Appl Biochem Biotechnol (2009) 158:20–24
DOI 10.1007/s12010-009-8629-5

Optimization of the Production Medium for Biosynthesis of Antifungal Antibiotic Ak-111-81 by Phosphate-Deregulated Mutant of *Streptomyces hygroscopicus*

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Received: 3 April 2008 / Accepted: 26 March 2009 /
Published online: 16 June 2009

Abstract Experimental mathematical designs were applied for optimization of a nutrient medium for biosynthesis of the antifungal antibiotic AK-111-81 by phosphate-deregulated mutant of *Streptomyces hygroscopicus* 111-81. Antifungal antibiotic AK-111-81 possesses well-expressed activity against *Fusarium graminearum* and other phytopathogenic fungi. The level of the production of the antibiotic AK-111-81 on this medium is more than three times higher than on the initial medium. The optimized quantitative composition of the nutrient culture media is (g/l): glucose, 20; soy meal, 18; ammonium succinate, 3; $CaCO_3$, 1.

Keywords *Streptomyces hygroscopicus* · Phosphate-deregulated mutant · Optimal production medium · Optimization · Antifungal antibiotic AK-111-81

Introduction

Actinomycetes are the most productive group of microorganisms with regard to their capability to synthesize secondary metabolites. Production of high yields of antibiotics depends on the fermentation media providing nutrients for cell proliferation and culture conditions for expressing genetic information favoring secondary metabolism [1–9].

Streptomyces hygroscopicus is a species which produces a variety of antibiotics belonging to different classes with distinct structure and possessing certain biological properties [5, 7, 10–14].

S. hygroscopicus strain 111-81 synthesizes antibacterial antibiotics, polyether IM-111-81, azalomycin B, and antifungal antibiotic AK-111-81 [12, 15] with well-expressed activity against *Fusarium graminearum* [16]. Earlier, the phosphate-deregulated mutant (*pho* 84) with higher antifungal antibiotic activity compared with that of the parent strain 111-81 was found [17].

This paper describes application of mathematical designs for optimization of the production medium for antifungal antibiotic biosynthesis of AK-111-81 by phosphate-deregulated mutant.

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Materials and Methods

Organism and Cultural Conditions

The strain used in this work was mutant *pho* 84 of *S. hygroscopicus* 111-81 [17]. The cultivation was carried out in 500 ml Erlenmeyer flasks containing 50 ml medium on a shaker (220 rpm) at 28 °C. The seed medium contained (g/l): glucose, 40; soy meal, 30; $CaCO_3$, 3; KH_2PO_4, 0.5; NaCl, 2.0; pH was adjusted to 7.2 before sterilization. Aliquots of seed were transferred after 40 h growth to flasks of production medium which were cultivated for 96 h. Concentrations of the components were changed in accordance to mathematical designs of two- and three-level factorial experiments [18]. The biomass was estimated by weighing to constant dry weight after drying at 105 °C.

Antibiotic Assay

Antifungal activity of methanol extracts from culture broths were assayed against *Candida utilis*, using as standard antibiotic AK-111-81 [12]. Component composition of AK-111-81 was monitored by thin-layer chromatography as described by Gesheva et al. [15]. Antibiotic was visualized by spraying with 3% solution of vanillin in 1.5 % sulfuric acid in ethanol and next heating at 110 °C for 15 min.

Results and Discussion

S. hygroscopicus 111-81 produces the nonpolyenic macrolide antibiotic AK-111-81 [12] with antibacterial and antifungal activity. AK-111-81 comprises five major components, niphimycin derivatives [15]. The component 3 is determined as demalonylniphimycin

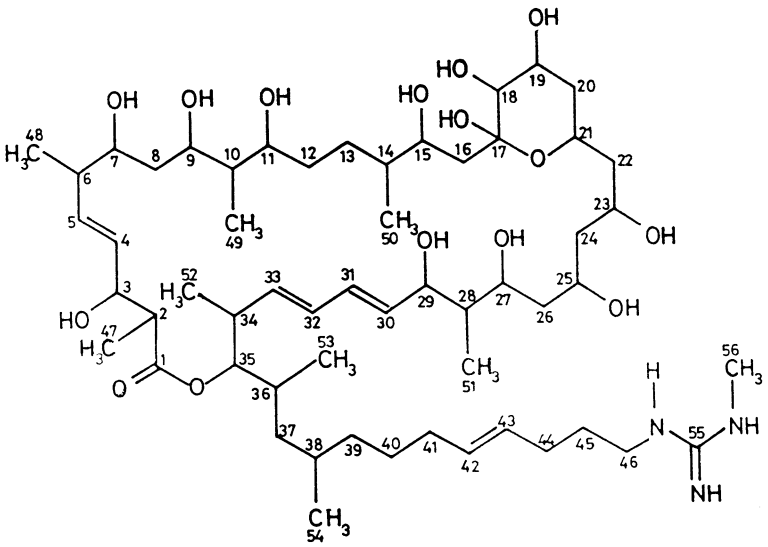

Fig. 1 Demalonylniphimycin

Table 1 Design matrix I.

No.	Glucose (*a*)	Soy meal (*b*)	Ammonium succinate (*c*)	$CaCO_3$ (*d*)	Antifungal activity (%)	Effect	Regression coefficient
1	−	−	−	−	159±16	1	141.4
2	+	−	−	−	121±11	*a*	17.5
3	−	+	−	−	198±23	*b*	27.2
4	+	+	−	−	288±28	*ab*	−4.4
5	−	−	+	−	95±9	*c*	−32.6
6	+	−	+	−	155±10	*ac*	−14.6
7	−	+	+	−	133±9	*bc*	−9.1
8	+	+	+	−	187±20	*abc*	−1.3
9	−	−	−	+	46±5	*d*	−25.7
10	+	−	−	+	224±25	*ad*	−3.2
11	−	+	−	+	164±19	*bd*	−7.4
12	+	+	−	+	191±21	*abd*	−19.5
13	−	−	+	+	69±4	*cd*	−8.1
14	+	−	+	+	44±3	*acd*	−22.4
15	−	+	+	+	126±8	*bcd*	7.8
16	+	+	+	+	61±2	*abcd*	15.1
17	0	0	0	0	100		

a(−,0,+)=10, 20, 30 g/l; *b*(−,0,+)=3, 7, 10 g/l; *c*(−,0,+)=3, 5, 7 g/l; *d*(−,0,+)=1, 3, 5 g/l

(Fig. 1). Observations on antibiotic biosynthesis by parent *S. hygroscopicus* 111-81 have shown that some carbon and nitrogen sources as lactose, soy meal, and ammonium succinate increased antifungal antibiotic productivity [15]. Thus, soy meal has a positive effect on antibiotic biosynthesis presumably due to its slow assimilation causing nitrogen limitation [1, 7, 8]. In contrast to parent strain, mutant *pho 84* required a presence of glucose in production medium which favored antifungal biosynthesis [17]. Usually, glucose is preferable carbon source because of its low cost.

Table 2 Design matrix II.

No.	Soy meal (*b*)	Ammonium succinate (*c*)	$CaCO_3$ (*d*)	Biomass (g/l)	Antifungal activity (μg/ml)
1	−	−	−	19.3	4,540±120
2	+	0	0	27.8	4,260±110
3	0	+	0	23.4	3,600±100
4	0	0	+	19.5	3,840±105
5	−	+	+	21.3	5,080±160
6	+	−	+	21.1	5,200±180
7	+	+	−	25.6	5,050±150
8	−	−	+	18.1	3,600±103
9	−	+	−	26.0	6,600±200
10	+	−	−	27.3	3,480±102

b(−,0,+)=18, 22, 26 g/l; *c*(−,0,+)=1, 2, 3 g/l; *d*(−,0,+)=1, 2, 3 g/l

Application of multifactorial experimental plan 2^4 to optimize desired metabolite yields allows the study of different parameters at distinct levels in a limited number of experiments. For each variable, two levels were chosen. Thus, 16 experimental trials were defined with either the level "−" or the level "+" (Table 1). Run 17 was performed at the central point "0."

Effects and interactions of individual factors were calculated by 16 regression coefficients. Regression analysis showed that regression coefficients for *b*, *c*, and *d* were significant, while other regression coefficients were not significant. The biosynthetic process could be described by the following lineal equation:

$$y = 141.4 + 27.2b - 32.6c - 25.7d$$

The sign of coefficient for *b* is positive. Hence, the concentration of soy meal was necessary to increase to achieve an optimum. The values of the coefficients for *c* and *d* are negative. Therefore, these parameters were changed also in order to obtain improved yield. The glucose was maintained at constant level, 20 g/l because its regression coefficient is not a significant parameter. Thus, the design matrix II with three levels was used with concentrations of soy meal (*b*), ammonium succinate (*c*), and $CaCO_3$ (*d*), (Table 2). From these results, it is concluded that the medium with highest value of antifungal activity is a variant 9. This medium contains (g/l): glucose, 20; soy meal, 18; ammonium succinate, 3; $CaCO_3$, 1. On this medium, the antifungal antibiotic reached 6,600 μg/ml which is more than a 3-fold increase of its activity compared to the initial medium, 2,200 μg/ml. Influence of particular nutrients on the antibiotic biosynthesis is determined by the chemical structures of antibiotic substances. Thus nitrogen source, soy meal, served as source of amino groups which are transferred to specific intermediate products. Succinate might be incorporated in biosynthetic path for the formation of elongation units of the oligoketide chain (methyl-CoA and methylmalonyl-CoA) and might serve as precursor of the nonpolyenic macrolide AK-111-81, a polyketide antibiotic.

Acknowledgment I express my thanks to Prof. A.L. Demain for constructive discussion, helpful comments, and critical reading of the manuscript.

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