Journal of Biotechnology **15**(4): 721-728, 2017

EFFECT OF CULTURE CONDITIONS FOR ANTIMICROBIAL ACTIVITY OF MARINE - DERIVED FUNGUS *ASPERGILLUS FLOCCULOSUS* 01NT.1.1.5

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Received: 05.9.2017
Accepted: 25.12.2017

SUMMARY

The biosynthesis of compounds with antibiotic activity produced by marine fungi, strongly depends on their growth conditions. A good understanding of the role of culture conditions in the biosynthesis of metabolites may lead to better exploitation of microbial metabolites. In this study, the influence of culture conditions including incubation period, initial pH and salinity on antimicrobial activity and secondary metabolites production of marine fungus 01NT.1.1.5 was investigated. This isolate, obtained from sponge *Stylissa* sp. in Nha Trang Bay, exhibited a broad spectrum of *in vitro* antimicrobial activity to *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19111, *Streptococcus faecalis* ATCC 19433 and *Candida albicans* ATCC 10231. According to morphological characteristics and sequence analysis of 28S rDNA, the fungus was identified as *Aspergillus flocculosus*. The results indicated that antimicrobial activity and metabolite amount were highest when the fungus was cultivated in rice medium with incubation period of 20 days. The optimum salinity of 35 g/L and initial pH of 6.0 were found for the maximum antibiotic production. The colony growth, antimicrobial activity and production of secondary metabolites of the strain *A. flocculosus* 01NT.1.1.5 varied depending on salt concentrations and initial pH of medium. Particularly, extract of this fungus only showed activity against *C. albicans* when it was cultured in medium with 30-35 g/L salinity and initial pH 4.0-8.0. The results indicate that salinity and initial pH along with cultivation period are important factors influencing antimicrobial activity and secondary metabolites of *A. flocculosus* 01NT.1.1.5, and might be for other marine fungi.

Keywords: *Aspergillus flocculosus, antimicrobial activity, culture conditions, marine fungi*

INTRODUCTION

In recent years, wide dissemination and emergence of multi-drug resistant bacteria have been concerned as great impact to public health. The rise in antibiotic resistance has been threatening to modern healthcare (Kalyani *et al.*, 2016; Wang *et al.*, 2011). Therefore, finding new antimicrobial agents, especially those from natural resources as well as biotechnological manipulation to increase their activities have been strongly pursued to develop efficient cure methods for treatment of infectious diseases. Marine fungi have been reported as potential sources of novel metabolites with bioactivities such as antibiotics, antiviral, anticancer and antioxidant (Saleem *et al*., 2007; Du *et al*., 2014). Particularly, fungal species belonging to the genus *Aspergillus* are ones of the major microbial sources of variety of compounds with antimicrobial activity (Li, 2010; Petersen *et al*., 2015). The antimicrobial potential of *Aspergillus* spp. against a panel of bacterial and fungal pathogens has been reported (Maria *et al*., 2005).

The production of antibiotics by microorganisms, including filamentous fungi can be enhanced by the nutritional factors such as carbon and nitrogen sources, inorganic salts with various cultivation factors, temperature, pH, incubation period (Barakat, Gohar, 2012). Optimization of culture conditions can impact the quantity and diversity of metabolic products of microbes and thus frequently has been

applied for the discovering new natural bioactive compounds (Bills *et al.*, 2008).

Aspergillus flocculosus 01NT.1.1.5, a marine fungus isolated from sponge *Stylissa* sp. at Nha Trang Bay. Our previous study showed that the fungus has considerable antimicrobial activity against a panel number of clinically significant pathogens. In present study, we conducted the effect of culturing conditions for this isolate in order to get the highest antibiotic production.

MATERIALS AND METHODS

Fungal isolate

The fungus *A. flocculosus* 01NT.1.1.5 was originally isolated from sponge *Stylissa* sp., which was collected at Nha Trang Bay, Vietnam, in February 2016. The fungus was identified according to its gene sequence of 28S rDNA. The genomic DNA of the isolate was extracted using a FastDNA spin kit for soil (Bio 101 Systems or Q-Bio gene) by following the company's protocol. DNA was amplified using primers NL209 (5'-AAGCGCAGGAAAAGAAACCAACAG-3') and NL912 (5'-TCAAATCCATCCGAGAACATCAG-3'), purified with a Geneclean III kit (Q-Bio gene), and sequenced using the fluorescent method and a Li-COR 4200 DNA sequencer (Amodia Bioservice GmbH, Braunschweig, Germany) (Zuccaro *et al.*, 2008). For identification, the sequence of the fungal 28S rDNA region were compared with those in the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). The strain was stocked in sterile seawater with 40% glycerol at -80 ^{o}C in the Marine Microorganism Collection at Nha Trang Institute of Technology Research and Application (NITRA).

Cultural and morphological properties of strain *A. flocculosus* 01NT.1.1.5

The cultural properties and morphological features of the spores and mycelia of strain *A. flocculosus* 01NT.1.1.5 were examined on Czapek medium (saccharose 30 g, $NaNO_3$ 3 g, K_2HPO4 1 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.1 g, agar 15 g, seawater 1000 mL) after culturing at 28^{o}C for 10 days (Vandermolen *et al.*, 2013). The conidiophores and conidia were observed with a B204 series biological microscope (Chongqing Optec Instrument Co., Ltd., Chongqing, China).

Antimicrobial assay

Antibacterial activity of ethyl acetate extracts from the marine fungus was determined against pathogens using disc diffusion assay (Becerro *et al.,* 1994). The crude extracts were impregnated at a concentration of 100 µg/disc on to 6 mm diameter sterile Whatman No1. discs and allowed to dry in the air at room temperature for solvent evaporation. The antimicrobial activity was assessed against six pathogens, including *B. cereus* ATCC 11778*, E. coli* ATCC 25922*, S. aureus* ATCC 25923*, L. monocytogenes* ATCC 19111, *S. faecalis* ATCC 19433 and *C. albicans* ATCC 10231. The test microorganisms were grown on nutrient agar media and their density was adjusted to standard McFarland 0.5 using a spectrophotometer at a wavelength of 625 nm. Ethyl acetate without extracts in the discs was used as negative control. The plates were incubated at 37 °C for 24 hours and results were recorded as zone of inhibition in mm.

Effect of culture conditions for antimicrobial activity

The fungal strain was grown stationary at 28 ^{o}C in 500 ml Erlenmeyer flasks, each containing 40 mL of natural seawater collected in Nha Trang Bay (pH 8.0, salinity of 30 g/L) supplemented with 20 g of rice, 20 mg of yeast extract, 10 mg of KH_2PO_4 (Sobolevskaya *et al.*, 2016). Effects of culture conditions on antimicrobial activity were investigated separately for each parameter (i.e. incubation period, pH and salinity). At the end of the incubation period, mycelia and media were homogenized and extracted two times with equal volume of ethyl acetate. The extracts were then concentrated by using rotary evaporator at 40 ^{o}C and used as crude extracts for the test of antimicrobial activity. Thus, growth time-dependent antimicrobial activity of the fungus was studied by growing it in rice media with cultivation time from 8 to 20 days with two day intervals. The effect of salinity was investigated at concentrations from 5 to 40 g sea salt/L (with intervals of 5 g sea salt/L) and initial culture medium pH from 4.0 to 9.0.

RESULTS AND DISCUSSION

Morphological characterizations and identification of the strain 01NT.1.1.5

The fungal strain 01NT.1.1.5 was cultured on Czapek medium for observation of morphological

characterization. After ten days of growth at 28°C, the fungus had colonies of about 20 mm in diameter, white to greyish white aerial mycelial, light yellow to olive to brownish orange sporulation, reddish brown soluble pigment in conspicuous and yellowish olive exudate. Observation under light microscopy revealed radiating conidial heads, biseriate conidiophores, yellow to brown hyaline stipes and globose vesicles (Figure 1).

Combination of macroscopic and microscopic characteristics and molecular methods remain commonly used and essential tools for identification of *Aspergillus* species (Samson *et al.*, 2014). The 28S rDNA sequence region (796 bp) was amplified by PCR and sequenced; BLAST search results indicated similarity to the sequence of *Aspergillus flocculosus* NRRL 5224 (GenBank accession number EU021616.1) with a 100% identity. Moreover, the observed morphological characteristics of this fungus are similar to features of *Aspergillus flocculosus* those were decribed by Samson *et al.,* (2014). Thus, the fungal strain 01NT.1.1.5 was assigned the name *Aspergillus flocculosus* 01NT.1.1.5.

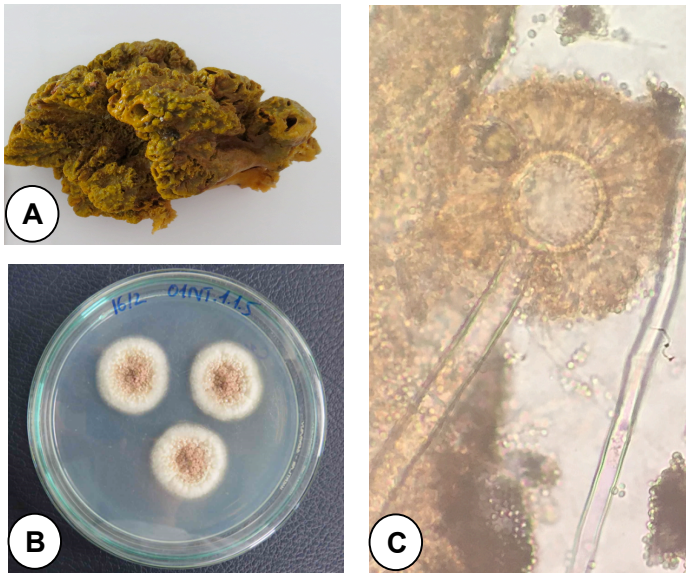

Figure 1. Colony appearance and micromorphology of *A. flocculosus* 01NT.1.1.5. and the sample of the sponge *Stylissa* sp. (A) Sample of the sponge *Stylissa* sp.; (B) Colony appearance after ten days on Czapek medium; (C) Conidiophores and conidia after ten days.

Effect of culturing conditions on antimicrobial activity of *A. flocculosus* 01NT.1.1.5

In this study, three parameters including incubation period, initial pH and salinity were investigated to achieve maximum antimicrobial activity and high yield of metabolite production by the fungal strain *A. flocculosus* 01NT.1.1.5.

Effect of incubation period

Antimicrobial metabolite production by this marine fungus was determined over a period of 26 days. The bioactive metabolite production increased from the 12th day, reaching the highest level on 20th day of incubation, then declined gradually (Figure 2). Therefore, 20 day incubation was selected for the fungus to reach maximum production of antimicrobial metabolites.

Generally fungal strains have different optimal culturing time for growth and synthesis of bioactive compounds. Particularly, the marine fungus *Aspergillus terreus* var. *africanus* showed optimal growth time with high antimicrobial activity at the 6th day (Barakat and Gohar, 2012). Similarly, production of bioactive compounds by marine fungus *Cladosporium sphaerospermum* was increased gradually until reached the maximum level after 8 days, then decreased (Kalyani *et al.*, 2016). However, Mabrouk *et al.,* (2011) reported that the marine fungus *Penicillium brevicompactum*, associated algae *Pterocladia* sp., showed maxium bioactivity after 12 days of cultivation.

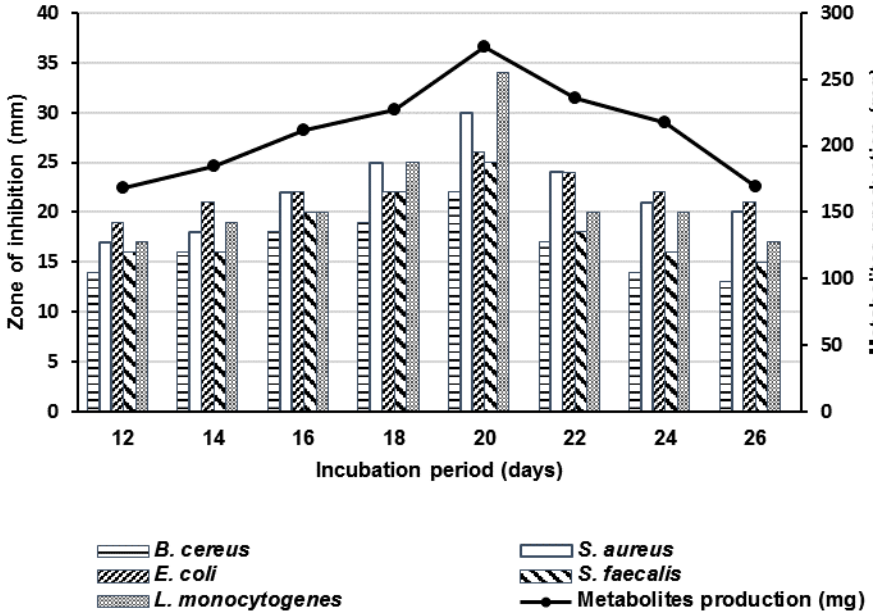

Figure 2. Effect of incubation period on antimicrobial activity and metabolites production.

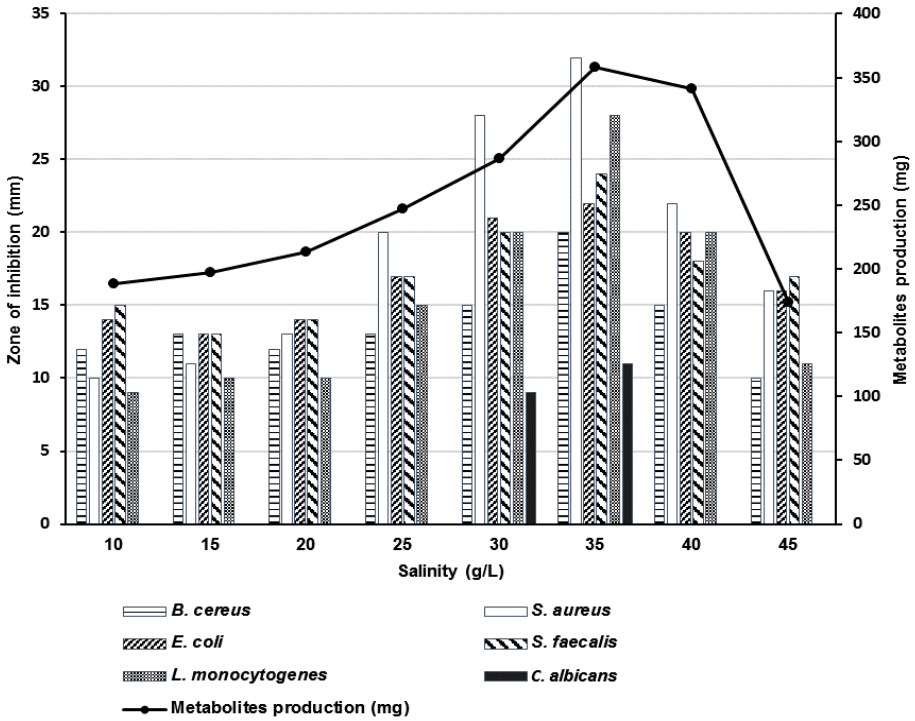

Figure 3. Effect of salinity on antimicrobial activity and metabolites production.

The differences in optimal incubation period for biosynthesis of bioactive compounds in many fungi might be due to different ecological characteristics and growth media as well. In particular, most of the strains reported were inoculated in broth medium with shaking. In our study, the isolate *A. flocculosus* 01NT.1.1.5 was cultured in semi-solid medium with static state. This could be the reason leading to a longer time for growth and biosynthesis of antibacterial compounds.

Effect of salinity

Growth and metabolite production by fungi, especially marine fungi are largely affected by salt concentration in the culturing media (Jingjing *et al.*, 2011). In our study, different concentrations of salt in the growth medium were tested. The results showed that strain *A. flocculosus* 01NT.1.1.5 obtained the highest antimicrobial activity and metabolite production at salinity of 35 g/L (Figure 3). Only at the salinity of 30 to 35 g/L this isolate had inhibitory effects against *C. albicans,* whereas at other salt concentrations this property was not observed. The higher salt concentration, the more rapid decrease of metabolite production by the strain *A. flocculosus* 01NT.1.1.5.

Several authors reported that NaCl concentration of 30 g/L was the optimal for maximum mycelia weight and antibacterial metabolite production (Kalyani *et al.*, 2016). Miao *et al.*, (2006) reported that medium at 34 g/L salt was the best condition for active metabolite production by the strain *A. saccharicola*. Beside, NaCl concentration of 3.0 % was also found to be optimum for maximum growth and production of bioactive metabolite by an fungus, *Fusarium* sp. (Gogoi *et al.*, 2008). Similarly, the marine fungus *Penicillium chrysogenum* was investigated by Trinh *et al*., (2016) and suggested that the fungal strain showed highest antimicrobial activity in rice medium at salinity of 35 g/L. Cantrell *et al.*, (2006) found that the marine fungi with dark cell wall can tolerate higher salinity than the moniliaceous fungi. According to Jingjing *et al.* (2011), the habitats of marine fungi had a strong influence on their adaptation to salt.

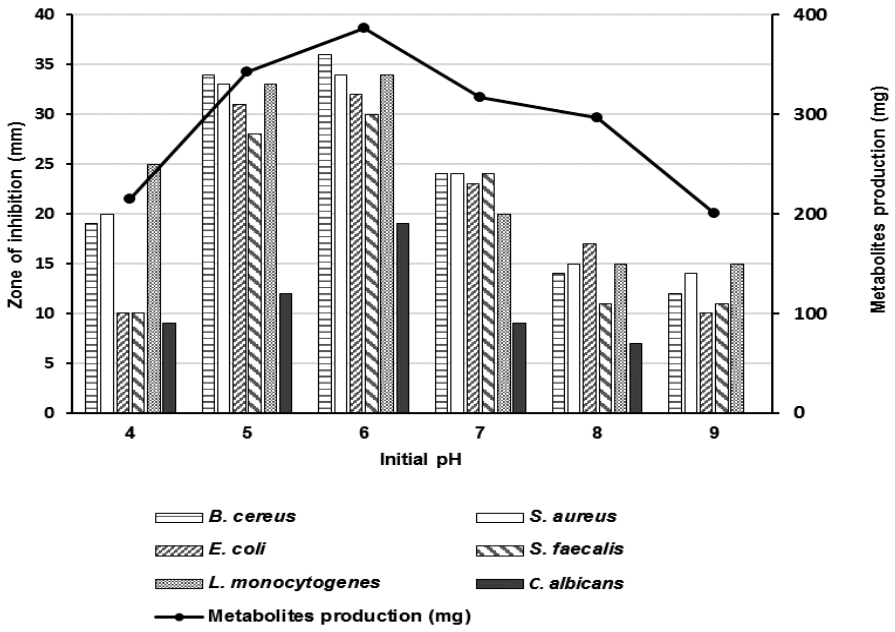

Figure 4. Effect of initial pH on antimicrobial activity and metabolites production.

Effect of initial pH

pH values regarding to hydrogen or hydroxyl ion concentration may affect directly on cell, or indirectly on a degree of dissociation of substances in the medium. Therefore, initial pH of the culture medium affects not only growth but also antimicrobial agent production (Singh *et al.*, 2017).

In the present study, different initial pH values

were experimentally tested. It has been observed that maximum antibiotic activity was obtained at pH 6.0 by *A. flocculosus* 01NT.1.1.5 and beyond the optimum pH the antimicrobial activity decreased (Figure 4). Low yield of metabolite and inactivation towards *C. albicans* was showed at pH 9.0.

Similar results were reported by Mabrouk *et al.* (2008), i.e. initial pH of the medium suitable for marine fungus *Varicosporina ramulosa* to get the highest bioactivity was pH 6.0. In another study, Jain and Pundir (2011) showed that the maximum antimicrobial activity of *A. terreus* against pathogens was also found at pH 6.0. Nevertheless, a more alkaline optimum pH of 7.5 was reported for antibacterial activity by marine-derived fungus *Arthrinium saccharicola* isolated from seawater in Yung Shue O, Hong Kong (Miao *et al*., 2006). The culture medium pH is usually not constant throughout fungal growth and the changes might affect the metabolite synthesis to a certain extension (Daryaei *et al.*, 2016; Padhi *et al.*, 2017).

Nowadays, the emergent drug resistance among pathogenic microorganisms, increasing the rate of microbial infections has been attracting much of public concern (Singh *et al.*, 2015). Discovering new and effective antimicrobial substances from varied natural resources, including microorganisms is an approach to overcome the problem.

Production of antimicrobial agents is often influenced by nutritional as well as cultivation factors, which are considered as important parameters for scale-up process in industrial production. The optimal culture conditions for the strain *A. flocculosus* 01NT.1.1.5 showed in this study could be the first steps in study on scaling up the production process (Bills *et al.*, 2008).

CONCLUSIONS

Our studies showed that *A. flocculosus* 01NT.1.1.5, a marine fungus isolated from sponge *Stylissa* sp., had optimum culture conditions for biosynthesis of antimicrobial metabolites in rice medium with salinity of 35 g/L, initial pH 6.0 after 20 days of incubation. The results also indicated that culture medium had a strong influence on antibiotic activity of the isolate. Further studies on optimization of nutritional composition for bioactivity and metabolite production by the strain will be carried out.

Acknowledgments: *This study was supported by the Grant of RV "Akademik Oparin" expedition, (VAST. HTQT.NGA.15-06/16-17).*

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ẢNH HƯỞNG CỦA ĐIỀU KIỆN LÊN MEN SINH HOẠT TÍNH KHÁNG SINH CỦA CHỦNG VI NẤM BIỂN *ASPERGILLUS FLOCCULOSUS* 01NT.1.1.5

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TÓM TẮT

Sự sinh tổng hợp các hợp chất có hoạt tính kháng khuẩn tạo ra bởi vi nấm biển phụ thuộc lớn vào điều kiện sinh trưởng của chúng. Việc làm rõ vai trò của điều kiện nuôi cấy trong quá trình tổng hợp các chất chuyển hóa sẽ thuận lợi cho các nghiên cứu về hợp chất từ vi sinh vật. Trong nghiên cứu này, ảnh hưởng của điều kiện nuôi cấy bao gồm thời gian nuôi cấy, pH ban đầu và độ mặn của môi trường lên hoạt tính kháng khuẩn cũng như quá trình tạo chất chuyển hóa thứ cấp của chủng vi nấm biển 01NT.1.1.5 đã được điều tra. Chủng vi nấm 01NT.1.1.5 được phân lập từ bọt biển *Stylissa* sp. thu thập tại vịnh Nha Trang, có hoạt tính kháng khuẩn phổ rộng đối với các chủng vi sinh vật kiểm định *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19111, *Streptococcus*

faecalis ATCC 19433 và *Candida albicans* ATCC 10231. Căn cứ vào đặc điểm hình thái và phân tích trình tự 28S rDNA, chủng vi nấm được xác định là *Aspergillus flocculosus*. Kết quả nghiên cứu cho thấy hoạt tính kháng sinh và hàm lượng chất chuyển hóa đạt cao nhất khi chủng vi nấm được nuôi cấy trên môi trường gạo với thời gian 20 ngày, độ mặn và pH môi trường nuôi cấy ban đầu tối ưu là 35 g/L và 6.0. Nồng độ muối và pH ban đầu khác nhau có ảnh hưởng rõ rệt tới sinh trưởng và hoạt tính kháng khuẩn của chủng *A. flocculosus* 01NT.1.1.5. Cụ thể, dịch chiết của chủng vi nấm chỉ thể hiện hoạt tính kháng *C. albicans* khi được nuôi trong môi trường có độ mặn 30-35 g/L và pH ban đầu 4.0-8.0. Các kết quả chứng minh rằng độ mặn và pH ban đầu cùng với thời gian lên men là những yếu tố quan trọng quyết định hoạt tính kháng khuẩn và sự sản sinh các chất chuyển hóa thứ cấp của chủng vi nấm biển.

Từ khóa: *Aspergillus flocculosus, hoạt tính kháng khuẩn, điều kiện nuôi cấy, vi nấm biển*