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EFFECT OF CULTURAL CONDITIONS ON ANTIMICROBIAL ACTIVITY OF MARINE-DERIVED FUNGUS *PENICILLIUM CHRYSOGENUM*

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SUMMARY

Marine fungi are a potential source of structurally diversified bioactive secondary metabolites that are not found in terrestrial sources. In our continuous investigation to search new antimicrobial agents from marine-derived fungi, we isolated fungal strain 045-357-2 from a soft coral sample collected from Ca Na bay, Ninh Thuan, Vietnam. The strain showed high antibacterial activity and was selected for further study. According to Internal Transcribed Spacer (ITS) rDNA molecular methods, the fungus was identified as *Penicillium chrysogenum* 045-357-2 and had a 100% homology (600/600 bp) with a GenBank sequence from a reference *P. chrysogenum* strain (NCBI accession no. EF200090). In the present investigation, the fungus was studied on effect of different culture incubation period, pH and salinity for antimicrobial activity against pathogenic microbes including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus cereus*, *Streptococcus faecalis*, *Listeria monocytogenes*, and *Candida albicans*. The ethyl acetate extracts of culture medium of the fungal isolate were determined antimicrobial activity by disc diffusion assay. The results showed that the optimum antimicrobial activity of the fungal strain *P. chrysogenum* 045-357-2 was obtained in medium containing 20 g rice, 20 mg yeast extract, 10 mg KH_2PO_4, and 40 mL natural seawater at 30°C on the 14th day of incubation. The strain also exhibited highest antimicrobial activity in the medium at initial pH 6.0 – 8.0 and salinity of 35 g/L. The findings indicate that cultural conditions significantly influenced on antimicrobial activity of the studied fungus. The optimization is potential use for further study on the mass cultivation and isolation of bioactive compounds for this fungus.

Keywords: *Penicillium chrysogenum, antimicrobial activity, marine fungi, secondary metabolites*

INTRODUCTION

Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities (Smetanina *et al*., 2007; Blunt *et al*., 2006). Due to their characteristic properties with reference to salinity, nutrition, high pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi (Liberra, Lindequist, 1995). Besides, marine fungi have proved to possess tremendous potential as a source of new medicines even at low concentrations of their secondary metabolites (Swathi *et al*., 2013).

In general, an effective screening process can be achieved through systematic manipulation of culture conditions for a small number of promising organisms. In fact, culture conditions have a major impact on the growth of microbes and the production of microbial products. As far as culture conditions are concerned, there is usually a dilemma between achieving maximal growth rates and maximal antibiotic yields because conditions that allow fast cell growth could be unfavorable to metabolite production (Fiedurek *et al*., 1996; Miao *et al*., 2006; Xu *et al*., 2008; Mohanty, Prakash, 2009; Kossuga *et al*., 2012). Changes in pH, temperature, incubation period, shaking and inoculum size of the antagonistic fungal strain can greatly influence antibiotic

biosynthesis (Calvo *et al*., 2002; Llorens *et al*., 2004). Antibiotic productivity can also decrease when media deficient in metal ions are used and culture vessels are incubated at high temperatures for long periods. Therefore, optimization and maintenance of proper culture conditions are necessary criteria to achieve maximum production of bioactive metabolites by an antagonistic microbial strain. However, there are fewer reports on effect of nutritional and cultural conditions on mycelia growth and antimicrobial metabolite production by the antagonistic fungal strains (Gogoi *et al*., 2008; Ritchie *et al*., 2009; Jain, Pundir, 2011). Therefore, the objective of this study was to optimize the culture conditions of *P. chrysogenum* 045-357-2 for enhanced bioactive agent production. Specifically, we empirically investigated the effects of incubation period, salinity and pH on bioactive metabolite production by the fungus.

MATERIALS AND METHODS

Isolation of marine fungus

The fungal strain 045-357-2 was isolated from an unidentified soft coral collected at Ca Na bay, Ninh Thuan, Vietnam. The soft coral sample was rinsed three times with sterile seawater in order to remove the non-attached bacteria. The sample (1.0 g) was triturated with sterile sea water and placed on the entire surface of modified Sabouraud medium (peptone 10 g, glucose 40 g, agar 18 g dissolved in 1000 ml sea water, pH 6.0-7.0). The plates were incubated for 5-7 days at 28°C, and colonies of strain 045-357-2 were isolated and stocked in 40% glycerol in seawater at -80^{o}C in the Marine Microorganism Collection, Nha Trang Institute of Technology Research and Application (NITRA).

Identification of marine fungus by ITS gene analysis

The fungus was identified based on rDNA sequence analysis of ITS region. Fungal DNA was extracted using the procedure described by Fredricks *et al.,* (2005) with modifications. In brief, the mycelial powder was transferred to a 1.5 ml eppendorf tube containing 400–500 µL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.3); equal volume of phenol solution was added to the tube. After brief mixing, the mixture was centrifuged at 12,000 g for 10 min at 4^{o}C. The aqueous phase was transferred to a new microtube and sequentially extracted with phenol solution and chloroform. RNA was removed in the aqueous phase using RNase. The sample was then again extracted with phenol solution and chloroform. Finally, DNA was precipitated by adding two volumes of ethanol. The DNA pellet was washed with 75% ethanol and resuspended in 50–100 µL of sterile water. The resulting genomic DNA was used as a template to amplify fungal ITS-rDNA fragments using the primers ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The reaction mixture contained 5 µL of 10 x reaction buffer with 1.5 mM $MgCl_2$ (Promega), 2 µL of 2.5 mM dNTPs, 2 µL of 10 pmol forward primer, 2 µL of 10 pmol reverse primer, 1 µL of fungal DNA (50 µg/mL), 0.5 µL of Taq DNA polymerase (5 UμL^{-1}, Promega), and 32.5 µL of sterile distilled H_2O. PCR conditions were as follows: initial denaturation (94^{o}C for 5min); 30 cycles of denaturation (94^{o}C for 50s), primer annealing (55^{o}C for 50s), and elongation (72^{o}C for 1 min), with a final elongation at 72^{o}C for 10 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and used directly for sequencing analysis. Alternatively, if satisfactory sequencing results were not obtained from PCR products, PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced using the plasmid DNA with the universal plasmid primers T7 and SP6.

Sequencing analyses were performed on an ABI 3730 XL (Applied Biosystems) automated sequencer using ITS1 and ITS4 primers for PCR templates or universal plasmid primers (T3 and T7) for plasmid templates. Sequence data were edited with Chromas Lite, version 2 (Technelysium). For preliminary identifications, sequences of fungal rDNA-ITS regions were compared with those in the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Fungal ITS-rDNA sequences in this study and the matched sequences from GenBank were edited and aligned with Seq-Man and Megalign (DNASTAR Package). The aligned sequences were imported into PAUP 4.0b10 (Swofford, 2002). Neighbor-joining (NJ) trees were estimated using pairwise genetic distances based on the basis of all substitutions with the Jukes– Cantor distance parameter. For maximum-parsimony (MP) analyses, we used a heuristic search strategy. A strict consensus tree was drawn when multiple best trees were obtained. The quality of the branching patterns for MP and NJ was assessed by bootstrap resampling of the data sets with 1000 replications.

Optimization of culture conditions for antimicrobial activity

The strain was fermented in Erlenmeyer flasks (500 ml), each flask containing 20 g of rice, 20 mg of yeast extract, 10 mg of KH_2PO_4, 40 mL of natural seawater (3.5 %), pH 7.0 for 20 days. To examine the effects of culture conditions (incubation period, pH and salinity) on antimicrobial activity, parameters of culture conditions were changed one at a time according to the experimental design described below while all other cultivation parameters remained unchanged. At the end of the incubation period, the mycelia and medium were homogenized and extracted two times with equal volume EtOAc. The extract of the fungus was concentrated to dryness using rotary evaporators at 40 ^{o}C and used as crude extract for antimicrobial activity.

Effect of incubation period

The effect of incubation period on antimicrobial activity of the fungus was studied by growing the fungal strain in rice medium with time course ranging from 8 to 20 days.

Effect of pH

The optimization of pH of medium for antimicrobial activity of the strain was done by carrying out the fermentation in rice medium at six different pH values 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. For each pH value, 40 ml of sea water was adjusted to desire pH by using either 50 mM citrate phosphate buffer (pH 4.0 to 7.0) or 50 mM Tris-HCl buffer (pH 8.0 to 9.0).

Effect of salinity

The effect of salinity on antimicrobial activity of the strain was determined by growing fungus in rice medium made of eight different salinities, including 5, 10, 15, 20, 25, 30, 35 and 40 (g/L).

Microbial target organisms

Seven clinical microbial pathogens including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *S. faecalis* ATCC 19433, *L. monocytogenes* ATCC 19111, and *C. albicans* ATCC 10231 were used in this study. The microorganisms were preserved at -80^{o}C in the presence of glycerol (30%, v/v) for longer periods.

Determination of antimicrobial activity using disc diffusion assay

A 100 µg fungal etyl acetate extract was loaded onto a sterile filter paper discs (6 mm in diameter). The paper discs were air-dried and placed onto the Muller Hinton Agar plate that had already been inoculated with lawn of the target microorganisms (approximatetly 10^8 CFU/mL). Paper discs contained only sovent (etyl acetate) which were served as negative control. After incubation for 24-48 h at 37^{o}C, the antimicrobial activity was evaluated by measuring the width of the growth inhibition zones in millimeter (Bauer *et al.*, 1966).

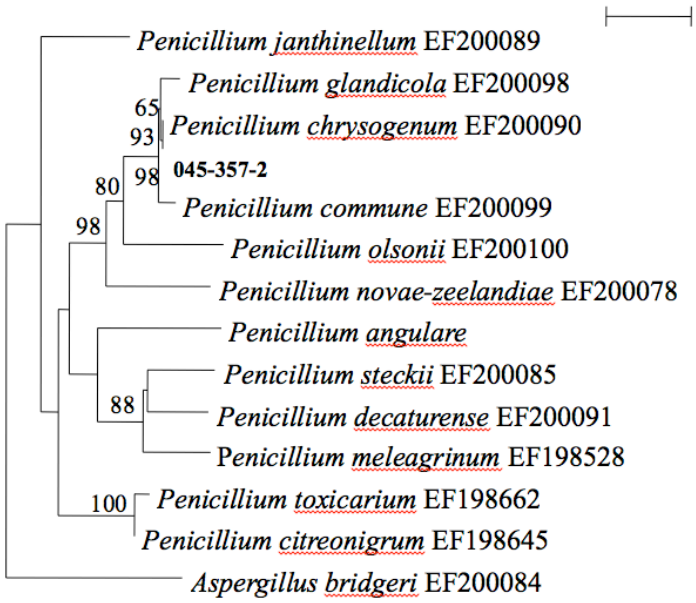

Figure 1. Phylogenetic tree based on rDNA gene sequences of ITS region and closely related members of the genus *Penicillium*. Numbers at nodes are levels of bootstrap support based on neighbor-joining analyses of 1000 replications.

RESULTS AND DISCUSSIONS

Identification of marine fungus

The analysis of the ITS gene sequences is an important tool for correct identification of fungal species (Wiese *et al.*, 2011). The length of the partial rDNA gene sequence of the isolate was 600 bp. The phylogenetic tree, which was constructed for comparison of the ITS gene sequences, indicated that strain 045-357-2 belonged to the genus of *Penicillium* (Figure 1). The fungus was phenotypically similar to *P. chrysogenum* with a 100 % homological reference (600/600 bp) to *P. chrysogenum* in GenBank (NCBI accession no. EF200090). Therefore, our fungus strain was named as *P. chrysogenum* 045-357-2.

Optimization of culture conditions for antimicrobial activity of marine fungus

Among 100 fungal strains were isolated from different marine sources including soft corals, sea cucumbers, seaweeds, sea grass, sediments, and punk collected at Con Son Island, Tho Chu Island and Ca Na Bay, 48 isolates shown antimicrobial activities against pathogenic microorganisms. Strain *P. chrysogenum* 045-357-2 represented highest antimicrobial activity, so it was selected for further study (Ngoc *et al.*, 2016).

In general, metabolite biosynthesis in microbes are tightly controlled by regulatory mechanisms to avoid over production; yet, these regulatory mechanisms often process to undesirably low levels. Incubation period (Gromov *et al.*, 1991), pH (Patterson, Boils, 1995) and salinity (Lehtimaki *et al.*, 1997) are the important factors influencing antimicrobial agent production.

Effect of incubation period on antimicrobial activity

The experiment was carried out to determine the optimum incubation period for antimicrobial activity of *P. chrysogenum* 045-357-2. The strain was incubated in flasks containing rice medium for 8 to 20 days. Antimicrobial activity was investigated for each two days.

The antimicrobial activity of *P. chrysogenum* 045-357-2 increased gradually by incubation time, reached maximum at 14 days, and then decreased (Table 1). Similarly, the marine derived fungus *P. chrysogenum* was investigated by Devi *et al*., (2009) and suggested that the fungus strain produced cinitrin highest after 15 days of incubation. Mabrouk *et al.,* (2011) reported that the marine fungus *P. brevicompactum*, associated algae *Pterocladia* sp., showed maxium bioactivity after culturing 12 days.

Table 1. The effect of time course (days) on antimicrobial activity (mm) of *P. chrysogenum* 045-357-2.

Microbes	Antimicrobial activity (zone of inhibition in mm)							
	NC	8 days	10 days	12 days	14 days	16 days	18 days	20 days
S. aureus	-	16	16	16	**28**	23	13	13
P. aeruginosa	-	8	8	8	**21**	18	7	7
E. coli	-	-	-	8	**12**	10	-	-
B. cereus	-	12	12	12	**20**	17	11	11
S. faecalis	-	12	14	12	**18**	15	13	12
L. monocytogenes	-	12	12	12	**18**	16	12	12
C. albicans	-	-	-	-	**-**	-	-	-

NC: Negative control

Effect of pH on antimicrobial activity

pH of a culture medium affects growth and other life processes of fungi. Thongwai and Kunopakarn (2007) pointed that most microorganisms synthetise antimicrobial compounds at pH from 5.5 to 8.5.

P. chrysogenum 045-357-2 grew under a wide range of pH (from 4.0 to 9.0). Antimicrobial activity showed a little difference regarding to pH conditions (Table 2).

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, it is important for antimicrobial agent production. In this study, *P. chrysogenum* 045-357-2 reached highest bioactivity at pH 7.0. Similar report on the marine-derived fungus *Arthrinium saccharicola*, isolated from seawater in Yung Shue O, Hong Kong, also demonstrated that the fungus achieved maximum

antibacterial activity at pH 7.5 (Miao *et al*., 2006). In the study of Jain and Pundir (2011), the maximum antimicrobial activity of *Aspergillus terreus* against pathogens was found at pH 6.0.

Table 2. Effect of pH of the culture medium on antimicrobial activity of *P. chrysogenum* 045-357-2.

Microbes	Antimicrobial activity (zone of inhibition in mm)						
	NC	pH4	pH5	pH6	pH7	pH8	pH9
S. aureus	-	13	13	13	**16**	14	14
P. aeruginosa	-	-	-	-	**-**	-	-
E. coli	-	7	8	8	**9**	8	8
B. cereus	-	12	12	12	**12**	12	11
S. faecalis	-	12	13	12	**13**	12	11
L. monocytogenes	-	11	13	13	**13**	12	11
C. albicans	-	-	-	-	**-**	-	-

Effect of salinity on antimicrobial activity

The effect of salinity on bioactivity was revealed by growing the fungus in media of seven different salinities of 5 to 40 g/L sea salt. The strain *P. chrysogenum* 045-3-57-2 grew vigorously in the medium with a wide range of salinity. Influence of sea salt on antimicrobial activity of the fungus is presented in table 3.

Marine fungal species have evolved metabolites to adapt with the salinity change. Strain *P. chrysogenum* 045-357-2 exhibited highest and lowest antimicrobial activity in the medium containing sea salt of 35 g/L and 5 g/L, respectively. The antimicrobial activity decreased slightly at 40 g/L. Miao *et al.,* (2006) also reported that medium at 34 g/L sea salt was optimal for active metabolite production of the strain *A. saccharicola*.

Culture condition is virtually important for industrial fermentation of bioactive products. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite productions. Various aspects of microbial media such as carbon and nitrogen sources, minimal salts, vitamins and pH have been reviewed (Rizk *et al*., 2007).

Table 3. Effect of salinity of the culture medium on antimicrobial activity of *P. chrysogenum* 045-357-2.

Microbes	Antimicrobial activity (zone of inhibition in mm)								
	NC	5 g/L	10 g/L	15 g/L	20 g/L	25 g/L	30 g/L	35 g/L	40 g/L
S. aureus	-	10	14	17	18	20	20	**22**	21
P. aeruginosa	-	-	-	-	-	-	-	**-**	-
E. coli	-	7	8	8	10	11	11	**12**	12
B. cereus	-	11	13	13	15	16	17	**20**	18
S. faecalis	-	10	18	18	18	18	18	**20**	19
L. monocytogenes	-	13	13	13	16	16	16	**18**	18
C. albicans	-	-	-	-	-	**-**	-	**-**	-

CONCLUSION

The marine fungus 045-357-2, that was isolated from an unidentified soft coral collected at Ca Na bay, Ninh Thuan, Vietnam, represented high antimicrobial activity and was identified as *P. chrysogenum* 045-357-2 based on rDNA sequence analysis of ITS region. The strain showed the highest antimicrobial activity in the rice medium with intial pH 7.0, 35 g/L sea salt and after 14 days of incubation. Our findings indicated that culture conditions influenced on antimicrobial activity of marine fungi. The present study will facilitate further researches for better understanding of acitive metabolite production from this fungus.

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ẢNH HƯỞNG CỦA ĐIỀU KIỆN NUÔI CẤY LÊN HOẠT TÍNH KHÁNG KHUẨN CỦA CHỦNG VI NẤM BIỂN *PENICILLIUM CHRYSOGENUM*

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TÓM TẮT

Vi nấm biển là một nguồn tiềm năng các chất chuyển hoá thứ cấp có hoạt tính sinh học và cấu trúc đa dạng mà không được tìm thấy ở các nguồn trên cạn. Trong quá trình nghiên cứu về các hợp chất mới có hoạt tính kháng sinh từ vi nấm biển, chúng tôi đã phân lập chủng vi nấm 045-357-2 từ một loài san hô mềm thu ở vịnh Cà Ná, Ninh Thuận, Việt Nam. Chủng vi nấm này thể hiện hoạt tính kháng khuẩn cao nên được chọn cho các nghiên cứu tiếp theo. Căn cứ vào phương pháp giải trình tự rDNA vùng ITS, chủng vi nấm được định danh là *Penicillium chrysogenum* 045-357-2 và tương đồng 100% (600/600 bp) với trình tự của *P. chrysogenum* trên Ngân hàng Gen (NCBI accession no. EF200090). Trong nghiên cứu này, chủng vi nấm được xác định ảnh hưởng của thời gian nuôi cấy, pH và nồng độ muối lên hoạt tính kháng khuẩn đối với các chủng gây bệnh bao gồm *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus cereus*, *Streptococcus faecalis*, *Listeria monocytogenes*, và *Candida albicans*. Dịch chiết ethyl acetate từ môi trường nuôi cấy của chủng vi nấm này được xác định hoạt tính kháng khuẩn theo phương pháp khuếch tán trên đĩa giấy. Kết quả cho thấy chủng *P. chrysogenum* 045-357-2 thể hiện hoạt tính kháng khuẩn tối ưu trong môi trường gồm 20 g gạo, 20 mg dịch chiết nấm men, 10 mg KH_2PO_4 và 40 mL nước biển ở 30^oC sau 14 ngày lên men. Chủng vi nấm này cũng thể hiện hoạt tính kháng khuẩn cao hơn khi được nuôi cấy trong môi trường gạo có pH ban đầu 6,0- 8,0 và nồng độ muối 35 g/L. Kết quả cho thấy điều kiện nuôi cấy có ảnh hưởng đáng kể đến hoạt tính kháng khuẩn của chủng vi nấm nghiên cứu. Sự tối ưu hóa này là số liệu hữu ích cho các nghiên cứu sâu hơn về việc nuôi cấy thu sinh khối cũng như phân lập các hợp chất có hoạt tính sinh học từ chủng vi nấm này.

Từ khoá: *Penicillium chrysogenum, chất chuyển hoá thứ cấp, hoạt tính kháng khuẩn, vi nấm biển*