Process Biochemistry 43 (2008) 1313–1317

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Production and statistical optimization of a novel olivanic acid by *Streptomyces olivaceus* MTCC 6820

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ARTICLE INFO

Article history:
Received 11 January 2008
Received in revised form 27 May 2008
Accepted 13 July 2008

Keywords:
Olivanic acid
Streptomyces olivaceus
Optimization
Plackett-Burman design
Response surface methodology
Response surface/contour plots

ABSTRACT

A microbial strain, isolated from soil samples, characterized as *Streptomyces olivaceus* MTCC 6820 showed broad-spectrum antibacterial activity. The compound produced was chemically characterized as a new form of olivanic acid. Olivanic acid production was optimized statistically by Plackett-Burman design (PBD) and response surface methodology (RSM). Effects of soybean meal, glycerol, $CaCO_3$ and DL-alanine were investigated with the help of PBD. The individual and interaction effects of the studied variables were evaluated by RSM using central composite design (CCD). By applying statistical design, antibiotic production was enhanced nearly 8 times (415 mg/l) as compared with the normal production medium (50 mg/l).

1. Introduction

During our extensive screening programme for the isolation of microbial cultures producing novel antimicrobial agents, an active strain of *Streptomyces olivaceus* was isolated from the soil samples collected from the hilly regions of India. The compound produced by the strain was purified and chemically characterized as olivanic acid.

S. olivaceus is reported to produce a diverse array of antibiotics such as elloramycin (polyketide), kanchanamycins (polyol macrolide), rabelomycin (anthraquinone) and beta-lactam ring containing carpetimycin or olivanic acids. Olivanic acids are reported to inhibit β-lactamases and show antimicrobial activity [1–4].

The traditional approach for the optimization of medium components takes into account one factor at a time, which is time consuming, as it does not depict the interaction of different medium components. Statistical optimization methods provide information about the optimum concentrations of various medium ingredients helpful in maximum product formation [5–7]. For the statistical analysis Plackett-Burman design is applied for the screening of most effective medium components and response surface methodology is used for the study of linear, square and interaction effect of the factors on the production of antibiotics.

2. Materials and methods

2.1. Microorganism and production conditions

The isolated culture was maintained on YMG slants containing (g/l) yeast extract 4.0, malt extract 10, glucose 4.0, $CaCO_3$ 2.0 and agar powder 20. Strain was taxonomically characterized on the basis of 16S rRNA homology and has been submitted at the Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India as *S. olivaceus* (accession number 6820).

Submerged fermentation was carried out by cultivating the culture for 3 days at 28 °C, 200 rpm in 1 l Erlenmeyer flask with 200 ml production medium comprising of (g/l) soybean meal 10, $CaCO_3$ 3, $MgSO_4{\cdot}7H_2O$ 0.5, $(NH_4)_2HPO_4$ 0.5, NaCl 3, K_2HPO_4 1, glycerol 15 ml, pH 6.9–7.0. Fermented broth was centrifuged at 11,086 × *g* for 20 min to separate the biomass. Extra-cellular compound was isolated through liquid–liquid extraction of the fermented broth.

2.2. Activity and chemical characterization of the active compound

Crude extract was subjected to Sephadex LH-20 column (Pharmacia) and finally purified by HPLC with reverse phase silica column (RP18). Identification of the compound as olivanic acid was done according to the method described by Box et al. [8]. Ultraviolet (UV) spectrum in methanol was determined with a PerkinElmer Lambda-25 UV spectrophotometer at 200–500 nm UV–vis range. Infra red (IR) spectrum was recorded on FT-IR PerkinElmer RX-1 spectrometer in the 200–4000 cm^{-1} range using KBr pellet technique. Mass spectrum was recorded on micromass Quattro II triple quadrupole mass spectrometer. For complete structure elucidation ^1H, ^{13}C and 2D nuclear magnetic resonance (NMR) spectra of the compound in deuterated chloroform ($CDCl_3$) were conducted with 600 MHz VARIAN INOVA instrument. Minimum inhibitory concentration (MIC) of the purified compound was estimated by serial dilution method recommended as NCCLS [9].

2.3. Plackett-Burman design for the screening of important components

Plackett-Burman design is used to screen out important nutrient components. In this design (Table 1) total number of runs is always greater than total number of

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doi:10.1016/j.procbio.2008.07.015

Table 1
Plackett-Burman design and result

Runs	(X1) Soybean	(X2) $CaCO_3$	(X3) DL-alanine	(X4) Glycerol	(D1) $(NH_4)_2HPO_4$	(D2) NaCl	(D3) K_2HPO_4	Yield (g/l)
1	20	6.0	2.0	5.0	0.5	3.0	1.0	0.030
2	1.0	6.0	2.0	30	0.5	3.0	1.0	0.040
3	1.0	0.1	2.0	30	0.5	3.0	1.0	0.020
4	20	0.1	0.1	30	0.5	3.0	1.0	0.005
5	1.0	6.0	0.1	5.0	0.5	3.0	1.0	0.050
6	20	0.1	2.0	5.0	0.5	3.0	1.0	0.020
7	20	6.0	0.1	30	0.5	3.0	1.0	0.014
8	1.0	0.1	0.1	5.0	0.5	3.0	1.0	0.029
Effects	−17.5	15	−2	−12.5	0.5	5.5	1.25	
S.E.	3.2691	3.2691	3.2691	3.2691	3.2691	3.2691	3.2691	
t-value	5.3530	4.5883	0.6118	3.8236	0.1529	1.6824	0.3824	
p-value	0.0005	0.001	0.5000	0.005	0.5000	0.1	>5.0	

X1, X2, X3, X4 are the independent variables and D1, D2, and D3 are the dummy variables. All the variables except glycerol were measured in g/l whereas glycerol was measured in ml/l. The *p*-values less than 0.05 are significant.

variables (medium ingredients) by one unit. Each variable represented in high and low levels defines the upper and lower limits of the range covered by variables [10]. Experiments were performed with various combinations of high and low values of the variables and analyzed for their effect on the product formation. In the present experiment, four independent and three dummy variables (shown in Table 1) were selected for the screening in eight trials. The results obtained with classical experiments (data not shown) helped in the selection of independent and dummy variables. Dummy variables are incorporated in the design to estimate experimental error. The effect of each variable was determined by following equation

$$E_{(X1)} = 2\frac{\sum M_{1H} - \sum M_{1L}}{N} \quad (1)$$

where $E_{(X1)}$ is the concentration effect of the tested variable. M_{1H} and M_{1L} are the antibiotic yield (g/l) from the trials where the variable present at high and low concentration, respectively, and N is the total number of trials. Experimental error was estimated by calculating the variance among the dummy variables

$$V_{eff} = \sum \frac{(E_d)^2}{n} \quad (2)$$

where V_{eff} is the variance of the concentration effect, n is the number of dummy variables and E_d is the concentration effect for the dummy variables.

The standard error (S.E.) was described by the square root of the variance of the effect, i.e.

$$\text{S.E.} = \sqrt{V_{eff}} \quad (3)$$

The significance level of each concentration effect was determined using Student's *t*-test

$$t_{(X1)} = \frac{E_{(X1)}}{\text{S.E.}} \quad (4)$$

The variables with confidence levels greater than 95% were considered to influence the olivanic acid production significantly. Three variables, which were found to be the most effective components for antibiotic production in PBD, were selected for further medium optimization studies using CCD and RSM.

2.4. Optimization of the selected medium components by RSM using CCD

Response surface designs are used to explore non-linear relationships between independent (medium components) and the dependent (antibiotic yield) variables. These relationships help in selecting the concentrations of the medium components producing maximum product. 2^{3-1} fractional factorial CCD proposed by Box et al. is the most accepted and widely used design to study the interaction effect of the medium components [11]. Total twenty experiments with eight cube points, six star points and six replicas of the central point were employed to fit the second order polynomial model. Design along with range and the levels of the three selected variables are shown in Table 2. Following regression equation was developed by the application of RSM showing an empirical relationship between the logarithmic values of antibiotic yield and the coded units of the test variables (medium components).

$$Y = b_0 + b_1x_i + b_2x_{ii} + b_3x_{iii} + b_4x_i^2 + b_5x_{ii}^2 + b_6x_{iii}^2 + b_7x_i \cdot x_{ii} + b_8x_i \cdot x_{iii} + b_9x_{ii} \cdot x_{iii} \quad (5)$$

Table 2
CCD for the study of three factors in concentration (conc.) units

Runs	Soybean meal		Glycerol		$CaCO_3$		Antibiotic yield (g/l)		
	Coded	Conc.	Coded	Conc.	Coded	conc.	observed	predicted	residual
1	+1	15	+1	0.059	+1	6	0.260	0.291	−0.031
2	+1	15	−1	0.019	−1	2	0.320	0.342	−0.022
3	−1	5	+1	0.059	−1	2	0.400	0.404	−0.004
4	+1	15	+1	0.059	−1	2	0.300	0.311	−0.011
5	+1	15	−1	0.019	+1	6	0.295	0.291	0.004
6	−1	05	+1	0.059	+1	6	0.350	0.331	0.019
7	−1	05	−1	0.019	+1	6	0.210	0.203	0.008
8	−1	05	−1	0.019	−1	2	0.326	0.302	0.024
9	−1.6	0.001	0	0.039	0	4	0.242	0.267	−0.025
10	0	10	−1.6	0.0001	0	4	0.211	0.217	−0.006
11	0	10	0	0.039	−1.6	0.001	0.378	0.372	0.006
12	+1.6	20	0	0.039	0	4	0.294	0.265	0.029
13	0	10	+1.6	0.079	0	4	0.315	0.304	0.011
14	0	10	0	0.039	+1.6	8	0.250	0.250	−0.00
15	0	10	0	0.039	0	4	0.421	0.411	0.010
16	0	10	0	0.039	0	4	0.410	0.411	−0.001
17	0	10	0	0.039	0	4	0.400	0.411	−0.011
18	0	10	0	0.039	0	4	0.410	0.411	−0.001
19	0	10	0	0.039	0	4	0.410	0.411	−0.001
20	0	10	0	0.039	0	4	0.421	0.411	0.010

Concentration units of soybean meal, and $CaCO_3$ were in g/l whereas glycerol was in g/ml of the medium. 0.019 g/ml = 15.1 ml/l; 0.039 g/ml = 30.9 ml/l; 0.059 g/ml = 46.8 ml/l; 0.079 g/ml = 62.65 ml/l.