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Optimization of compactin fermentation

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A compactin producer *Penicillium* sp strain was isolated from soil in our screening program. The compactin-biosynthesising capacity of the strain was improved from 5 μg ml^{-1} to 250 μg ml^{-1} by mutation-selection method. We investigated the effect of the medium composition on compactin productivity. A central, orthogonal three-factor experimental design by Box and Wilson was used in the investigation. The results were analysed by non-linear regression analysis. The composition of the production medium was optimized according to the calculated mathematical model using the steepest ascent method. The compactin productivity was increased to 400 μg ml^{-1} by applying this method.

Keywords: compactin; optimization; regression analysis; *Penicillium*

Introduction

Compactin was isolated first from the fermentation broth of *Penicillium brevicompactum* in 1976 as an antifungal metabolite [3], and its hypocholesterolemic activity was published also that year [5,6]. Compactin is the precursor of pravastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, applied in the clinical treatment of hypercholesterolemia [4,8,9]. Pravastatin is produced by a two-step fermentation process, in which the first step is the production of compactin by a fungal strain and the second is the microbial hydroxylation of compactin [7]. Because of the microbial processes, it is very important to reduce the cost of fermentation. One possible way is to determine the optimal composition of the production medium. Previously we have found that the most effective carbon source is glucose and the most effective nitrogen source is yeast extract. Magnesium-sulphate in the production medium also increases the compactin yield. It is difficult to investigate the quantitative effects of three factors in a single experiment. Therefore we used a central composite experimental design, non-linear regression analysis and the steepest ascent method to determine the optimal concentration of the medium components [1,2].

Materials and methods

Microorganism

Penicillium sp IDR-629 was maintained on slants of malt extract-yeast extract medium, which contained malt extract 1% (w/v), yeast extract 0.4% (w/v), glucose 0.4% (w/v) and agar 2% (w/v) in distilled water (pH adjusted to 7.0 before sterilization, the medium was sterilized at 121°C for 25 min). Cultivation was carried out at 25°C for 10 days.

Media

CI1 (glycerol 3% (w/v), glucose 1% (w/v), yeast extract 0.6% (w/v), $NaNO_3$ 0.2% (w/v), $MgSO_4 \cdot 7H_2O$ 0.1% (w/v), in tap water, pH adjusted to 6.5 before sterilization) was used for seed medium. CT1 (glucose 3% (w/v), glycerol 3% (w/v), peptone 0.4% (w/v), $NaNO_3$ 0.2% (w/v), $MgSO_4 \cdot 7H_2O$ 0.1% (w/v), in tap water, pH adjusted to 6.5 before sterilization) was used as fermentation medium in the screening work and in the strain improvement. Media, containing glucose, yeast extract and magnesium-sulphate were used for production media, in which the concentration of the ingredients was calculated according to the central, orthogonal experimental design. Media were prepared using tap water and the pH was adjusted to 6.5 before sterilization. All media were sterilized at 121°C for 25 min.

Mutagenic treatment

The spores of the 10-day-old fungal strain were washed from the slant and treated with 1 mg ml^{-1} *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Sigma, St Louis, MO, USA) in 0.05 M phosphate buffer pH 8.0 at 28°C for 45 min. Then the spores were centrifuged at 4000 × *g* for 10 min, washed with sterile distilled water and plated on MS agar. After incubation at 25°C for 7 days, colonies were isolated and their compactin biosynthesising ability was investigated in shaken flask experiments.

Fermentation procedure

Experiments were carried out in 500-ml Erlenmeyer flasks containing 100 ml medium on a rotary shaker operating at 250 rpm. The seed medium was inoculated with 10^6 spores ml^{-1} and incubated at 25°C for 72 h. Five per cent of this preculture was used to inoculate the production medium. Fermentation was carried out at 25°C for 7 days.

Determination of compactin

The fermentation broth was diluted four times with ethanol. After shaking for 5 min with high frequency bath, the samples were centrifuged at 4000 × *g* for 20 min. Ten microliters from the supernatant phase were injected for HPLC analysis on a BST Nucleosil C_{18} 10-μm column (240 × 4 mm) eluted by a solvent acetonitrile : water (pH = 2.0 with H_3PO_4) 60 : 40. The flow rate was 1 ml min^{-1}, the detection was at 237 nm.

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Experimental design

A central, orthogonal three-factor experimental design by Box and Wilson consisting of 15 treatments was used to investigate the quantitative effects of the following variables: glucose, yeast extract and magnesium-sulphate concentration. The experimental design is shown in Table 1.

Statistical analyses

The quantitative effects of glucose, yeast extract and magnesium-sulphate concentration on compactin productivity were analysed by non-linear regression analysis. The regression coefficients and the constant response plot were calculated by computer.

Results

Selection of the strain and mutagenic treatments

Screening work was carried out to isolate microorganisms, which produce biological active compounds. A *Penicillium* sp strain, having compactin-producing ability (5 μg ml^{-1}), was isolated in this program. Mutagenic treatments were carried out to improve the compactin-biosynthesising ability of this wild-type strain. More than 3000 isolates obtained with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine were investigated. A mutant strain (*Penicillium* sp IDR-629), which produced 250 μg ml^{-1} compactin, was selected from the examined mutants.

Medium optimization

We investigated the compactin productivity on different carbon and nitrogen sources. We found that the most effective carbon source is glucose and the most effective nitrogen source is yeast extract. The quantitative effects of glucose, yeast extract and magnesium-sulphate concentration were analysed on the compactin productivity by using the composite design, shown in Table 1. In the first set of experiments the base points of the variables were: glucose concentration, 5%; yeast extract concentration, 0.95%; magnesium-sulphate concentration, 0.1%. The range of the tested variables was: glucose concentration, 3.8–6.2%; yeast extract concentration, 0.7–1.2%; magnesium-sulphate concentration, 0.04–0.16%. Coded values were used to calculate the regression coefficients. The coded values ranged from −1.215 to +1.215.

Formulae for converting actual values to coded values are:

$$\text{Glucose} = \text{concentration}(\%) - 5$$
$$\text{Yeast extract} = (\text{concentration}(\%) - 0.95) \times 5$$
$$\text{Magnesium} - \text{sulphate} = (\text{concentration}(\%) - 0.1) \times 20$$

Results of the experiment shown in Table 1 (column A) were analysed by non-linear regression analysis according to the following equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{1,2}X_1X_2 + b_{1,3}X_1X_3 + b_{2,3}X_2X_3 + b_{1,1}(X_1)^2 + b_{2,2}(X_2)^2 + b_{3,3}(X_3)^2$$

where Y = dependent variable, compactin concentration; X_1 = glucose concentration (coded level); X_2 = yeast extract concentration (coded level); X_3 = magnesium-sulphate concentration (coded level); b_0–$b_{3,3}$ = regression coefficients. We arrived at the following equation:

$$\text{Compactin conc.} = 216.7 + 60.7X_1 + 20.4X_2 + 3.18X_3 + 68.3X_1X_2 - 1.8X_1X_3 - 0.6X_2X_3 - 38.8(X_1)^2 - 38.9(X_2)^2 + 16.2(X_3)^2$$

The calculated compactin concentration may also be determined from the constant response plot shown in Figure 1. Concentration of magnesium-sulphate is near to the optimum and it has weak effect on the compactin productivity. According to this observation, only the glucose and yeast extract concentration were optimised by the steepest ascent method. The gradient vector of the function, which direct to the optimum, was determined from the regression coefficients. For each 0.5% by which glucose concentration is increased, yeast extract concentration should be increased

Table 1 The experimental design and the results of the first (A) and the second (B) set of experiments

No.	Coded level			Compactin (μg ml^{-1})	
	Glucose	Yeast extract	$MgSO_4$	A	B
1	+1	+1	+1	310	271
2	−1	+1	+1	56	259
3	+1	−1	+1	123	321
4	−1	−1	+1	156	320
5	+1	+1	−1	307	320
6	−1	+1	−1	59	230
7	+1	−1	−1	131	314
8	−1	−1	−1	143	382
9	−1.215	0	0	59	272
10	+1.215	0	0	230	352
11	0	−1.215	0	126	411
12	0	+1.215	0	162	320
13	0	0	−1.215	213	397
14	0	0	+1.215	238	362
15	0	0	0	262	390

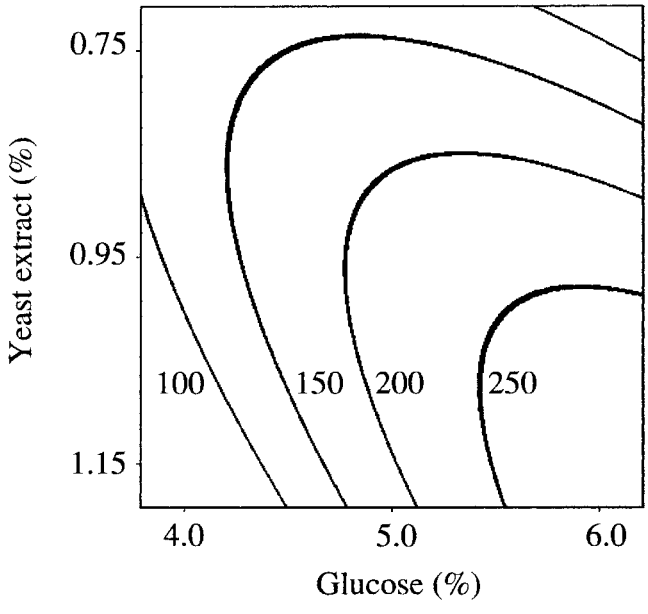

Figure 1 Constant response plot for compactin concentration (μg ml^{-1}), magnesium-sulphate at 0.1%.

Table 2 Concentration of medium ingredients and the obtained compactin yields in the path to the optimum

No.	Glucose (%)	Yeast extract (%)	Compactin (μg ml^{-1})
1	5.0	0.95	241
2	5.5	0.98	259
3	6.0	1.01	293
4	6.5	1.04	349
5	7.0	1.07	370
6	7.5	1.10	332
7	8.0	1.13	289
8	8.5	1.16	276
9	9.0	1.19	270
10	9.5	1.22	254

by 0.03%. Concentration of magnesium-sulphate was not changed. The path and the obtained compactin concentration are shown in Table 2. In the Experiments (4) and (5), the obtained yields were 349 and 370 μg ml^{-1}. Although the first value was lower than the second no real significance could be attributed to this, and the second set of experiments was carried out to determine the optimal concentration exactly. The conditions corresponding to Experiment (5) were used as base point: glucose concentration, 7%; yeast extract concentration, 1.07%; magnesium-sulphate concentration, 0.1%. The experimental design shown in Table 1 was used, the range of the tested variables being: glucose concentration, 5.8–8.2%; yeast extract concentration, 0.94–1.2%; magnesium-sulphate concentration, 0.075–0.125%. The concentration of the medium ingredients was converted to the coded values according to the following equations:

$$\text{Glucose} = \text{concentration}(\%) - 7$$
$$\text{Yeast extract} = (\text{concentration}(\%) - 1.07) \times 10$$
$$\text{Magnesium} - \text{sulphate} = (\text{concentration}(\%) - 0.1) \times 50$$

Results are shown in Table 1 (column B). The difference among the values is comparable with the variability of the process, therefore the regression analysis of the results is inapplicable. It can be proved from the following results:

(a) The optimal concentration of glucose is 7%.
(b) The concentration of magnesium-sulphate has weak effect on the compactin productivity and it is approximately in the optimum.
(c) The optimal concentration of the yeast extract is between 0.95% and 1.07%. According to the variability of the natural processes, the optimal concentration can not be determined exactly. Because of this, the fermentation medium contains 1% yeast extract.

Discussion

The fungal metabolites, compactin and related compounds are potent competitive inhibitors of HMG-CoA reductase enzyme. Pravastatin, which is the hydroxylated derivative of compactin, has been developed as one of the most prominent drugs of this family. In our study, we optimised the fermentation medium for compactin production applying non-linear regression analysis and the steepest ascent method, which has proved to be a quick and efficient technique. Glucose, yeast extract, selected in our earlier experiments as most effective carbon and nitrogen source, and magnesium-sulphate concentration were optimised using these processes. The mathematical model of the effects of glucose, yeast extract and magnesium-sulphate on the compactin productivity was determined in the first set of experiments. The path to near of the optimum was calculated from the regression coefficients using the steepest ascent method. The best result, obtained on the path, was 370 μg ml^{-1}. A second set of experiments was performed at this point to determine the optimal concentrations exactly. We obtained that the optimal concentrations are: glucose, 7.0%; yeast extract, 1.0%; magnesium-sulphate, 0.1%. Compactin yield was 390–410 μg ml^{-1} in this concentration of medium ingredients.

Compactin productivity was increased from 240–260 μg ml^{-1} to 390–410 μg ml^{-1} using central, orthogonal three-factor experimental design by Box and Wilson, nonlinear regression analysis and the steepest ascent method. It can be proved from the results described in this paper that this method is applicable for the optimization of the fermentation medium to enhance compactin production.

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