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Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi

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Abstract
The study investigated the effects of salinity on growth, antimicrobial activities and secondary metabolites of 47 marine filamentous fungi isolated from the East China Sea near the western shore of the Taiwan Straits. The results indicate that NaCl promoted the growth up to 91.5% of test strains. However, only 14.9% of them showed a significant increase of antimicrobial activity against *Candida albicans*. When incubated in different concentrations of NaCl, the colony growth, antimicrobial activities and composition of secondary metabolites of the strain Ty01b-8 of *Penicillium* sp. varied. Treatment with KCl also showed a similar effect. An alkaloid isolated from the fermentation broth of Ty01b-8 was identified as chrysogine, inhibition activity of which against Hela cells was 15.05% at 20 μg/ml, and yield was 4.4 and 4.9 times higher in 3 percent and 6 percent NaCl treatments, respectively, compared with the non-salt culture condition. These findings prove that salinity is an important factor influencing growth and secondary metabolites of some marine fungi, which can be used to screen for new metabolites from marine fungi, and to enhance their metabolites production in industrial fermentation.

Key words: marine fungi, salinity, secondary metabolites, antimicrobial activities

1 Introduction

The sea is a unique living environment of high salinity, high pressure, low temperature and limited nutrition. To adapt to the environment, marine microorganisms have evolved a distinct metabolism different from the terrestrial microorganisms. In the last twenty years, a large number of bioactive compounds have been found from marine microorganisms. More than 270 and 300 new compounds were isolated from marine fungi and marine cyanobacteria, respectively (Tan, 2007; Bugni and Ireland, 2004), showing that marine microorganisms are a prolific source of natural compounds. Meanwhile, how the metabolism of marine microorganisms responds to salinity environments have received increased attention in recent years. Two New compounds, thiocoraline and salinosporamide, as antitumor agents have been already under the clinical and preclinical trials, respectively (Chauhan et al., 2006; Newman and Cragg, 2004). Producer of them have been proved belonging to obligate marine actinomycetes.

Only limited publications dealt with the effects of salinity on growth and secondary metabolites of marine fungi. Bugni and Ireland (2004) compared the antimicrobial activities and metabolic products of marine fungi in 0%–100% artificial sea water. Yang et al. (2007) found that salinity improved the growth and compound production of marine fungus UST030110-009 strain. Dela Cruz et al. (2006) proved that there were no significant correlations between the origination of marine fungi *Dendryphiella* spp. and their responses to the investigated salt concentrations. These reports promoted our further study on the impact of salts on marine fungi isolated from the sea near western shore of the Taiwan straits.

In the present study, 47 antimicrobial strains of marine fungi, isolated from the sea near western shore of the Taiwan straits, were used to investigate the impact of salts on the growth, secondary metabolism and antimicrobial activities of marine fungi.

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2 Materials and methods

2.1 *Survey and identification of strains*

Twenty-nine samples, including three soil samples from Brine Pan, seven mangrove plant samples, six seaweed samples, and thirteen sediment samples, were collected from Xiamen and the Jinjiang Sea area near the western shore of the Taiwan Straits. Three hundred and ninety filamentous fungal strains were isolated from the samples using potato dextrose agar (PDA) medium supplemented with 6% (w/v) NaCl. Antimicrobial activity against *Candida albicans* as 2.538 was tested using the double-layer method (Hentschel et al., 2001). Forty-seven strains displaying antimicrobial activities were obtained and used in the experiments. Based on morphology identification (Barnett and Hunter, 1977), these strains were assigned to *Penicillum* (32 strains), *Aspergillus* (10 strains), *Mycellia sterilia* (3 strains), *Fusarium* (1 strain), *Paecilomyces* (1strain), respectively.

2.2 *Effects of NaCl on growth of marine fungi*

The optimal growth of marine fungi were determined at four salt levels (0%, 3%, 6% and 9% NaCl), respectively. Two different methods were used for sporulating and non-sporulating fungi. In case of sporulating fungi, the conidia of test strains were diluted with distilled water to a concentration of 10^{5-6} conidia per millilitre. Then, 2 μl of the suspensions was spot-inoculated in dishes containing 15 ml modified Czapek's medium with 0%, 3%, 6% and 9% NaCl, respectively. The composition and the preparation of the medium are as follows: NH_4NO_3(AR), 0.71 g; $(NH_4)H_2PO_4$(AR), 0.66 g; $MgSO_4$(AR), 0.5 g; $FeSO_4$ (AR), 0.01 g; sucrose(AR), 30 g; agar (purity 99.9%) 20 g, the volume is top to 1 000 ml by distilled water, and the pH value is adjusted to 6.8 by ammonia. In case of non-sporulating fungi, agar with fresh mycelia of test strains were cut into small chunks with a diameter of 0.5 cm, and then inoculated on the medium mentioned above. After 5 d incubation at 28 °C (7 d for the strains growing slowly), the diameter of colony was measured. For non-sporulating fungi, the longest and shortest diameters of colony were measured and the mean was recorded as the size of the colony. Six replicates were performend for each strain.

To evaluate the effect of habitats on the adaptation of marine fungi to salinity, colony diameters were treated by standardizing method. Standardized colony diameter (D_s) was calculated by the following equation: $D_s = (D_x - D_{min})/(D_{max} - D_{min}) \times [(D_{max} + D_{min})/2]$, where D_x was the colony diameter of strain at each NaCl concentration tested; D_{max} and D_{min} were the maximum and minimum colony diameters of strain among four NaCl concentrations tested.

2.3 *Effects of NaCl on antimicrobial activities*

The antimicrobial activities against *C. albicans* were determined by double-layer method (Hentschel et al., 2001). The strains tested were inoculated on modified Czapek's agar plate with 0%, 3%, 6% and 9% NaCl. After 7 d incubation at 28 °C, the conidia of the fungi were killed and scraped off with a cotton swab containing 75% ethanol. Then, plugs of 0.7 cm in diameter were stanced out with a corkborer and placed on agar seeded with the indicator *C. albicans* As 2.538. Following 48 h incubation at 28 °C, the plates were inspected for the formation of inhibition zones around the agar plug and the diameter of the inhibition zones was recorded. Five replicates were performed for each strain.

2.4 *The effects of NaCl on metabolite composition*

The strains tested were cultured in modified Czapek's liquid medium with 0%, 3%, 6% and 9% NaCl in a rotary shaker (150 r/min) at 28 °C. After 10 d, the mycelia were separated by filtration, and 100 ml filtrate were precisely measured and extracted with an equal volume of ethyl acetate (purity>99.5%) in a rotary shaker with 180 r/min. The extraction was performed twice (12 h each time). The combined organic solution was concentrated under vacuum at 45 °C to produce a crude extract, which was dissolved with methanol to a final volume of 10 ml. A 1100 series HPLC from Agilent Technologies (Palo Alto, CA) with UV detection was used for the separation of the crude extract (HPLC column: Merck RP18, 5 μm particle size, 250 mm×4.6 mm dimensions; mobile phases: 25% methanol). All the solvents used were high-quality HPLC grade solvents. The flow rate of mobile phase was set at 1.0 ml/min. Ten microlitres injections were performed and the column temperature was maintained at 26 °C. Three replicates were performend for each strain.

2.5 *Isolation of compound H1 from strain Penicillium Ty01b-8*

Penicillium Ty01b-8 was cultured in modified Czapek's liquid medium with 3% NaCl in a rotary shaker with 150 r/min at 28 °C. After 10 d, the mycelia

were separated by filtration, and the filtrate was extracted twice (12 h each time) with an equal volume of ethyl acetate. The combined organic solution was concentrated under vacuum at 45 °C to produce a crude extract. The extract was fractionated by medium-pressure liquid chromatography (MPLC) RP-18 column chromatography, Sephadex LH-20 column chromatography and silica gel column. A colorless crystal was obtained and named H1, which was analyzed by NMR (600M, Bruker).

2.6 *Cytotoxic and anti-microbes activities of the compound H1*

The cytotoxicity of the compound H1 on Hela and HepG2 cells were detected by the MTT method (Mosmann, 1983). Growth inhibition rate was calculated as described by Lin et al. (2005). Cisplatin (Jinzhou Pharmaceutical Factory, China) was used as positive control.

Antimicrobial activities of H1 were determined by the double-layer method (Hentschel et al., 2001) with indicator organisms including *Bacillus subtilis* ATCC 9372, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *C. albicans* As 2.538 and *Aspergillus niger* ACCC 30005. Each test was performed in triplicate.

2.7 *Statistical analysis*

The comparison of the colony growth and the antimicrobial activities at different NaCl concentrations were performed using t-test. The results were expressed as means ±SD and analyzed using SPSS statistical software. P values less than 0.05 were considered significant.

3 Results

3.1 *NaCl affected the growth of marine fungi*

The growth of all 47 test strains was affected by different NaCl concentrations and displayed three types of growth characteristics. Four strains whose colony was the largest in the medium without NaCl, were grouped into type A, representing 8.5% of all tested strains; 32 strains (68.1%) were grouped into type B, having the largest colony diameter in 3% NaCl. Eleven strains (23.4%) were grouped into type C. They had the largest colony diameter in 6% NaCl. Forty-three strains in total (91.5%) showed the optimal growth in 3% or 6% NaCl. Among them, 38 strains (80.9%) displayed significant differences compared with the non-salt condition ($P<0.05$ or $P<0.01$).

The habitats give a significant effect on the adaptation of marine fungi to salinity. Table 1 shows the strain numbers for optimal growth at different salt concentrations. There were 8 (50.0%), 14 (77.8%), 5 (71.4%) and 5 (83.3%) isolates derived from Brine Pans, sediments, seaweeds and mangrove plants with an optimal growth at 3% NaCl, respectively. However, except the strains derived from Brine Pans, whose proportion for optimal growth was 43.8% at 6% NaCl, most of the strains derived from three other sites were striking inhibited when NaCl concentration increased from 3% to 6%. We also compared the colony size at various salt concentrations (Table 2). The results show that the strains derived from Brine Pans had a largest colony diameter of 1.69–1.63 cm at NaCl concentrations between 3%–6%, whereas the colony diameters for the strains from three other sites significantly reduced when NaCl concentrations increased from 3% to 6%.

Table 1. The numbers of marine fungi for optimal growth at different concentrations of NaCl

Habitat	Numbers of strain			
	0%	3%	6%	9%
Brine pans	1(6.3)	8(50.0)	7(43.8)	0(0.0)
Sediments	2(11.1)	14(77.8)	2(11.1)	0(0.0)
Seaweeds	1(14.3)	5(71.4)	1(14.3)	0(0.0)
Mangrove plants	0(0.0)	5(83.3)	1(16.7)	0(0.0)

Table 2. The colony diameters of marine fungi grown at different concentrations of NaCl

Habitat	Standardized colony diameter/cm[1)]			
	0%	3%	6%	9%
Brine pans	0.91	1.69	1.63	0.08
Sediments	0.92	1.99	1.38	0.20
Seaweeds	0.85	1.97	1.70	0.18
Mangrove plants	1.23	2.40	1.66	0.00
Average	0.95	1.94	1.55	0.13

Note: [1)]The colony diameters were treated by the standardizing method described in the text.

3.2 *NaCl affected the antimicrobial activities of marine fungi*

The antimicrobial activities of 29 strains (61.7%) were significantly higher in the medium without NaCl (defined type I, Table 3) while only seven strains (14.9%) showed higher activities in the medium supplemented with NaCl (defined type II). Eleven strains did not show statistically significant difference between the medium without NaCl and with 3% NaCl

Table 3. The inhibition activities of marine fungi against *C. albicans* at different concentrations of NaCl[1)]

Strain No.	Diameter of inhibition zone/cm				Type[2)]
	0%NaCl	3%NaCl	6%NaCl	9%NaCl	
Ty01a-3	1.23(±0.054 8)	1.63**(±0.173 2)	2.10**(±0.083 7)	2.07**(±0.141 4)	II
Ty01b-8	1.04(±0.181 7)	1.22(±0.192 4)	1.42**(±0.192 4)	2.50**(±0.255 0)	II
JSSb-4	1.56(±0.336 2)	1.96**(±0.151 7)	2.52**(±0.130 4)	2.20**(±0.200 0)	II
ZH02b-1	1.06(±0.054 8)	1.28**(±0.130 4)	0.94*(±0.0548)	0.80**(±0.000 0)	II
Ty01b-3	2.08(±0.148 3)	2.00(±0.308 2)	2.54**(±0.194 9)	2.22(±0.109 5)	II
ZH03a-1	0.87(±0.115 5)	0.80(±0.000 0)	1.40**(±0.141 4)	0.95(±0.070 7)	II
ZL02b-6	1.54(±0.230 2)	2.34**(±0.151 7)	1.16**(±0.134 2)	1.27**(±0.251 7)	II
Ty01a-7	3.28(±0.263 0)	3.13(±0.386 2)	2.90(±0.216 0)	2.46**(±0.114 0)	III
Ty01b-12	2.60(±0.100 0)	2.54(±0.114 0)	2.62(±0.238 7)	0.98**(±0.109 5)	III
ZH03a-5	1.08(±0.083 7)	1.08(±0.083 7)	1.12(±0.083 7)	0.92(±0.130 4)	III
ZL01b-17	4.08(±0.125 8)	3.98(±0.125 8)	3.78(±0.464 6)	3.15**(±0.251 7)	III
HQGb-4f	0.83(±0.057 7)	0.90(±0.100 0)	1.00(±0.122 5)	–	III
ZH02b-4	0.96(±0.089 4)	0.88(±0.083 7)	0.84*(±0.054 8)	0.90(±0.100 0)	III
Tda-5	2.48(±0.258 8)	2.42(±0.216 8)	1.50**(±0.070 7)	1.00**(±0.100 0)	III
Ty01a-9	1.38(±0.216 8)	1.22(±0.083 7)	1.38(±0.258 8)	1.32(±0.164 3)	III
Ty02b-1	0.96(±0.054 8)	0.93(±0.095 7)	0.90(±0.081 7)	0.82(±0.044 7)	III
ZH07a-4	1.06(±0.089 4)	1.06(±0.054 7)	0.96(±0.089 4)	1.00(±0.000 0)	III
ZL02b-4	3.15(±0.129 1)	2.95(±0.173 2)	1.70**(±0.336 7)	1.30**(±0.264 6)	III
Ty01a-4	3.10(±0.100 0)	2.76**(±0.114 0)	2.26**(±0.151 7)	1.82**(±0.327 1)	I
Ty03b-7	2.88(±0.150 0)	2.50**(±0.081 7)	2.48**(±0.206 2)	1.78**(±0.148 3)	I
Ty03b-13	2.60(±0.122 5)	2.40*(±0.200 0)	2.50(±0.070 7)	1.20**(±0.081 7)	I
ZL01a-9	2.75(±0.129 1)	2.38**(±0.130 4)	1.74**(±0.181 7)	–	I
ZL02b-9	2.84(±0.181 7)	2.56**(±0.403 7)	2.10**(±0.100 0)	1.14**(±0.260 8)	I
hy01a-3	2.52(±0.044 7)	2.32**(±0.083 7)	1.35**(±0.495 0)	–	I
HSGb-2	3.00(±0.158 1)	2.44**(±0.181 7)	2.28**(±0.216 8)	2.22**(±0.216 8)	I
ZH11a-4	3.60(±0.081 7)	3.38*(±0.095 7)	3.02**(±0.130 4)	2.32**(±0.130 4)	I
ZH03b-3	3.15(±0.129 1)	2.60**(±0.070 7)	2.46**(±0.089 4)	2.46**(±0.167 3)	I
Ty01a-10	3.43(±0.095 7)	1.94**(±0.089 4)	1.90**(±0.346 4)	1.92**(±0.258 8)	I
Ty01b-6	1.34(±0.114 0)	1.08**(±0.083 7)	1.02**(±0.148 3)	1.28(±0.130 4)	I
ZL02b-11	2.74(±0.114 0)	2.44**(±0.134 2)	2.34**(±0.114 0)	1.30**(±0.282 8)	I
ZL03b-12	3.28(±0.221 7)	2.48**(±0.083 7)	2.32**(±0.258 8)	2.30**(±0.200 0)	I
Ty01a-6	3.13(±0.050 0)	2.78**(±0.083 7)	2.70**(±0.122 5)	2.52**(±0.148 3)	I
hy01b-2	2.58(±0.083 7)	2.28**(±0.083 7)	2.14**(±0.054 8)	1.60**(±0.141 4)	I
Tda-1	3.43(±0.095 7)	2.92**(±0.083 7)	2.82**(±0.109 5)	1.40**(±0.000 0)	I
Tja-1	3.30±(0.081 7)	2.94**(±0.114 0)	2.64**(±0.260 8)	2.28**(±0.083 7)	I
Tja-4	2.44±(0.151 7)	1.80**(±0.244 9)	1.20**(±0.100 0)	0.83**(±0.050 0)	I
ZH11a-1	3.73±(0.095 7)	3.40**(±0.141 4)	3.00**(±0.081 7)	2.73**(±0.050 0)	I
Tdb-9	3.30±(0.081 7)	2.98**(±0.083 7)	2.60**(±0.100 0)	0.80**(±0.000 0)	I
Tjb-3	3.05±(0.129 1)	1.70**(±0.308 2)	1.02**(±0.083 7)	0.88**(±0.050 0)	I
ZH03b-4	3.48±(0.095 7)	2.88**(±0.216 8)	2.80**(±0.255 0)	2.78**(±0.263 0)	I
ZH05b-2	3.30±(0.141 4)	2.90**(±0.173 2)	2.62**(±0.130 4)	1.32**(±0.148 3)	I
ZH06b-2	3.00±(0.070 7)	2.68**(±0.130 4)	2.64**(±0.181 7)	2.28**(±0.083 7)	I
ZH08b-7	3.43±(0.150 0)	2.70**(±0.081 7)	2.68**(±0.083 7)	1.38**(±0.130 4)	I
Ty02a-2	3.10±(0.163 3)	1.56**(±0.357 8)	1.78**(±0.719 0)	1.50**(±0.244 9)	I
Ty02b-8	1.36±(0.114 0)	1.22*(±0.044 7)	1.02**(±0.044 7)	–	I
Ty03b-12	3.02±(0.216 8)	1.30**(±0.158 1)	0.80**(±0.000 0)	0.80**(±0.000 0)	I
HQGb-1	2.60±(0.070 7)	1.92**(±0.277 5)	1.64**(±0.305 0)	1.04**(±0.151 7)	I

Notes: [1)]Compared with the non-salt culture condition (* means $P<0.05$ and ** $P<0.01$). [2)]I represents higher antimicrobial activity in medium without NaCl, II higher antimicrobial activity in medium with NaCl, III antimicrobial activity no significant difference in medium without NaCl and with 3% NaCl.

(defined type III). Among the seven strains whose activities were promoted by NaCl (Ty01a-3, Ty01b-3, Ty01b-8, ZL02b-6, JSSb-4, ZH03a-1 and ZH02b-1), only ZH02b-1, JSSb-4, Ty01b-3 and Ty01b-8 grew better in saline medium. But surprisingly, the Ty01a-3 strain isolated from Brine Pan grew best in a non-salt medium while its antimicrobial activity reached the peak in the medium with 6%–9% NaCl ($P<0.01$).

3.3 *NaCl affected the metabolite composition of marine fungi*

The composition of second metabolites treated with different NaCl concentrations were compared by HPLC for those seven strains whose antimicro-

bial activity was elevated by NaCl. Both the types and amount of metabolites were affected by different concentrations of salt, especially the strain Ty01b-8, ZL02b-6 and JSSb-4. For example, three new HPLC peaks of retention times at 12 min, 14 min and 17 min appeared in the extracts from the strain Ty01b-8 when cultured in 3% NaCl while two new peaks at approximately 14 min and 17 min appeared when cultured in 6% NaCl (details not shown). Besides, the peak area at retention times 16 min also increased significantly with increasing salt concentration. The peak area was 4.4 times and 4.9 times in 3% and 6% NaCl concentrations compared with the non-salt medium, respectively.

3.4 *KCl affected the growth and antimicrobial activities of Ty01b-8*

Different concentrations of KCl in modified Czapek's medium promoted the growth and the production of antimicrobial substances of the Ty01b-8 strain. The colony growth and the antimicrobial activities with increasing KCl concentrations displayed the same trends as under NaCl influence (Fig. 1). However, the composition of metabolites obviously decreased in the medium with KCl, especially with 9% KCl (details not shown).

3.5 *The isolation and identification of compound H1 from Ty01b-8*

Based on the HPLC analysis of Ty01b-8, with increasing NaCl concentration, the peak area of P16 (retention time of 16 min) increased significantly. A crystal compound H1 was obtained by isolating and purifying the fermentation broth of this strain when growing in a modified Czapek's liquid medium with 3% NaCl. This compound was identified as chrysogine by NMR. In the same HPLC condition, the retention time of component H1 and the P16 from the fermentation broth of Ty01b-8 cultured in 3% NaCl was similar. The peak area of P16 expanded proportionally when H1 was added into the ferment extracts. A new HPLC peak was absent, proving the P16 peak of the fermentation broth being composed of H1. As compared with the non-salt medium, the amount of this compound was 4.4 times and 4.9 times higher in 3% and 6% NaCl respectively. The inhibition rate of H1 against Hela cell was 15.05% at 20 μg/ml, but inhibition activities against HepG2 cells and the other indicator organisms were not observed.

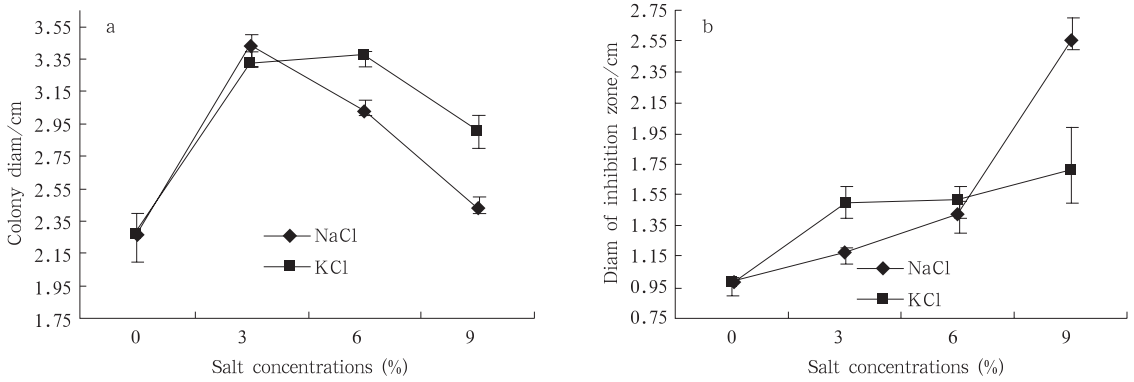

Fig.1. The effects of NaCl and KCl on the growth and antimicrobial activity of the strain Ty01b-8. a. Growth, and b. antimicrobial activity.

4 Discussion

The present study shows a remarkable impact of salt on the growth of marine fungi. Among the 47 strains tested, 91.5% grew better at 3% or 6% NaCl than in the medium without NaCl. The result that NaCl can promote growth of most marine fungi supports the conclusion of Masuma et al. (2001) who proved that marine fungi grew more abundantly as the seawater concentration increased. Masuma et al. also found that the marine fungi grew rapidly in the medium containing 4% NaCl and the growth of terrestrial strains was suppressed by 4% NaCl. The characteristic that marine fungi can tolerance higher salinity than that of their habitats, was also reported by other authors (Cantrell et al., 2006; Dela Cruz et al., 2006). In the study, we used the medium containing 6% NaCl to isolate marine fungi to inhibit growth of terrestrial strains. However, all of 47 strains tested were still able to grow in the medium without NaCl, suggesting that

all of the isolates belong to facultative marine fungi (Kohlmeyer and Kohlmeyer, 1979), which may have been washed from the beaches into the sea, displaying the characteristics of halotolerant fungi after a long-term evolution which helped them to adapt the new salty environment.

It is still unclear why marine fungi have different degree of tolerances to salt. Dela Cruz et al. (2006) considered that there were no significant correlations between geographical locations of marine *Dendryphiella* species and their responses to salt. Cantrell et al. (2006) found that the marine fungi with dark cell wall can tolerate higher salinity than the moniliaceous fungi. The present results show that the habitats of marine fungi had a strong influence on their adaptation to salt. Forty-three point eight percent of the isolates collected from Brine Pan can obtain optimal growth at the concentration of 6% NaCl, showing better adaptation to salt than the strains from the other sites. The standardizing analysis of colony diameters also showed that their fastest growth took place at NaCl concentrations between 3%–6%, having the largest colony diameters of 1.69–1.63 cm. Although most strains from other habitats can also grow fastest at 3% NaCl, but their colony diameters outstanding reduced in the medium containing 6% NaCl. We speculate that the differences may be related to the salinity of surrounding habitats, but further investigation will be necessary to confirm this.

The HPLC analysis of the metabolites from seven marine fungi in different NaCl concentrations showed that the changes in HPLC peak area may be observed, indicating that NaCl is able to affect the amount of metabolites. The peak area of P16 of the strain Ty01b-8 increased 4.4 times and 4.9 times in 3% and 6% NaCl, respectively, compared with the non-salt condition. NaCl also promoted the synthesis of new compounds. New HPLC peaks appeared at the retention time of 14 and 17 min when Ty01b-8 cultured in 3% and 6% NaCl. Therefore, new metabolites or substances with new bioactivities may be obtained by regulating salt concentrations in fermentation of secondary metabolites of some marine fungi.

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