Vol. 17(21), pp. 668-679, 23 May, 2018
DOI: 10.5897/AJB2017.16378
Article Number: D4CB5FF57196
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Marine killer yeast *Metschnikowia saccharicola* active against pathogenic yeast in crab and an optimization of the toxin production

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Received 23 December, 2017; Accepted 7 May, 2018

A marine yeast strain, DD21-2 was isolated from sediments in Dandong, China, which has killer activity against yeast *Metschnikowia bicuspidata* WCY (pathogenic to crab, *Portunus trituberculatus*). Routine identification, sequence analysis of 26S rDNA and ITS sequencing showed that the strain was *M. saccharicola* DD21-2, and has not been previously reported as having killer activity against *M. bicuspidata* WCY. To optimize the production of the killer toxin by *M. saccharicola* DD21-2, the interaction effects of fermentation process variables were investigated by Response Surface Methodology (RSM). The following reaction factors were selected in screening experiments: Inoculum concentration (2 to 6%), pH (5.0 to 6.0), temperature (20 to 28°C), and fermentation time (2 to 3 days), and the diameter of the inhibition zone produced by the killer toxin was used as a response variable. A quadratic regression model of killer toxin activity was established by regression analysis and significance testing (P test). The results identified the following parameters as optimal for maximal production of the killer toxin by *M. saccharicola* DD21-2: Fermentation temperature 28°C, pH 5.5, fermentation time 2.7 days, and inoculum concentration 4.1% (v/v). Thus, RSM was effective in determining the best conditions for killer toxin production, suggesting the practical usage of this marine yeast in the investigation of process variables.

Key words: Marine killer yeast, killer toxin, response surface methodology (RSM), fermentation, optimization.

INTRODUCTION

Many studies have shown that some marine yeast species are pathogenic to marine animals (Xu, 2005; Wang et al., 2008; Kaewwichian et al., 2012). A condition called 'milky disease' caused by yeast *Metschnikowia*

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bicuspidata has been detected in crab *Portunus trituberculatus* cultured in 2001 in Zhoushan, Zhejiang Province, China (Xu et al., 2003). However, anti-yeast compounds such as nystatin, benzalkonium bromide, and extracts of gold thread root and garlic are toxic to the crab, so killer toxins produced by some yeast species against pathogenic yeasts may be a good alternative (Phillliskink and Young, 1975). Studies have shown that killer yeasts and the killer toxins they produce have antimicrobial activity and can be used to control the growth of pathogenic yeasts in humans, animals and plants (Comitini et al., 2004; Magliani et al., 2008; Chi et al., 2010; Wang et al., 2013).

Yeast-derived killer toxins have been widely used to control harmful yeast growth in industrial fermentation systems, food production, animal breeding, agriculture, and medicine as antifungal drug formulations (Magliani et al., 2008; Wang et al., 2013). In the food industry, killer toxins inhibit the growth of wild yeast and prevent contamination, thus providing a good system environment and improving product quality (Schmitt and Breinig, 2002). In aquaculture, some diseases of marine animals, including *P. trituberculatus* can be controlled by certain marine killer toxin-producing yeast (Wang et al., 2007; Chi et al., 2010).

Optimizing the process of yeast fermentation is important to obtain maximum yield of killer toxins (Bandeira et al., 2006; Çorbacı and Uçar, 2017). However, the reaction system cannot be comprehensively analyzed using a traditional one-variable-at-a-time technique if more than one variable is present (Bezerra et al., 2008). Therefore, it is necessary to employ an appropriate statistical approach to evaluate the relationship among measurable variables. Response surface methodology (RSM) comprises mathematical and statistical techniques aimed at optimizing system response influenced by several independent variables (Bezerra et al., 2008), such as fermentation temperature and the amount of starter culture to obtain the best performance during fermentation (Yaakob et al., 2011). The objective of this study was to screen marine yeast species for the production of killer toxins against pathogenic yeast and determine the optimal conditions for killer toxin production in culture by investigating the interactions between process variables providing a basis for further research of disease prevention and control in marine aquaculture.

MATERIALS AND METHODS

Yeast strains and media

M. bicuspidata WCY (collection number 2E00088 at the Marine Microorganisms Culture Collection of China) was confirmed pathogenic yeast in *P. trituberculatus* (Wang et al., 2007). *Candida tropicalis* and *Candida albicans* isolated from different marine environments were used as susceptible yeast strains. Yeast strains were grown in yeast extract peptone dextrose (YPD) medium containing 1.0% yeast extract, 2.0% glucose, 2.0% peptone, and 3% agar. The medium for killer toxin production was prepared aseptically by adding 2% NaCl and 15% glycerol to YPD, and medium pH was initially adjusted to 4.5 with 0.05 mol/L citric acid-hydrogen phosphate disodium buffer. Killer toxin activity was assayed in YPD agar supplemented with 3 mg/mL methylene blue dissolved in ethanol (final concentration 0.003%) (Guo et al., 2013a).

Screening of marine killer yeast

Each yeast strain from the slants was grown in YPD liquid medium at 28°C, 140 rpm for 24 h; then, 2 ml of culture was centrifuged at 4°C for 5 min (5,000 × g) and washed three times with sterile water. Cell suspension was adjusted to 1×10^7 cells/ml, and 0.2 ml was inoculated into assay YPD agar plates seeded with susceptible pathogenic yeast strains. After 2 to 3 days of incubation at 28°C, a clear killing zone was observed around the colonies of killer yeast, which were then selected based on killing activity (determined as the ratio of the inhibition-zone diameter to colony diameter) (Wang et al., 2007; Peng, 2010).

Yeast identification

Routine yeast identification was conducted as described previously (Kurtzman and Fell, 2011; Kaewwichian et al., 2012). DNA extraction, PCR for the amplification of yeast 26S rDNA, and ITS were performed as previously reported by Chi et al. (2007). Primers for the D1/D2 domain of 26S rDNA were: Forward NL-1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and reverse NL-4 5′-GGTCCGTGTTTCAAGACGG-3′ (Sugita et al., 2003). Primers for ITS1/ITS4 were: Forward ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and reverse ITS4 5′-TCCTCCGCTTATTGATATGC-3′(Guo et al., 2013b). The reaction volume (50 mL) contained 25 mL SuperMix (2 ´ Easy Taq), 0.1 mmol/L NL-1 or NL-4 (1 mL), 0.1 mmol/L ITS1 or ITS4 (1 mL), 50 ng/mL template DNA (2 mL), and H_2O (21 mL). PCR was performed in the Eppendorf Gradient Mastercycler (Shanghai Eternal Medical Instrument Co., Ltd., Shanghai, China) under the following conditions: Initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated via agarose gel electrophoresis and the ITS and D1/D2 26S rDNA fragments were sequenced by Invitrogen Biotechnology (Shanghai, China).

Phylogenetic analysis

Yeast phylogenetic analysis was performed by the neighbor joining method using MEGA 5.1 (Tamura et al., 2011). Evolutionary distances were calculated using the p-distance model of MEGA 5.1, and bootstrap analysis was performed on 1,000 random resamplings. Reference sequences were retrieved from GenBank (accession numbers are indicated on the tree).

Measurement of killer toxin activity

Killer toxin activity was determined by a diffusion test using Oxford cups (6.0 × 10.0 mm) placed on the surface of assay agar plates (Santos et al., 2000) seeded with strain WCY. Killer toxin supernatant (250 µL) was added to each cup, and plates were incubated at 28°C for 2 days; the diameter of the inhibition zone was used as a measure of killer toxin activity (Peng, 2010). And each experiment was repeated 3 times.

Killer toxin production

Before designing the RSM experiment, selecting the inoculum

Table 1. Factors and levels of RSM test.

Factor	Level			
	Symbol	-1	0	+1
Inoculum concentration (%)	X_1	2	4	6
pH value of fermentation broth	X_2	5.0	5.5	6.0
Fermentation time (days)	X_3	2	2.5	3
Fermentation temperature (°C)	X_4	26	28	30

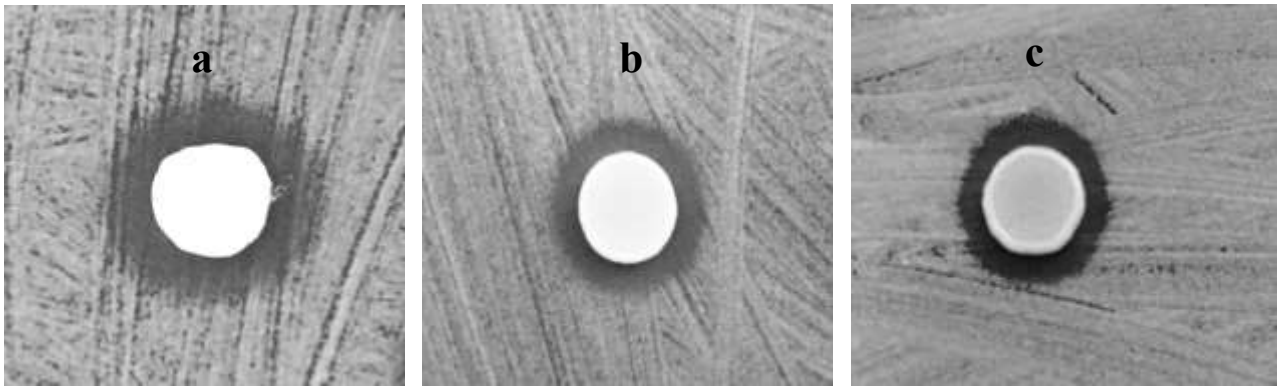


Figure 1. Clear zones formed on the assay agar seeded with pathogenic yeast. Sensitive strains: (A) *Metchnikowia bicuspidata* WCY, (B) *Candida tropicalis* Ct, and (C) *Candida albicans* YTS-03.

concentration, pH of the fermentation broth, fermentation time, and temperature as four independent variables for single-factor screening experiments were done. *M. saccharicola* was seeded at different inoculum concentrations (2 to 12%) in 500-mL Erlenmeyer flasks containing 100 mL of toxin production medium (pH 4.5) in a rotary bed shaker (140 rpm) at 28°C for 48 h. Based on the results, medium pH was varied from 3.5 to 6.5 using 0.05 M citric acid-hydrogen phosphate disodium buffer, and yeast was cultured at different temperatures (20 to 35°C) for 12 to 72 h.

Experimental design and statistical analysis

According to the results of single factor tests, a three-level-four-factor Box-Behnken Design (BBD) was applied to optimize fermentation conditions using the Design Expert (V 8.0.5) software. Inoculum concentration (X1), pH of the fermentation broth (X2), and fermentation time (X3) and temperature (X4) were the independent variables (Table 1), and killer toxin activity was used as an endpoint in the response surface tests (Su et al., 2013). Each variable was assigned three levels: Low (−1), medium (0), and high (+1).

The diameter of the inhibition zone (Y) was taken as a response of the design experiment. Full quadratic models (Bezerra et al., 2008) for killer toxin activity were established using the following mathematical equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j=2}^{4} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response (diameter of inhibition zone, cm), β0 is the intercept, βi, βii, and βij are regression coefficients of the linear, quadratic, and interactive terms, respectively; and Xi and Xj represent coded independent variables. The fitted polynomial equation can be expressed as surface plots to visualize the relationships between responses and investigated parameters (Lwa et al., 2015). ANOVA was employed to evaluate the empirical mathematical model at 5% significance level and to test the significance of the difference between two or more sample mean differences. The statistical significance was considered by P-value，where the calculated P-value should be greater than the tabulated P-value to reject the null hypothesis and all the regression coefficients were 0 (Devore and Farnum, 2004).

RESULTS AND DISCUSSION

Screening of marine killer yeast against pathogenic yeast strains

The yeast strain DD21-2 isolated from river bed sediments could secrete killer toxins onto culture medium and kill pathogenic yeast *M. bicuspidata* WCY, *C. tropicalis* Ct, and *C. albicans* YTS-03 (Figure 1). The killer activity of strain DD21-2 against *M. bicuspidata* WCY was higher than that against Ct and YTS-03, as evidenced by the ratio of diameter of inhibition zone to diameter of the colony been ≥1.5 (Figure 1); therefore, *M. bicuspidata* WCY was used as a sensitive strain in further experiments.

Identification of yeast strain DD21-2

Based on colony and cell morphology (Figure 2), fermentation and carbon source assimilation (Table 2),

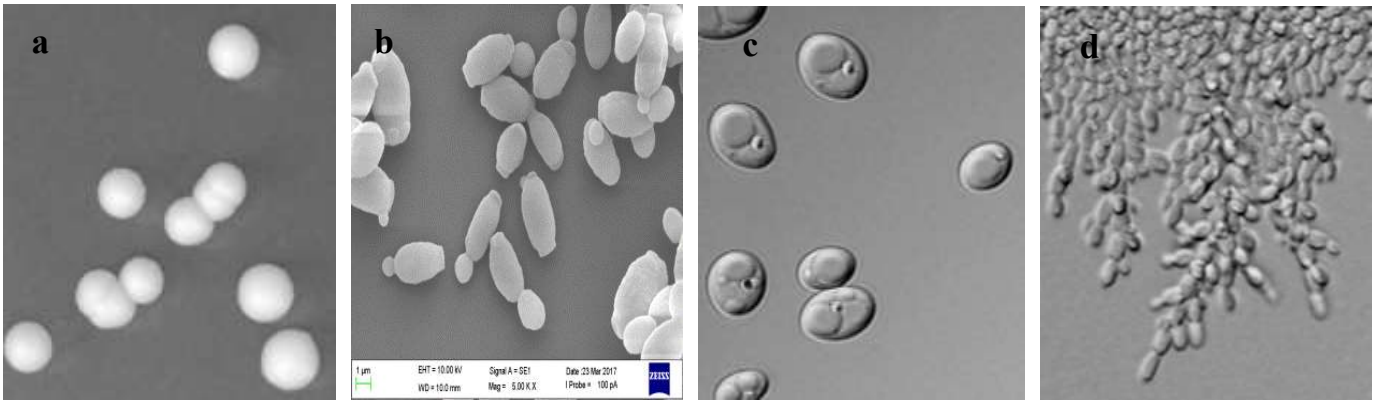


Figure 2. Images of colonies (a), scanning electron microscopy (b), budding cells (c), and pseudohyphae (d) of the *M. saccharicola* DD21-2 strain.

Table 2. Carbohydrate fermentation and assimilation by strain DD21-2.

Assimilation	Reaction	Fermentation	Reaction
Glucose	+	Glucose	+
Maltose	+	Maltose	–
Galactose	–	Galactose	l
Sucrose	+	Sucrose	–
Lactose	–	Lactose	–
Raffinose	+	Raffinose	–
Melibiose	–	Melibiose	–
Amidulin	–	Trehalose	–
Trehalose	+		
Cellobiose	+		
D-arabinose	–		
L-arabinose	–		
D-xylose	w		

+, Positive; -, Negative; l, Delayed positive; w, Weak.

and strain types of marine yeasts (Kurtzman and Fell, 2011), the yeast strain DD21-2 was related to *M. saccharicola*. Phylogenetic analysis based on ITS and the D1/D2 domain of 26S rDNA showed that many phylogenetically related yeast species were similar to the marine yeast strain and confirmed that DD21-2 was closely related to *M. saccharicola* (Figures 3 and 4). Although the phylogenetic analysis of 26S rDNA showed that Metschnikowia is similar to Candida, they differ greatly in morphology and physiology (Kurtzman and Fell, 2011; Kaewwichian et al., 2012). Moreover, the nucleotide blast on NCBI also showed that only Metschnikowia saccharicola has 100% similarity to reference sequence. Phylogenetic analysis of ITS sequencing also fully proved this point (Figure 4). Therefore, the topology of the phylogenetic in Figures 3 and 4 confirms that strain DD21-2 was *M. saccharicola* (NCBI accession number KY849584 and MF115995).

Single-factor screening experiments

Individual fermentation variables significantly influenced the activity of the yeast-derived killer toxin. Thus, killer activity was highest at an inoculum concentration of 4% (v/v; Figure 5a) and pH 5.5 (Figure 5b), but further increases inhibited toxin activity. Killer toxin activity was maximum at 54 h of fermentation (Figure 5c) and 27.5°C (Figure 5d), which is consistent with previous reports (Zhou et al., 2014; Wu et al., 2015). Therefore, the independent factors were inoculum concentration (X1, 2 to 6%, w/v), pH value (X2, 5.0 to 6.0), fermentation temperature (X3, 26 to 28°C), and fermentation time (X4, 2 to 3 days) (Table 1).

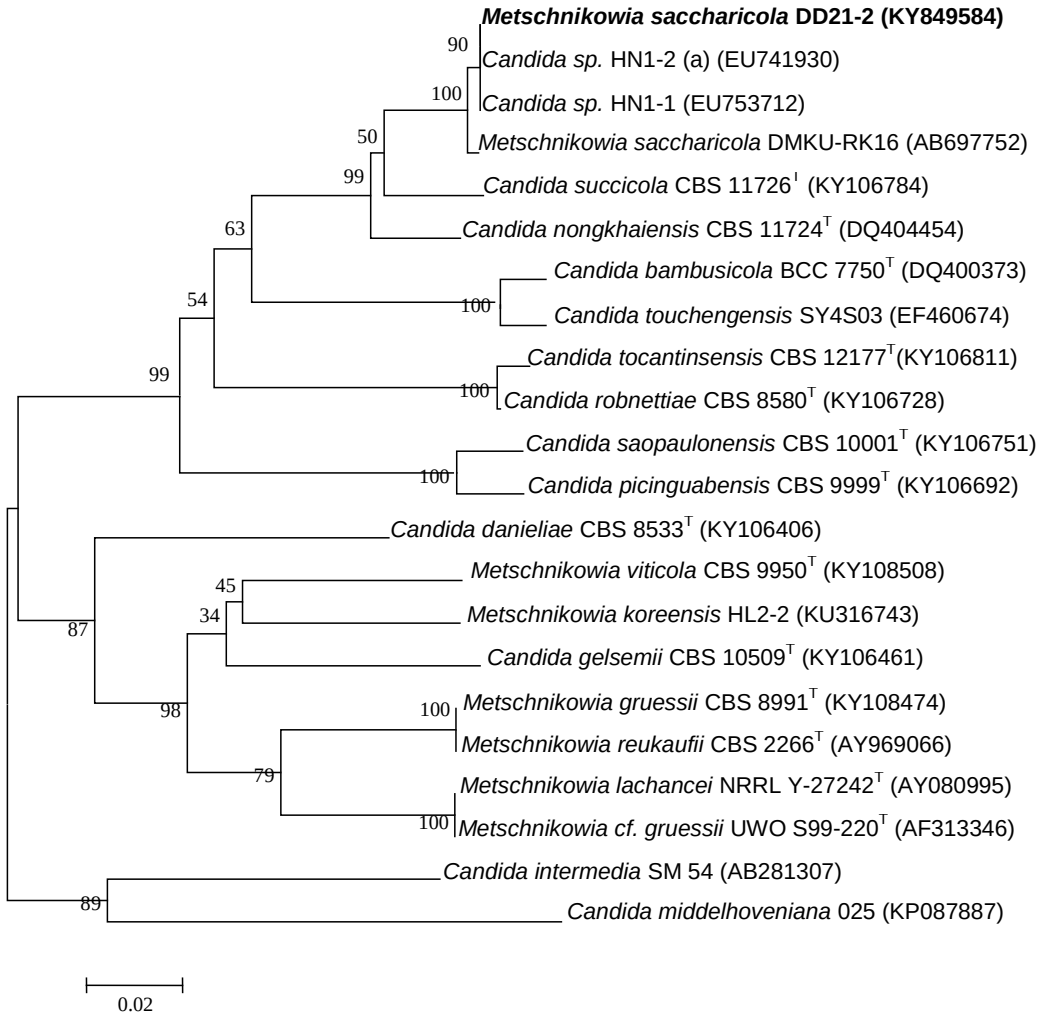


Figure 3. Phylogenetic tree of yeast strain DD21-2 and 21 of its closest relatives (type strains) constructed using neighbor parsimony analysis of the D1/D2 domain of 26S rDNA. Bootstrap support values were calculated based on 1,000 pseudoreplications; values ≥50% are shown above the branches. Strain and sequence accession numbers are shown.

RSM model analysis

Model fitting for RSM

The complete design matrix of the Box-Behnken design and the interaction effect of variables on the response are given in Table 3. A total of 29 experiments were conducted to determine the optimum fermentation conditions, and the data indicated that different levels of variables resulted in different response.

Multiple regression fitting was performed using Design Expert (V 8.0.5) (Shen et al., 2010), and full quadratic models were established using Equation 1. Through regression analysis, the relationship between inoculum concentration (X_1), pH of the fermentation broth (X_2), fermentation time (X_3), fermentation temperature (X_4), and the diameter of the inhibition zone (Y) was described by quadratic regression Equation 2:

$$Y = -45.62 + 0.62X_1 + 7.10X_2 + 1.68X_3 + 1.74X_4 + -0.08X_1X_2 + 0.11X_1X_3 - 0.01X_1X_4 + 0.10X_2X_3 - 0.02X_3X_4 - 0.03X_1^2 - 0.65X_2^2 - 0.37X_3^2 - 0.03X_4^2 \quad (2)$$

ANOVA showed that the *P* value of the model was 0.0228 (Table 4), indicating that regression was significant and that the experimental data and model were well fitted. The *P* test showed that the quadratic effect or interaction of independent variables significantly influenced the diameter of the inhibition zone. The coefficient of determination (R^2) for the quadratic model was 0.903, indicating that it was a well-fitting model that could adequately describe the relationships between the response (inhibition-zone diameter) and variables within the studied ranges. These results suggested that the empirical models for killer toxin activity provided good predictions for optimization.

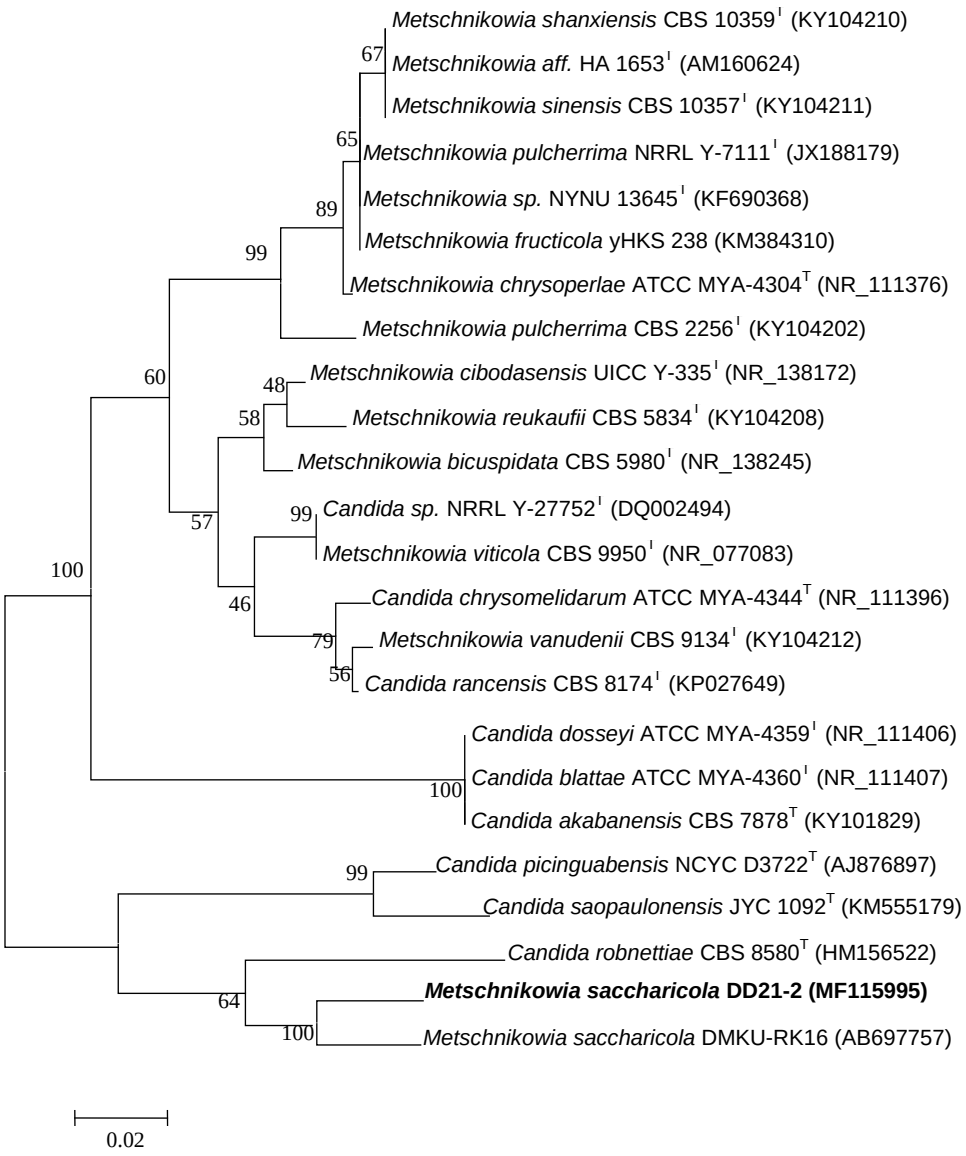


Figure 4. Phylogenetic tree of yeast strain DD21-2 and 23 of its closest relatives (type strains) based on the neighbor parsimony analysis of ITS sequences. Bootstrap support values were calculated based on 1,000 pseudoreplications; values ≥50% are shown above the branches. Strain and sequence accession numbers are shown.

Interaction effect of process variables on the diameter of the inhibition zone

The three-dimensional (3D) surfaces and the corresponding contour plots generated by the Design Expert software were used to study the effects of the parameters and their interactive effects on the killer toxin activity (Figure 6). According to the equation, the coefficients of the quadratic terms were negative, and the parabolic opening in 3D surfaces was downward, which indicated a maximum value point. Contour plots revealed significant interactive effects of the four factors on the diameter of the inhibition zone. The increment of the inhibition zone diameter with the increase of inoculum concentration and pH up to a critical point (Figure 6a) is in agreement with the fact-finding results of killer toxin' s optimal condition of Liu et al. (2012), and the ellipsoid contour plot of the inhibition-zone diameter indicated that the interaction between inoculum concentration and pH was considerable (Figure 6b).

The interaction effects of inoculum concentration and fermentation time on the inhibition zone diameter revealed that longer fermentation time produced a larger inhibition zone (Figure 6c). However, as the inoculum concentration and fermentation time increased, killer toxin activity decreased (Figure 6c), which could be

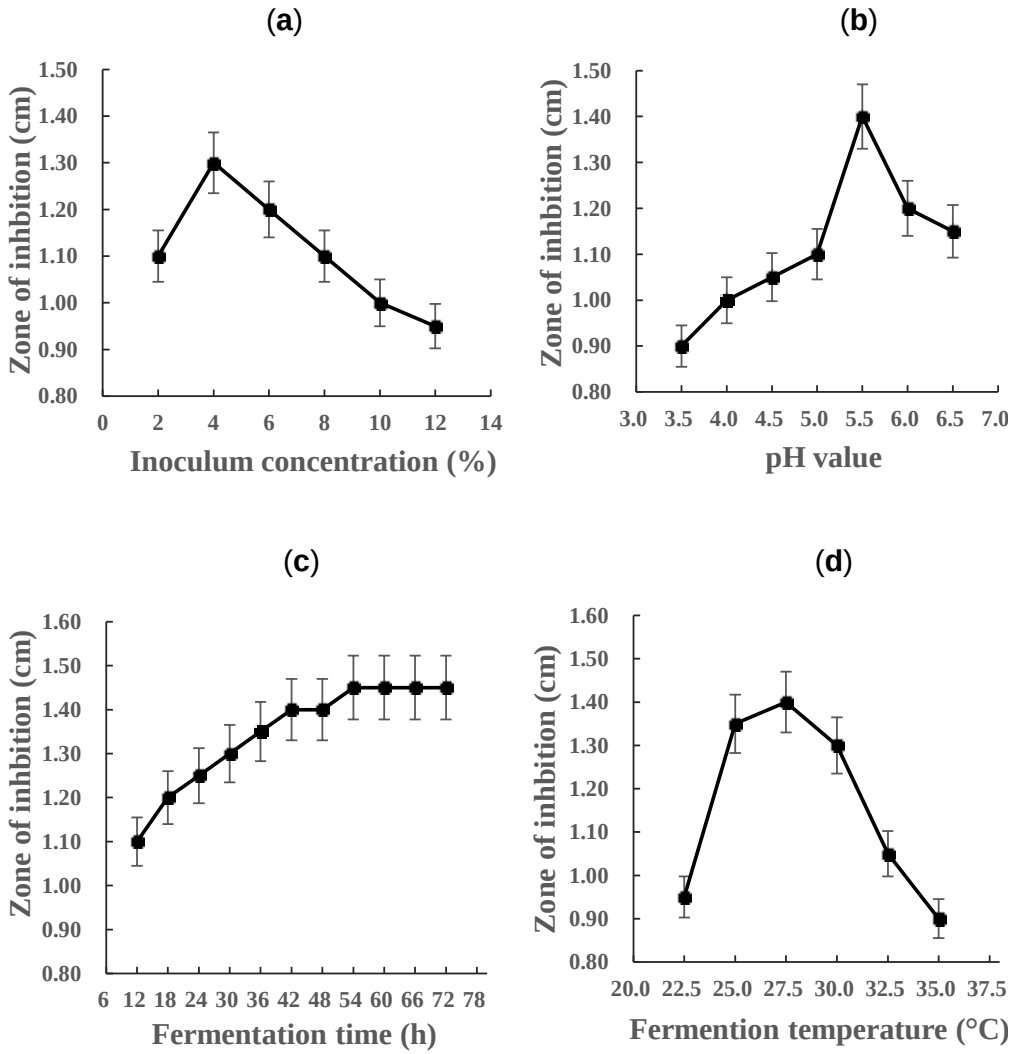


Figure 5. Effects of inoculum concentration (a), pH (b), and fermentation time (c) and temperature (d) on the activity of the killer toxin.

Table 3. Design and results of central composite tests.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response[1)]
	X_1	X_2	X_3	X_4	Y
	A: Inoculum concentration (%, v/v)	B: pH of fermentation broth	C: Fermentation time (d)	D: Fermentation temperature (°C)	Diameter of inhibition zone (cm)
1	0	0	0	0	1.45
2	1	0	1	0	1.20
3	0	1	0	1	0.95
4	0	0	-1	1	1.10
5	0	1	0	-1	1.05
6	0	0	0	0	1.40
7	0	0	1	-1	1.30
8	1	0	0	1	1.00
9	-1	0	0	1	1.30
10	0	0	0	0	1.35
11	0	0	0	0	1.35

Table 3. Contd.

12	0	0	0	0	1.40
13	0	1	1	0	1.30
14	4	-1	0	-1	1.20
15	-1	-1	0	0	1.05
16	0	1	-1	0	1.05
17	1	0	0	-1	1.15
18	0	-1	-1	0	1.10
19	-1	0	0	-1	1.30
20	-1	1	0	0	1.15
21	0	-1	0	1	1.10
22	1	-1	0	0	1.20
23	0	0	-1	-1	1.00
24	0	-1	1	0	1.25
25	-1	0	1	0	1.00
26	0	0	1	1	1.30
27	1	1	0	0	1.00
28	1	0	-1	0	1.05
29	-1	0	-1	0	1.30

[1)]Data are presented as means of 3 replications.

Table 4. ANOVA of Box-Behnken test results.

Source	Sum of squares (SS)	Degree of freedom (DF)	Mean squares (MS)	*F*-value	*P*-value[b]
Model[a]	0.4400	14	0.0320	3.05	0.0228*
A-Inoculum concentration	0.0210	1	0.0210	2	0.1793
B-pH value of fermentation	0.0130	1	0.0130	1.28	0.2771
C-Fermentation time	0.0470	1	0.0470	4.5	0.0523
D-Fermentation temperature	0.0052	1	0.0052	0.5	0.4913
AB	0.0230	1	0.0230	2.16	0.1639
AC	0.0510	1	0.0510	4.86	0.0448*
AD	0.0056	1	0.0056	0.54	0.4747
BC	0.0025	1	0.0025	0.24	0.6319
BD	0.0000	1	0.0000	0	1.0000
CD	0.0025	1	0.0025	0.24	0.6319
A^2	0.1000	1	0.1000	9.59	0.0079**
B^2	0.1700	1	0.1700	16.26	0.0012**
C^2	0.0560	1	0.0560	5.37	0.0361*
D^2	0.0900	1	0.0900	8.65	0.0107*
Residual	0.1500	14	0.0100		
Lack of fit	0.1400	10	0.0140	7.94	0.0303
Pure error	0.0070	4	0.0018		
Cor total	0.5900[c]	28[d]			

*P < 0.05, significant difference; **P < 0.01, very significant difference; [a] R^2 = 0.903; [b] Confidence level of 95%, α = 0.05; [c] Cor total sum of squares: Sum of squares total corrected for the mean; [d]Cor total degree of freedom: degree of freedom total corrected for the mean number of runs minus one.

attributed to inactivation of the killer toxin during longer fermentation (Jia, 2012). In the interaction of the inoculum concentration and fermentation temperature, the diameter of the inhibition zone increased with the increase of the inoculum concentration and temperature up to the optimum level (Figure 6e). Similarly, the

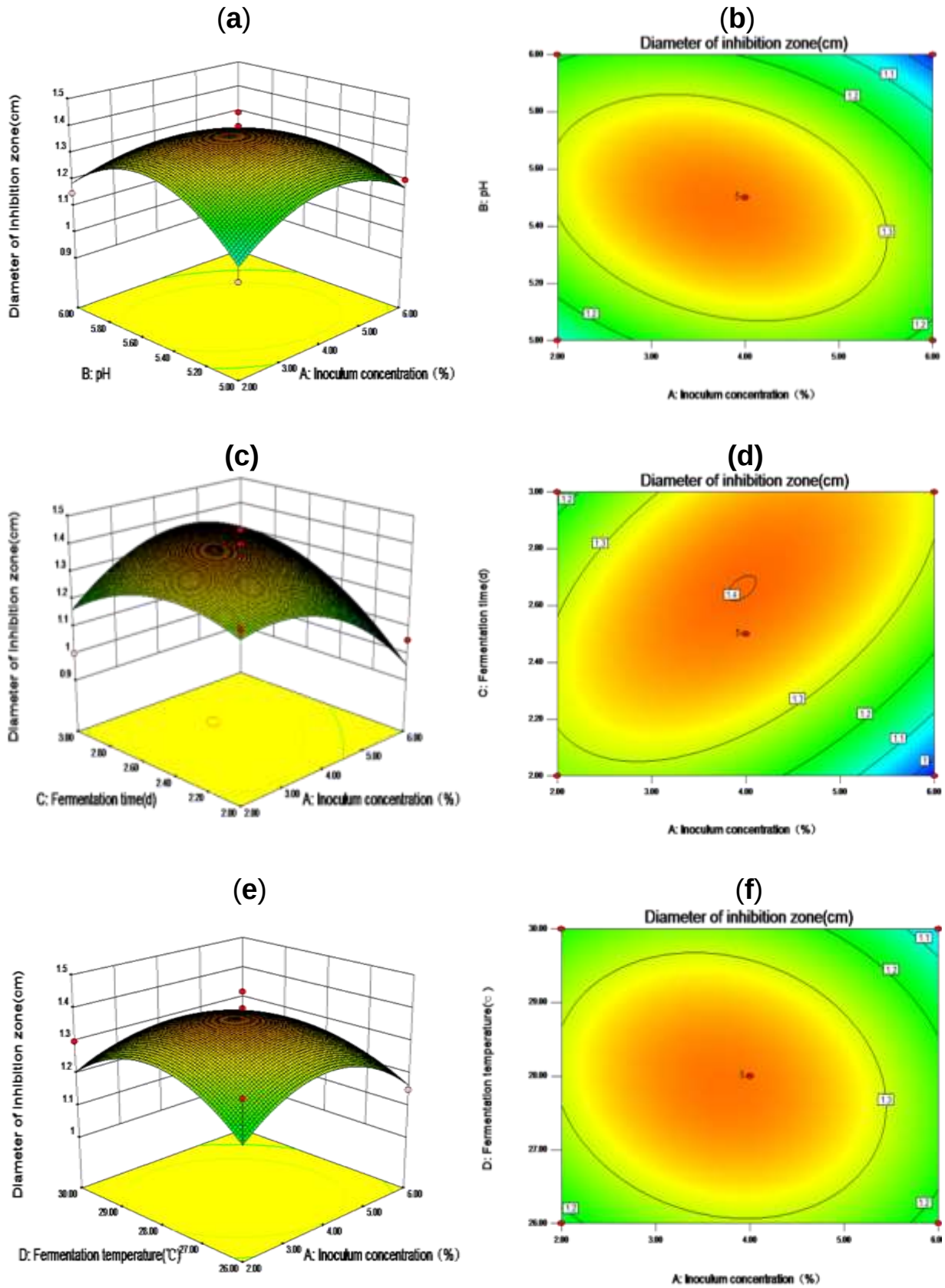
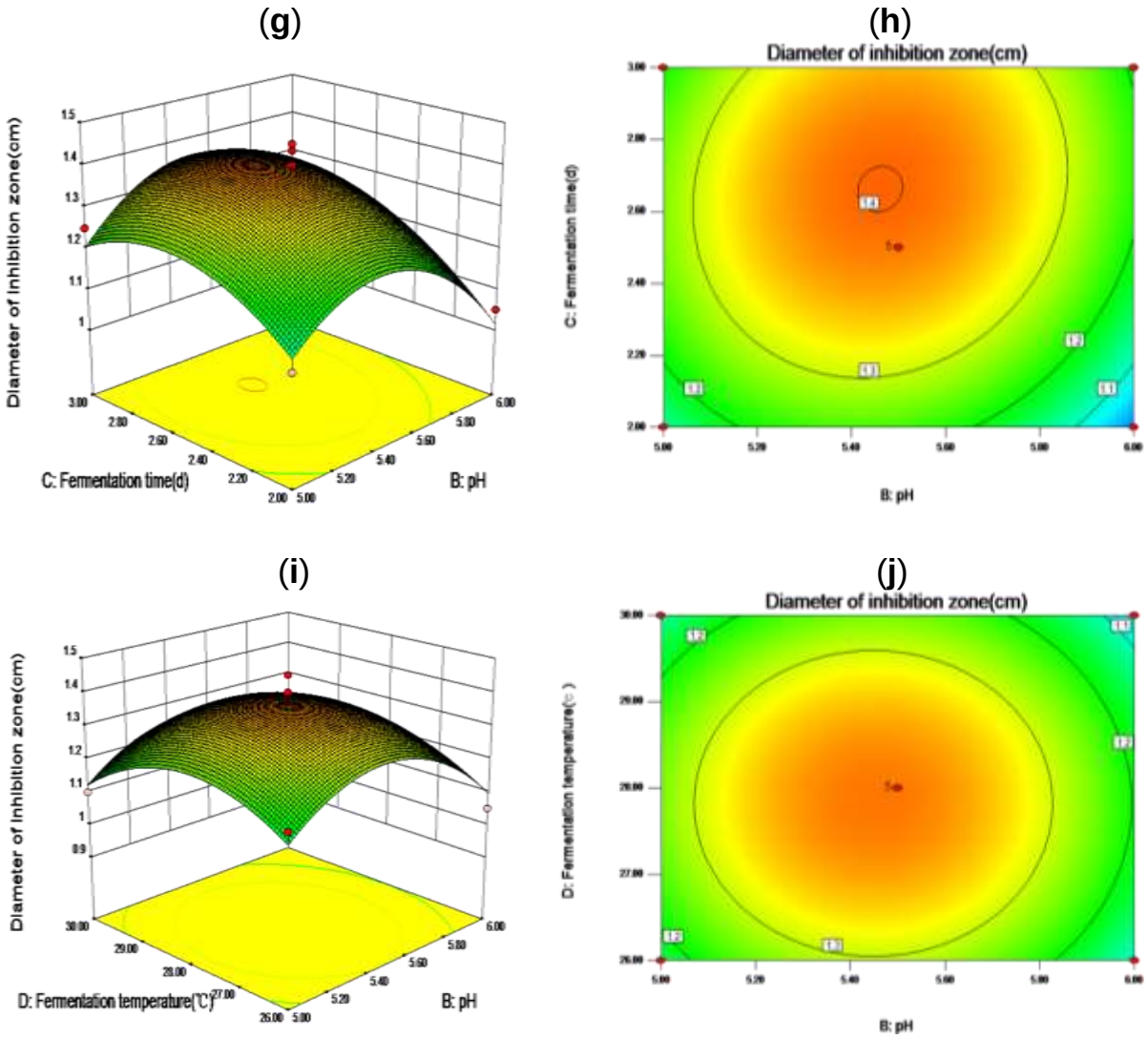


Figure 6. The three-dimensional surface map and contour map of interactions between two variables on killer toxin production by yeast strains.



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interaction of pH and fermentation time significantly influenced the diameter of the inhibition zone (Figure 6g). The inhibition zone diameter was found to decrease linearly with the increase in the fermentation temperature above 27.5°C and pH above 5.5 (Figure 6i), consistent with the results obtained for *Kloeckera apiculata* KY-13c with optimal growth condition at pH 5.5 (Wu et al., 2015). Thus, high pH and fermentation temperature inhibited killer toxin activity. However, the DD21-2 strain could exert killer effects in a wider range of fermentation conditions compared with some other marine yeast, such as *Williopsis saturnus* WC91-2, which showed optimum toxin production at pH 3 to 3.5 and 16°C (Wang et al., 2012). Furthermore, fermentation time and temperature also had a significant effect on DD21-2 killer activity; the activity first increased and then decreased (Figure 6k), which was similar to that observed for *Saccharomyces cerevisiae* (Zhou et al., 2014). Thus, the interaction among the analysed factors significantly affected the inhibition-zone diameter, that is, killer toxin production.

Optimization of killer activity

Killer toxin production by *M. saccharicola* DD21-2 was optimized with RSM based on the four-factor, three-level BBD method. The results indicate that the four factors were significant in affecting killer activity, and the best fermentation conditions were determined for killer toxin production: Inoculum concentration 4.1% (v/v), pH 5.5, fermentation temperature 28°C, and fermentation time 2.7 days. These conditions were verified experimentally, and the diameters of the obtained triplicate inhibition zones were 1.45, 1.42 and 1.45 cm, which were very

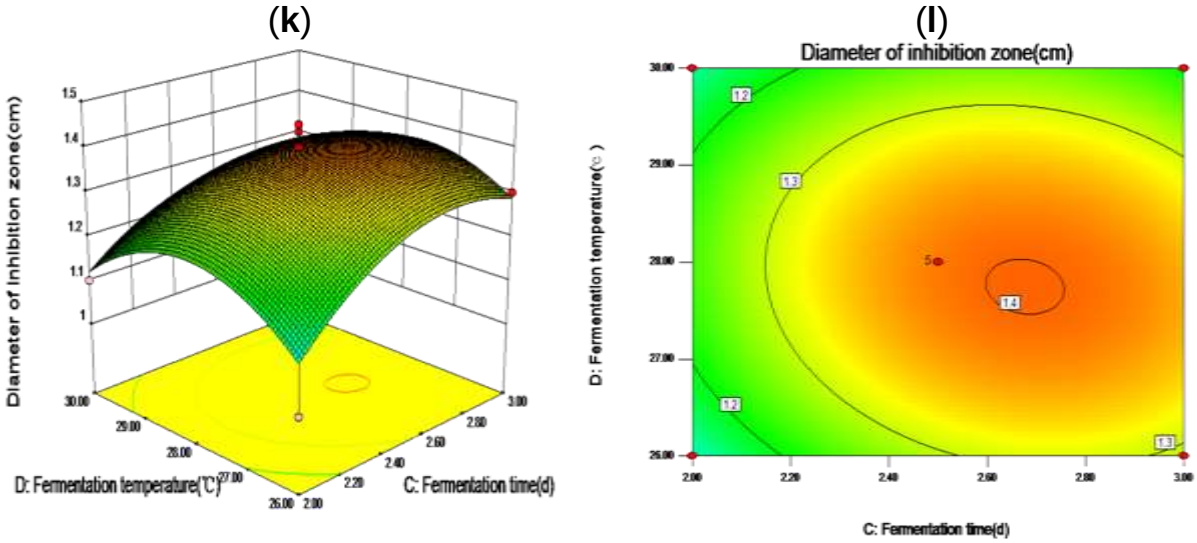

Figure 6. Contd.

close to the predicted value of 1.41 cm. The error between the predicted and experimental values (2.84%) was within the 5% level of significance, indicating that the model is acceptable and the response surface optimization of fermentation conditions is effective. Thus, higher killer toxin yields can be achieved by the aforementioned methodology and the study suggests practical directions for further research in purification and characterization, and finally provides a basis for disease prevention and control in marine aquaculture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This research was financially supported by the Marine Fishery Science and Technology and Industry Development Special Project of Guangdong Province (A201501C13). Technology and equipment were provided by the Shandong International Biological Science and Technology Park. The authors would like to thank Professor Zhenming Chi, who provided the conditional pathogenic yeast strain, *Metschnikowia bicuspidata* WCY.

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