Optimization of culture conditions for ergosterol production by a marine fungus *Cladosporium cladosporioides* M-40

Huange Zhao, Yingying Lin, Min Wang, Songlin Zhou*

Key Laboratory of Tropical Diseases and Translation Medicine of the Ministry of Education & Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571199, China.

Received: February 12, 2018; accepted: May 28, 2018.

This research aims at screening a newly isolated ergosterol-producing marine fungus and optimizing culture conditions. The fungus was identified as *Cladosporium cladosporioides* via the phenotypic and molecular methods. Single factor experiments and response surface methodology were employed to examine the variables and obtain the optimum values. Statistical-mathematical experimental results demonstrated that the optimized medium for the maximum ergosterol yield of 356.98 mg/L should contain 4.41 g/L bran, 10.27 g/L sucrose, 7.69 g/L crude sea salt, with the appropriate cultivation conditions of pH 7.54 and fermentation time 25.7 d. The optimization process led to an overall 4.28-fold increase in the ergosterol yield. Our results indicate that this fungus with high ergosterol production is an alternative and promising candidate for commercial and industrial applications.

Funding: The work was funded by the National Natural Science Foundation of China (Project No. 81560484), and the Natural Science Foundation of Hainan Province, China (Project No. 817147).

Keywords: Marine fungi; *Cladosporium cladosporioides* M-40; Ergosterol; Optimization of fermentation; Response surface methodology (RSM).

Abbreviations: BBD: Box-Behnken design; CC: Column chromatography; CCD: Central composite design; ESI-MS: ElectroSpray ionization-mass spectrometry; HPLC: High performance liquid chromatography; MFUM: Marine fungal universal medium; NMR: Nuclear magnetic resonance; RSM: Response surface methodology; TLC: Thin-layer chromatography; UV: Ultraviolet spectra.

***Corresponding author:** Songlin Zhou. Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571199, China. Phone: +86 898 6689 3322; Fax: +86 898 6689 3335; E-mail: zhousonglin106@163.com.

Introduction

As one of the fungal sterols, ergosterol (provitamin D_2) was proved to have significant cellular functions such as participating in the endocytosis [1], maintaining permeability, integrity, and fluidity of the membranes [2], homotypic vacuole fusion [3]. Nowadays, ergosterol has become a research hotspot. Many studies have indicated that ergosterol is involved in a wide range of biological activities, including ameliorate the progression of chronic obstructive pulmonary disease [4], anti-inflammatory activity [5, 6], and anti-hepatic fibrosis activity [7]. To achieve successful real-life applications of ergosterol, some efforts have been made to obtain alternative and continuous sources of ergosterol. Considering that a great number of unique pharmaceutical agents have been isolated from marine fungi in recent years [8]. We tried to find an ergosterol-producing fungus in various marine-derived fungi strains. In previous work, we had screened 195 marine-derived fungal strains to measure antibacterial and antitumor cell activity for secondary metabolites of fungi, which were isolated from different habitats in coastal regions of Haikou [9]. We found that one strain *Cladosporium cladosporioides* M-40 could produce high

productivity of ergosterol as the biologically active constituent (Figure 1).

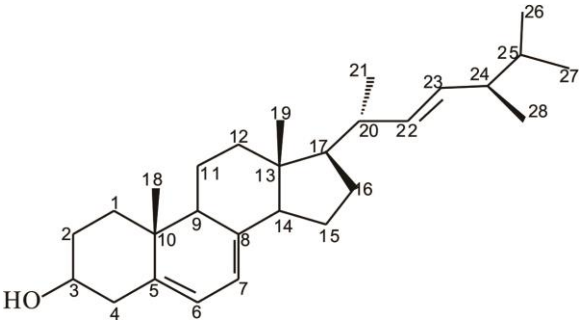

Figure 1. Chemical structures of ergosterol.

For the successful use of microbes in industries, the optimal design of the fermentation is of great importance as medium composition can affect product yield considerably [10]. Response surface methodology (RSM) is a powerful statistical tool by which the optimal culture conditions can be determined. Also, the interactive effect of process variables can be explored, and the overall process can be precisely revealed by making a mathematical model [11]. As the most common design, Box Behnken design (BBD) has been widely utilized for the optimization of bioprocess variables such as fermentation culture media, cultivation and process conditions [12]. In our preliminary study, it was the first time that the ergosterol isolated from marine fungus *C. cladosporioides*. Therefore, the objective of the present study was to obtain the optimal culture conditions for increasing the ergosterol production from *C. cladosporioides* M-40 by RSM and single factor test, which could enhance the potential of *C. cladosporioides* in commercial and industrial ergosterol production.

Materials and methods

General experimental procedures

The data of ElectroSpray ionization-mass spectrometry (ESI-MS) was obtained on an AB Sciex API4000 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were measured by a Bruker AV 400 MHz spectrometer. Ultraviolet spectra (UV) were determined on a Hitachi U-2900 spectrophotometer. Sephadex LH-20 (Amersham Biosciences) was employed in column chromatography (CC). Reverse phase HPLC was carried out on a Hitachi L-2000 system equipped with a RP-C^{18} (LaChrom, 5µm, 4.6 mm × 150 mm) column.

Fungal culture

The marine derived strain (M-40) was isolated from a rotted leaf sample collected from the coastline of Haikou, Hainan province, China. This fungus grew slowly on solid marine fungal universal medium (MFUM) The medium was composed of 10 g/L glucose, 1 g/L yeast extract, 2 g/L tryptone, 20 g/L agar, and 600 mL/L aged seawater that can be replaced by 2.1 g/L crude sea salt. After being kept at 25°C for 7 d, the yellow-white colored hypha bearing spores was observed. Fungal identified was performed via optical election microscope and ITS region sequencing following Zhou *et al.* [13]. Briefly, the chromosomal DNA from M-40 strains was extracted using a fungus genomic DNA extraction kit, and the isolated DNA was amplified by PCR using universal primers ITS 1 and ITS 4, corresponding to a 5.8S rDNA sequence. The PCR thermal conditions were set as follows: preheating for denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 60 s, and additional extension at 72°C for 7 min. The sequencing analysis data obtained from Sinogenomax Co., Ltd. (Beijing, China) showed that M-40 was most closely related to the partial sequence of *Cladosporium cladosporioides*. The phylogenetic analysis was done using the neighbor-joining method by Molecular Evolutionary Genetics Analysis 7.0 software.

Fermentation, extraction, purification and identification of Ergosterol

The fungal strain was cultured under shaking conditions at 120 rpm, 25°C for 20 d in 500 mL Erlenmeyer flasks contain with liquid MFUM. At

harvest time, all broth culture (10 L) of each flask had been collected, then added to 1 L of MeOH for 24 h. Next, the mixture was filtered through four layers of gauze to separate supernatant and hyphae. The supernatant was extracted three times using ethyl acetate (AcOEt) and the extract was obtained by a rotary evaporator. Besides, the hyphae were extracted three times with 60% methanol. The methanol suspension was extracted three times with AcOEt to yield another EtOAc extract after removing the solvent under vacuum. Both EtOAc extracts (8.7 g) were applied to Sephadex LH-20 CC (120 cm × 3 cm i.d.) with 2 L petroleum ether/MeOH/ CH_2Cl_2 (2:1:1, v/v/v), and then recrystallized with MeOH/CH_2Cl_2 to gain the compound 1 (80.2 mg) that was identified by spectroscopic methods.

Single factor experiment for ergosterol production and mycelial biomass

For shake flask culture, this marine fungus *C. cladosporioides* M-40 was cultured on MFUM plate at 25°C for 7 d and about 7 mm diameter of plugs were inoculated into each 250 mL Erlenmeyer flask with 100 mL liquid MFUM (as a seed starting medium). After being cultured at 25°C, 150 rpm for 5 d, 2% (v/v) seed culture was inoculated into 100 mL different nutrient medium for single factor experiment.

The fungal growth medium was composed of defined amounts of carbohydrates, nitrogen, and inorganic salt sources. To investigate the influences of variability of media composition on the production of ergosterol, various carbohydrates, nitrogen, and inorganic salt supplements were used. Specifically, the same amounts (10 g/L) of carbohydrate supplements including cornmeal, maltose, lactose, glucose, soluble starch, and sucrose were chosen to replace glucose in the medium. Similarly, the influences of nitrogen and inorganic salt sources on the production of ergosterol were evaluated. Various organic nitrogen supplements (e.g. silkworm chrysalis power, bran, soybean flour, beef extract, peptone, and yeast extract) and inorganic salt source supplements (e.g. KH_2PO_4, K_2HPO_4, Na_2HPO_4, NaCl, KCl, and crude sea salt) were used. Nitrogen supplements were individually added at 0.3% (w/v) concentration to replace original nitrogen source (1 g/L yeast extract, 2 g/L tryptone), and inorganic salt supplements were individually added at 0.21% (w/v) concentration to replace original aged seawater in MFUM. All fermentation experiments were run on a rotary shaker at 25°C and 120 rpm for 20 d.

To increase the production of ergosterol, the diverse culture factors were optimized when being cultured in a rotary shaker at 25°C and 120 rpm, including aged seawater concentration (0%, 20%, 40%, 60%, 80%, and 100%), the initial pH (6.0, 6.5, 7.0, 7.5, and 8.0) and fermentation time (5, 10, 15, 20, 25, and 30 d).

Experimental Design

To achieve the efficient production of ergosterol, a central composite design (CCD) combined with RSM was employed to optimize the production of ergosterol by the marine fungus. Five parameters (i.e. the initial pH, fermentation time, sucrose concentration, bran concentration, and crude sea salt concentration) were selected as independent variables to investigate the effect on the ergosterol production. Design parameter levels were coded as +1 (high), 0 (central point), and -1 (low). A total of 46 combinations of variables were made to match a full quadratic formula model, including six replicates of the center points. The second-order polynomial formula (equation 1) was given as follow:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j + \delta$$

where, Y is dependent variable; X_i and X_j are the independent coded variables; β_0, β_j, β_{jj}, and β_{ij} are the intercept, linearity coefficient, square coefficient, and interaction coefficient, respectively; k is the number of variates and δ is the random error. The actual and coded levels of the independent variables applied the experimental design were shown in Table 1.

Table 1. The design and results of the central point design for the ergosterol production.

Run	Factors					Ergosterol (mg/L)		
	X_1 (pH)	X_2 (Time, d)	X_3 (Sucrose, g/L)	X_4 (Bran, g/L)	X_5 (Crude sea salt, g/L)	observed	predicted	Residual
22	-1 (7.0)	-1 (20)	0 (10)	0 (3)	0 (7.0)	285.42	283.91	1.51
42	1 (8.0)	-1 (20)	0 (10)	0 (3)	0 (7.0)	274.17	267.06	7.11
21	-1 (7.0)	1 (30)	0 (10)	0 (3)	0 (7.0)	281.30	284.75	-3.45
14	1 (8.0)	1 (30)	0 (10)	0 (3)	0 (7.0)	296.10	293.96	2.15
18	0 (7.5)	0 (25)	-1 (8)	-1 (1)	0 (7.0)	243.05	249.98	-6.94
44	0 (7.5)	0 (25)	1 (12)	-1 (1)	0 (7.0)	249.29	255.32	-6.03
5	0 (7.5)	0 (25)	-1 (8)	1 (5)	0 (7.0)	332.13	330.64	1.49
3	0 (7.5)	0 (25)	1 (12)	1 (5)	0 (7.0)	340.67	338.27	2.39
13	0 (7.5)	-1 (20)	0 (10)	0 (3)	-1 (3.5)	160.83	160.39	0.44
32	0 (7.5)	1 (30)	0 (10)	0 (3)	-1 (3.5)	170.48	172.15	-1.66
25	0 (7.5)	-1 (20)	0 (10)	0 (3)	1 (10.5)	231.43	233.05	-1.61
19	0 (7.5)	1 (30)	0 (10)	0 (3)	1 (10.5)	245.32	249.04	-3.72
41	-1 (7.0)	0 (25)	-1 (8)	0 (3)	0 (7.0)	296.62	295.45	1.17
4	1 (8.0)	0 (25)	-1 (8)	0 (3)	0 (7.0)	286.54	292.61	-6.07
38	-1 (7.0)	0 (25)	1 (12)	0 (3)	0 (7.0)	305.63	302.91	2.72
36	1 (8.0)	0 (25)	1 (12)	0 (3)	0 (7.0)	293.58	298.11	-4.52
28	0 (7.5)	0 (25)	0 (10)	-1 (1)	-1 (3.5)	142.90	129.78	13.12
10	0 (7.5)	0 (25)	0 (10)	1 (5)	-1 (3.5)	195.14	200.03	-4.88
27	0 (7.5)	0 (25)	0 (10)	-1 (1)	1 (10.5)	205.64	192.99	12.64
34	0 (7.5)	0 (25)	0 (10)	1 (5)	1 (10.5)	281.01	286.37	-5.36
33	0 (7.5)	-1 (20)	-1 (8)	0 (3)	0 (7.0)	287.08	285.53	1.55
16	0 (7.5)	1 (30)	-1 (8)	0 (3)	0 (7.0)	304.30	297.82	6.48
45	0 (7.5)	-1 (20)	1 (12)	0 (3)	0 (7.0)	291.40	290.43	0.97
2	0 (7.5)	1 (30)	1 (12)	0 (3)	0 (7.0)	311.79	305.89	5.90
23	-1 (7.0)	0 (25)	0 (10)	-1 (1)	0 (7.0)	245.46	249.33	-3.86
35	1 (8.0)	0 (25)	0 (10)	-1 (1)	0 (7.0)	233.19	230.98	2.21
37	-1 (7.0)	0 (25)	0 (10)	1 (5)	0 (7.0)	319.00	316.60	2.40
7	1 (8.0)	0 (25)	0 (10)	1 (5)	0 (7.0)	335.80	327.32	8.48
20	0 (7.5)	0 (25)	-1 (8)	0 (3)	-1 (3.5)	177.47	178.29	-0.82
26	0 (7.5)	0 (25)	1 (12)	0 (3)	-1 (3.5)	181.24	183.94	-2.70
12	0 (7.5)	0 (25)	-1 (8)	0 (3)	1 (10.5)	255.38	252.24	3.14
11	0 (7.5)	0 (25)	1 (12)	0 (3)	1 (10.5)	260.81	259.55	1.26
24	-1 (7.0)	0 (25)	0 (10)	0 (3)	-1 (3.5)	170.23	169.76	0.48
39	1 (8.0)	0 (25)	0 (10)	0 (3)	-1 (3.5)	163.53	167.49	-3.96
46	-1 (7.0)	0 (25)	0 (10)	0 (3)	1 (10.5)	245.12	246.08	-0.96
40	1 (8.0)	0 (25)	0 (10)	0 (3)	1 (10.5)	235.32	240.71	-5.40
6	0 (7.5)	-1 (20)	0 (10)	-1 (1)	0 (7.0)	225.46	232.10	-6.64
1	0 (7.5)	1 (30)	0 (10)	-1 (1)	0 (7.0)	238.99	243.50	-4.50
9	0 (7.5)	-1 (20)	0 (10)	1 (5)	0 (7.0)	308.11	311.43	-3.33
15	0 (7.5)	1 (30)	0 (10)	1 (5)	0 (7.0)	326.59	327.78	-1.19
43	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	330.22	338.24	-8.03
17	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	351.52	338.24	13.28
8	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	331.98	338.24	-6.26
30	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	338.87	338.24	0.63
31	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	349.31	338.24	11.07
29	0 (7.5)	0 (25)	0 (10)	0 (3)	0 (7.0)	327.56	338.24	-10.69

The experimental design result and estimation of predicted responses were evaluated utilizing Design-Expert 8.05b procedure (State-Ease, Inc., Minneapolis, MN, USA). Analysis of variance (ANOVA) was implemented to determine and simulate the optimum culture conditions for production of ergosterol.

Quantification of ergosterol

After fermentation, 10 mL of MeOH was added to each flask and the broth culture (100 mL) was filtered through four layers of gauze to separate supernatant and hyphae. The filtrate was extracted three times using 150 mL $CHCl_3$, and the crude $CHCl_3$ extract was acquired after removing the solvent under vacuum. The hyphae were dried and weighed in order to determine the fungal mycelial biomass. While the dried hyphae were extracted with 60% methanol, the suspension of methanol solution was extracted three times with $CHCl_3$ to yield another $CHCl_3$ extract under vacuum. The ergosterol content in crude extract was measured by HPLC RP-18 (column temperature: 35°C; injection volume: 10 µL; flow rate: 1.0 mL/min). The composition of the mobile phase was kept constant ("isocratic elution mode"), and consisted of acetonitrile (A, 85%) and ultra water (B, 15%); the ultraviolet detector was set at 245 nm for acquiring chromatograms. The retention time of ergosterol was about 12.89 minutes (Figure 2).

Statistical analysis

All the experiments were conducted in triplicate and the data were determined by mean ± standard deviation (SD) (n=3). Statistical significance was determined based on a one-way ANOVA. Significant difference was determined when p was < 0.05.

Results

Ergosterol producing marine fungus identification

Generally, the strain M-40 colonies appeared grey-yellow on MFUM plates in 7 d and grew to 10–20 mm diameter on MFUM plates, with reverse tawny color and white margins (Figure 3a). A majority of the mycelia existed in the substratum, and the hyphae were sparse, unbranched and septate, with numerous conidia catenated in long branched chains (Figure 3b). According to the above morphological characteristics, this strain of fungus was preliminary identified as *Cladosporium. sp* [14]. Subsequently, the fungus was identified based on a molecular biological protocol of the internal transcribed spacer (ITS1-5.8S-ITS4) region analysis. The ITS sequence (560 bp, GenBank accession number: F08U01PD015) exhibited exceed 99% homology to one *Cladosporium cladosporioides* sequences (GenBank accession number: AY291273.1). Therefore, the fungal strain was finally identified as *C. cladosporioides*. (Figure 3c and 3d). The strain M-40 is now preserved at the Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College.

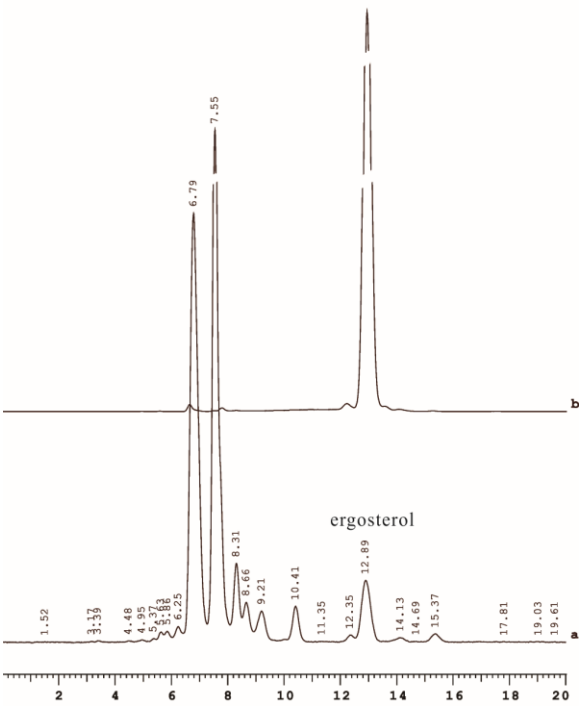

Figure 2. HPLC analysis and culture optimization of ergosterol producing *C. cladosporioides* M-40 strain: (a) the fermented broth of M-40 stain by HPLC; (b) Standard substance of ergosterol by HPLC. The peak heights are normalized to the height of the standard.

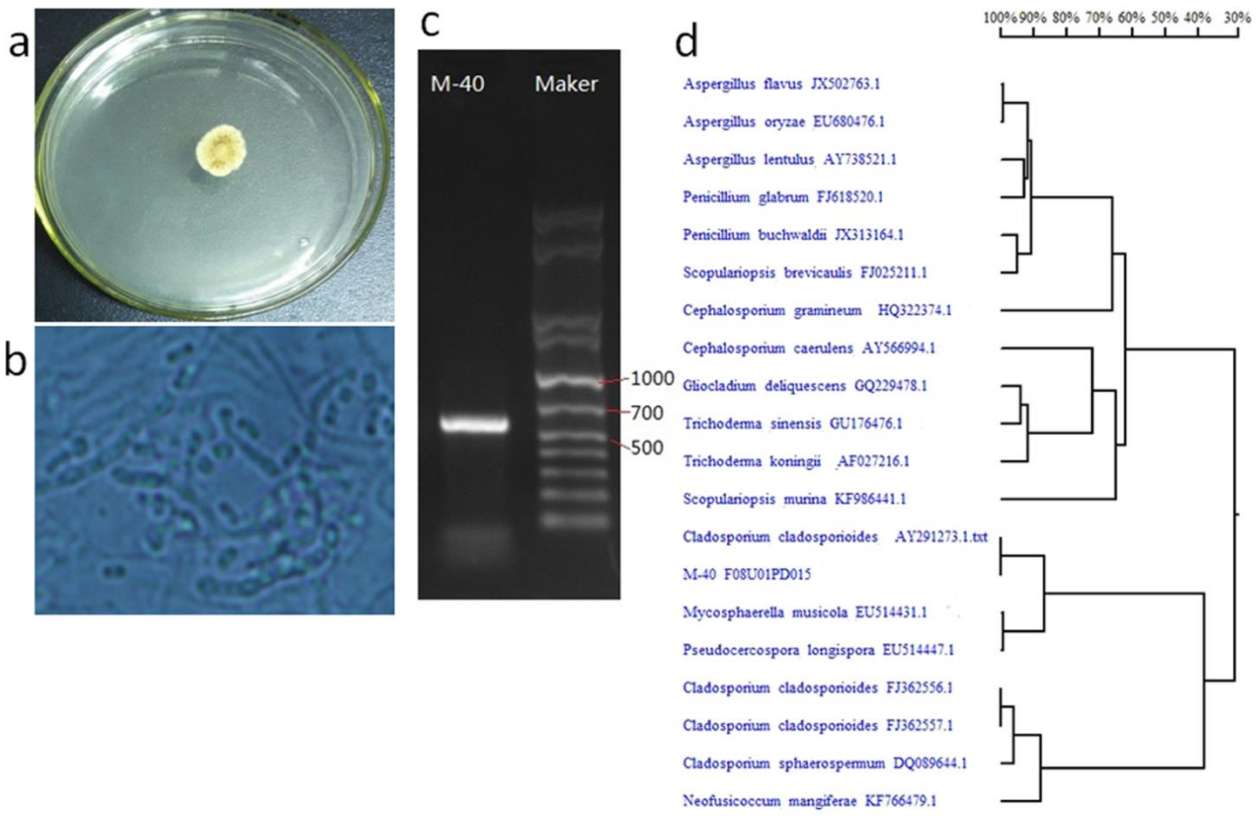

Figure 3. Ergosterol producing marine fungus M-40 isolated from the coastline of Haikou: (a) and (b) represented the marine fungus M-40 of colonial morphology and micrographic characteristics (×400) respectively; (c) PCR product of ITS rDNA sequence of strain; (d) phylogenetic tree constructed by the program neighbor-joining (NJ) based on ITS1-5.8S-ITS2 sequences of ergosterol producing marine fungus. Bootstrap values (1,000 tree interactions) are indicated at the nodes.

Identification of ergosterol

Compound 1: The compound 1 was obtained as an achromatous acicular crystal with a molecular formula of $C_{28}H_{44}O$ (Mw 396.66). The ^{1}H- and ^{13}C-NMR spectroscopic data of the compound 1 in $CDCl_3$ was listed as follows: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.57 (1H, d, J = 6.2 Hz, H-6), 5.38 (1H, d = 6.2 Hz, H-7), 5.16 (1H, dd, J = 15.5, 8.0 Hz, H-22), 5.20 (1H, dd, J = 15.5, 6.8 Hz, H-23), 3.64 (1H, m, H-3), 2.03 (1H, m, H-20), 0.63(3H, s, H-18), 0.93 (3H, s, H-19), 1.03 (3H, d, J = 6.5 Hz, H-21), 0.81 (3H, d, J = 6.8 Hz, H-26), 0.83 (3H, d, J = 6.8 Hz, H-27), 0.91 (3H, d, J = 6.8 Hz, H-28). ^{13}C-NMR (100 MHz, $CDCl_3$): δ140.3 (C-8), 138.8 (C-5), 134.5 (C-22), 130.9 (C-23), 118.4 (C-6), 115.3 (C-7), 69.4 (C-3), 54.7 (C-17), 53.5 (C-14), 45.2 (C-9), 41.8 (C-24), 41.8 (C-13), 39.4 (C-20), 38.1 (C-12), 37.3 (C-1), 36.0 (C-10), 39.7 (C-4), 32. 0 (C-25), 31.0 (C-2), 27.3 (C-16), 22.0 (C-15), 20.1 (C-21), 20.1 (C-11), 18.5 (C-27), 18.6 (C-26), 16.6 (C-28), 15.3 (C-19), 11.0 (C-18). Compared with the data published [15], compound 1 was identified as ergosterol (Figure 1).

The effect of single factor test for ergosterol production and mycelial biomass

Figure 4 showed the results of single factor experiment. The ergosterol production reached the highest value of 242.56 ± 24.23 mg/L, when the aged seawater concentration was 20%. The mycelia yield increased with increasing aged seawater content, and the highest yield was obtained when using 100% aged seawater (Figure 4a). The data in figure 2b indicate that the mycelial biomass had the highest productivity at pH 6.5, while the maximum ergosterol yield was acquired at pH 7.5. As shown in figure 4c, the ergosterol was produced

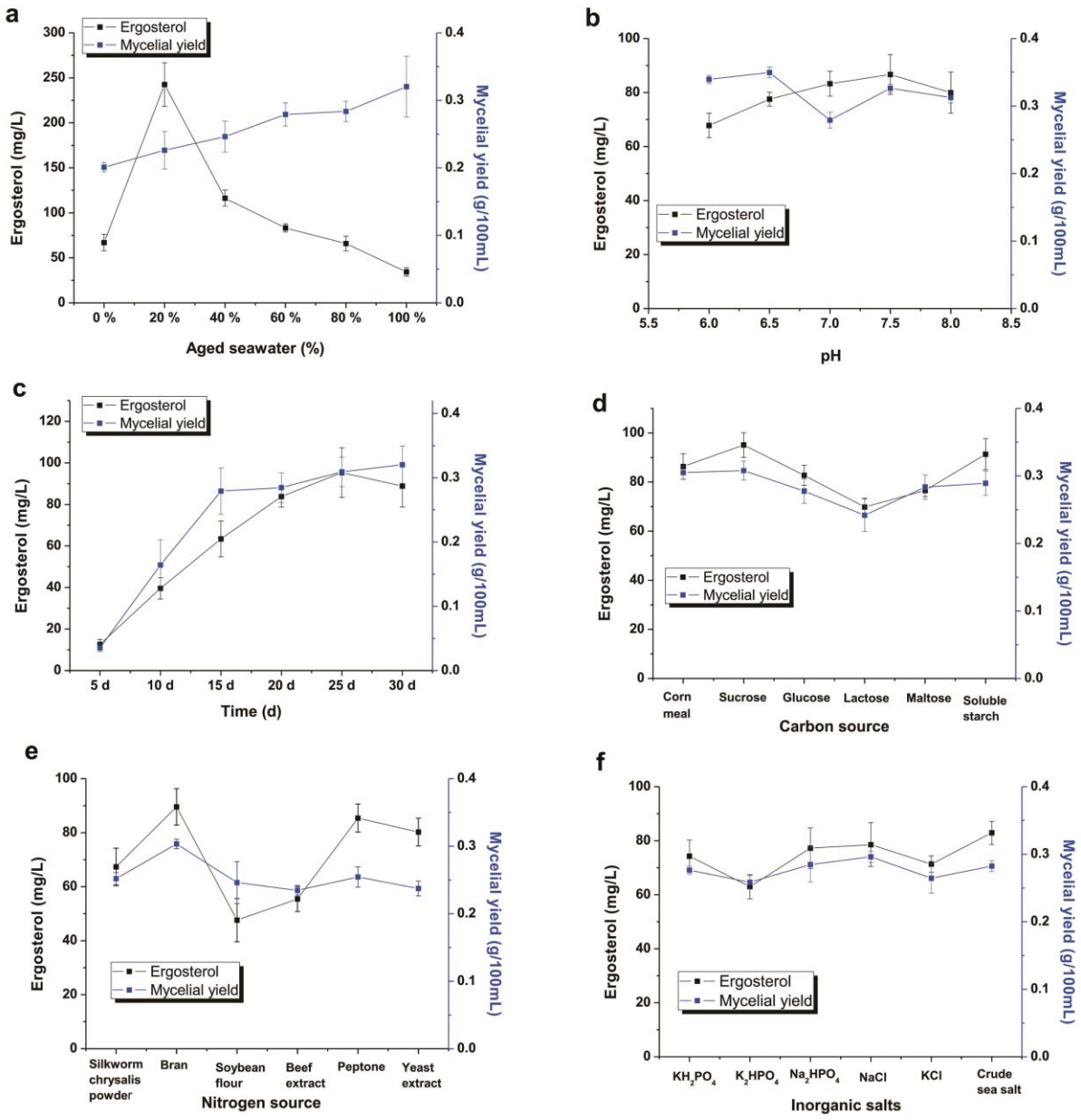

Figure 4. Effects of nutrient and fermentation factors on ergosterol production and mycelial biomass by *C. cladosporioides* M-40. (a) Effect of initial aged seawater concentration on ergosterol production and mycelial biomass. (b) Effect of initial pH values on ergosterol production and mycelial biomass. (c) Effect of fermentation time on ergosterol production and mycelial biomass. (d) Effect of carbon sources on ergosterol production and mycelial biomass. (e) Effect of nitrogen sources on ergosterol production and mycelial biomass. (f) Effect of inorganic source on ergosterol production and mycelial biomass. All experiments are done by changing one independent variable while fixing others at certain levels.

by *C. cladosporioides* M-40 on the fifth day and the production reached the maximum (95.31 ± 11.98 mg/L) on the 25th day. However, the mycelia yield increased with increasing fermentation time. When the sucrose and bran as the sources of carbon and nitrogen respectively, both ergosterol production and mycelia yield were achieved the highest values (Figure 4d and 4e). Although crude sea salt was found to be the optimal inorganic salts source for ergosterol production, mycelia yield achieved the highest value when NaCl was used as the inorganic salt source (Figure 4f).

Table 2. ANOVA of the response surface quadratic model for the ergosterol production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	150086.3	20	7504.317	127.7409	< 0.0001	significant
X_1-pH	58.3811	1	58.3811	0.9938	0.3284	
X_2-Time	769.7712	1	769.7712	13.1033	0.0013	
X_3-Sucrose	168.0394	1	168.0394	2.8604	0.1032	
X_4-Bran	26770.11	1	26770.11	455.6895	< 0.0001	
X_5-Crude sea salt	22364.53	1	22364.53	380.6962	< 0.0001	
$X_1 X_2$	169.6637	1	169.6637	2.8881	0.1016	
$X_1 X_3$	0.9575	1	0.957462	0.0163	0.8994	
$X_1 X_4$	211.1645	1	211.1645	3.5945	0.0696	
$X_1 X_5$	2.39941	1	2.39941	0.0408	0.8415	
$X_2 X_3$	2.49780	1	2.4980	0.0425	0.8383	
$X_2 X_4$	6.1454	1	6.1454	0.1046	0.7491	
$X_2 X_5$	4.4880	1	4.4880	0.0767	0.7845	
$X_3 X_4$	1.3110	1	1.3110	0.0223	0.8824	
$X_3 X_5$	0.6872	1	0.6872	0.0117	0.9147	
$X_4 X_5$	133.7492	1	133.7492	2.2767	0.1439	
X_1^2	6238.384	1	6238.384	106.1918	< 0.0001	
X_2^2	7384.597	1	7384.597	125.703	< 0.0001	
X_3^2	1769.473	1	1769.473	30.1205	< 0.0001	
X_4^2	8092.492	1	8092.492	137.753	< 0.0001	
X_5^2	97135.54	1	97135.54	1653.472	< 0.0001	
Residual	1468.66	25	58.7464			
Lack of Fit	951.5778	20	47.5789	0.46	0.9019	not significant
Pure Error	2.8797	5	0.5759			
R^2	0.9903					
Adj R^2	0.9826					
Pred R^2	0.9700					
Adeq Precision	40.2583					

Statistical mathematical optimization of ergosterol production by *C. cladosporioides* M-40

In this study, we found that fermentation time, initial pH, sucrose concentration, bran concentration, and crude sea salt concentration were five important parameters for ergosterol production. Therefore, CCD was employed to determine the optimal levels of the five selected parameters. As per the experimental data from CCD (Table 1), a second-order polynomial equation (equation 2) was obtained for ergosterol production:

$$Y = +338.24 - 1.91X_1 + 6.94X_2 + 3.24X_3 + 40.90X_4 + 37.39X_5 + 6.51X_1X_2 - 0.49X_1X_3 + 7.27X_1X_4 - 0.77X_1X_5 + 0.79X_2X_3 + 1.24X_2X_4 + 1.06X_2X_5 + 0.57X_3X_4 + 0.41X_3X_5 + 5.78X_4X_5 - 26.74X_1^2 - 29.09X_2^2 - 14.24X_3^2 - 30.45X_4^2 - 105.50X_5^2$$

where, Y is the production of ergosterol (mg/L); X_1 is the initial pH of culture medium; X_2 is the culture time (d); X_3 is the sucrose concentration (g/L); X_4 is the bran concentration (g/L); and X_5 is the crude sea salt concentration (g/L).

The statistical model data of ANOVA were summarized in Table 2. The value of Adj.R^2

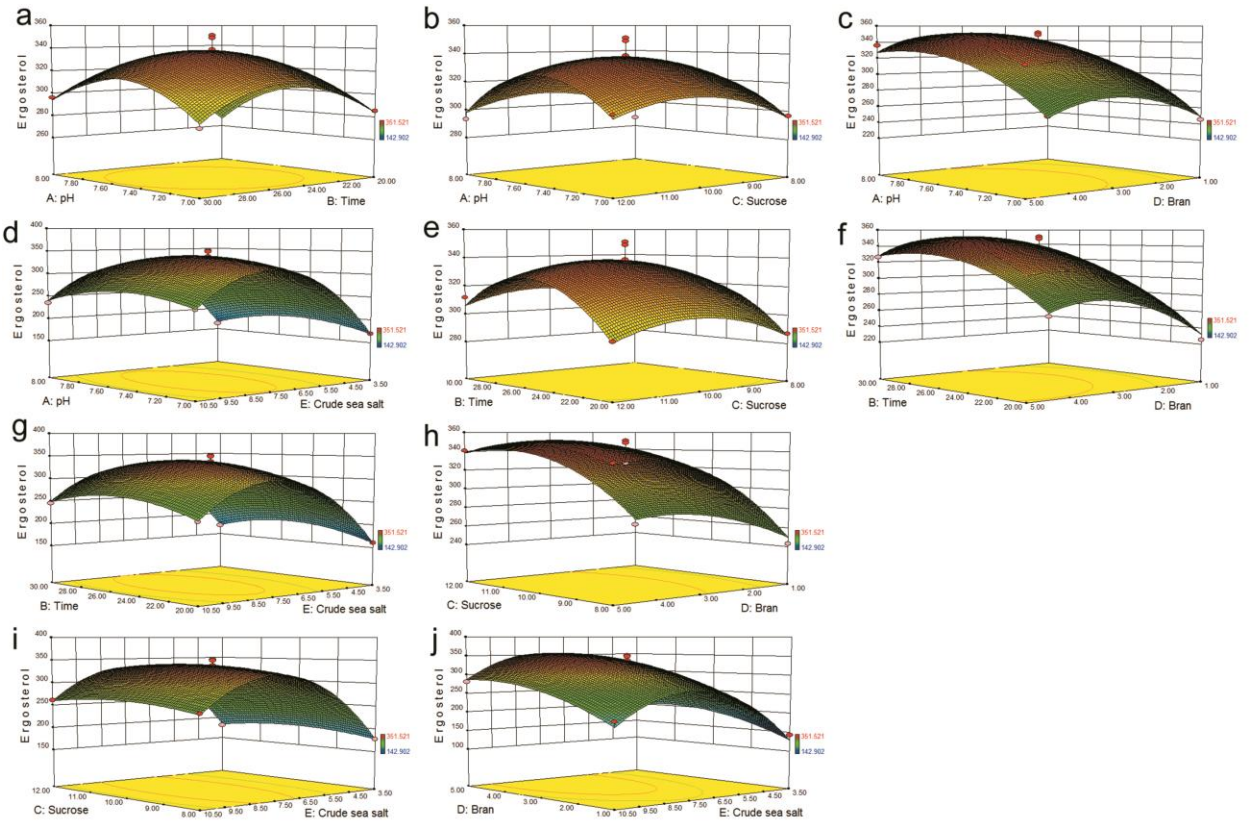

Figure 5. Response surfaces plots for the ergosterol production by *C. cladosporioides* M-40: (a) varying the fermentation time and the initial of pH; (b) varying the initial of pH and the concentration of sucrose; (c) varying the initial of pH and the concentration of bran; (d) varying the initial of pH and the concentration of crude sea salt; (e) varying the fermentation time and the concentration of sucrose; (f) varying the fermentation time and the concentration of bran; (g) varying the fermentation time and the concentration of crude sea salt; (h) varying the fermentation the concentration of sucrose and bran; (i) varying the fermentation the concentration of sucrose and crude sea salt; (j) varying the fermentation the concentration of bran and crude sea salt.

(0.9826) and the value of R^2 (0.9903) indicated that most of the variability could be well-explained by our model and there was a high correlation between the predicted and the experimental values in the model. Also, the Pred.R^2 of 0.9700 was in good agreement with the Adj.R^2 of 0.9826. Besides, "Adeq Precision" detects the signal-to-noise ratio, which is reliable when it is bigger than 4. The ratio of 40.26 revealed a reliable signal and proved again that our model design was fitted. The model F-value of 127.74 means a high reliability of the experiment. The lack of fit F-value of 0.46 implied that the mode could be desirably predicted through the variation. Considering that the low P-value ($P < 0.05$) implies a highly significant level of model terms, the linear terms of X_2, X_4, and X_5, and quadratic terms of X_1^2, X_2^2, X_3^2, X_4^2, and X_5^2 are significant.

The response surface curves in figure 5 demonstrated the regression model for ergosterol production by the marine fungus *C. cladosporioides* M-40, which explained the interaction of five variables and determined the optimal level of each variable for the maximum response. The shape of all the 3-dimensional surfaces in figure 5 shows obvious interactions among the parameters. Specifically, the dome shape of the plots in figure 5 (a, b, and e) illustrated that there were shared interactions between pH and time, pH and sucrose concentration, time and sucrose concentration. However, in the response surfaces of figure 5 (c,

d, f, g, h, i, and j), there were obvious interactions among the parameters investigated. In addition, 2-dimensional contour plot is the visual description of the regression equation and helps to understand the interactions among selected parameters [16]. Each contour curve indicates an unlimited number of combinations of the two test parameters. The elliptical contour plots in figure 5 (d, g, i, and j) indicated striking significant interactions among selected factors, while the circular contour plots of the response surfaces in figure 5 (a, b, and e) suggested that the interactions among selected factors were negligible. The hyperbolic contour plot for the interaction between initial pH and concentration of bran, time and the concentration of bran, as well as the concentration of sucrose and bran in figure 5 (c, f, and h) indicated that the center was neither a maximum nor a minimum point.

The optimum values of the five factors selected variables for the fermentation process were obtained by solving equation 2 using the Design-Expert software package. The optimum culture conditions of the ergosterol production by *C. cladosporioides* M-40 were statistically predicted as follows: fermentation time, 25.7 d; pH, 7.54; sucrose, 10.27 g/L; bran, 4.41 g/L; crude sea salt, 7.69 g/L (or salinity 7.69 ‰). Under this optimal condition, the predicted ergosterol production was 356.98 mg/L, that was a 4.28-fold increase compared to that using the original conditions.

Discussion

Genus Cladosporium is known as produced of a series of Cladosporols secondary metabolites [17]. In this research, ergosterol was first isolated from the marine fungus *C. cladosporioides* M-40 with high productivity of ergosterol. Meanwhile, our experimental data illustrate that optimization of fermentation conditions is a reproducible and non-expensive way to attain the provitamin D_2, ergosterol.

In single factor experiment, overall, the result shown in figure 4 demonstrated that the ergosterol yield was proportional to the cellular mass. However, the proportionality was significantly disturbed by the salinity concentration – the addition of seawater at 20% remarkably promoted ergosterol yield but not cellular growth. On the other hand, the addition of seawater at 40% or more even decreased the production yield. Hence, the salinity played a key role in the production of ergosterol by *C. cladosporioides* M-40 fermentation. This reason is that salinity can influence the secondary metabolites of marine-derived fungi, compared to the terrestrial fungi [18]. Besides, due to the residential characteristic environments for marine fungi, they grow up more slowly compared to terrestrial fungi, and thus the production of secondary metabolites reached the maximum in 20 to 30 d [18, 19]. During the process of fermentation, the nutrients can be gradually used in the medium, which would influence the fungi physiology and bring about accumulated secondary metabolites. These nutrients also have an important effect on the metabolic activity of fungal cells. Such as Tan *et al.* [20] reported that sucrose can facilitate the production of ergosterol by *Saccharomyces cerevisiae*. Bran was found to be the optimum nitrogen source for *C. cladosporioides* M-40 fermentation. A similar result was reported by Shang *et al.* [21] who found that bran could support the maximum ergosterol production by *S. cerevisiae*.

Secondary metabolite formation in fungi is a complex process usually linked with cellular differentiation and morphological development. Many genes and physiological mechanisms are influenced by chemical and physical environmental factors. Several common means of improving secondary metabolite yield include the screening of highly productive strains, the optimization of cultivation conditions, and the overexpression of genes by gene mutation or recombination [22]. We selected this high-yielding ergosterol producing marine fungus *C. cladosporioides* M-40 via screening various

marine fungi, which ergosterol production could statistically predict reached to 356.98 mg/L through optimized five factors that affect ergosterol production based on MFUM medium. The *C. cladosporioides* M-40 produced ergosterol was inferior to other recombinant commercial ergosterol producing strains *S. cerevisiae* [21, 23]. However, its yield was superior to some wild types ergosterol producing strains *S. cerevisiae* [24]. Hence, further research might be required through metabolic engineering and directed mutations to explore the molecular regulation mechanism of the marine fungus *C. cladosporioides* M-40.

In short, the newly isolated marine fungus *Cladosporium cladosporioides* M-40 was identified using phenotypic and molecular approach. The structure of ergosterol was established by extensive spectroscopic analysis. Production of ergosterol was calculated through HPLC quantitatively. The optimum culture conditions were: modified MFUM in 100 mL/250 mL flasks; initial pH 7.54; sucrose, 10.27 g/L; bran, 4.41 g/L; crude sea salt, 7.69 g/L (or salinity 7.69 ‰); culture time 25.7 days; temperature 25°C; rotary shake at 120 rpm. under such conditions the production of ergosterol increased to 356.976 mg/L, which was 4.28-fold increasing of production comparing to its prior fermentation. Further research might be required through metabolic engineering and directed mutations to explore the molecular regulation mechanism of *C. cladosporioides* M-40.

Reference

1. Munn AL, Heese-Peck A, Stevenson BJ, Pichler H, Riezman H. 1999. Specific sterols required for the internalization step of endocytosis in yeast. Molecular biology of the cell. 10(11):3943-3957.
2. Sturley SL. 2000. Conservation of eukaryotic sterol homeostasis: new insights from studies in budding yeast. Biochimica et biophysica acta. 1529(1-3):155-163.
3. Kato M, Wickner W. 2001. Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion. The EMBO journal. 20(15):4035-4040.
4. Huan W, Tianzhu Z, Yu L, Shumin W. 2017. Effects of Ergosterol on COPD in Mice via JAK3/STAT3/NF-kappaB Pathway. Inflammation. 40(3):884-893.
5. Nallathamby N, Guan-Serm L, Vidyadaran S, Abd Malek SN, Raman J, Sabaratnam V. 2015. Ergosterol of Cordyceps militaris Attenuates LPS Induced Inflammation in BV2 Microglia Cells. Natural product communications. 10(6):885-886.
6. Zhang SY, Xu LT, Li AX, Wang SM. 2015. Effects of Ergosterol, Isolated from Scleroderma Polyrhizum Pers., on Lipopolysaccharide-Induced Inflammatory Responses in Acute Lung Injury. Inflammation. 38(5):1979-1985.
7. Tai CJ, Choong CY, Lin YC, Shi YC, Tai CJ. 2016. The anti-hepatic fibrosis activity of ergosterol depended on upregulation of PPARgamma in HSC-T6 cells. Food & function. 7(4):1915-1923.
8. de la Calle F. 2017. Marine microbiome as source of natural products. Microbial biotechnology. 10(6):1293-1296.
9. Zhou S, Wang M, Feng Q, Lin Y, Zhao H. 2016. A study on biological activity of marine fungi from different habitats in coastal regions. SpringerPlus. 5(1):1966.
10. Asisipho N, Kunle O, Ademola O, Nwodo U, Mabinya L, Okoh A. 2017. Optimization of growth parameters for cellulase and xylanase production by *Bacillus* species isolated from decaying biomass. J Biotech Res. 8:33-47.
11. Managamuri U, Vijayalakshmi M, Poda S, Ganduri VS, Babu RS. 2016. Optimization of culture conditions by response surface methodology and unstructured kinetic modeling for bioactive metabolite production by Nocardiopsis litoralis VSM-8. 3 Biotech. 6(2):219.
12. Zhao HG, Wang M, Lin YY, Zhou SL. 2018. Optimization of culture conditions for penicilazaphilone C production by a marine-derived fungus *Penicillium sclerotiorum* M-22. Letters in applied microbiology. 66(3):222-230.
13. Zhou SL, Wang M, Zhao HG, Huang YH, Lin YY, Tan GH, Chen SL. 2016. Penicilazaphilone C, a new antineoplastic and antibacterial azaphilone from the Marine Fungus *Penicillium sclerotiorum.* Archives of pharmacal research. 39(12):1621-1627.
14. Torres DE, Rojas-Martinez RI, Zavaleta-Mejia E, Guevara-Fefer P, Marquez-Guzman GJ, Perez-Martinez C. 2017. *Cladosporium cladosporioides* and *Cladosporium pseudocladosporioides* as potential new fungal antagonists of Puccinia horiana Henn., the causal agent of chrysanthemum white rust. PloS one. 12(1):e0170782.
15. Nowak R, Drozd M, Mendyk E, Lemieszek M, Krakowiak O, Kisiel W, Rzeski W, Szewczyk K. 2016. A New Method for the Isolation of Ergosterol and Peroxyergosterol as Active Compounds of *Hygrophoropsis aurantiaca* and in Vitro Antiproliferative Activity of Isolated Ergosterol Peroxide. Molecules. 21(7):946.
16. Taheri M, Moazeni-Pourasil RS, Sheikh-Olia-Lavasani M, Karami A, Ghassempour A. 2016. Central composite design with the help of multivariate curve resolution in loadability optimization of RP-HPLC to scale-up a binary mixture. Journal of separation science. 39(6):1031-1040.

17. Peng X, Wang Y, Zhu G, Zhu W. 2018. Fatty acid derivatives from the halotolerant fungus Cladosporium cladosporioides. Magnetic resonance in chemistry: MRC. 56(1):18-24.
18. Xu R, Xu GM, Li XM, Li CS, Wang BG. 2015. Characterization of a Newly Isolated Marine Fungus Aspergillus dimorphicus for Optimized Production of the Anti-Tumor Agent Wentilactones. Marine drugs. 13(11):7040-7054.
19. Zhao C, Guo L, Wang L, Zhu G, Zhu W. 2016. Improving the yield of (+)-terrein from the salt-tolerant Aspergillus terreus PT06-2. World journal of microbiology & biotechnology. 32(5):77.
20. Tan TW, Zhang M, Gao H. 2003. Ergosterol production by fed-batch fermentation of Saccharomyces cerevisiae. Enzyme Microb Tech. 33:366–370.
21. Shang F, Wen S, Wang X, Tan T. 2006. Effect of nitrogen limitation on the ergosterol production by fed-batch culture of Saccharomyces cerevisiae. Journal of biotechnology. 122(3):285-292.
22. Nahlik J, Hrncirik P, Mares J, Rychtera M, Kent CA. 2017. Towards the design of an optimal strategy for the production of ergosterol from Saccharomyces cerevisiae yeasts. Biotechnology progress. 33(3):838-848.
23. Thomas AB, Shetane TD, Singha RG, Nanda RK, Poddar SS, Shirsat A. 2017. Employing Central Composite Design for Evaluation of Biomass Production by Fusarium venenatum: In Vivo Antioxidant and Antihyperlipidemic Properties. Applied biochemistry and biotechnology. 183(1):91-109.
24. Lamacka M, Sajbidor J. 1977. Ergosterol determination in Saccharomyces cerevisiae. Comparison of different methods. Biotechnology Techniques. 11(10):273-275.