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Antimicrobial activity and optimisation of aureobasidin A production by *Aureobasidium pullulans* PA-2 against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: This work aims to separate the antimicrobial lipopeptides from fermentation by *Aureobasidium pullulans* PA-2, and to verify its antimicrobial activity and the optimum conditions of the lipopeptide production. Using a high--performance liquid chromatography analysis, the lipopeptides with antimicrobial activity were evaluated with the agar well diffusion test. Response surface methodology was used to determine the optimum conditions for lipopeptides from *A. pullulans* PA-2. The lipopeptides with antimicrobial activity in the fermentation supernatant of the PA-2 strain was found to be aureobasidin A (AbA). The *in vitro* antagonistic tests showed that the minimal inhibitory concentration of AbA against *Staphylococcus aureus* and *Escherichia coli* was 0.5 and 1.0 mg/mL, respectively. The optimal fermentation conditions were an inoculum size of 6.8% (v/v) (OD_{600} = 0.25), a rotation speed of 216 rpm, a culture temperature of 26 °C, a liquid volume of 125 mL and an initial pH of 7. Under these conditions, the predicted yield of the antimicrobial lipopeptides by the model was 940 mg/L, the observed yield was 920 mg/L, which was 51% more than that before the optimisation (610 mg/L).

Keywords: *Aureobasidium pullulans* PA-2; antimicrobial activity; lipopeptides; response surface methodology

Aureobasidium pullulans is a species of yeast-like fungus, which has both yeast-like and mycelium forms (Schoch et al. 2006; Slepecky & Starmer 2009; Gostinčar et al. 2011). *A. pullulans* has attracted interest due to its abilities to be used to produce considerable biotechnological applications (Prasongsuk et al. 2018). *A. pullulans* can be used to produce many fermentation products, such as pullulan, aureobasidin A, siderophores, melanin, and heavy oils, etc. that have applications in medicine, agriculture and the food industry (Leathers 2003; Manitchotpisit et al. 2014; Sugumaran & Ponnusami 2017). Moreover, *A. pullulans* is an available biocontrol species that is effective against both

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bacterial and fungal plant pathogens (Johnson & Temple 2013; Janisiewicz et al. 2014; Spadaro & Droby 2016). Aureobasidin A (AbA) produced by *A. pullulans* BP-1983 shows strong fungicidal activity against *Cryptococcus neoformans*, *Candida* species, and some *Aspergillus* species (Takesako et al. 1991, 1993). Zhang et al. (2010) demonstrated that the application of *A. pullulans* PL5 could suppress the post-harvest grey mould, brown rot, and blue mould on fruits by secreting lytic enzymes and including competition for nutrients to the pathogen in the fruits. It was reported that the *A. pullulans* L1 and L8 strains, isolated from peach fruit, showed a high efficacy against *Penicillium expansum*, *Botrytis cinerea*, and the *Colletotrichum acutatum* responsible for the rot and mould of apple fruits (Mari et al. 2002).

The antibiotic aureobasidin A is a cyclic nonadepsipeptide produced by *A. pullulans* (Takesako et al. 1991) that shows strong fungicidal activity against *Candida* species, *Cryptococcus neoformans*, and some *Aspergillus* species (Takesako et al. 1993). Recent research has focused on the characterisation of the biosynthesis, inhibitory mechanism and properties of aureobasidin A (Slightom et al. 2009). However, studies on its production are still limited.

Previously, our group has reported on the spore production and inhibitory activity by *A. Pullulans* PA-2 (Yang et al. 2019; Guo et al. 2020). In the present study, an indigenous strain of *A. pullulans* PA-2 was examined for its antimicrobial activity against four different targets strains. Acid hydrolysis, high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) were performed to verify the structure of the antimicrobial agent. Moreover, the minimal inhibitory concentration (MIC) of the AbA was evaluated using the well diffusion assays. Finally, the optimum conditions suitable for the formation of the AbA production was determined.

MATERIAL AND METHODS

Microorganisms. Test strain: *Aureobasidium pullulans* PA-2 was provided by the Key laboratory of agricultural integrated pest management of Qinghai Province at Academy of Agriculture and Forestry Science, Qinghai University. This yeast strain was maintained in a solid potato dextrose agar (PDA) medium containing glucose 20.0 g/L, potato extract 20.0 g/L, dextrose 20.0 g/L, and agar 18.0 g/L at 4 °C. The rDNA internal transcribed spacer sequence and the 28S rDNA sequence of the PA-2 strain were deposited in the GenBank under accession numbers MH304262 and MT965771, respectively. Target strains: *Staphylococcus aureus* ATCC6538, *Saccharomyces cerevisiae* ATCC9098, *Escherichia coli* ATCC8739, and *Candida albicans* ATCC10231 were maintained on a Luria-Bertani (LB) solid medium containing tryptone 10.0 g/L, yeast extract 5.0 g/L, and NaCl 5.0 g/L at 4 °C. *Mycosphaerella cerasella* LD-2 and *Pyrenophora teres* HZ-2 were maintained in a PDA medium at 4 °C.

Culture conditions. *A. pullulans* PA-2 was grown on a PDA medium and incubated at 25 °C for seven days. One mycelial agar plug with a diameter of Φ = 0.5 cm was picked then inoculated into the fermentation medium [glucose 20 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $CaCl_2$ 0.1 g/L, NaCl 0.1 g/L, $FeCl_3$ 0.5 g/L, $ZnSO_4$ 0.5 mg/L] and incubated at 25 °C for five days with shaking at 180 rpm. The resulting fermentation broth was centrifuged at 10 000 rpm at 4 °C for 10 min, the supernatant was filtered through a 0.22 μm filter membrane and the filtrate was stored at 4 °C for the subsequent experiments.

Purification and identification of the antimicrobial lipopeptide. The crude lipopeptide was extracted according to the protocols of Yan et al. (2020). Briefly, the supernatants were precipitated by adding 6 M of HCl to obtain a final pH of 2.0, and then the entire sample was allowed to stand overnight at 4 °C. The samples were centrifuged at 10 000 rpm for 10 min at 4 °C. The precipitates were collected and then extracted with 20 mL of methanol for 2 h using an ultrasonic oscillator. The extracts were filtered through Whatman No. 1 filter paper, and then the solvents were removed by an evaporator to obtain the crude lipopeptide. The crude lipopeptide was weighed and the yield (m/v) was 610 mg/L. The crude lipopeptide was dissolved in 1 mL of methanol and analysed by HPLC. The solution was analysed by chromatography on an analytical ODS-BP gel column (SinoChrom C18, 4.6 mm × 250 mm, 5 μm), and the column was eluted with 75% methanol containing 0.1% trifluoroacetic acid (TFA) to separate component 1 and 2. Component 1 was purified by a preparative C18 column (20 mm × 250 mm, 10 μm) with 70% methanol containing 0.1% TFA to obtain the peak 3 component.

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Antimicrobial activity of AbA on the test strains. The antimicrobial activity of AbA against the test strains was measured using the agar well diffusion method (Nanjundan et al. 2019). The sterilised medium was poured into Petri plates and allowed to solidify. The concentration of the bacterial inoculum was 10^4 CFU/mL, and that of the fungi was 10^5 CFU/mL. A 0.5 mL inoculum of the test strains was pipetted into a Petri dish and was then spread on the surface of LB (for the bacteria) or PDA (for the fungi) by a glass spreading rod and air-dried, after which wells with an 8 mm diameter were made in the plate using a sterile cork borer. For each test organism, 100 µL of AbA was loaded in each well in triplicate, with the methanol alone as control 1. The test samples were prepared at a concentration of 1 mg/mL. Distilled water was used as control 2. Three replications were maintained for each treatment. All the Petri dishes were incubated either at 28 ± 2 °C for 24 h (for the bacteria) or at 25 ± 2 °C for 96 h (for the fungi), followed by measuring the size of the inhibition zone. The evaluation of the antimicrobial activity was carried out in triplicate. The minimum inhibitory concentration (MIC) of AbA against the test strains refers to the above method. The MIC was recorded as the lowest concentration of AbA that completely inhibited the visible growth of the test strains.

Optimisation of the fermentation conditions of *A. pullulans* PA-2. A single factor-screening test was used to ascertain the optimal culture conditions for the highest production of AbA. First, the production of AbA was considered as the optimisation factors to select the optimal environmental conditions, including the inoculum size, rotation speed, temperature, liquid volume, and initial pH value.

Response surface methodology (RSM) is a statistical experimental design for optimising biological processes, which is used to establish continuous variable surface models to evaluate the factors affecting the biological processes and their interactions, given the range of optimal levels and the relatively small number of test groups required, this approach has been successfully applied to a wide range of biological process optimisations (Oktay et al. 2023). RSM was employed to study the interaction among independent variables and their contribution towards the AbA production. The process variables and the responses were determined by single-factor experiments. The effects of the variables, inoculum size (χ_1), rotation speed (χ_2), and temperature (χ_3), liquid volume (χ_4) and initial pH value (χ_5) on the production of AbA were implemented. Each variable was coded at five levels: –2.378, –1, 0, 1, 2.378 (Table 1).

The variables were coded according to the following equation:

$$\chi_i = (X_i - \overline{X}_l)/\Delta\overline{X}_l \quad (1)$$

where: χ_i – dimensionless value of an independent variable; X_i – real value of an independent variable; $\overline{X}_l$ – real value of an independent variable at the centre point; $\Delta\overline{X}_l$ – step change.

The investigated response function was $\overline{Y}_l$ = mg AbA/L of fermented media. A central composite experimental design (CCD) was arranged to allow the second-order model to be fitted (Shi et al. 2011) (Table 2). The CCD combined the vertices of the hybercube whose coordinates were given by the 2^n factorial design with the star points. The star points were added to the factorial design to provide the curvature estimation of the model. Five replicates at the centre point of the design were used to estimate of the pure-error sum of squares. All

Table 1. Values of the independent variables of the processes and their corresponding levels and the passage from the coded variables level to the natural variable level*

Independent variable	Symbol		Coded variables levels				
	uncodified	codified	–2.378	–1	0	+1	+2.378
Inoculum size (%)	X_1	χ_1	2.24	5	7	9	11.76
Rotation speed (rpm)	X_2	χ_2	118.66	160	190	220	261.34
Temperature (°C)	X_3	χ_3	20.24	23	25	27	29.80
Liquid volume (mL)	X_4	χ_4	31.10	100	150	200	268.90
Initial pH value	X_5	χ_5	4.62	6	7	8	9.38

*Given by the following equations: $\chi_1 = (X_1 - 7)/2$; $\chi_2 = (X_2 - 190)/30$; $\chi_3 = (X_3 - 25)/2$; $\chi_4 = (X_4 - 150)/50$; $\chi_5 = (X_5 - 7)/1$

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Table 2. Composite experimental design arrangement, responses, and predicted values for the aureobasidin A (AbA) yield

Run	Coded variables					Uncoded variables					AbA yield (Y) (mg/L)		
	X_1	X_2	X_3	X_4	X_5	χ_1	χ_2	χ_3	χ_4	χ_5	experimental (Y_0)	predicted (Y_i)	Y_0–Y_i
1	0	–2.4	0	0	0	7	118	25	150	7	320	310	10
2	0	2.4	0	0	0	7	261	25	150	7	820	830	–10
3	1	–1	1	1	–1	9	160	27	200	6	260	250	10
4	1	–1	1	1	1	9	160	27	200	8	250	260	–10
5	0	0	0	0	0	7	190	25	150	7	830	860	–30
6	0	0	0	2.4	0	7	190	29.8	268.9	7	250	240	10
7	0	0	0	0	0	7	190	25	150	7	890	860	30
8	0	0	0	0	0	7	190	25	150	7	820	860	–40
9	–1	–1	1	–1	1	5	160	27	100	8	450	480	–30
10	1	–1	–1	1	–1	9	160	23	200	6	460	450	10
11	–1	1	1	–1	–1	5	220	27	100	6	770	760	10
12	–1	–1	–1	1	1	5	160	23	200	8	450	440	10
13	–1	–1	–1	–1	1	5	160	23	100	8	280	270	10
14	1	1	1	1	1	9	220	27	200	8	480	450	30
15	1	1	–1	–1	1	9	220	23	100	8	520	530	–10
16	–1	1	–1	1	–1	5	220	23	200	6	560	510	50
17	1	1	–1	1	–1	9	220	23	200	6	590	580	10
18	0	0	0	0	2.4	7	190	25	150	9.3	360	340	20
19	–1	1	1	1	–1	5	220	27	200	6	500	520	–20
20	1	1	1	–1	–1	9	220	27	100	6	780	750	30
21	–2.4	0	0	0	0	2.2	190	25	150	7	450	420	30
22	–1	–1	1	1	–1	5	160	27	200	6	340	280	60
23	–1	1	–1	1	1	5	220	23	200	8	340	550	–10
24	–1	–1	1	1	1	5	160	27	200	8	330	340	–10
25	–1	1	–1	–1	1	5	220	23	100	8	450	470	–20
26	0	0	0	–2.4	0	7	190	25	31.1	7	360	360	0
27	–1	–1	1	–1	–1	5	160	27	100	6	390	430	–40
28	1	–1	–1	–1	1	9	160	23	100	7	330	330	0
29	–1	1	1	–1	1	5	220	27	100	7	810	770	40
30	–1	1	–1	–1	–1	5	220	23	100	6	430	450	–20
31	–1	1	1	1	1	5	220	27	200	8	490	540	–50
32	0	0	–2.4	0	0	7	190	20.2	150	7	380	390	–10
33	1	–1	1	–1	–1	9	160	27	100	6	420	430	–10
34	1	1	1	1	–1	9	220	27	200	6	450	480	–30
35	1	–1	–1	–1	–1	9	160	23	100	6	340	310	30
36	0	0	2.4	0	0	7	190	29.8	268.9	7	520	510	10
37	2.38	0	0	0	0	11.8	190	25	150	7	420	450	–30
38	0	0	0	0	–2.4	7	190	25	150	4.6	280	300	–20
39	1	–1	1	–1	1	9	160	27	100	8	440	430	10
40	0	0	0	0	0	7	190	25	150	7	840	860	–20
41	1	–1	–1	1	1	9	160	23	200	8	460	470	–10

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Table 2 to be continued

Run	Coded variables					Uncoded variables					AbA yield (Y) (mg/L)		
	X_1	X_2	X_3	X_4	X_5	χ_1	χ_2	χ_3	χ_4	χ_5	experimental (Y_0)	predicted (Y_i)	Y_0-Y_i
42	–1	–1	–1	–1	–1	5	160	23	100	6	230	210	20
43	0	0	0	0	0	7	190	25	150	7	850	860	–10
44	–1	–1	–1	1	–1	5	160	23	200	6	300	370	–70
45	1	1	1	–1	1	9	220	27	100	8	710	720	–10
46	1	1	–1	1	1	9	220	23	200	8	600	580	20
47	0	0	0	0	0	7	190	25	150	7	860	860	0
48	0	0	0	0	0	7	190	25	150	7	900	860	40
49	0	0	0	0	0	7	190	25	150	7	910	860	50
50	1	1	–1	–1	–1	9	220	23	100	6	550	550	0

X_1 – inoculum size; X_2 – rotation speed; X_3 – temperature; X_4 – liquid volume; X_5 – initial pH value

the experiments were carried out in a randomised order to minimise the extraneous factors on the observed responses.

The model proposed for the response (Y_i) was:

$$Y_i = b_{0+} b_1\chi_1 + b_2\chi_2 + b_3\chi_3 + b_4\chi_4 + b_5\chi_5 + \\ + b_{11}\chi_1^2 + b_{22}\chi_2^2 + b_{33}\chi_3^2 + b_{44}\chi_4^2 + b_{55}\chi_5^2 + \\ + b_{12}\chi_1\chi_2 + b_{13}\chi_1\chi_3 + b_{14}\chi_1\chi_4 + b_{15}\chi_1\chi_5 + \\ + b_{23}\chi_2\chi_3 + b_{24}\chi_2\chi_4 + b_{25}\chi_2\chi_5 + b_{34}\chi_3\chi_4 + \\ + b_{35}\chi_3\chi_5 + b_{45}\chi_4\chi_5 \quad (2)$$

where: Y_i – predicted response; b_0 – offset term; b_1, b_2, b_3, b_4, and b_5 – liner effect terms; b_{11}, b_{22}, b_{33}, b_{44}, and b_{55} – squared effects; b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, and b_{45} – interaction effects.

Surface plots were generated by assigning constant (zero) values to two of the three variables and solving the fitted equations as a quadratic equation in the remaining variable.

The statistical significance of the model equation was examined using the F-test and the proportion of variance explained by the model which was indicated by the R^2-value (Song et al. 2011). For each variable, the quadratic model was expressed by the response surface graph generated using Design-Expert (version 10.0.3). The generated three-dimensional response surface plots facilitate the understanding of the effect of the variables individually and in combination. To verify the predicted optimal cultivation conditions, the flasks were used in triplicate.

Statistical analysis. The data were processed using Microsoft Excel 2019. The data were expressed as the mean ± standard deviation (SD) with at least three independent experiments. The statistical significance was analysed by a one-way analysis of variance (ANOVA) using SPSS (version 17.0). The differences were considered statistically significant at $P < 0.05$.

RESULTS

Purification and identification of the antimicrobial lipopeptides. Fifty-nine grams of crude extract was obtained from 50 L of the fermentation broth. The results of the antimicrobial activity of the crude extracts are summarised in Table 3. In all of the tested species, the crude extract strongly inhibited *Staphylococcus aureus*, with a 34.5 ± 0.03 mm inhibition zone, while *Saccharomyces*

Table 3. Inhibition effect of the crude lipopeptide on the different test microorganisms

Test strains	Average inhibitory zone diameter (mm)
Staphylococcus aureus	34.5 ± 0.03[a]
Saccharomyces cerevisiae	18.5 ± 0.05[b]
Escherichia coli	23.5 ± 0.06[b]
Candida albicans	0.00 ± 0.00[d]
Mycosphaerella cerasella	12.5 ± 0.03[bc]
Pyrenophora teres	20.5 ± 0.04[b]

[a–d]Different letters in the same column indicate a difference at a 0.05 level

Experiments were performed in triplicate and the results are presented as the mean ± SD

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Table 4. Inhibition effect of the component on the different test microorganisms

Test strains	Component 1	Component 2	Peak 3	Peak 4	Peak 5
Staphylococcus aureus	32.0 ± 0.08[a]	0.00 ± 0.00	27.5 ± 0.06	20.0 ± 0.04	25.0 ± 0.08
Escherichia coli	28.0 ± 0.05	0.00 ± 0.00	25.0 ± 0.03	16.0 ± 0.03	22.0 ± 0.02

Data are given as the average inhibitory zone diameter (mm). Experiments were performed in triplicate and the results are presented as the mean ± SD

cerevisiae showed far lower activity. The crude extract had no effect on *Candida albicans*. The crude extracts that showed antimicrobial activity were subjected to HPLC. Two components were collected and assayed for their antimicrobial activity. Component 1 had an obvious inhibition zone against *S. aureus* (32.0 ± 0.08 mm) and *E. coli* (28.0 ± 0.05 mm) (Table 4), whereas component 2 had no inhibition zone (Figure 1). Component 1, which showed significant antimicrobial activity, was further purified by the preparative HPLC. The HPLC profile exhibited three major peaks (peak 3, 4, 5), which were later assayed for antimicrobial activity (Figure 2). Peak 3 exhibited the most inhibitory effect against *S. aureus* and *E. coli*. The inhibition zone diameter was 27.0 ± 0.06 mm and 25.0 ± 0.03 mm, respectively, followed by the peak 5 component, with an inhibition zone diameter of 25.0 ± 0.08 mm and 22.0 ± 0.02 mm (Table 4), respectively.

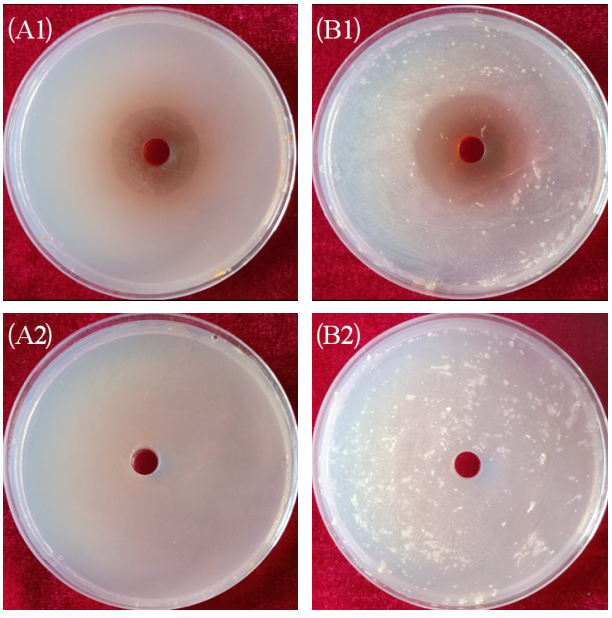

Figure 1. Inhibitory effects of the component from the crude extract against *Escherichia coli* and *Staphylococcus aureus* on LB agar (28 °C, 3 days)
Inhibitory effects of component 1: (A1) *E. coli*; (B1) *S. aureus*
Inhibitory effects of component 2: (A2) *E. coli*; (B2) *S. aureus*

In contrast, the peak 4 component showed the least antimicrobial activity against *S. aureus* and *E. coli* (Figure 3). Ultimately, the profiles suggest that the peak 3 was identified as a single compound by the analytical HPLC (Figure 4).

The peak 3 component (pure compound) was characterised by using spectroscopic [mass spectrometry (MS) and NMR] methods as aureobasidin A. It has the molecular formula $C_{60}H_{92}N_8O_{11}$, and was obtained as white needle crystals with the following spectral characteristics: MS *m*/*z* 1 100.694 (M + H), 1 123.696 $(M + Na)^+$, 1 099.694 $(M\text{-}H)^-$. ^{1}H-NMR ($CDCI_3$) δ 8.90~7.59 (total 3H, NH), 7.32~7.10 (m, 9H, MePhe, Phe), 6.52 (d, 1H, *j* = 7.5 Hz, MePhe), 5.80 (s) and 5.77 (s) (total 1H, α-CH Hmp), 4.20 (s, 1H, HO HOMeVal), 3.41 (s, 1H, α-CH HOMeVal), 3.31 (s), 3.18 (s), 3.16 (s), 3.07 (s), 2.67 (s) and 2.50 (s) (total 12H, N-CH_3), 1.39 (s, 3H, γ-CH_3 HOMeVal), 1.19 (s, 3H, γ-CH_3 HOMeVal). The same compound was also characterised by Ikai et al. (1991) and Yoshikawa et al. (1993) from *A. pullulans* R106.

Determination of the antimicrobial activity of AbA. The inhibitory zone diameter of AbA from PA-2 against *S. aureus*, *S. cerevisiae*, *E. coli*, *M. cerasella*, and *P. teres* were 36.5 ± 0.09 mm, 19.5 ± 0.04 mm, 21.5 ± 0.08 mm, 13.5 ± 0.03 mm, and 21.8

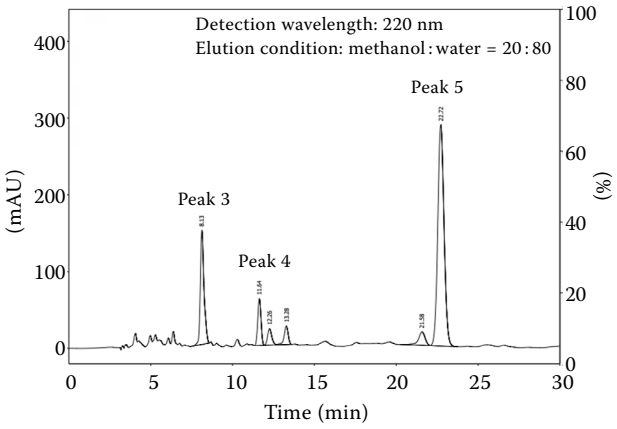

Figure 2. High-performance liquid chromatography chromatogram obtained from peak 3, 4, and 5 produced by *Aureobasidium pullulans* PA-2

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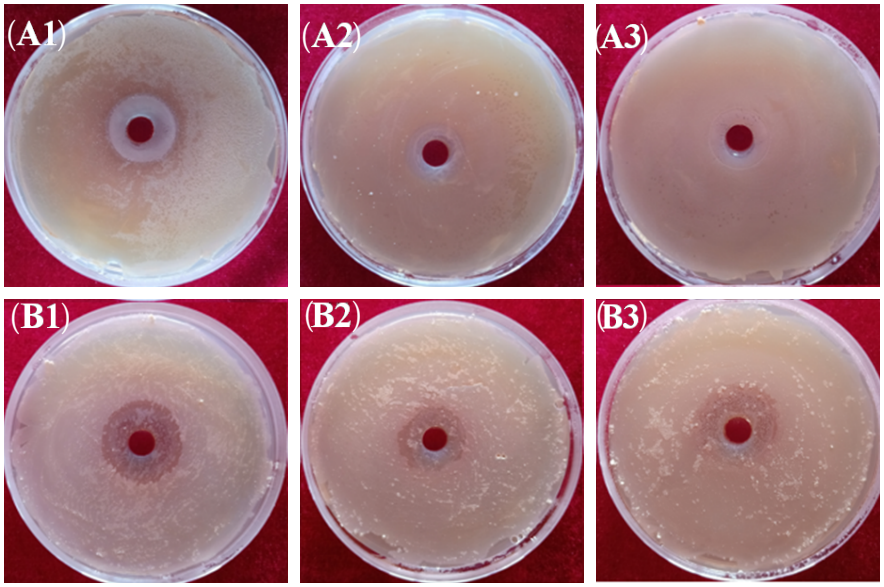

Figure 3. Inhibitory effects of peak 3, 4 and 5 from component 1 against *Escherichia coli* and *Staphylococcus aureus* on LB agar (28 °C, 3 days)
Inhibitory effects of peak 3: (A1) *E. coli*; (B1) *S. aureus*
Inhibitory effects of peak 4: (A2) *E. coli*; (B2) *S. aureus*
Inhibitory effects of peak 5: (A3) *E. coli*; (B3) *S. aureus*

± 0.02 mm, respectively (Table 5). However, AbA had no inhibitory activity on *C. albicans* (Figure 5). As the *S. aureus* and *E. coli* are the main pathogens that affect food globally, these two strains were selected as the test strains for further study.

In this study, we reported the MIC value of the AbA isolated from PA-2 tested against *S. aureus* and *E. coli* (Figure 6). Different concentrations (2.0, 1.5, 1.0, 0.5, and 0.25 mg/mL) of AbA were applied to test for the antimicrobial activity. As shown in Figure 6, the antimicrobial activity of AbA against *S. aureus* was better than that against *E. coli*. Among them, at the concentrations of 2.0, 1.5, and 1.0 mg/mL, obvious inhibition zones could be observed in the culture media for culturing *E. coli*, whereas there was no obvious inhibition zone at the concentrations of 0.5 and 0.25 mg/mL. This indicates that the MIC value of AbA against *E. coli* was 1.0 mg/mL. No inhibition zone appeared on the media for culturing *S. aureus* at a concentration of 0.25 mg/mL, so the MIC value of AbA was 0.5 mg/mL.

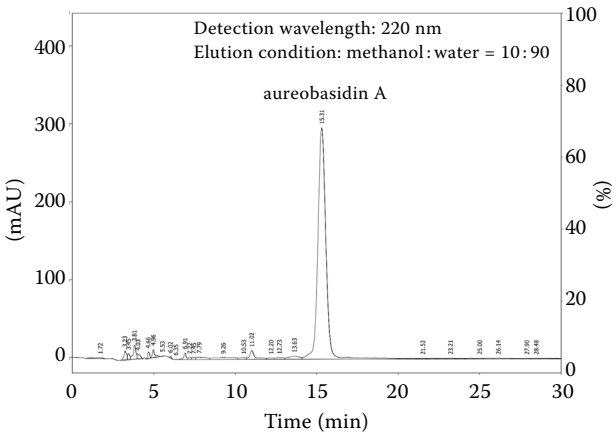

Figure 4. High-performance liquid chromatography chromatogram obtained from the aureobasidin A compound produced by *Aureobasidium pullulans* PA-2

Optimisation conditions for the production of AbA from *A. pullulans* PA-2. Figure 7A indicates that 7% (ratio of the volume) was the optimum inoculum size for the production of AbA, with a maximum production of 530 mg/L. More inoculum size did not show any positive effect on the AbA production. As shown in Figure 7B, the AbA production at a rotation speed of 190 rpm exhibited a relatively higher yield than that at the other four rotation speeds, indicating that 190 rpm

Table 5. Inhibition effect of aureobasidin A on the different test microorganisms

Test strains	Average inhibitory zone diameter (mm)
Staphylococcus aureus	36.5 ± 0.09[a]
Saccharomyces cerevisiae	19.5 ± 0.04[b]
Escherichia coli	21.5 ± 0.08[b]
Candida albicans	0.00 ± 0.00[d]
Mycosphaerella cerasella	13.5 ± 0.03[bc]
Pyrenophora teres	21.8 ± 0.02[b]
Methanol (control 1)	0.00 ± 0.00[d]
Distilled water (control 2)	0.00 ± 0.00[d]

[a–d]Different letters in the same column indicate a difference at a 0.05 level
Experiments were performed in triplicate and the results are presented as the mean ± SD

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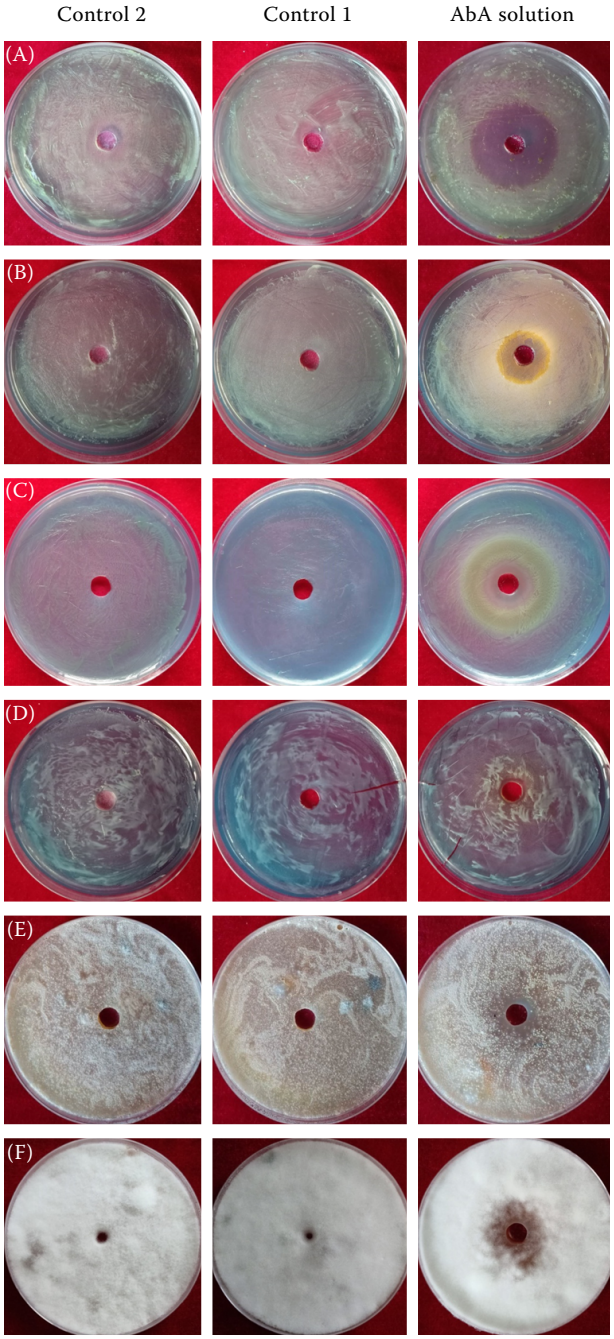

Figure 5. The inhibition effect of the aureobasidin A solution on the different test microorganisms
Staphylococcus aureus (A), *Saccharomyces cerevisiae* (B), *Escherichia coli* (C), *Candida albicans* (D), *Mycosphaerella cerasella* (E) and *Pyrenophora teres* (F)
Control 1 – methanol; Control 2 – sterile distilled water

was a good choice for the rotation speed. There are obvious differences in the production of AbA between 21 and 29 °C. *A. pullulans* PA-2 cultured at 25 °C exhibited the highest production at about 730 mg/L (Figure 7C), and was significantly higher

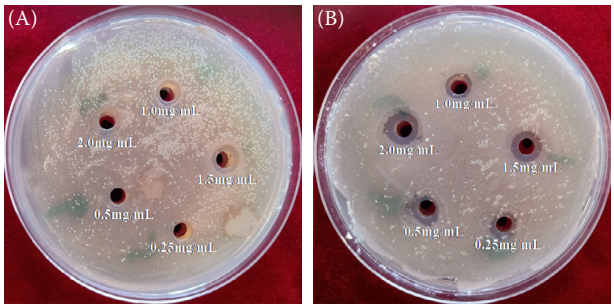

Figure 6. Determination of the minimum inhibitory concentration of aureobasidin A against *Escherichia coli* (A) and *Staphylococcus aureus* (B) on LB agar (28 °C, 3 days)

than that at the other temperature groups. For the production of AbA, 150 mL of the liquid volume was the optimum and the maximum AbA yield was 500 mg/L by *A. pullulans* PA-2 (Figure 7D). Further increasing the liquid volume has no obvious effect on increasing the AbA yield. As shown in Figure 7E, the highest production of AbA reached 650 mg/L when the pH value of the fermentation medium was 7.0 and was higher than the other samples in the pH group. Considering all the above results, the optimal culture conditions of *A. pullulans* was determined at a 7% inoculum size, a rotation speed of 190 rpm, a temperature of 25 °C, a liquid volume of 150 mL, and an initial pH value of 7.

The results of the CCD experiment with the different environmental conditions are presented in Table 2. The application of RSM yields the following regression equation, which is an empirical relationship between the AbA production and the test variable in coded units, as given in the following equation.

$$\begin{aligned} Y = {} & 0.862\,4 + 0.005\,7\chi_1 + 0.108\,2\chi_2 + \\ & + 0.025\,7\chi_3 - 0.025\,4\chi_4 + 0.009\,5\chi_5 - \\ & - 0.001\,9\chi_1\chi_2 - 0.028\,1\chi_1\chi_3 - 0.007\,5\chi_1\chi_4 - \\ & - 0.010\,6\chi_1\chi_5 + 0.022\,5\chi_2\chi_3 - 0.024\,4\chi_2\chi_4 - \\ & - 0.008\,8\chi_2\chi_5 - 0.078\,1\chi_3\chi_4 - 0.003\,8\chi_3\chi_5 + \\ & + 0.001\,9\chi_4\chi_5 - 0.075\,6\chi_1^2 - 0.051\,7\chi_2^2 - \\ & - 0.072\,9\chi_3^2 - 0.098\,5\chi_4^2 - 0.095\,9\chi_5^2 \end{aligned} \quad (3)$$

The statistical testing of the model was undertaken by the Fisher's statistical test for the analysis of variance (ANOVA) and the result is listed in Table 6. The analysis of variance indicated the model terms of χ_2, χ_3, χ_4, $\chi_1\chi_3$, $\chi_2\chi_3$, $\chi_2\chi_4$, and $\chi_3\chi_4$ for the desired response, i.e., the AbA production was significant ("Prob > *F*" less than 0.05). The interaction between χ_1 and χ_5 was not significant (higher *P*-

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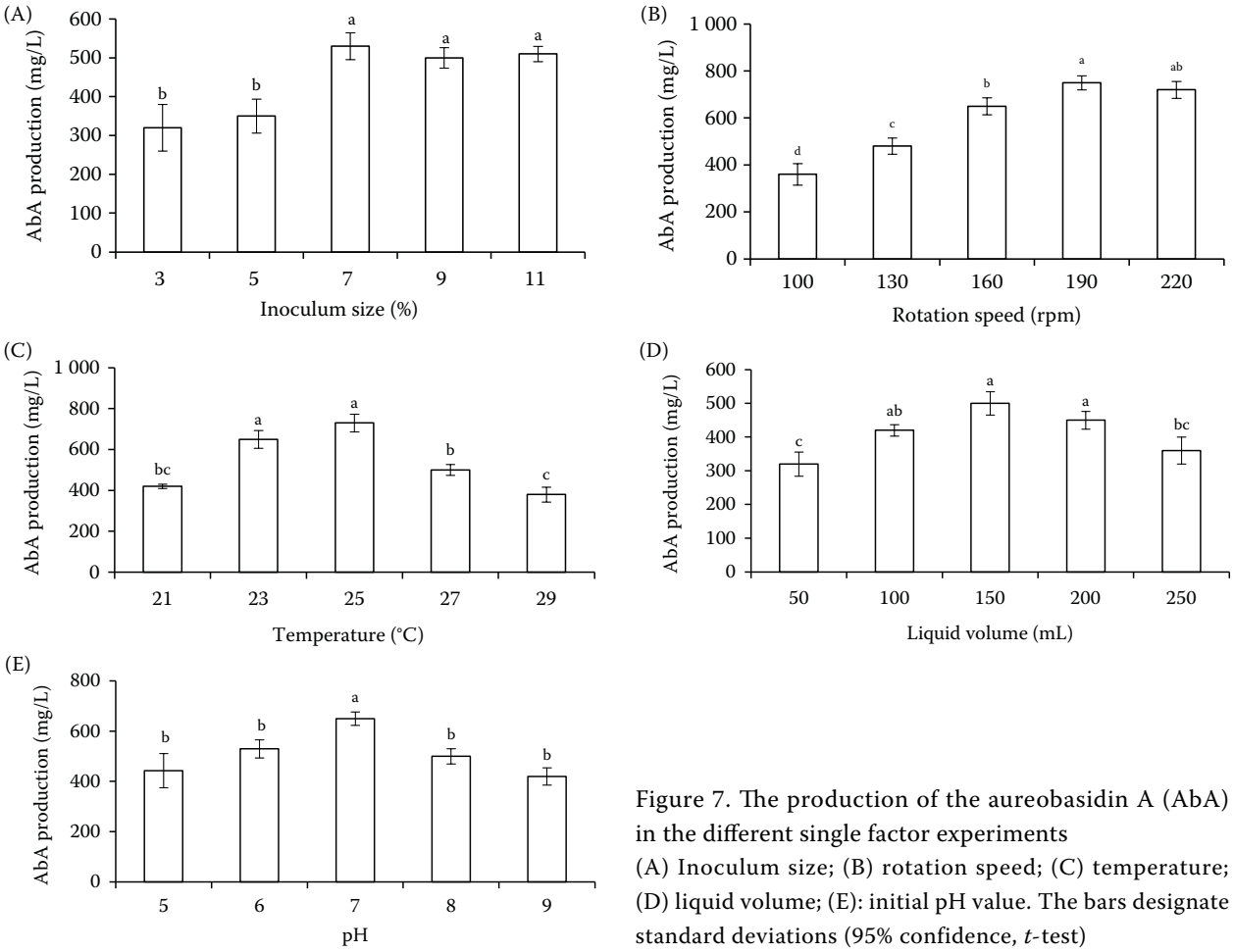

Figure 7. The production of the aureobasidin A (AbA) in the different single factor experiments
(A) Inoculum size; (B) rotation speed; (C) temperature; (D) liquid volume; (E): initial pH value. The bars designate standard deviations (95% confidence, *t*-test)

value). The model *F*-value is 89.28 which suggests that the model is significant and there is only 0.01% chance that a model *F*-value this large could occur due to noise. The "lack of fit" of 1.02 implies the lack of fit is not significant relative to the pure-error due to noise. In addition, the coefficient of variation was calculated as 0.001, indicating that the performed experiments are more reliable.

The quality of the model can be tested by the determining coefficient (R^2). In our case, the regression equation demonstrated that the correlation coefficient of R^2 is 0.984 0 and the adjusted R^2 and predicted R^2 values for the model were 0.973 0 and 0.952 4, respectively (Table 6). Thus, the model is expected to predict the response more accurately in the current case.

The response surface plots were obtained as shown in Figure 6, which illustrates the interactive effects of the independent variables on the dependent one. These graphs were drawn by keeping two variables at coded zero levels while varying the remaining two variables and predicting the AbA production. Figure 8A represents the interaction between the inoculum size (χ_1) and temperature (χ_3) by keeping the other variables at their zero levels for the AbA production. This figure indicated that at a lower level of the inoculum size and temperature, the AbA production was lower and it increased gradually when the level of the inoculum size and temperature was increased in the medium. In Figure 8B, the response for the interactive factors of the rotation speed (χ_2) and temperature (χ_3) is shown when the other variables were kept at a fixed level. It should be noted that at a lower rotation speed, the AbA production was lower, but increased with an increase in the rotation speed. However, the effect of the rotation speed on the AbA production is more significant as compared to the temperature. Similarly, it can be seen from Figure 8C that the influence of the rotation speed on the AbA production is more significant than that of the liquid volume (χ_4). Figure 8D displays the interaction between the liquid volume (χ_4) and the temperature (χ_3) when the other variables were kept constant.

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Table 6. Analysis of variance (ANOVA) for the regression equation

SV	SS	DF	MS	*F*-value	Probe > *F*	Significance
Model	2.06	20	0.10	89.28	< 0.000 1	**
χ_1	1.426×10^{-3}	1	1.426×10^{-3}	1.23	0.275 6	
χ_2	0.51	1	0.51	439.53	< 0.000 1	**
χ_3	0.029	1	0.029	24.76	< 0.000 1	**
χ_4	0.028	1	0.028	24.26	< 0.000 1	**
χ_5	3.887×10^{-3}	1	3.887×10^{-3}	3.37	0.076 9	
$\chi_1\chi_2$	1.125×10^{-4}	1	1.125×10^{-3}	0.097	0.757 2	
$\chi_1\chi_3$	0.025	1	0.025	21.92	< 0.000 1	**
$\chi_1\chi_4$	1.800×10^{-3}	1	1.800×10^{-3}	1.56	0.221 9	
$\chi_1\chi_5$	3.613×10^{-3}	1	3.613×10^{-3}	3.13	0.087 5	
$\chi_2\chi_3$	0.016	1	0.016	14.03	0.000 8	**
$\chi_2\chi_4$	0.019	1	0.019	16.46	0.000 3	**
$\chi_2\chi_5$	2.450×10^{-3}	1	2.450×10^{-3}	2.12	0.156 0	
$\chi_3\chi_4$	0.20	1	0.20	169.10	< 0.000 1	**
$\chi_3\chi_5$	4.500×10^{-4}	1	4.500×10^{-4}	0.39	0.537 4	
$\chi_4\chi_5$	1.125×10^{-4}	1	1.125×10^{-4}	0.097	0.757 2	
χ_1^2	0.32	1	0.32	275.38	< 0.000 1	**
χ_2^2	0.15	1	0.15	129.08	< 0.000 1	**
χ_3^2	0.30	1	0.30	256.42	< 0.000 1	**
χ_4^2	0.54	1	0.54	468.04	< 0.000 1	**
χ_5^2	0.51	1	0.51	443.22	< 0.000 1	**
Residual	0.033	29	1.155×10^{-3}			
Lack of fit	0.026	22	1.161×10^{-3}	1.02	0.528 6	
Pure error	7.950×10^{-3}	7	1.136×10^{-3}			
Total	2.10	49				

DF – degrees of freedom; MS – mean squares; SS – sum of squares; SV – source of variations

*$P < 0.05$; **$P < 0.01$

R^2(Adjust) = 0.973 0; R^2(predict) = 0.952 4

Considering all the available results, an initial pH value of 7.17, a temperature of 25.80 °C, a rotation speed of 216.24 rpm, an inoculum size of 6.85%, and a liquid volume of 124.87 mL were found to be optimal for the AbA production from *A. pullulans*. The predicted maximum yield of AbA was 940 mg/L under the optimum conditions. Correspondingly, the actual values were an initial pH value of 7, a temperature of 26 °C, a rotation speed of 216 rpm, an inoculum size of 6.8%, and a liquid volume of 125 mL. The observed yield of AbA was 920 mg/L, which was 51% more than that before optimisation (610 mg/L).

DISCUSSION

Some yeasts show antimicrobial activity on food and plant pathogens that may be used as biological control agents, with the main antimicrobial substances being lipopeptides (Takesako et al. 1991; Slightom et al. 2009). PA-2 previously identified as *A. pullulans* (Li et al. 2014; Guo et al. 2020), was assayed for its activity against four bacterial and two fungal pathogens: *S. aureus*, *S. cerevisiae*, *E. coli*, *M. cerasella*, *P. teres*, and *C. albicans*. The results herein correspond well to the previous report of antimicrobial activity against *S. aureus*, *S. cerevisiae*, and *E. coli* in terms of the inhibition of growth (Takesako et al. 1993). The antimicrobial component used in this study was also identified as lipopeptides (AbA) by the HPLC, MS, and NMR analyses. Lipopeptides, such as AbA, can be efficiently used as biocontrol agents towards other species (Prasongsuk et al. 2013). AbA is capable of inhibiting fungal pathogens and providing the effective control of bacterial diseases. Major

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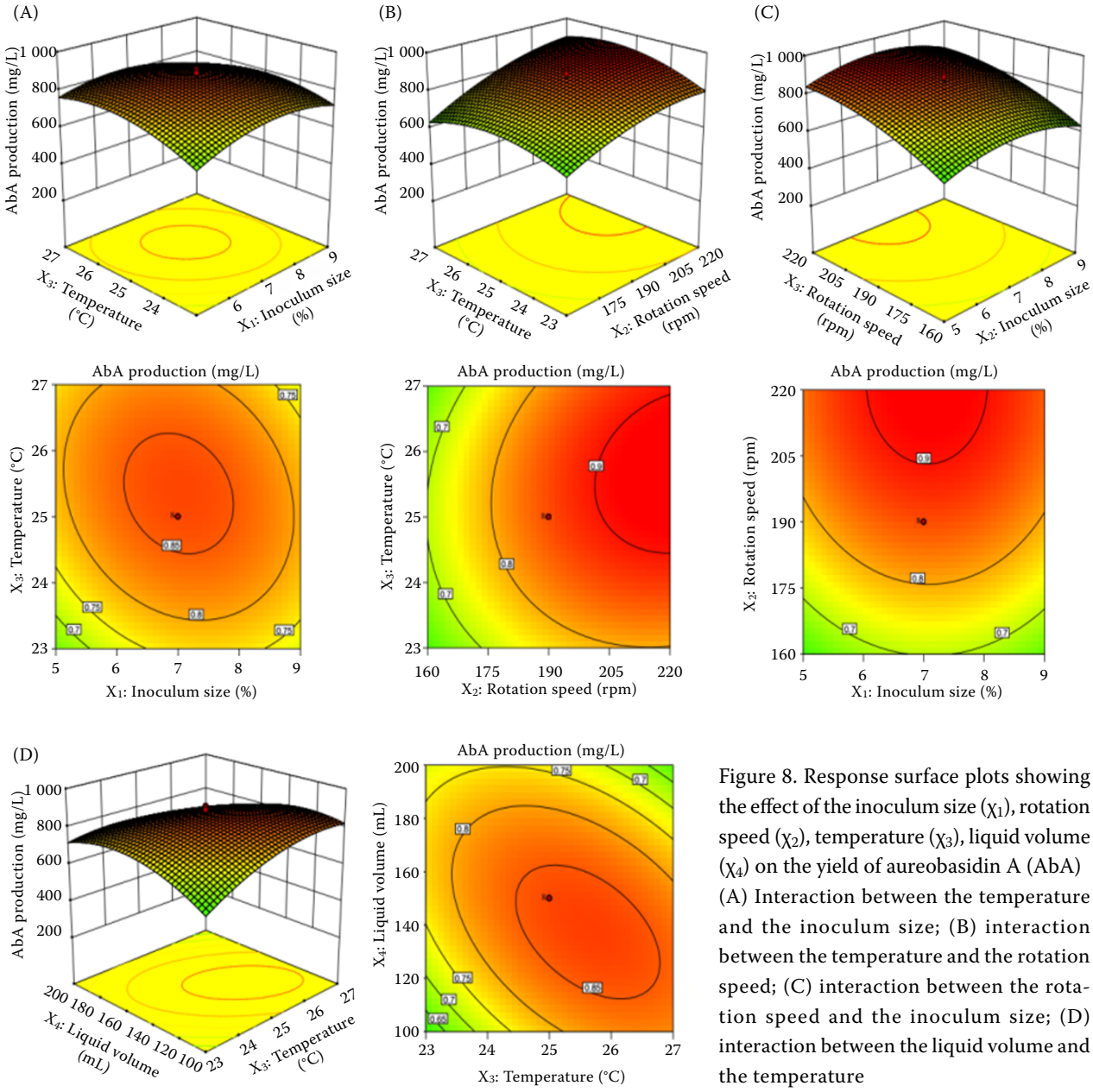

Figure 8. Response surface plots showing the effect of the inoculum size (χ_1), rotation speed (χ_2), temperature (χ_3), liquid volume (χ_4) on the yield of aureobasidin A (AbA)
(A) Interaction between the temperature and the inoculum size; (B) interaction between the temperature and the rotation speed; (C) interaction between the rotation speed and the inoculum size; (D) interaction between the liquid volume and the temperature

reports of aureobasidin appeared to have come from the *A. pullulans* strain R106 (Takesako et al. 1991, 1993; Kurome et al. 1996). This finding corresponded with earlier reports by Slightom et al. (2009), showing that the major antimicrobial agent produced by *A. pullulans* R106 was AbA.

Among the six pathogens examined, *S. aureus* was the most sensitive to AbA, whereas *C. albicans* exhibited the least sensitivity. However, in comparison with previous reports (Takesako et al. 1993; Endo et al. 1997), the sensitivities of these investigated pathogens to AbA are quite different in the sensitivity levels and species. It was reported that the low sensitivity of some *Aspergillus* spp. and *C. albicans* to AbA was associated with the increased the resistance efflux caused by the ATP-binding cassette (ABC) transporters, and that the co-treatment of AbA and the mammalian multidrug resistance (MDR) modulator dramatically increased the sensitivity of *A. fumigatus* to AbA (Ogawa et al. 1998; Zhong et al. 2000). Whether the low sensitivity of *C. albicans* is due to the increased efflux of AbA by some ABC transporters remains to elucidated upon in future studies. Such studies should include (1) the measurement of the radio-labelled aureobasidin A accumulation in the presence and absence of MDR modulators to confirm that the efflux is indeed involved and (2)

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the identification and deletion of the ABC transporter gene YOR1 (mediates pleiotropic drug resistance) in *C. albicans* to confirm its involvement in the AbA resistance of this organism.

The AbA yield, however, is low, to the extent that it constitutes a strong obstacle against the development of this compound into products for biocontrol agent use. Therefore, it is important to increase the production of AbA in the fermentation process, which will make the process economically feasible. The yield of the AbA product during fermentation depends on several factors and one of them is the interaction among the cultures (Liu et al. 2019). Classically, the single point optimisation technique has been used to enhance the yield during fermentation (Chi & Zhao 2003; Choudhury et al. 2012). However, the classical single variable or one-point optimisation methods are tedious and time-consuming and tend to overlook the interaction among the different factors and, therefore, may often lead to the misinterpretation of the results (Francis et al. 2003). RSM is an important statistical optimisation method, which can optimise many factors simultaneously, and can extract a large amount of quantitative information with only a few experimental tests, and has been widely used in the optimisation of medium and process conditions in different biological processes (Oskouei et al. 2008; Song et al. 2011). In this study, an initial pH value of 7.17, a temperature of 25.80 °C, a rotation speed of 216.24 rpm, an inoculum size of 6.85%, and a liquid volume of 124.87 mL were found to be optimal for the AbA production from *A. pullulans*. Up to now, there is still a lack of knowledge concerning the conditions of AbA production from *A. Pullulans* by statistical optimisation techniques, but similar studies using other products with *A. pullulans* have been reported. Li et al. (2011) worked on polymalic acid from *A. pullulans* and found the temperature had a significant effect and concluded that the optimum conditions were: temperature of 25 °C, inoculum size 8%, and an initial pH value of 7.17. Jin et al. (2012) reported that the optimal conditions for the polymalic acid production of *A. pullulans* were as follows: temperature of 25 °C, shaking speed of 220 rpm, and an initial pH of 5.5. Shen et al. (2019) stated that the significant effects of the pH, liquid volume, temperature, and rotation speed for the polysaccharide production from *A. pullulans* RM1603 had been found and concluded that the optimum fermentation conditions were: an initial pH of 7.0, a temperature of 28 °C, a rotation speed of 160 rpm, a liquid volume 50 mL in a 150 mL flask, and an inoculum size of 1%.

In conclusion, research on the biological activities of the *A. pullulans* lipopeptide in this study showed that the lipopeptide was found to be active against *S. aureus*, *E. coli*, *S. cerevisiae*, *M. cerasella* and *P. teres*. The structure of the lipopeptide was established by NMR spectroscopic and MS data and determined to be aureobasidin A. The MIC of AbA was 0.5 mg/mL, indicating that the AbA of *A. pullulans* exhibited obvious inhibition activity against *S. aureus*. An initial pH value of 7.17, a temperature of 25.80 °C, a rotation speed of 216.24 rpm, an inoculum size of 6.85%, and a liquid volume of 124.87 mL were found to be optimal for the AbA production from *A. pullulans* PA-2 which could obtain a yield of 920 mg/L. To conclude, our results on the production of AbA with antimicrobial properties are encouraging for the elimination of bacterial infections.

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