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APPLICATION OF FACTORIAL DESIGN FOR THE OPTIMIZED PRODUCTION OF ANTISTAPHYLOCOCCAL METABOLITE BY AUREOBASIDIUM PULLULANS

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Abstract

Background: Antimicrobial substances are mainly produced by bacteria and lower fungi, and have great roles in the treatment of most infectious diseases.

Purpose: Production of antistaphylococcal metabolite from *Aureobasidium pullulans* by development of a cultural medium using response surface methodology. Methods: Production of antistaphylococcal metabolite from *Aureobasidium pullulans* was optimized in shake flasks using a statistical experimental design approach. Effect of various components in the basal medium, glucose, peptone, KH_2PO_4 as well as initial pH and temperature were statistically combined using a 2 level, 4 factor experimental design and tested for their influence on maximal antistaphylococcal metabolite production. Results were analyzed using response surface methodology (RSM) software. Results: Optimum production of antistaphylococcal metabolite occurred at glucose 2.0%, peptone 2.5%, KH_2PO_4 0.15%, pH 4.0 and temperature 30°C. The maximum amount of antistaphylococcal metabolite 900 U/flask from about 0.85 g of dry weight biomass was extracted. Conclusion: The antistaphylococcal activity of *Aureobasidium pullulans* seemed to be associated with primary metabolite rather than secondary metabolite. However, this conclusion should be taken with caution because both secondary metabolites as well as antibiotics are heterogeneous group and our knowledge regarding the exact definitions and of secondary metabolite / antibiotics are far from the perfection.

Keywords:

Aureobasidium, Antistaphylococcal activity, Production, Factorial design.

Introduction

Aureobasidium pullulans (de Bary) is cosmopolitan yeast like fungus that occurs in diverse habitats including the phyllosphere of many crop plants and due to production of melanin, it is popularly known as black yeast (1-3). Literature survey shows few reports on production of antimicrobial compounds from *Aureobasidium pullulans* (3,4). Despite extensive use of antibiotics and vaccination programs, infectious diseases continue to be leading cause of morbidity and mortality worldwide. Widespread antibiotic resistance, the emergence of new pathogens in addition to the resurgence of old ones, and the lack of effective new therapeutics exacerbate the problem (5). The need for safe and effective antimicrobial compounds increases in parallel with the expanding number of immuno-compromised patients at risk for invasive fungal / bacterial infections. One of the most common operations in the study of production of antimicrobial agents by microorganisms is the development of a medium to obtain

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maximum cell and metabolic product yield (6,7).

The selection of media for microorganism's growth and metabolic products is usually based on a combination of experimentation and logic (8). Often such medium screening strategies involve the "one factor at a time" technique. This approach is tedious and time consuming, especially for a large number of variables. Moreover, it does not guarantee the determination of optimal conditions (9). The experimental design constitutes an efficient tool and is well adapted for treating problems with a large number of variables. In particular, response surface methodology can be used when presence of complex interaction is suspected (10). In our preliminary studies in the development of the production medium, various parameters were found to be important factors in enhancing the antistaphylococcal metabolite formation. However, no systematic study to achieve optimum medium composition and process conditions has been reported for the production of antistaphylococcal metabolite. This work reports production of antistaphylococcal metabolite from *A. pullulans* by development of a cultural medium using response surface methodology.

Materials and methods

Antistaphylococcal metabolite from *A. pullulans* extracted out as described in our previous article (3). Briefly, 1 mL of inoculums was added to 100 mL of broth medium containing glucose, 2.0 g / 100 mL; $(NH_4)_2 SO_4$, 0.5 g / 100 mL; KH_2PO_4, 0.15 g / 100 mL; $MgSO_4$, 0.5 g / 100 mL; $CaCl_2$, 0.01 g / 100 mL and 5 µg / mL $FeCl_3$ and $ZnSO_4$ in a 500 mL flask and incubated at 28°C (120 rpm) on shaker. After 2 days of incubation, the medium was replenished with 20 mL of concentrated medium supplemented 5% peptone and growth was continued for additional 4 days.

Extraction medium of antimicrobial metabolite from A. pullulans

The broth was centrifuged at 5000 rpm for 20 min. Plates were suspended in equal volume of ethanol and ground in mortar and pestle with coarse silica for 15 – 20 min and the alcoholic extract was collected as supernatant after centrifugation at 5000 rpm. The ethanol extract was dried under the stream of nitrogen gas. The dry residue obtained at the end of extraction was dissolved in 2 mL of 95% ethanol, and was loaded to paper disc as follows: 10 discs (Whatman paper No. 1, disc diameter – 5 mm) were soaked in 100 µL of ethanolic extract, kept at room temperature overnight. The discs were placed on nutrient agar plates seeded with the target culture. Plates were incubated at 37°C for 48 h and the diameters of inhibition zones were measured. Discs containing 10 µL of ethanol were kept as control.

Optimization of media for production of antistaphylococcal metabolite from A. pullulans using factorial design

A two level factorial design-experiment was carried out for five variables glucose (0.4, 2.0 and 4.0 g%), peptone (0.5, 2.5 and 5.0 g%), KH_2PO_4 (0.015, 0.15 and 0.3 g%), pH (3.0, 4.0 and 5.0) and temperature (25, 30 and 35^{0}C) affecting the production of antistaphylococcal metabolite by *A. pullulans*.

Results

Optimization of media for production of antistaphylococcal metabolite by A. pullulans

To observe the effect of five variables namely glucose, peptone, KH_2PO_4, pH and temperature on production of antistaphylococcal metabolite, statistically designed experiments were performed. The variables having significant effect on production were evaluated by conducting 40 experiments which included two replicates of a 2^4 factorial experiments

with all the four factors and eight center points. Effect of temperature could not be established because at 35°C there was no response in terms of production of antistaphylococcal metabolite. Therefore, we dropped it from the analysis. Results were analyzed using response surface methodology (RSM) software. Table 1 gives the responses obtained in the form of production of antistaphylococcal metabolite (U/flask).

Table 1: 2- level factorial design for production of antistaphylococcal metabolites by *A. pullulans*

Run	A	B	C	D	Response* (Units / flask)
1	-1	-1	-1	-1	200
2	1	-1	-1	-1	170
3	-1	1	-1	-1	240
4	1	1	-1	-1	210
5	-1	-1	1	-1	190
6	1	-1	1	-1	170
7	-1	1	1	-1	230
8	1	1	1	-1	200
9	-1	-1	-1	1	190
10	1	-1	-1	1	150
11	-1	1	-1	1	190
12	1	1	-1	1	220
13	-1	-1	1	1	180
14	1	-1	1	1	0
15	-1	1	1	1	180
16	1	1	1	1	200
17	-1	-1	-1	-1	210
18	1	-1	-1	-1	180
19	-1	1	-1	-1	220
20	1	1	-1	-1	190
21	-1	-1	1	-1	200
22	-1	-1	1	-1	230
23	-1	1	1	-1	190
24	1	1	1	-1	240
25	-1	-1	-1	1	180
26	1	-1	-1	1	260
27	-1	1	-1	1	160
28	1	1	-1	-1	260
29	-1	-1	1	1	240
30	1	-1	1	1	0
31	-1	1	1	1	220
32	1	1	1	1	220
33	0	0	0	0	380
34	0	0	0	0	420
35	0	0	0	0	460
36	0	0	0	0	480
37	0	0	0	0	430
38	0	0	0	0	380
39	0	0	0	0	450
40	0	0	0	0	420

*Response is in term of production of antistaphylococcal metabolite per flask. The data of runs is the mean of three independent experiments.

A= Glucose C= KH_2PO_4

B= Peptone D= pH

The experimental results obtained showed that all the variables had significant effect on production of antistaphylococcal metabolite from *A. pullulans* strain. Based on the identification of variables by the 2-level factorial design, a central composite design was developed for variables significantly affecting production of antistaphylococcal metabolite. These studies revealed that the optimum production of antistaphylococcal metabolite occurred at 2.0% glucose, 2.5% peptone, and 0.15% KH_2PO_4, pH 4.0 and temperature 30°C. The responses obtained were statistically evaluated and the model was built based on the variables with confidence levels more than 95% (Table 2). The generated model was of the quadratic type, the selected P-values of linear and interactive variables have been mentioned in Table 3. The standard error in production of antistaphylococcal metabolite was estimated to be 35.75.

Table 2: Estimates of the model coefficients with their *p*- values

Variables		Estimate of Coefficient	*p* – values*
A	Glucose	-10	0.4363
B	Peptone	19.375	0.2542
C	KH_2PO_4	-10.625	0.4175
D	pH	-13.125	0.3546
AB	Glucose - Peptone	16.875	0.2872
AC	Glucose - KH_2PO_4	-13.125	0.3546
AD	Glucose - pH	-4.375	0.6872
BC	Peptone - KH_2PO_4	10	0.4363
BD	Peptone - pH	8.75	0.4784
CD	KH_2PO_4 - pH	-12.5	0.3687
AA	Glucose - Glucose	-59.0625	0.0492
BB	Peptone - Peptone	-59.0625	0.0492
CC	KH_2PO_4 - KH_2PO_4	-59.0625	0.0492
DD	pH- pH	-59.0625	0.0492

A = Glucose, B = Peptone, C = KH_2PO_4, D = pH

***** *p*- values less than 0.05 indicate significant variables.

Table 3: Analysis of variances of model

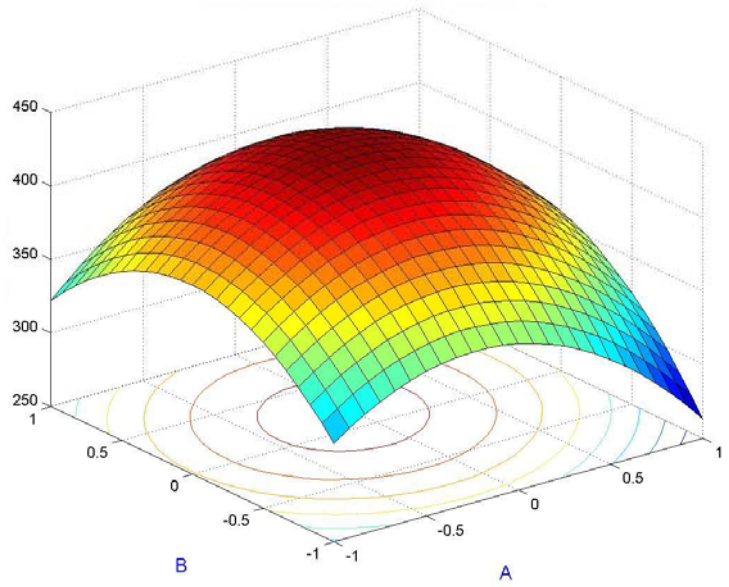
Source	D.F.	Sum of Squares	Mean Square	F	*p* values*
Model	11	407435	37039.5455	17.3212	0
Linear	4	24337.5	6084.375	2.8453	0.0426
A	1	3200	3200	1.4965	0.2314
B	1	12012.5	12012.5	5.6175	0.2049
C	1	3612.5	3612.5	1.6894	0.2043
D	1	5512.5	5512.5	2.5779	0.1196
Interactions	6	25887.5	4314.5833	2.0177	0.0967
AB	1	5512.5	9112.5	4.2614	0.0484
AC	1	5512.5	5512.5	2.5779	0.1196
AD	1	612.5	612.5	0.2846	0.5967
BC	1	3200	3200	1.4965	0.2314
BD	1	2450	2450	1.1457	0.2936
CD	1	5000	5000	2.3382	0.1375
Quadratic	2	357210	178605	83.523	0.0003
AA	1	357210	357210	167.046	0
BB	1	0	0	0	1
CC	1	0	0	0	1
DD	1	0	0	0	1
Pure Error	7	8950	1278.5714	0	0
Lack of Fit	21	50925	2425	1.8966	0.196
Error	28	59875	2138.3929	0	0
Total	39	467310	0	0	0

***** *p* – Values less than 0.05 indicate significant variables.

Figs. 1-3 indicate that glucose-peptone, KH_2PO_4- peptone, pH-peptone, KH_2PO_4-glucose, pH-glucose, pH- KH_2PO_4 have a quadratic relationship. With increase in any of them the antistaphylococcal metabolite production increased.

A = Glucose
B = Peptone
C = KH_2PO_4

Activity (Unit / mL)



Activity (Unit / mL)

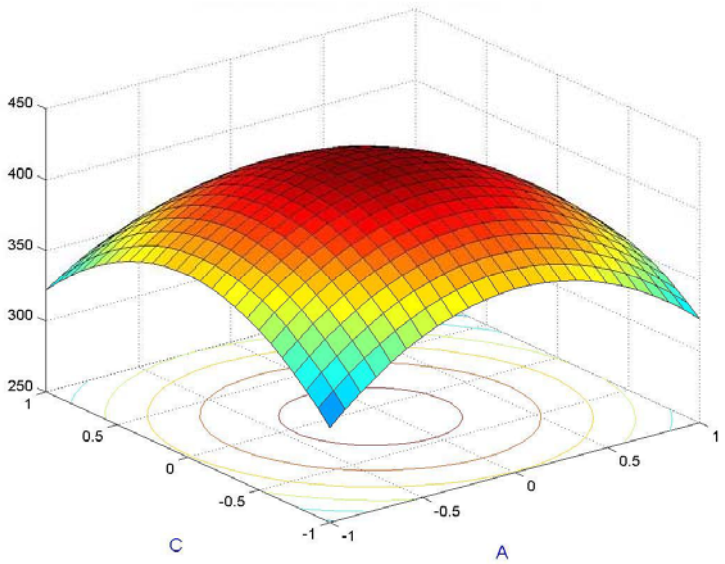
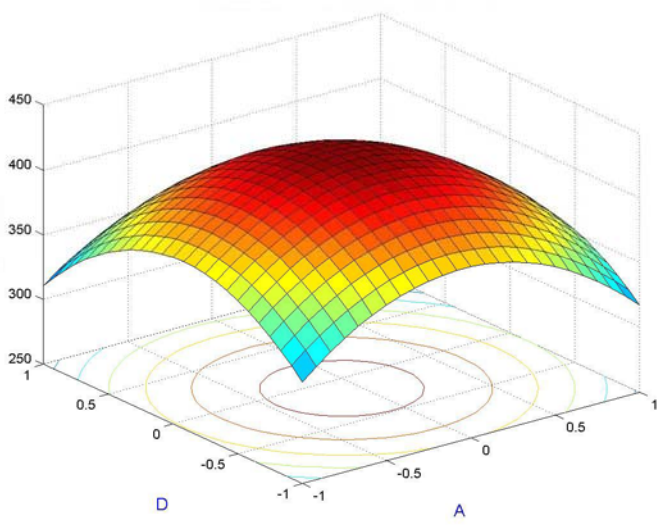


Fig. 1: Optimization of antistaphylococcal metabolite from *A. pullulans* using factorial design. A three level factorial design was carried out for optimization of production of antistaphylococcal metabolite for four variables glucose (0.4, 2 and 4 %), peptone (0.5, 2.5 and 5%), KH_2PO_4 (0.015, 0.15 and 0.3%) and pH (3,4,5).

The model has a high correlation coefficient (R^2=0.8719), a significant F-value (17.3212), an insignificant lack of fit F-value (1.8966) and standard error less than 10 in all the factors. Based on the model equation, three-dimensional surface plots were constructed, which gave the optimal level of the variables and their linear, interactive or quadratic responses. The plots represent interaction of two variables while keeping others constant.

A = Glucose
B = Peptone
C = KH_2PO_4
D = pH



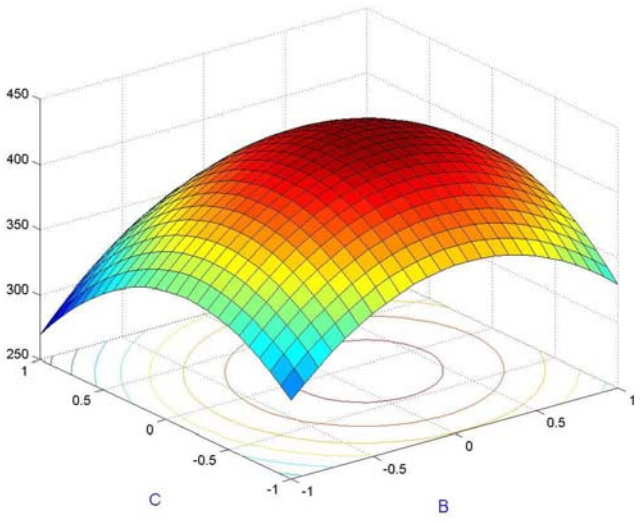
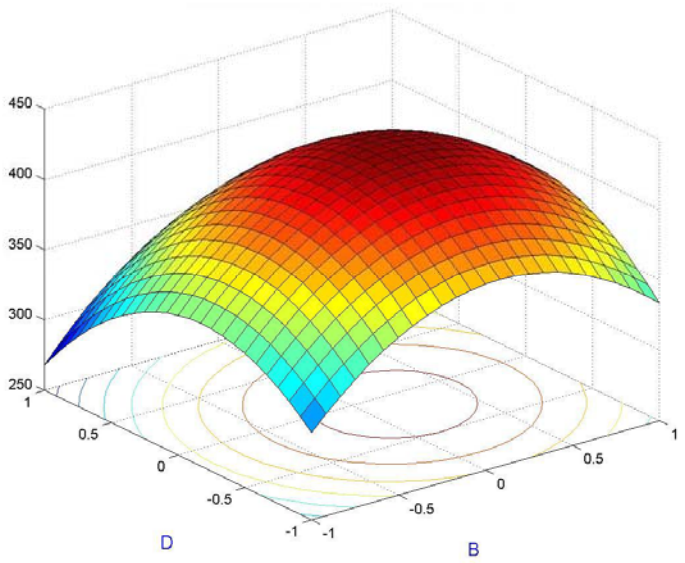


Fig. 2: Optimization of antistaphylococcal metabolite from *A. pullulans* using factorial design.

B = Peptone
C = KH_2PO_4
D = pH

Activity (Unit / mL)



Activity (Unit / mL)

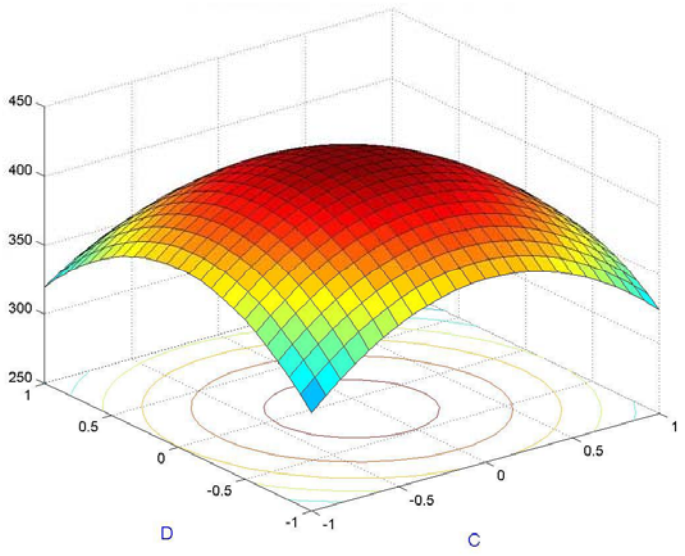


Fig. 3: Optimization of antistaphylococcal metabolite from *A. pullulans* using factorial design.

Discussion

In order to optimize the parameters of production of antistaphylococcal metabolite by *A. pullulans* factorial design was used. In the traditional methods of optimization, since each parameter is independently investigated, the interaction effect is missed. Moreover, it is tedious and time consuming, especially for a large number of variables (11,12). According to Adinarayana and Ellaiah, factorial experiments are good way of judging the relative significance of the influencing factors and give a quantitative measure of the contribution of each factor to the overall response (10). In the present investigation, a 2^4 factorial design was chosen to investigate the effect of parameters namely; glucose, peptone, KH_2PO_4, and pH. Under optimized conditions *A. pullulans* produced 900 units of antistaphylococcal metabolite from the biomass grown in 100 mL medium under the optimum conditions.

The production of antistaphylococcal metabolite was parallel to log phase of growth, though, the initial lag of one day was observed before the beginning of accumulation of intracellular antistaphylococcal metabolite. The antistaphylococcal activity of *A. pullulans* seemed to be associated with primary metabolite rather than secondary metabolite. However, this conclusion should be taken with caution because both secondary metabolites as well as antibiotics are heterogeneous group and our knowledge regarding the exact definitions and of secondary metabolite / antibiotics are far from the perfection.

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