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Optimization of Medium for Ascochlorin Production by the Leafhopper Pathogenic Fungus *Microcera* sp. BCC 17074

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Ascochlorin is a potent antiviral, antitumor and breast cancer suppressor compound. The optimization of the fermentation conditions for the production of this compound by *Microcera* sp. BCC 17074 was conducted using a multi-step strategy. The first step was the selection of carbon and nitrogen sources using a full factorial design. This was followed by a Plackett-Burman design to select the most important factors among the nine medium components and a central composite design to determine the optimal five key components. The utility of the optimized medium was confirmed in a 5-L bioreactor. Fructose and yeast extract were the most preferable carbon and nitrogen sources, respectively. The results of the Plackett-Burman design yielded a medium containing 60 g/L fructose, 6 g/L yeast extract, 2 g/L NaCl, 0.2 g/L $CaCl_2.2H_2O$ and 2 g/L K_2HPO_4. Evaluating the levels of the main factors using the central composite design, an ascochlorin production of 41.12±0.62 mg/L was reached with a medium containing 40 g/L fructose, 4 g/L yeast extract, 3 g/L NH_4Cl, 3 g/L NaCl and 4 g/L KH_2PO_4. The chloride ions drastically affected the ascochlorin production. The production of ascochlorin in a 5-L bioreactor gave an ascochlorin production of 68.35±1.17 mg/L at 144 h and a highest biomass production of 19.6±0.99 g/L at 193 h. These results demonstrated that *Microcera* sp. BCC 17074 serves as a useful cell factory for ascochlorin production.

Keywords: Ascochlorin, *Microcera*, cell factory, Plackett-Burman, central composite design.

Ascochlorin is an isoprenoid antibiotic structurally related to ubiquinol (Figure 1). It was first isolated from *Ascochyta viciae* LIBERT by Tamura *et al.*[1] and Sasaki *et al.*[2] and was described as antiviral agent. Ascochlorin has also been isolated from several other genera such as *Nectria*[3], *Acremonium*[4], *Verticillium*[5], *Cylindrocarpon*[6] and *Microcera*[7], among others. Ascochlorin is an unusual cytochome bc_1 inhibitor that acts at both of the active sites of the enzymes Q_o and Q_i. It is a specific inhibitor that acts on mitochondria[8]. Ascochlorin has also shown antiviral and antitumor activities[1, 9]. Interestingly, significant effects on *in vivo* breast cancer propagation could be shown using this compound[10]. Effects on hypertension were also demonstrated[11].

Our collaborative research group at the National Center for Genetic Engineering and Biotechnology (Thailand) recently reported the isolation of ascochlorin and related compounds from cultures of the leafhopper pathogenic fungus *Microcera* sp. BCC 17074 [7]. With this strain, ascochlorin was produced as a major secondary metabolite with a low amount of other compounds. The yield of ascochlorin using BCC 17074, based on the volume of the fermentation medium, was a

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relatively efficient level when compared with the data from experimental procedures in other ascochlorin isolation papers, in which a yield of 37 mg was obtained from *Verticillium hemipterigenum* BCC 2370 [5]. The goal of this project was to increase the productivity of ascochlorin using our strain and to confirm the results in the bioreactor to demonstrate the applicability of the procedure for production. A full factorial design was used to determine the optimal carbon and nitrogen sources, which allows for a clear selection of the best carbon and nitrogen sources. Following this selection, a fractional factorial design at 2 levels (Plackett-Burman design) was used for the influential quantitative factors, and biomass and ascochlorin production were taken into consideration as secondary responses in order to evaluate the relationship between growth and production. The last step of optimization was performed using a central composite design to obtain the optimal range of the most influential factors, and then the optimal conditions were confirmed in a 5-L bioreactor.

EXPERIMENTAL

Materials and Methods

Fungal strain

The fungus used in this study, *Microcera* sp., is preserved at the BIOTEC Culture Collection (BCC) as BCC 17074.

Inoculum

The fungus was initially grown on potato dextrose agar (PDA) at 25 °C for 3-5 days. An agar block of 1 cm^3 containing *Microcera sp.* BCC 17074 was cut into small pieces, transferred to a 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth (Becton, Dickinson and company, MD, USA) and incubated at 25 °C for 3-5 days on a rotary shaker at a shaking speed of 200 rpm (New Brunswick, NJ, USA). This primary culture was transferred to a 1 L Erlenmeyer flask containing 250 ml of PDB, incubated at 25 °C for 3-5 days on a rotary shaker at a shaking speed of 200 rpm and was used as the inoculum in all experiments.

Experimental design

A general factorial design was used to determine the optimal carbon and nitrogen sources. The optimization was performed in 250 mL Erlenmeyer flask containing 50 mL of medium. Approximately 20 g/L of 6 different carbon sources (maltose, trehalose, glucose, fructose, galactose and sorbitol) and 4 g/L of 5 nitrogen sources (ammonium chloride, malt extract (Becton, Dickinson and company, MD, USA), yeast extract (Becton, Dickinson and company, MD, USA), meat extract (Merck KGaA, Damstadt, Germany) and tryptone (Becton, Dickinson and company, MD, USA)) were used. When the optimal carbon and nitrogen sources were obtained, a two-level fractional factorial design of 2^{n-1} (Plackett-Burman design) was applied to 9 selected factors influencing ascochlorin production: fructose (20-60 g/L), yeast extract (2-6 g/L), ammonium chloride (0-4 g/L), NaCl (0-2 g/L), $CaCl_2‡2H_2O$ (0-0.2 g/L), KH_2PO_4(0-2 g/L), K_2HPO_4(0-2 g/L), KCl (0-2 g/L) and trace elements (0-2 mL/L). Experiments were conducted in duplicate and with 3 center points using a fold-over augment (27 runs and 3 center points). When the factors from the Plackett-Burman design were selected, a response surface of the central composite design was conducted with 5 selected factors influencing ascochlorin production: fructose (20-60 g/L), yeast extract (2-6 g/L), ammonium chloride (2-4 g/L), NaCl (2-4 g/L) and KH_2PO_4(2-4 g/L), with a practical alpha factor (1.49535) and a small size of central composite design, were used. A quadratic model was obtained allowing the determination of the levels of the 5 selected nutritional factors. All results were analyzed with Design Expert software (Version 7.0.b1.1, Stat-Ease Inc., Minneapolis, USA).

Fermentation condition

For scale-up studies, the fungus fermentation was conducted in a 5-L bioreactor (B.E. Marubishi, Pathum Thani, Thailand). The fermenter equipped with 6-blade turbine, baffled pyrex jacket, and round shape sparger. The medium used a working volume of 4 L containing 40 g/L fructose, 4 g/L yeast extract, 3 g/L ammonium chloride, 3 g/L NaCl and 4 g/L KH_2PO_4. The cultivation was performed at 25 °C, an agitation speed of 300 rpm, an aeration rate of 1 vvm and a pH that was not controlled.

Biomass determination

Biomass content was determined by harvesting 1.6 ml of sample, which was centrifuged at 12000 rpm for 2 min. The filter cakes were washed with distilled water and dried at 105–110 °C for 24–48 h until a stable weight was achieved. The culture

filtrate was then subjected to metabolite and sugar analyses.

Ascochlorin extraction and quantification

The culture broth was harvested and filtered using Whatman No 1 filter paper. The filter cake was immersed in 40 ml of methanol for 24 h. The extracted supernatant was then filtered through Whatman No 1 filter paper. The filtrate was poured into a separation funnel, an equal volume of hexane was added and the layers were separated. The bottom (methanol) layer was evaporated, and the residue was diluted with distilled water (25 mL) and extracted with ethyl acetate (3 × 25 mL). The combined organic layer was concentrated under reduced pressure to obtain the crude mycelial extract, which was subjected to HPLC analysis for quantification of ascochlorin. The extract was dissolved in 1 ml of methanol, and a 20 mL portion was injected. Ascochlorin was detected using HPLC employing a reverse phase NovaPak C18 column, 3.9x150 mm with a 5 mm particle size (Waters, MA, USA) and acetonitrile:methanol,:0.05% TFA in water (Composition 25:25:50) as mobile phases at a flow rate of 0.45 mL/min, monitored spectrophotometrically at 220 nm (Waters 996 Photodiode Array Detector). Standard ascochlorin was isolated and purified from the same fungal strain[7]. The concentration of ascochlorin was determined from a standard curve (0 to 1000 mg/L).

Table 1. Biomass and ascochlorin production by *Microcera sp.* BCC 17074 on different carbon and nitrogen sources using general factorial design

Carbon sources(20 g/L)	Nitrogen sources (4 g/L)	Biomass (g/L)	Ascochlorin (mg/L)	Ascochlorin (mg/g DW)
maltose	ammonium chloride	0.85±0.02	0.56±0.02	0.66±0.02
trehalose	ammonium chloride	1.25±0.04	2.60±0.04	**2.08±0.03***
glucose	ammonium chloride	1.0±0.07	1.97±0.05	1.97±0.05
fructose	ammonium chloride	1.70±0.01	3.13±0.05	1.84±0.03
galactose	ammonium chloride	0.8±0.04	1.27±0.14	1.59±0.18
sorbitol	ammonium chloride	0.85±0.04	1.06±0.02	1.25±0.02
maltose	malt extract	6.7±0.07	0.57±0.04	0.09±0.01
trehalose	malt extract	1.65±0.04	1.02±0.01	0.62±0.01
glucose	malt extract	1.65±0.11	2.55±0.03	1.55±0.02
fructose	malt extract	1.75±0.04	1.04±0.01	0.60±0.01
galactose	malt extract	1.8±0.04	2.32±0.01	1.29±0.01
sorbitol	malt extract	2.35±0.11	3.39±0.01	1.44±0.01
maltose	yeast extract	6.95±0.04	1.92±0.01	0.28±0.01
trehalose	yeast extract	12.5±0.21	14.64±0.01	1.17±0.01
glucose	yeast extract	10.9±0.07	6.63±0.01	0.61±0.01
fructose	yeast extract	12.1±0.64	**20.19±0.01***	1.67±0.01
galactose	yeast extract	8.2±0.57	7.71±0.02	0.94±0.01
sorbitol	yeast extract	8.55±0.32	2.69±0.01	0.32±0.01
maltose	meat extract	4.85±0.11	0.08±0.01	0.02±0.01
trehalose	meat extract	12.1±1.34	13.26±0.01	1.10±0.01
glucose	meat extract	9.9±0.07	12.14±0.01	0.96±0.01
fructose	meat extract	**12.6±2.40***	11.24±0.01	0.89±0.01
galactose	meat extract	10.55±1.03	5.83±0.01	0.55±0.01
sorbitol	meat extract	7.35±0.46	4.77±0.01	0.65±0.01
maltose	tryptone	7.55±0.67	6.88±0.01	0.91±0.01
trehalose	tryptone	6.55±0.32	2.98±0.01	0.46±0.01
glucose	tryptone	5.95±0.04	1.35±0.01	0.23±0.01
fructose	tryptone	4.95±0.04	2.71±0.01	0.55±0.01
galactose	tryptone	6.45±0.39	2.50±0.01	0.39±0.01
sorbitol	tryptone	4.55±0.32	0.13±0.01	0.03±0.01

*Maximum values

The retention time of ascochlorin was at 6.3-6.4 min.

Sugar determination

The medium was centrifuged at 12,000 rpm for 2 min, and the supernatant was filtered through a 0.22 µm MCM syringe filter. The filtrate was subjected to HPLC analysis using an Aminex Resin-Based column with 5 mM sulfuric acid as a mobile phase at a flow rate of 0.6 ml×min^{-1}. Sugars were detected refractometrically with a Waters 2414 Refractive Index Detector. A standard curve for sugar determination was prepared by using a standard sugar solution in the concentration range of 1.25 - 20.0 g/L.

RESULTS

The productions of biomass and ascochlorin by *Microcera* sp. BCC 17074 were evaluated on different carbon and nitrogen sources using a general factorial design: 6 carbon sources and 5 nitrogen sources were used. The model of ascochlorin production on different carbon and nitrogen sources are shown in equations 1 and 2, respectively. Fructose and yeast extract had the highest positive effects on ascochlorin production, respectively.

Ascochlorin (mg/L)=5.09-3.08A+1.81B+2.57C-

Table 2. Analysis of variance (ANOVA) for the general fractional factorial design of biomass production by *Microcera* sp. BCC 17074

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model* A-Carbon source	460.4316.15	106	46.042.69	13.290.78	< 0.00010.5958
B-Nitrogen source	444.27	4	111.07	32.06	< 0.0001
Residual	83.15	24	3.46		
Corrected total	543.58	34			

R^2 = 0.8470, adj-R^2 = 0.7833, SD = 1.86, Mean = 5.89, %CV = 31.59

*significant

Table 3. Analysis of variance (ANOVA) for the general fractional factorial design of ascochlorin production by *Microcera* sp. BCC 17074

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model* A-Carbon source	2.123E61.675E5	106	2.123E527912.33	8.451.11	< 0.00010.3855
B-Nitrogen source	1.956E6	4	4.889E5	19.45	< 0.0001
Residual		6.033E5	24	25139.30	
Corrected total	2.726E6	34			

R^2 = 0.7787, adj-R^2 = 0.6865, SD = 158.55, Mean = 143.64, %CV = 110.38

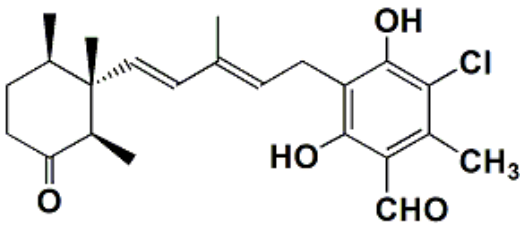

Fig. 1. Ascochlorin structure

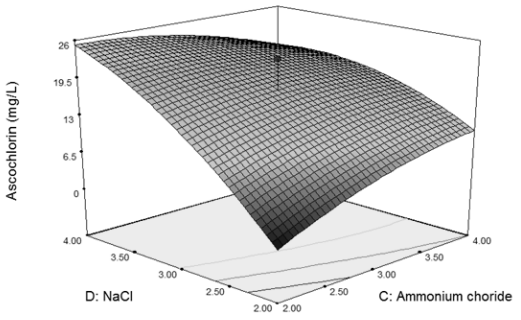

Fig. 2. Effects of ammonium chloride and sodium chloride on ascochlorin

Table 4. Biomass and ascochlorin production by *Microcera sp.* BCC 17074 using a Plackett-Burman design with 9 factors (2^{n-1}) and 2 dummies

STD	Factors									Biomass	Ascochlorin	Ascochlorin
	Fructose (g/L)	(g/L)Yeast extract (g/L)	Ammonium chloride (g/L)	NaCl (g/L)	$CaCl_2.2H_2O$ (g/L)	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)	KCl (g/L)	Trace elements (mL/L)		(mg/L)	(mg/g DW)
1	60	6	0	2	0.2	2	0	0	0	15.75±0.21	24.60±0.76*	1.56±0.40
2	20	6	4	0	0.2	2	2	0	0	7.95±0.86	2.40±0.09	0.30±0.16
3	60	2	4	2	0	2	2	2	0	13.05±0.64	19.43±0.74	1.49±0.54
4	20	6	0	2	0.2	0	2	2	2	8.90±0.28	8.41±0.75	0.95±0.08
5	20	2	4	0	0.2	2	0	2	2	8.25±0.07	11.24±0.15	1.36±0.29
6	20	2	0	2	0	2	2	0	2	3.60±0.14	0.94±0.02	0.26±0.20
7	60	2	0	0	0.2	0	2	2	0	7.75±0.78	0.27±0.02	0.04±0.01
8	60	6	0	0	0	2	0	2	2	10.80±0.71	2.36±0.30	0.22±0.03
9	60	6	4	0	0	0	2	0	2	12.00±1.41	22.26±2.89	1.86±0.22*
10	20	6	4	2	0	0	0	2	0	11.00±3.39	3.15±0.11	0.29±0.01
11	60	2	4	2	0.2	0	0	0	2	9.50±1.27	12.44±0.56	1.31±0.06
12	20	2	0	0	0	0	0	0	0	6.20±0.57	0.24±0.04	0.04±0.01
13	40	4	2	1	0.1	1	1	1	1	12.05±0.50	10.07±0.16	0.84±0.01
14	40	4	2	1	0.1	1	1	1	1	11.50±1.84	5.83±0.04	0.51±0.01
15	40	4	2	1	0.1	1	1	1	1	11.75±0.07	12.12±0.27	1.03±0.02
16	20	2	4	0	0	0	2	2	2	8.90±0.71	3.72±0.23	0.42±0.02
17	60	2	0	2	0	0	0	2	2	7.00±2.83	3.15±0.06	0.45±0.01
18	20	6	0	0	0.2	0	0	0	2	9.70±0.14	0	0
19	60	2	4	0	0	2	0	0	0	12.75±1.91	10.87±0.12	0.858±0.01
20	60	6	0	2	0	0	2	0	0	23.40±1.84*	0	0
21	60	6	4	0	0.2	0	0	2	0	16.15±0.78	6.16±0.22	0.38±0.01
22	20	6	4	2	0	2	0	0	2	17.15±9.69	4.24±0.07	0.25±0.01
23	20	2	4	2	0.2	0	2	0	0	8.70±0.71	1.81±0.02	0.02±0.01
24	20	2	0	2	0.2	2	0	2	0	6.25±2.19	0	0
25	60	2	0	0	0.2	2	2	0	2	6.65±0.07	1.71±0.09	0.26±0.01
26	20	6	0	0	0	2	2	2	0	9.90±1.70	3.95±0.12	0.40±0.01
27	60	6	4	2	0.2	2	2	2	2	14.55±1.49	4.24±0.02	0.29±0.01
28	40	4	2	1	0.1	1	1	1	1	11.75±2.10	13.31±5.05	1.13±0.36

*Maximum values

1.16D-0.16E-3.05F-3.27G-3.29H+5.00I+3.88J-2.51K ...(1)

and

biomass (g/L) =+5.89-0.51A+0.92B+0.73C-0.33D-0.01E-1.61F-4.68G-3.32H+4.14I+3.64J+0.22K ...(2)

where A-maltose, B-trehalose, C-fructose, D-galactose, E-glucose, F-sorbitol, G-ammonium chloride, H-malt extract, I-yeast extract, J-meat extract and K-tryptone.

The highest biomass production of 12.6±2.40 g/L was obtained on a fructose/meat extract, while the highest ascochlorin production of 20.19±0.01 mg/L was obtained on a fructose/yeast extract (Table 1). The highest ascochlorin

Table 5. Analysis of variance (ANOVA) for a Plackett-Burman design of biomass production by *Microcera* sp. BCC 17074

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model*	411.85	22	18.72	6.32	0.0249
A-Fructose	76.51	1	76.51	25.81	0.0038
B-Yeast extract	143.33	1	143.33	48.35	0.0009
C-Ammonium chloride	24.10	1	24.10	8.13	0.0358
D-Sodium chloride	19.89	1	19.89	6.71	0.0488
E- $CaCl_2.2H_2O$	10.21	1	10.21	3.44	0.1227
F- KH_2PO_4	0.27	1	0.27	0.091	0.7746
G-K_2HPO_4	1.11	1	1.11	0.37	0.5682
H-KCl	4.91	1	4.91	1.65	0.2546
J-Trace elements	19.89	1	19.89	6.71	0.0488
Curvature	5.79	1	5.79	1.95	0.2212
Residual	14.82	5	2.96		
Lack of fit**	5.87	1	5.87	2.63	0.1804
Pure error**	8.95	4	2.24		
Corrected total	455.51	29			

R2 = 0.9653, adj-R2 = 0.8124, SD = 1.72, Mean = 10.88, %CV = 15.82

*significant, **not significant

Table 6. Analysis of variance (ANOVA) for a Plackett-Burman design of ascochlorin production by *Microcera* sp. BCC 17074.

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model**	990.0	22	45.0	2.12	0.2061
A-Fructose	189.20	1	189.20	8.93	0.0305
B-Yeast extract	10.61	1	10.61	0.50	0.5107
C-Ammonium chloride	132.19	1	132.19	6.24	0.0546
D-Sodium chloride	12.38	1	12.38	0.58	0.4791
E- $CaCl_2.2H_2O$	0.045	1	0.045	2.12E-3	0.9650
F- KH_2PO_4	24.79	1	24.79	1.17	0.3288
G-K_2HPO_4	3.60	1	3.60	0.17	0.6973
H-KCl	9.91	1	9.91	0.47	0.5245
J-Trace elements	0.14	1	0.14	6.59E-3	0.9385
Curvature	44.13	1	44.13	2.08	0.2085
Residual	105.93	5	21.19		
Lack of fit**	34.26	1	34.26	1.91	0.2389
Pure error**	71.67	4	17.92		
Corrected total	1297.98	29			

R^2 = 0.9033, adj-R^2 = 0.4781, SD = 4.60, Mean = 6.76, %CV = 68.14

**not significant

yield of 2.08±0.01 mg/g DW was obtained on trehalose and ammonium chloride. The analysis of variance of the general factorial design for biomass and ascochlorin production by *Microcera* sp. BCC 17074 is shown in Tables 2 and 3. After this selection, biomass and ascochlorin production were then subjected to a Plackett-Burman design using 9 factors. The influence of two levels of fructose and yeast extract along with chloride-containing compounds (NH_4Cl, $CaCl_2 \cdot 2H_2O$, NaCl and KCl) was evaluated on ascochlorin production. The highest biomass production of 23.40±1.84 g/L was obtained on 60 g/L fructose, 6 g/L yeast extract, 2 g/L NaCl and 2 g/L K_2HPO_4, but there was no production of ascochlorin. In contrast, the highest ascochlorin production of 24.60±0.76 mg/L was obtained on 60 g/L fructose, 6 g/L yeast extract, 2 g/L NaCl, 0.2 g/L $CaCl_2 \cdot 2H_2O$ and 2 g/L KH_2PO_4, in which 15.75±0.21 g/L of biomass was obtained. The highest ascochlorin yield of 1.86±0.22 mg/g DW was obtained on 60 g/L fructose, 6 g/L yeast extract, 4 g/L NaCl, 2 g/L K_2HPO_4 and 2 mL/L of trace elements solution (Table 4). The models of ascochlorin and biomass production are shown in equation 3 and 4, in which fructose, yeast extract, ammonium chloride, NaCl and KH_2PO_4 were the most positive influential factors on the production of ascochlorin. The standard deviation of 4.60 and R^2 of 0.90 were obtained for ascochlorin production, while curvature and the lack of fit were not significant (Table 5 and 6).

Ascochlorin (mg/L) =6.15+2.81A+0.67B+ 2.35C+0.72D-0.043E+1.02F-0.39G-0.64H+0.076J...(3)

and

biomass (g/L) = 10.66+1.79A+2.44B+1.00C+0.91D-0.65E-0.11F0.21G-0.45H-0.91J ...(4)

where A-fructose, B-yeast extract, C-ammonium chloride, D-NaCl, E-$CaCl_2.2H_2O$, F-KH_2PO_4, G-K_2HPO_4, H-KCl and J-trace elements.

The optimization was carried out using a central composite design with the 4 influential factors, and the addition of NH_4Cl. NH_4Cl was

Table 7. Biomass and ascochlorin production by *Microcera sp.* BCC 17074 using a central composite design

STD	Factors					Biomass	Ascochlorin	Ascochlorin
	Fructose (g/L)	Yeast extract (g/L)	Ammonium chloride (g/L)	NaCl (g/L)	KH_2PO_4 (g/L)	(g/L)	(mg/L)	(mg/g DW)
1	60	6	2	4	2	19.4±0.42	13.12±0.62	0.68±0.03
2	60	2	4	4	2	12.55±1.03	7.55±0.40	0.59±0.03
3	20	6	4	2	4	10.7±0.92	1.48±0.02	0.14±0.01
4	60	6	4	2	2	**19.9±0.78***	21.74±0.18	1.09±0.01
5	60	6	2	2	4	18.3±0.49	14.22±0.55	0.78±0.03
6	60	2	2	4	4	14.95±0.74	1.21±0.07	0.08±0.01
7	20	2	4	4	4	8.1±0.64	1.75±0.04	0.22±0.01
8	20	6	2	4	4	12.5±0.35	2.38±0.09	0.19±0.01
9	60	2	4	2	4	12.25±1.24	6.14±0.26	0.50±0.02
10	20	6	4	4	2	11.25±0.53	1.86±0.03	0.17±0.01
11	20	2	2	2	2	6.2±0.57	1.22±0.06	0.20±0.01
12	10.09	4	3	3	3	2.9±0.78	0.03±0.01	0.01±0.00
13	69.91	4	3	3	3	4.3±0.49	0.09±1.950.01	0.02±0.00
14	40	1.01	3	3	3	7.5±1.06	5.45±0.39	0.73±0.05
15	40	6.99	3	3	3	15.8±1.56	14.20±0.57	0.90±0.04
16	40	4	1.5	3	3	15.0±0.71	14.97±0.02	1.00±0.01
17	40	4	4.5	3	3	14.5±1.06	17.78±0.16	1.23±0.01
18	40	4	3	1.5	3	16.25±1.24	0.80±0.14	0.05±0.01
19	40	4	3	4.5	3	17.2±1.56	25.60±0.28	1.49±.02
20	40	4	3	3	1.5	14.4±1.70	29.20±0.57	2.03±0.04
21	40	4	3	3	4.5	18.8±1.14	41.12±0.62*	2.19±0.03*
22	40	4	3	3	3	14.12±0.65	15.46±0.05	1.10±0.01

*Maximum values

chosen to lower the cost of yeast extract and also because the chloride ion appeared to affect ascochlorin production (Figure 2). The highest biomass production of 19.90±0.78 g/L was obtained on 60 g/L fructose, 6 g/L yeast extract, 4 g/L NH_4Cl, 2 g/L NaCl and 2 g/L KH_2PO_4, whereas the highest ascochlorin production and yield of 41.12±0.62 mg/L and 2.19±0.03 g/g DW, respectively, were obtained on 40 g/L fructose, 4 g/L yeast extract, 3 g/L NH_4Cl, 3 g/L NaCl and 4 g/L KH_2PO_4 (Table 7). The models of ascochlorin and biomass production obtained from the central composite design are shown in equation 5 and 6, with sodium chloride and yeast extract having the highest positive effects on the production of ascochlorin and biomass, respectively. The interaction between yeast extract/ammonium chloride and fructose/yeast extract had the highest positive effects on ascochlorin and biomass production, respectively. The standard deviation of 6.94 and an R^2 of 0.95 were obtained for ascochlorin production, and the lack of fit was not significant (Table 8 and 9).

Ascochlorin (mg/L) =18.48+0.018A+2.92B +0.94C+8.29D+3.99E+11.62AB +10.16AC-0.58AD+3.67AE+4.70BC-4.59BD-0.10BE-4.18CD0.30CE-8.66DE-8.97A2-4.60B2-1.68C2-3.09D2+6.72E2 ...(5)

and

biomass (g/L) = 13.44+0.47A+2.78B-0.17C+0.32D+1.47E+2.44AB+2.15AC +2.22AD+0.72AE-0.87BC-1.03BD-2.59BE-0.90CD-2.26CE-1.62DE-4.24 A2 -0.64B2+0.75C2+ 1.63D2+1.58E2 ...(6)

where A-fructose, B-yeast extract, C-ammonium chloride, D-NaCl and E-KH_2PO_4.

The model was then confirmed in a 5-L bioreactor with the selected optimized medium obtained from the central composite design; the highest biomass production of 19.6±0.99 g/L was obtained at 193 h, and the highest ascochlorin

Table 8. Analysis of variance (ANOVA) for a central composite design of biomass production by *Microcera* sp. BCC 17074

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model*	486.27	20	24.31	18.95	0.0165
A-Fructose	0.98	1	0.98	0.76	0.4464
B-Yeast extract	34.44	1	34.44	26.85	0.0140
C-Ammonium chloride	0.12	1	0.12	0.097	0.7754
D-Sodium chloride	0.45	1	0.45	0.35	0.5949
E- KH_2PO_4	9.68	1	9.68	7.55	0.0709
AB	14.19	1	114.19	11.06	0.0449
AC	11.09	1	11.09	8.64	0.0605
AD	11.77	1	11.77	9.17	0.0564
AE	1.22	1	1.22	0.95	0.4010
BC	1.79	1	1.79	1.40	0.3223
BD	2.52	1	2.52	1.96	0.2559
BE	16.06	1	16.06	12.52	0.0384
CD	1.92	1	1.92	1.50	0.3086
CE	12.23	1	12.23	9.53	0.0538
DE	6.29	1	6.29	4.90	0.1137
A^2	191.57	1	191.57	149.33	0.0012
B^2	4.31	1	4.31	3.36	0.1642
C^2	6.02	1	6.02	4.70	0.1188
D^2	28.52	1	28.52	22.23	0.0181
E^2	26.61	1	26.61	20.74	0.0198
Residual	3.85	3	1.28		
Lack of fit**	3.0	1	3.0	7.04	0.1176
Pure error	0.85	2	0.43		
Corrected total	490.11	23			

R^2 = 0.99921, adj-R^2 = 0.9398, SD = 1.13, Mean = 13.13, %CV = 8.63

*significant, **not significant

Table 9. Analysis of variance (ANOVA) for a central composite design of ascochlorin production by *Microcera* sp. BCC 17074

Source	Sum of squares	D.F.	Mean square	F-value	Probability (P) > F
Model*	2589.28	20	129.46	2.69	0.0112
A-Fructose	1.49E-3	1	1.49E-3	3.10E-5	0.9959
B-Yeast extract	38.22	1	38.22	0.79	0.4384
C-Ammonium chloride	3.95	1	3.95	0.082	0.7931
D-Sodium chloride	307.48	1	307.48	6.39	0.0855
E- KH_2PO_4	71.09	1	71.09	1.48	0.3110
AB	322.54	1	322.54	6.71	0.0811
AC	246.82	1	246.82	5.13	0.1084
AD	0.80	1	0.80	0.017	0.9053
AE	32.27	1	32.27	0.67	0.4727
BC	52.72	1	52.72	1.10	0.3720
BD	50.31	1	50.31	1.05	0.3817
BE	0.025	1	0.025	5.30E-4	0.9831
CD	41.77	1	41.77	0.87	0.4201
CE	0.21	1	0.21	4.37E-3	0.9514
DE	179.17	1	179.17	3.73	0.1491
A^2	859.49	1	859.49	17.87	0.0242
B^2	226.46	1	226.46	4.71	0.1185
C^2	30.04	1	30.04	0.62	0.4870
D^2	102.22	1	102.22	2.13	0.2409
E^2	482.95	1	482.95	10.04	0.0505
Residual	144.29	3	48.10		
Lack of fit**	59.53	1	59.53	1.40	0.3577
Pure error	84.76	2	42.38		
Corrected total	2733.57	23			

R^2 = 0.9472, adj-R^2 = 0.5953, SD = 6.94, Mean = 11.17, %CV = 62.07

*significant, **not significant

production of 68.35±1.17 mg/L was achieved at 144 h (Figure 3).

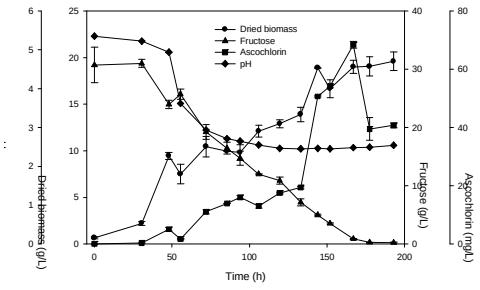

Fig. 3. Time profile of biomass and ascochlorin production by *Microcera sp.* BCC 17074 in a 5-L bioreactor.

DISCUSSION

Microcera sp. BCC 17074 produced ascochlorin as a major compound unlike *Verticillium hemipterigenum* BCC 2370, which produced a lower level of ascochlorin together with other ascochlorin derivatives (5, 7). Chloride ions directly affected the production of ascochlorin produced by *Microcera sp.* BCC 17074, as the ascochlorin structure contains chloride on the benzene ring (1,7). Because ascochlorin is a chlorine atom-containing molecule, it was suspected that the addition of a chloride ion source to the medium would enhance the production of this compound by *Microcera* sp. BCC 17074. The present results were consistent with this hypothesis. Thus, increased the levels of NH_4Cl and NaCl

concentrations resulted in the higher production of ascochlorin, and the results showed that the enriched chloride-containing compounds in the medium enhanced the ascochlorin production. The production of ascochlorin by *Microcera* sp. BCC 17074 in a 5-L bioreactor (68.35±1.17 mg/L) was higher compared with the screening medium (20.19±0.01 mg/L) and was 1.9 times higher than that from *Verticillium hemipterigenum* BCC 2370 (37 mg/L) (5). Similar findings were reported on the effect of substrate compositions on mycelial growth and secondary metabolites in *Penicillium janthinellum* and *P. declauxii* (12). Among all of the carbon sources used, fructose was the most preferable sugar for ascochlorin production. Thus, yeast extract favored both biomass and ascochlorin production. Carbon and nitrogen sources are the most influential parameters on secondary metabolites of entomopathogenic fungi (13). One study with a Plackett-Burman design showed that sucrose, maltose, glucose and $NaNO_3$ were significant factors in zofimarin production (14). The use of the Plackett-Burman design and the central composite design for anhydromevalonolactone production has been reported, and the results suggested that sucrose, $NaNO_3$, yeast extract and K_2HPO_4 were the key factors affecting anhydromevalonolactone production in a complex medium, whereas the major components required for a defined medium were $NaNO_3$, K_2HPO_4, KH_2PO_4 and trace elements, where a maximum anhydromevalonolactone production of 250 mg/L in the complex was obtained[15]. Ascochlorin is a potent antiviral and antitumor antibiotic and is known to suppress breast cancer propagation (1, 7,8 9): the use of *Microcera sp.* BCC 17074 as a cell factory for ascochlorin production is of great interest for the production of a high amount of ascochlorin compared with other reported microorganisms. The cost of production medium in this study was approximately 0.5 $/L compared with the production in potato dextrose broth for 2.5 $/L. The production of ascochlorin by *Microcera sp.* BCC 17074 in this study has the potential for mass scale production, as the medium composition obtained from this study is very useful for enhancing its production. The high chloride-containing substrate might be corrosive to the stainless steel of the bioreactor, so perhaps feeding the chloride-containing substrate at the stationary phase of growth might be possible for process optimization of ascochlorin production. Furthermore, the major production of ascochlorin by *Microcera sp.* BCC 17074 is also beneficial for the downstream and purification processes, as it can avoid unwanted derivatives. This makes *Microcera sp.* BCC 17074 very attractive for ascochlorin production (7). The present study revealed that *Microcera* sp. BCC 17074 is a highly efficient cell factory for the ascochlorin production. The optimized medium conditions were successfully applied to incubation in a 5-L bioreactor, which demonstrated the feasibility of ascochlorin mass production.

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