrichment culture and strain isolation and purification

Totally 90 mL sterile distilled water was added to 10 g soil sample. After evenly oscillation, 1 mL of solution was added to the enrichment medium for the culture at 30 °C and shaking speed of 200 r/min. Upon culture for 48 h, the gradient diluted bacterial solution was coated in selective medium with DL-HPH as the sole nitrogen source. The colony with uniform size and excellent growth status were inoculated on the common bacterial plate. Subjected to purification with several steps, a single colony with high purity was stored in common bacterial slant medium for future use.

Screening of Dhase-producing strain

Microporous rapid screening method: 0.2% substrate DL-HPH in 0.9% Tris-HCl buffer (pH 9.0) was prepared. Totally 100 µL of substrate solution was added to each microwell, and the well containing Tris-HCl without substrate was used as the control. The target positive strains were transferred to the slants of inducing medium with the culture at 30 °C for 24 h. A small amount of bacteria were inoculated into both wells. After reaction at 37 °C for 30 min, 15 mL of 100 g/L dimethylaminobenzaldehyde was added to each well for 5-10 s reaction. The strain in the well with specific yellow but colorless in the control well was identified as the positive Dhase-producing strain.

Screening of Dcase-producing strain

Preliminary screening of selective medium using CpHPG as the sole nitrogen source: Dhase-producing bacterial strains were inoculated to the preliminary screening medium, and the colonies with consistent shape, uniform size and excellent growth as well as the capability to use CpHPG as the sole nitrogen source were selected for future rescreening.

Rescreening of paper chromatography: The screening of positive strains were inoculated to inducing medium plate at 30 °C for 24 h after adding 5 mL of saline-washed bacteria through the centrifugation at 3800 r/min for 30 min. The supernatant was removed, and the bacteria were washed by normal saline. The bacteria were harvested and 500 mL of 0.9% phosphate buffer (pH 8.0) was added to dissolve the bacteria at 37 °C with shaking for 30 min. Totally 200 mL of supernatant was centrifuged with paper chromatography. Paper chromatography conditions included Xinhua No. 1 filter paper, 100 mL of loading amount, developing system with n-butanol-formic acid-water of 15:3:2, standard D-HPG, and development time of 5 h, The positive strain was identified based on the locations of standard sample.

The properties of Dhase/Dcase-producing strains

The selected Dhase/Dcase-producing positive strains were inoculated in the common bacterial culture plate and stored for future use after multiple-step purification. By morphological, physiological and biochemical identification, as well as the reference from Bergey's bacterial identification manual (8^{th} edition), the properties and number of Dhase/Dcase-producing positive strains have been confirmed. Meanwhile, the strains were selected through flask-shaking fermentation to obtain the strain with excellent growth status and the highest productivity of D-HPG as the starter strain of fermentation process investigation.

Fermentation process optimization for D-HPG production through Dhase/Dcase-producing strain

Overall experimental design

The starter strain was inoculated to seed culture medium (200 r/min, 35 °C) until logarithmic growth phase. The inoculated cell culture was transferred to fermentation cultivation medium using D-HPG yield as the evaluation index of fermentation efficiency. The effects of inoculation amount, carbon resource concentration, nitrogen resource concentration, inducer amount, loading amount, fermentation pH, fermentation temperature

and fermentation time on fermentation efficiency were explored by using Plackett-Burman design of response surface methodology, which was used to explore the optimal fermentation conditions for Dhase/Dcase-producing strains for D-HPG.

Effect of carbon source on fermentation efficiency

On the basis of the basic fermentation medium, the carbon source type was changed to explore the effect of carbon resource type on fermentation efficiency.

Effect of nitrogen source type on fermentation efficiency

On the basis of the basic fermentation medium, the nitrogen source type was changed to explore the effect of nitrogen source type on fermentation efficiency.

Plackett-Burman experimental design for key factors

Upon the screening of optimal carbon source and nitrogen source, the effects of 8 major factors on fermentation efficiency were investigated by using D-HPG yield as the response value to screen key factors from the significantly changed response value. The factors, levels and codes of Plackett-Burman experimental design were shown in Table 1.

Experimental optimization through Box-Behnken response surface design

Based on the results of Plackett-Burman experiments, the key factors with significantly changed response values were selected to conduct optimal fermentation process condition exploration through Box-Behnken response surface design.

Determination of D-HPG yield

After fermentation completion, the yield of D-HPG in fermentation broth was measured to evaluate the fermentation efficiency. The concentration of D-HPG was determined by high performance liquid chromatography [20].

RESULTS

Screening of Dhase/Dcase-producing strains

After enrichment culture, preliminary screening of selective culture medium using DL-HPH as the single nitrogen source, rapid

Table 1. Factors, levels and codes of Plackett-Burman experimental design

Factors	Codes	Lower (-)	Higher (+)
Seed inoculation (%) (v/v)	X_1	3	5
Optimal carbon source amount (g/L)	X_2	10	30
Optimal nitrogen source amount (g/L)	X_3	10	20
Inducer (DL-HPH amount (g/L)	X_4	2.0	3.0
Loading amount (mL/250 mL)	X_5	50	100
pH	X_6	6.0	7.0
Fermentation temperature (!)	X_7	30	35
Fermentation time (h)	X_8	40	60

Table. 2. Design and response values of Plackett-Burman experiments

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Response values Yield of D-HPG, g/L
1	-1	-1	1	1	1	-1	1	1	0.201
2	1	-1	1	-1	-1	-1	1	1	0.155
3	1	1	1	-1	1	1	-1	1	0.229
4	-1	1	1	-1	1	-1	-1	-1	0.164
5	-1	-1	-1	-1	-1	-1	-1	-1	0.189
6	-1	1	-1	-1	-1	1	1	1	0.216
7	-1	-1	-1	1	1	1	-1	1	0.267
8	1	1	-1	1	1	-1	1	-1	0.208
9	1	1	-1	1	-1	-1	-1	1	0.256
10	1	-1	-1	-1	1	1	1	-1	0.148
11	1	-1	1	1	-1	1	-1	-1	0.237
12	-1	1	1	1	-1	1	1	-1	0.236

microporous rescreening, totally 56 positive strains with the capability to produce Dhase were isolated from 33 soil samples. Then, subjected to preliminary screening using selective medium with CpHPG as the single nitrogen source and rescreening by paper chromatography, 23 positive strains with the capability to produce Dcase were isolated from 56 Dhase-producing strains.

Characterization of Dhase/Dcase-producing strains

Twenty Dhase/Dcase-producing strains were identified by morphological observation, physiological and biochemical property analysis, and searching in Bergey's bacterial identification manual (8th edition) to reveal 10 *Agrobacterium*, 4 *Pseudomonas*, 3 *Arthrobacter sp.*, 4 *Blastomyces spp* and 2 *Achromobacter*. Considering the growth status and substrate conversion, a *Pseudomonas* was selected as the starter strain to explore the optimal fermentation process.

Optimal fermentation process of Dhase/Dcase-producing strain for the production of D-HPG

Effect of carbon source type on fermentation efficiency

As shown in Fig. 1, different carbon sources revealed a significant impact on the fermentation efficiency. Polysaccharides such as dextrin and starch are difficult to be utilized; however, sorbitol, glycerol, sucrose and glucose exhibited excellent fermentation efficiency. Based on the comprehensive consideration of production cost and fermentation efficiency, industrial glucose

Table 3. Factors and results analysis of Plackett-Burman experiments

Factors	Effect	Coefficient	Standard error	T	P	Importance	Significance
		0.20878	0.004025	51.87	0.000		
X_1	-0.00655	-0.00328	0.004025	-0.81	0.475	8	
X_2	0.01845	0.00923	0.004025	2.29	0.106	5	
X_3	-0.01025	-0.00513	0.004025	-1.27	0.293	7	
X_4	0.05035	0.02518	0.004025	6.26	0.008	1	Remarkable
X_5	-0.01205	-0.00602	0.004025	-1.50	0.231	6	
X_6	0.02655	0.01328	0.004025	3.30	0.046	3	Remarkable
X_7	-0.02965	-0.01483	0.004025	-3.68	0.035	2	Remarkable
X_8	0.02375	0.01188	0.004025	2.95	0.060	4	

Table 4. Design and results of Box-Benhnken experiments

Test number	A Inducer (DL-HPH amount (g/L)	B pH	C Fermentation temperature (°C)	Yield of D-HPG (g/L)
1	1(3.0)	0(6.5)	-1(30)	0.175
2	0(2.5)	1	-1	0.168
3	0	0	0(32.5)	0.252
4	0	-1(6.0)	1(35)	0.201
5	-1(2.0)	0	-1	0.166
6	0	0	0	0.259
7	0	0	0	0.248
8	1	1(7.0)	0	0.252
9	-1	-1	0	0.208
10	0	0	0	0.238
11	1	0	1	0.206
12	0	0	0	0.259
13	-1	0	1	0.165
14	0	1	1	0.197
15	0	-1	-1	0.185
16	1	-1	0	0.259
17	-1	1	0	0.251

can be as the optimal carbon source during the industrial production of D-HPG.

As shown in Fig. 2, different nitrogen sources also exhibited a significant impact on fermentation efficiency. Inorganic nitrogen sources such as ammonium sulfate and sodium nitrate are difficult to be utilized; however, yeast extract and corn syrup as the nitrogen sources revealed excellent fermentation efficiency. Similarly, based on the comprehensive consideration of production cost and fermentation efficiency, corn syrup can be the optimal nitrogen source.

Screening of key factors using Plackett-Burman experimental design

The data in Table 1 from Plackett-Burman experimental design was subjected to fitting and variance analysis using Minitab 15 software. As shown in Table 2 and Table 3, the order the factors for affecting D-HPG fermentation efficiency from strong to weak was inducer amount (X4), fermentation temperature (X7), fermentation pH (X6), fermentation time (X8), optimal carbon source (industrial glucose) concentration (X2), flask-loading amount (X5), optimal nitrogen source (corn syrup) concentration (X3) and inoculation amount (X1). Among these factors, inducer amount (X4), fermentation temperature (X7) and fermentation pH (X6) revealed significant effect on fermentation efficiency. Therefore, three factors were selected for further Box-Behnken response surface analysis. However, the optimal combination of other factors was generated by using Minitab 15 software to be

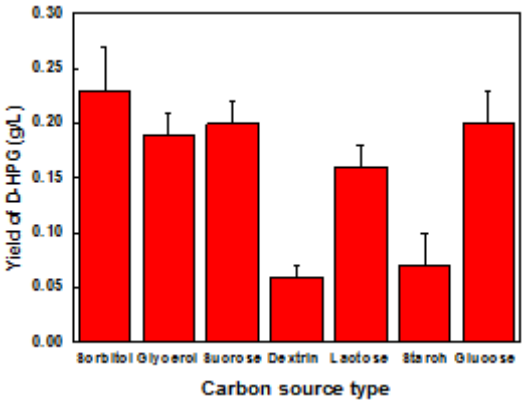
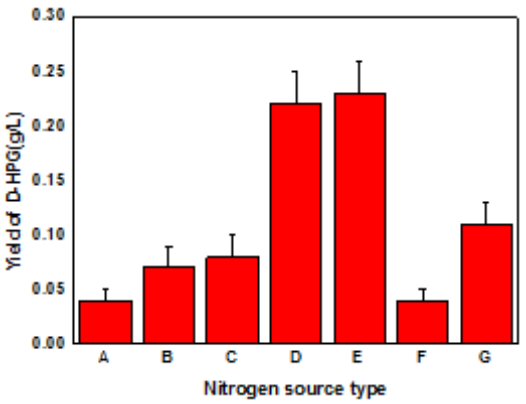


Fig. 1. Effect of carbon source type on fermentation efficiency



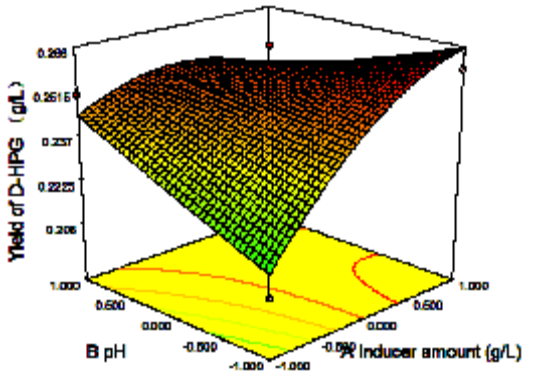
Note: A: Ammonium sulfate, B: Fish peptone, C: Bean cake powder, D: Yeast extract, E: Corn syrup, F: Sodium nitrate, G: Gluten powder

Fig. 2. Effect of nitrogen source type on fermentation efficiency

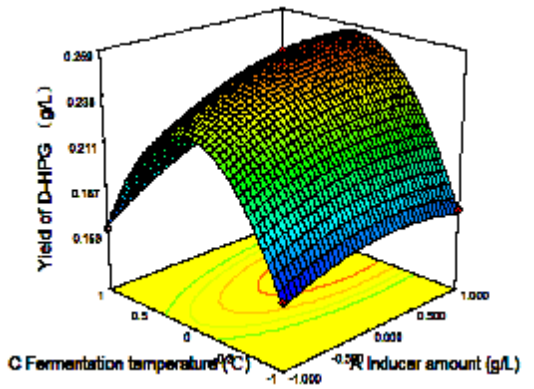
Table 5. Variance analysis and results of regression model

Source	Sum of squares	Df	Mean square	F Value	P Value	Significance
Model	0.021	9	2.32E-03	21.93	0.0003	* *significant*
A	1.30E-03	1	1.30E-03	12.28	0.0099	**significan*
B	2.81E-05	1	2.81E-05	0.27	0.6222	
C	7.03E-04	1	7.03E-04	6.64	0.0367	**significan*
AB	6.25E-04	1	6.25E-04	5.9	0.0455	**significan*
AC	2.56E-04	1	2.56E-04	2.42	0.1640	
BC	4.23E-05	1	4.23E-05	0.40	0.5478	
A^2	3.58E-04	1	3.58E-04	3.38	0.1085	
B^2	1.16E-06	1	1.16E-06	0.011	0.9196	
C^2	0.017	1	0.017	162.67	< 0.0001	**significan*
Residual	7.42E-04	7	1.06E-04			
Lack of Fit	4.35E-04	3	1.45E-04	1.89	0.2725	*not significant*
Pure Error	3.07E-04	4	7.67E-05			
Cor Total	0.022	16				

inoculum size (X1) of 3%(v/v) , industrial glucose concentration (X2) of 30 g/L, corn syrup concentration (X3) of 10 g/L , flask-loading volume (X5) 50 mL in a 250 mL flask and fermentation time (X8) of 60 h.



(a)



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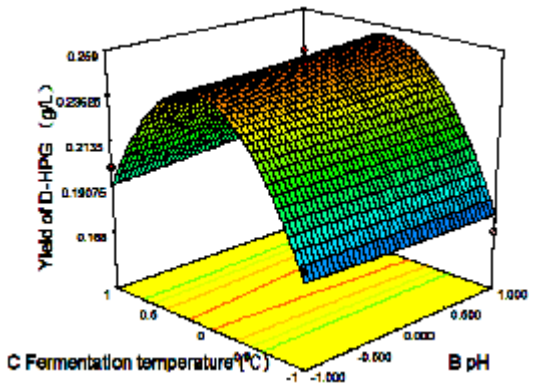

(c)

Fig. 3. Response surface plots for the effects of cross-interactions among factors on fermentation

Optimal design and results of Box-Behnken response surface experiments

Taking D-HPG yield as the response value, the response surface experiments with three factors and three levles were conducted by Box-Benhnken design and the results were shown in Table 4. Meanwhile, the expertimental data were subjected to regression analysis by Expert7.0 software, and the regression equation between D-HPG yield and each factor was achieved to be Y =+0.25+0.013 * A+1.875E-003 * B+9.375E-003* C-0.013* A * B+8.000E-003* A * C+3.250E-003* B * C-9.225E-003* A2+5.250E-004 * B2-0.064* C2

After analysing the regression model, as shown in Table 5, this model had statistically significant (P=0.0003<0.05), and the item with fitting loss was no significant difference (P=0.2725>0.05), indicating that the regression equation was in good fitting condition with small error, so that it can better describe the relationship between each factor and response value. In addition, this equation also can be used to determine the optimal fermentation conditions of Dhase/Dcase-producing strain for the production of D-HPG. From ANOVA analysis, the order of factors for affecting D-HPG yield from strong to weak was inducer amount (A), fermentation temperature (C), and fermentation pH (B), in which inducer amount (A) and fermentation temperature (C) exhibited a significant impact on fermentation efficiency.

As shown in Fig. 3, based on the response surface analysis results, the predicted optimal fermentation conditions of Dhase/Dcase-producing strain for the production of D-HPG were inducer amount of 3.0 g/L, fermentation pH of 6.0 and fermentation temperature of 32.8 °C. Under the optimal fermentation conditions, the yield of D-HPG reached up to 0.267 g/L. In addition, 3 parallel experiments were conducted on the basis of the optimal fermentation conditions, the average yield of D-HPG was 0.262 g/L, which is close to the theoretical prediction value of 0.267 g/L. Therefore, response surface methodology is suitable for exploring the optimal fermentation process conditions with high accuracy, reliability, feasibility and reproducibility.

CONCLUSION

Thirty-three soil samples collected from Longyan City, Fujian Province were subjected to enrichment culture, Dhase-producing strain screening and Dcase-producing strain screening. Totally 23 positive strains were identified by morphological, physiological and biochemical analysis as 10 *Agrobacterium*, 4 *Pseudomonas*, 3 *Arthrobacter sp*, 4 *Blastomycosis* and 2 *Colorless spp*. Taking comprehensive consideration of bacterial growth status and substrate conversion, a *Pseudomonas* was selected as the starter strain for exploring the optimal fermentation process of D-HPG production.

Industrial glucose and corn syrup are the optimal carbon and nitrogen sources of starter fermentation strain. Plackett-Burman design in response surface methodology has used to explore the effects of carbon source concentration, nitrogen source concentration, inducer amount, flask-loading amount, fermentation pH, fermentation temperature and fermentation time on the yield of D-HPG during the fermentation process of Dhase/Dcase-producing strain. The optimal fermentation process parameters include 3% inoculum size, 30 g/L industrial glucose, 10 g/L corn syrup, 3.0 g/L inducer, 50 mL of loading volume in 250 mL flask, pH 6.0, fermentation temperature at 32.8 °C and fermentation time of 60 h. Among these factors, inducer amount, fermentation pH and fermentation temperature are major factors. Moreover, inducer amount and fermentation temperature have significant impact on the response value in Box-Behnken design.

DISCUSSION

â-Lactam antibiotics are the development focus of pharmaceutical industries in China. However, as the material sources of â-lactam antibiotics, D-HPG synthesis has important practical significance [21]. Especially in recent years, the demand of D-HPG in antibiotics market reveals an increasing trend; therefore, its big potential market value and huge economic benefits will stimulate the development and production of D-HPG in many domestic and foreign institutions and enterprises [22-23]. Moreover, dual enzymatic process realized by single bacterial strain is simple process, low energy consumption, high productivity and activity and low pollution, which can realize the clean and highly efficient production of D-HPG for meeting international development trend [24].

Currently, according to the reports at home and abroad, the Dhase/Dcase-producing strains are mainly distributed in *Agrobacterium* and *Pseudomonas* [25]. In this paper, through fermentation cultivation of Dhase/Dcase-producing *Pseudomonas*, the yield of D-HPG can reach up to 0.262 g/L, which is better than or close to D-HPG levels from the fermentation by various strains reported at home and abroad [26-27]. In addition, our fermentation culture medium is relatively cheap, and the operation is simple and controllable. These studies will provide references and theoretical basis for the industrial production of D-HPG.

Moreover, in order to improve dual enzymatic production of D-HPG, the fermentation process conditions have been optimized. Furthermore, the yield of D-HPG can also be enhanced through recombinant enzyme with optimal Dhase/Dcase ratio[28], protein engineering to improve Dhase/Dcase stability and stereoselectivity[29]. As the continuous advancement of life science and gradual maturation of fermentation engineering technology, a dual-enzyme technology for the production of D-HPG will bring important influence and promotion role in the industrial production of D-amino acids.

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