

Regulation and Activity Evaluation of Secondary Metabolism of an Oyster Symbiotic Fungus *Schizophyllum* sp. YS-08

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Abstract: Marine microorganisms are the important resources for natural drug discovery. However, most of genes are usually in silent and could not express under the condition of traditional culture, which limits the discovery of lead compounds. In the present research, marine fungus *Schizophyllum* sp. YS-08 was selected as the research object, which was from Yellow Sea of China, to activate its silent genes by changing the medium of nutrients and environmental conditions. The crude extracts from metabolites of marine fungi were analyzed by HPLC, and their antioxidant activity and acetylcholinesterase (AChE) inhibitory activity were studied by DPPH radical-scavenging and Ellman's method, respectively. The results indicated that metabolic pathway of fungus was regulated effectively in different culture conditions, especially in the normal sea water medium with peptone. The antioxidant activity and AChE inhibitory activity increased significantly in comparison with wild strain and increase of the quantity of chemical constituents were even more. It is also observed that the strains showed relatively slow growth in the high salinity situation, while this adversely environmental condition could promote and produce more active metabolites. Furthermore, 6 mutant strains were obtained under salt stress and were identified. Interestingly, we found that all mutant strains had potency toward antioxidant and AChE inhibitory activity. Among them, YS-08-2 was the most potent, scavenging more than 56.02% towards DPPH free radicals, and YS-08-1, YS-08-4 and YS-08-5 exhibited more than 40% inhibition against AChE. HPLC analysis of extracts showed the metabolites of most fungi were more abundant after regulation of culture conditions.

Keywords: Symbiotic Fungi, OSMAC Strategy, Secondary Metabolites, Antioxidant Activity, AChE Inhibitory Activity

1. Introduction

Secondary metabolites of marine microorganisms provide the most important sources of lead compounds in drug discovery. Because of the special environment for the growth of microorganisms, their secondary metabolites are usually very special and these compounds have high structural complexity and diversity [1-3]. However, currently, problems also existed in marine microorganisms such as few species, high repetition rate, single metabolites, etc, thus the development and utilization of marine natural products were limited under laboratory conditions [4-6]. Recently, research

has found that there were still many silent genes existing in biosynthetic pathway of microorganisms and the activation of silenced genes again could enhance metabolites production or synthesis of novel compounds, which may provide new opportunity to the development of marine microorganism drugs [7-12].

One strain many compounds (OSMAC) strategy has been proposed recently, which become a convenient and effective method to investigate and explore marine microorganism natural products. The strategy is to activate the expression of silent genes in microorganisms by the change the composition of culture medium or culture conditions, so as to

enhance the ability of microorganisms to synthesize secondary metabolites and then obtain more novel bioactive compounds [14-16]. At present a large number of researches indicated that OSMAC strategy has been successfully applied to induction and optimization of secondary metabolites of marine microorganisms, especially fungi, because this method is simple, convenient and practicable [17-24]. In the present work, symbiotic fungus (*Schizophyllum* sp. YS-08) in wild oysters from Yellow Sea of China was used as the research object. To activate its silent metabolic pathway, the culture conditions and fermentation conditions were optimized by changing the medium of nutrients and environment. Moreover, the crude extracts from metabolites of endophytic fungi were analyzed by HPLC, and then their antioxidant activity and AChE inhibitory activity were evaluated by DPPH radical scavenging assay and Ellman's method, respectively. The purpose of this study is to provide a theoretical basis for the development of secondary metabolites of marine fungi.

2. Materials and Methods

2.1. Materials

Vitamin B1, Vitamin B2, Vitamin B3, Vitamin B5, Vitamin B6, Inositol, 4-aminobenzoic acid, Vitamin H, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), Acetylthiocholine iodide (ATCI), Acetyl cholinesterase (AChE), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1,1-diphenyl-2-picrylhydrazyl

(DPPH), Ascorbic Acid, Glucose were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Peptone and Agar powder were from Beijing Laboratory Biology Technology Co., Ltd (Beijing, China). MNIQ-10 Column Fungi Genomic DNA Purification Kit was purchased from Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China). All other chemical agents were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Fungal Materials

Schizophyllum sp. YS-08 was isolated from wild oysters in Yellow Sea of China (longitude 119.45, latitude 35.42) and stocked at School of Pharmacy, Jining Medical University, Rizhao, China.

2.3. Regulation of Secondary Metabolism of Oyster Symbiotic Fungus YS-08

The effects on the secondary metabolism of fungus YS-08 with different growth factors were investigated. Oyster symbiotic fungus YS-08 was cultured in potato dextrose agar (PDA) medium, supplemented with peptone, multi-vitamins or Fe^{2+} , respectively, as described in Table 1. The morphology and color of symbiotic fungi were observed after 5-7 days incubated at 28°C [25]. Next, we studied the effect on the secondary metabolism of fungus YS-08 with different salinities. The fungus YS-08 was incubated in the medium with different concentrations of sea salt (as described in Table 2), and cultured for 5-7 days.

Table 1. The fungus medium with different growth factors (seawater, 100 mL).

No.	Glucose (g)	Agar (g)	Peptone (g)	Multi-vitamins (mL)	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg)
1-1	2	1.5	-	-	-
1-2	2	1.5	-	1	-
1-3	2	1.5	-	-	5
1-4	2	1.5	-	1	5
1-5	2	1.5	1	-	-
1-6	2	1.5	1	1	-
1-7	2	1.5	1	-	5
1-8	4	1.5	-	-	-
1-9	4	1.5	-	1	-
1-10	4	1.5	-	-	5

Table 2. The fungus medium with different salinities (seawater, 100 mL).

No.	Glucose (g)	Agar (g)	Peptone (g)	Multi-vitamins (mL)	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg)	Sea salt (g)
2-1-1X	2	1.5	-	-	-	3
2-1-2X	2	1.5	-	-	-	6
2-2-1X	2	1.5	-	1	-	3
2-2-2X	2	1.5	-	1	-	6
2-3-1X	2	1.5	-	-	5	3
2-3-2X	2	1.5	-	-	5	6
2-4-1X	2	1.5	1	-	-	3
2-4-2X	2	1.5	1	-	-	6

2.4. Stability Study of Mutant Strains

The oyster symbiotic fungi YS-08 was cultured in PDA medium with different growth factors (Table 1) or different salinities (Table 2). The mutant strains were isolated by the streak plate method and cultured in PDA medium. After the

cultivation of three generations, they were very steady and hereditary, indicating that the mutant strains had a good genetic stability.

2.5. Identification of Mutant Strains

The mutant strains were inoculated to the potato dextrose

broth (PDB) medium for spores growing, and the culture was shaken for 5–7 d at 150 r/min. Then the spores were collected and ground to no particles. The genomic DNA was extracted from the spores with MNIQ-10 column fungi genomic DNA purification kit and was further amplified by PCR with fungal universal primers of ITS4 and ITS5. After amplification, agarose gel electrophoresis of the PCR products was performed to make sure the products were the wanted fragments. At the same time, the verified products were submitted to Genewiz Corporation to confirm the sequencing. After the sequencing BLAST in the NCBI database, the phylogenetic tree was constructed based on Neighbor-Joining methods using the MEGA 7.0 software [26].

2.6. Liquid Fermentation

All the fungi were propagated in the potato-dextrose broth (PDB) medium and incubated at 28°C for 7–10 d. Then the precipitate was removed by centrifugation, and the clear supernatant extract was extracted for 3 times with ethyl acetate. The crude extract was obtained after concentrating and stored at 4°C.

2.7. HPLC Analysis of Metabolites

The crude extract was weighed and dissolved in methanol at concentration of 10 mg/mL. The fingerprints of metabolites of symbiotic fungi were determined by high performance liquid chromatography (HPLC, Shimadzu), whose detector is DAD and whose mobile phase was methanol-water solution. HPLC method with gradient elution: 5%–100% methanol (0–40 min), 100% methanol (40–50 min), 100%–5% methanol (50–60 min).

2.8. Bioactivity Screening for Secondary Metabolite

The antioxidant activity of each crude extract was determined by using DPPH free radical scavenging method in vitro. A stock solution (10 mg/mL) of crude extract was prepared in DMSO. To a solution of 100 μ L DPPH (0.4 mmol/L) was added 2 μ L of crude extract, and then 98 μ L 75% ethanol was added in a 96-well plate in sextuplicate, and 2 μ L of Vc was added into positive control group, and 0.2 μ L of DMSO was added into the control group. After 30 min of incubation in the dark at room temperature, the absorbance was recorded at 517 nm on a microplate reader of multi-wavelength measurement system [27]. The DPPH free radical scavenging activity (%) was calculated by the formula $(A_0 - (A_i - A_i'))/A_0 \times 100\%$, where A_0 is the absorbance value of control group, A_i is the absorbance value of test group, and A_i' is the absorbance value of the crude extract itself.

The inhibitory activity of AChE was carried out by Ellman's method. A volume of 25 μ L of AChE solution (0.05 μ L), 16 μ L of DTNB solution (0.0667 mmol/L) and 10 μ L of ATCI solution (0.1 mmol/L) were added to 213.6 μ L of PB buffer solution (pH = 8.0), and then added with 5.4 μ L of each crude extract (10 mg/mL). After incubation of 4 min 20 s at 37°C, the reaction mixture was treated with 10 μ L of SDS solution (4 μ mol/L). The absorbance value of

the reaction mixture was recorded at 412 nm [28]. The AChE inhibitory activities (%) was measured according to the formula $((A_0 - A_1) - (A_i - A_i'))/(A_0 - A_1) \times 100\%$, where A_0 is the absorbance value of control, A_1 is the absorbance value of suppression group, A_i is the absorbance value of test group, and A_i' is the absorbance value of the crude extract itself.

3. Results

3.1. Morphological Analysis of Fungus YS-08 in Different Growth Conditions

To make silence gene of oyster symbiotic fungus express again, and to study the chemical diversity and bioactivity of its secondary metabolites, fungus YS-08 was cultured in PDA medium with different growth factors or different salinities. The strain was analyzed by color and morphological observation. The results showed that there was very little difference of morphological characteristic of strain when different growth factors were added in PDA medium (Figure 1A). However, the strains grew more vigorously and mycelium was dense hairy coronal. The color of strain was obviously deepened when peptone or Fe^{2+} was added. Interestingly, the addition of sea salt in the medium resulted in the inhibition of growth of the fungus, even to the extent, it could led to changes in colony morphology and color (Figure 1B), especially in 2X salinity culture media. Therefore, although growth of fungus was inhibited under the adverse conditions of salt stress, this could results in the appearance of some new mutant strains.

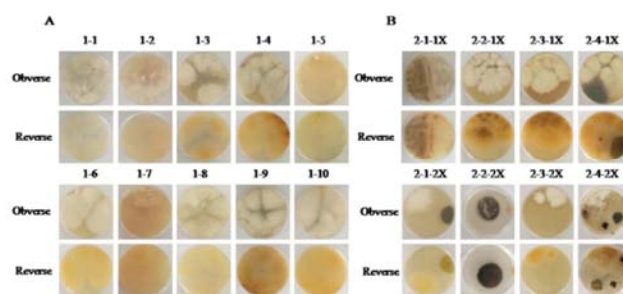


Figure 1. Morphological analysis on fungus YS-08 in different growth factors (A) or different salinities (B).

3.2. HPLC Analysis of Crude Extract of Fungus YS-08 in Different Growth Conditions

The fingerprint of main chemicals in crude extract of fungus YS-08 in different growth conditions was investigated by HPLC. As shown in Figure 2, the main peak (t_R 33 min) of fingerprint of fungus YS-08 increased significantly when cultured in the PDB medium with multi-vitamins and iron ions (1–4), not single multi-vitamins (1–2) or iron ions (1–3). In addition, with the peptone addition, the number of peaks of fungus metabolites increased obviously (1–5, 1–6 and 1–7). Under the control of different salinities, the strains showed relatively slow growth speed and less aerial hyphae. Nevertheless, there were significant differences of the

metabolites compared with cultured in normal medium. Furthermore, when the multi-vitamins (2-2-1X, 2-2-2X) or peptone (2-4-1X, 2-4-2X) was added in the high salinity cultured medium, the metabolites were even more abundant.

Consequently, the concentration of sea salt was too high in the medium was unfavorable to fungal growth, but to stimulate the expression of metabolites by activating silence gene of wild fungus [29].

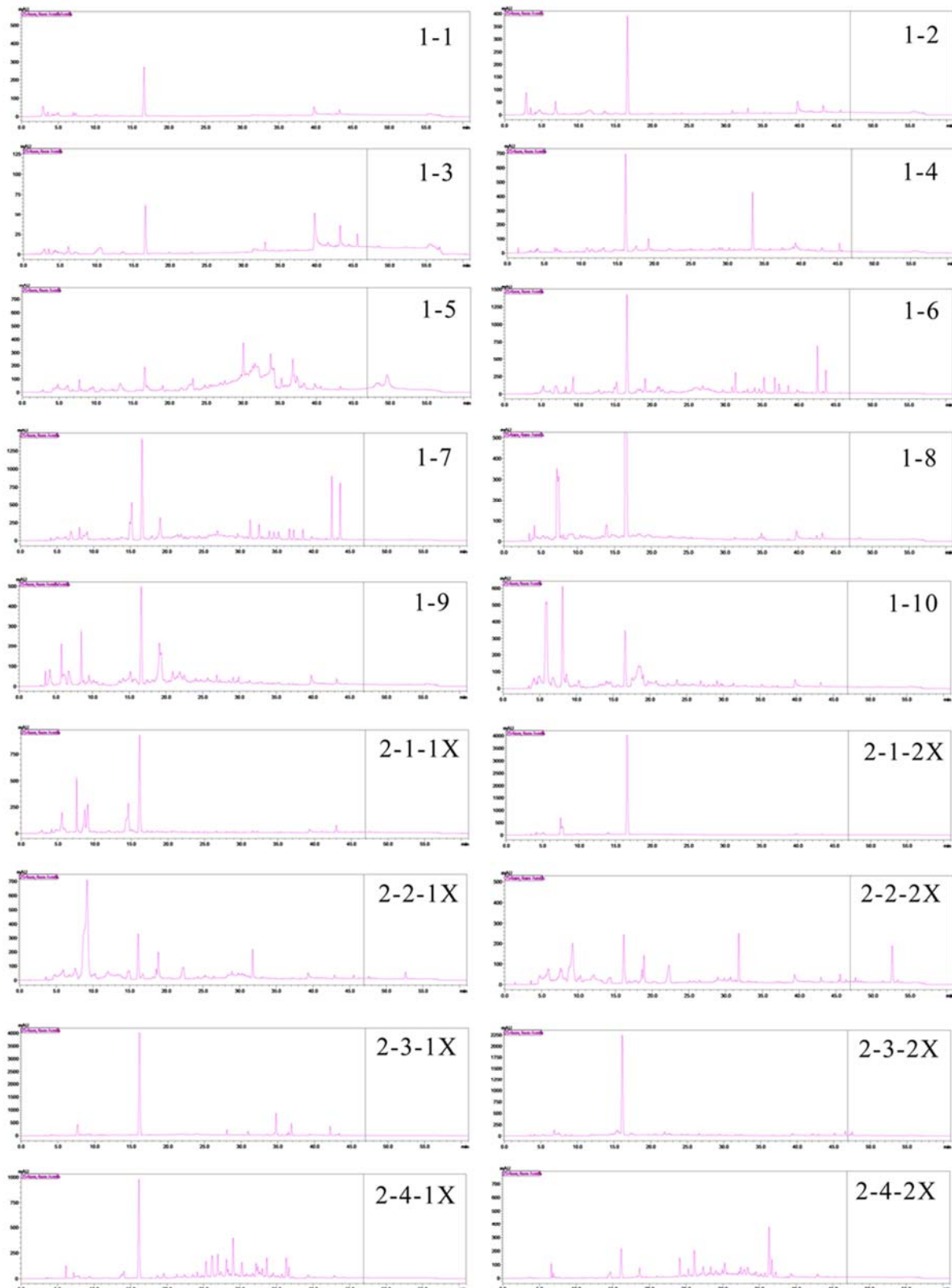


Figure 2. HPLC analysis on metabolites of fungus YS-08 in different growth conditions.

3.3. Biological Activity of Crude Extract of Fungus YS-08 in Different Growth Conditions

The antioxidant effects and AChE inhibitory activities of crude extract of fungus YS-08 in different growth conditions were tested by DPPH radical scavenging assay and Ellman assay *in vitro*. The results indicated that the antioxidant activity of metabolites was significantly increased in the dual

role of multi-vitamins and iron ion, and the scavenging rate was over 18.8% (Figure 3A). What is more, peptone in the culture medium has great effect on metabolites of endophytic fungus, and DPPH free radical scavenging rates were 11.52%, 18.44% and 15.32% (Figure 3A), and the inhibitory rate of AChE were 19.04%, 31.95% and 27.54% (Figure 3B), respectively.

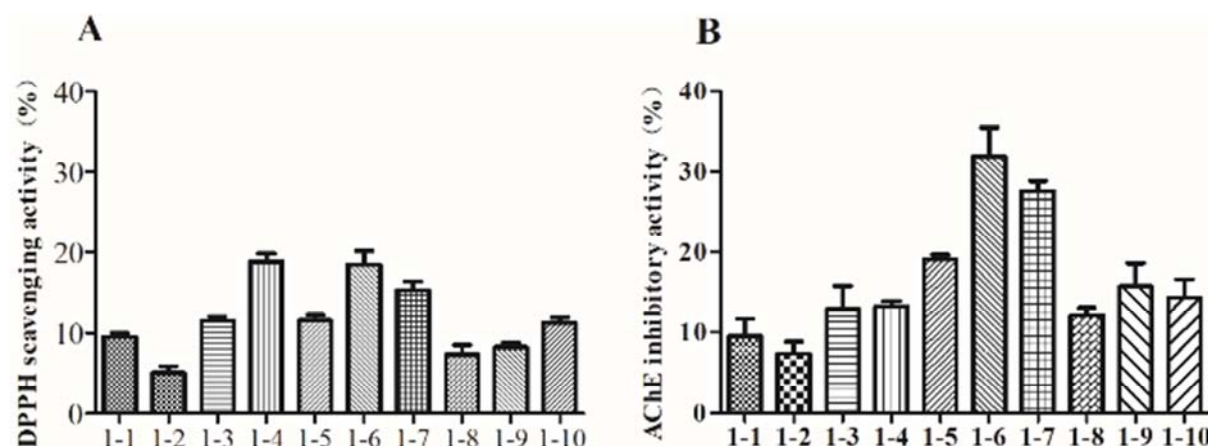


Figure 3. The influence of different growth factors on metabolites of endophytic fungus YS-08. A: DPPH free radical scavenging activity; B: AChE inhibitory activity.

Furthermore, the influence of different salinity conditions on metabolites of endophytic fungi was studied and the antioxidant activity and AChE inhibitory activity were investigated. Under 1X salinity conditions, Fe^{2+} or peptone was added into the medium could promote the activity of metabolites, and the scavenging rates of DPPH free radical were 24.15% and 19.15% (Figure 4A), and the inhibition rate

of AChE activity were 44.79% and 38.58% (Figure 4B). Under higher salinity stress (2X) the growth of fungi was restrained; however, the activities of metabolites increased, which might be regarded as appropriate salinity was beneficial to stimulate its silent metabolic pathway and produce new natural active products [30].

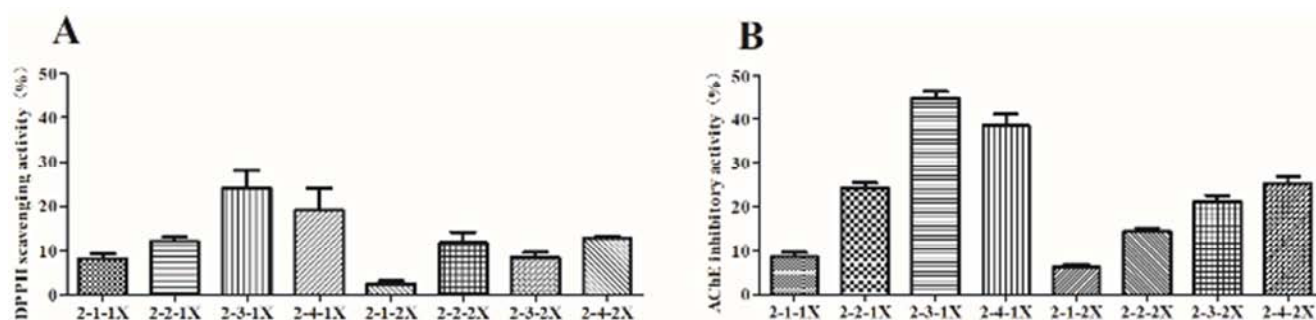


Figure 4. The influence of different salinity on metabolites of endophytic fungi YS-08. A: DPPH scavenging activity; B: AChE inhibitory activity.

3.4. Identification of Mutant Strains

Six species of mutant strains were isolated and purified when wild fungi (YS-08) was cultured in the above different salinity conditions. Subsequently, these mutant strains were preliminary identified through 16S rDNA sequence analysis and locus in the development process (Figure 6) combined with their morphological features and culture characters (Figure 5). The results show that the species of the mutant strains were determined as *Cladosporiumphaerospermum* (YS-08-1), *Penicilliumrubens* (YS-08-2, YS-08-4), *Curvularialunata* (YS-08-3), *Aspergillusflavus* (YS-08-5) and

Aspergillus sp. (YS-08-6), respectively.

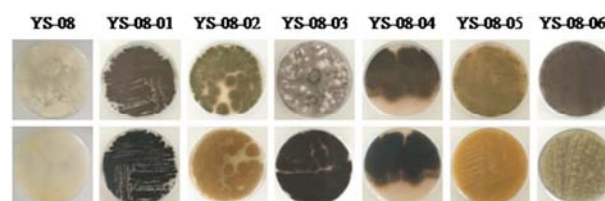


Figure 5. Morphological analysis of the mutant strains.

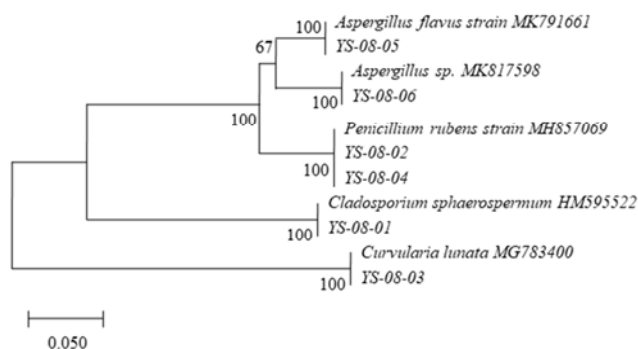


Figure 6. System development tree of the mutant strains.

3.5. Biological Activity of Metabolites of Mutant Strains

The bioactivity of metabolites of 6 mutant strains was further studied. As shown in Figure 7, compared with wild strain YS-08, the antioxidant activity of 4 mutant strains (YS-08-1, YS-08-2, YS-08-5 and YS-08-6) were significantly increased, and the scavenging rate of DPPH radical were 31.14%, 56.02%, 40.48% and 31.16%, respectively (Figure 7A). Inhibitory activity on AChE of all mutant strains were markedly enhanced, and the inhibitory rate of YS-08-1, YS-08-4 and YS-08-5 were up to 40.41%, 47.99% and 42.36%, respectively.

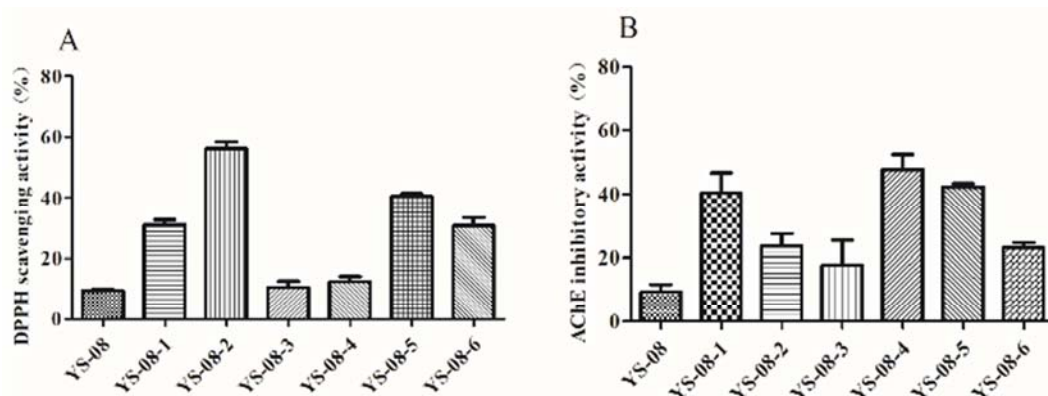


Figure 7. DPPH scavenging activity (A) and inhibitory activity on AChE (B) of metabolites of the mutant strains.

3.6. HPLC Analysis on Metabolites of Mutant Strains

The metabolites of 6 mutant strains were also analyzed by HPLC and we found that the marker peaks increased remarkably in comparison with YS-08 (Figure 2, Figure 8).

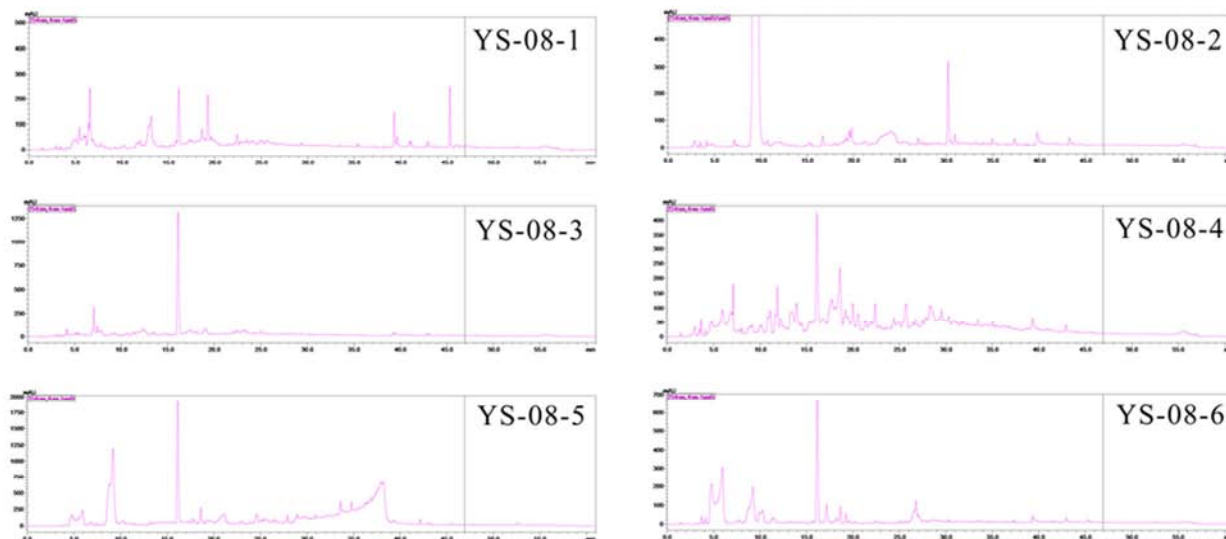


Figure 8. HPLC analysis on metabolites of the mutant strains.

4. Discussion

Chemical novelty and diversity of biological activities of the secondary metabolites from marine-derived microorganisms made a prominent contribution to the new

The results indicated that new secondary metabolites have been produced in mutant strains. According to the results of activity, structures of these compounds may be tightly related to the biological activities.

lead compounds. Therefore, researching on the secondary metabolites of marine microorganisms have attracted the attentions of researchers [1, 31]. However, under the laboratory conditions, most of genes of marine microorganisms were usually in silent and could not express and resulted in failure to produce more novel structural

compounds. The OSMAC strategy was first proposed by Zeeck [32] and have been widely used in the cultivation of microorganisms. This strategy can systematically change the growth parameters of microorganisms, and thus be beneficial for the diversity of their metabolites to some extent. Recent studies have shown that the change of fungal culture conditions can effectively regulate the production of secondary metabolites, and more compounds with novel structure and good biological activity could be obtained [19, 23]. For a better understanding of the impact of endophytic fungi on secondary metabolites under different culture conditions, oyster symbiotic fungi *Schizophyllum* sp. YS-08 was selected as the object in the present study.

The results showed that growth factor had significant effects on metabolic pathway of fungus, especially in the normal sea water salinity medium with peptone. In comparison with wild strain, the antioxidant activity and AChE inhibitory activity dramatically increased and the chemical constituents were even more abundant. Research has also found that the genes can be activated even under the adverse conditions, such as salt stress [29]. Consistently, the results of studies indicated that the bioactive metabolites produced by marine fungi are abundant in the high salinity situation despite the strains showed relatively slow growth speed. Furthermore, regulation of metabolites of fungi was more significant by adding Fe^{2+} or peptone to the previous salt stress condition. Meanwhile, 6 mutant strains were obtained under salt stress. Interestingly, we found that all mutant strains had potency toward antioxidant and AChE inhibitory activity. Among them, the DPPH free radical scavenging rates of YS-08-1, YS-08-2, YS-08-5, YS-08-6 were 31.14%, 56.02%, 40.48% and 31.16%, and AChE inhibition rate of YS-08-1, YS-08-4 and YS-08-5 were 40.41%, 47.99% and 42.36%. Analysis of extracts by HPLC showed the metabolites of most strains were more abundant after regulation of culture conditions.

5. Conclusion

The metabolic pathway of the oyster symbiotic fungi *Schizophyllum* sp. YS-08 was regulated effectively in different culture conditions. Under salt stress conditions, 6 mutant strains were obtained under salt stress and were identified to belong to *Cladosporium sphaerospermum* YS-08-1, *Penicillium rubens* YS-08-2 and YS-08-4, *Curvularia lunata* YS-08-3, *Aspergillus flavus* YS-08-5, *Aspergillus* sp. YS-08-6, respectively. Furthermore, these strains could produce more active metabolites. Therefore, the OSMAC strategy has been successfully applied in the activation of silencing genes of oyster endophytic fungi. This study will supply a new approach to obtaining lead compounds with novel structures and diverse bioactivities in drug discovery.

Conflicts of Interest

There are no conflict of interest.

Acknowledgements

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