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Full Length Research Paper

A new screening method for discovering antibacterial agents from filamentous fungi

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Rapid development of resistance among major bacterial pathogens renders antibiotics more and more ineffective and it is crucial to find novel antibiotics for controlling these pathogens. Since high-throughput screening (HTS) that selects antibacterial agents according to targets *in vitro* rather than whole-cell have not proven effective in the discovery of new antibiotics; new approaches for discovering the next generation of antibiotics are urgently needed. Filamentous fungi are an important source for many of the antibiotics currently used, but screening them for novel antibiotics is difficult primarily due to the lack of efficient screening methods capable of maintaining whole bacterial cell metabolism. In this study, mixed agar plate culture (MAPC) screening method is described. The method maintains the advantages of traditional whole-cell screening but offers increased screening efficiency. Furthermore, its simplicity and convenience makes it suitable for many laboratories. MAPC screening increases the probability of discovering novel antibacterial agents from filamentous fungi under laboratory conditions.

Key words: Drug-resistant bacterial pathogens, novel antibiotics; screening method, filamentous fungi products.

INTRODUCTION

Evidence of antibiotic resistance is accumulating, increasing the urgency to develop new antibiotics (Knapp et al., 2010; Xuan et al., 2010; Alagesaboopathi, 2011). However, antibacterial drug discovery and development has slowed considerably in recent years. Traditional approaches have not continued to yield novel classes of antibacterial compounds and despite a wealth of new bacterial targets, the current framework of high-throughput screening (HTS) in major pharmaceutical industries has not been successful in discovering inhibitory compounds for therapeutic use (Opar, 2007; Payne, 2008). There is excitement whenever a new antibiotic target or a new antibiotic mechanism has been identified because it could lead to a new treatment for drug-resistant bacterial pathogens by the HTS method (Bax et al., 2010). However, many technical difficulties remain as indicated by the low probability of success in creating a new antibiotic approved for clinical use (Payne et al., 2007). An isolated target gives no indication of the complexity of developing a successful antimicrobial drug. As a result, some companies have turned away from HTS and returned to more traditional approaches (Opar, 2007). The discovery of penicillin in the 1940s was soon followed by the discovery of a large number of antibiotics from microbes using traditional approaches, in particular from fungi and actinomycetes. However, it is difficult to discover new antibiotics from natural products because core technologies had not evolved (Lam, 2007). Success in discovering new antibiotics from microbial communities should focus on how to maximize screening efficiency, especially at the early stage that is the slowest, most labor intensive, step in the screening process used to discover novel drugs (Casenghi,et al., 2007). To meet this urgent medical need, new drug discovery technologies

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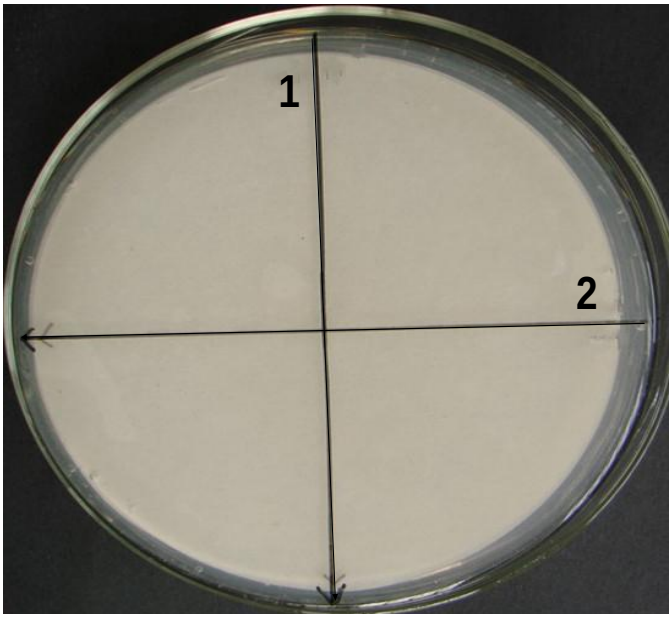

Figure 1. Perpendicular lines and their numbers on the filter paper and the base of Petri dish.

must be developed that improve the efficiency of the discovery process (Peláez, 2006). Untapped biological resources coupled with “smart screening” methods could provide the means for so doing (Li and Vederas 2009).

Fungi are one of the major antibiotic-producing organisms and one of the most diverse groups of organisms, so the search for novel antibiotics from fungi is promising. In 1995, six of the world’s 20 best-selling pharmaceuticals are fungal derived compounds (Ho et al., 2003). In recent years fungi have been shown to produce a plethora of bioactive secondary metabolites, some of them featuring new carbon frameworks hitherto unknown in nature. These compounds are of interest as they provide new structural frameworks for medical research, and the pharmaceutical industry has become increasingly interested in screening fungi for novel antibiotic activity (Kjer et al., 2010). In the past, discovery of novel medical compounds from fungi has often resulted from accidental discovery or from a ubiquitous fungus at the beginning of an antibiotic screening by pure culture. However, this approach is difficult to turn up new medical compounds again due to its unsystematic screening. The question is how to detect antibacterial agents produced by a filamentous fungus in nature, particularly if it is produced in the soil, the ultimate but highly complex reservoir of fungal diversity.

Moreover, due to the large number of species in nature (Hawksworth, 2001), it is not feasible to evaluate the antibacterial activity of every pure strain, particularly since many filamentous fungi do not produce useful anti-bacterial agents. This may partially explain the failure of conventional screening to discover new antibiotics from filamentous fungi. Nevertheless, since filamentous fungi have proven to be a potent group for the discovery of new antibiotic products (Ho et al., 2003), they were selected for the research leading to this paper. A rapid screening method using mixed agar plate culture (MAPC) with the help of systematic screening by appropriate culture conditions, increases the chance of finding novel antibacterial agents by avoiding pure cultures as the first stage to increase screening efficiency.

MATERIALS AND METHODS

Preparing mixed culture plates

Soil samples were collected from different locations covered with plant litter in the south of China, and all of these locations are common places, so every Chinese citizen is permitted to observe and study in field according to the Law of the People's Republic of China. Cultures were maintained on Potato Sucrose Agar (PSA, 20% potato, 3% sucrose, 1.5% agar). Potatoes were chopped, cooked, and the boiled solution was filtered. Sucrose and agar were added to the filtered solution to make PSA, and then 15 ml of warm liquid PSA was poured into a 100×15 mm sterilized Petri dish. When the agar solidified, two pieces of sterilized filter paper (diameter 9 cm) were laid on the surface and another 20 ml warm liquid PSA was poured into the Petri dish to cover the filter papers. Before the paper was laid, two perpendicular lines were drawn through center of the lower paper, number 1 or number 2 was given to each line, and this face was laid downward on the dish. After the second agar layer solidified, two perpendicular lines were also drawn and numbers were given to each line under base of the dish according to the filter paper (Figure 1). Each soil sample was spread on the surface of the PSA and cultured at 28°C for 8 days, allowing filamentous fungi to form colonies in MAPC. After 8 days of incubation, the upper layers, including the filter papers, upper PSA medium and microbial community were lifted up. The lower filter paper was carefully taken out and the other layers returned to its original position for later study.

Evaluation of antibacterial activity from MAPC

Antibacterial activity of fungal MAPC was evaluated using the lower filter paper by the conventional diffusion method. The lower filter paper was cut into two parts with zigzag shapes (Figure 2) and “line 1” was used as a symmetry line. Gram-positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*) were used at this stage and the assays were carried out using Tryptic soy agar (TSA) medium (Tryptic Soy Broth, Guangdong Huankai Microbial. Sci. & Tech. Co., LTD., China; Agar, Tianjin Kermel Chemical Reagent Co., Ltd., China). Briefly, 25 ml of sterilized TSA was poured into a Petri dish (100×15 mm). After the medium was solidified, the test bacterium was streaked on the TSA surface using a sterile cotton swab. The zigzag-shaped paper was laid on the surface of the agar plate. Antibacterial activity was examined after 18 h of incubation at 37°C. Inhibition zones implied that there were antibacterial substances on the paper secreted by filamentous fungi (Figure 2).

Isolation of filamentous fungi producing antibacterial agents

If the zigzag-shaped paper expressed antibacterial activity, its source was located. The relevant Petri dish was picked out and the active areas in this plate were marked according to the relative areas in the antibacterial test plate (Figure 3). Then the fungal

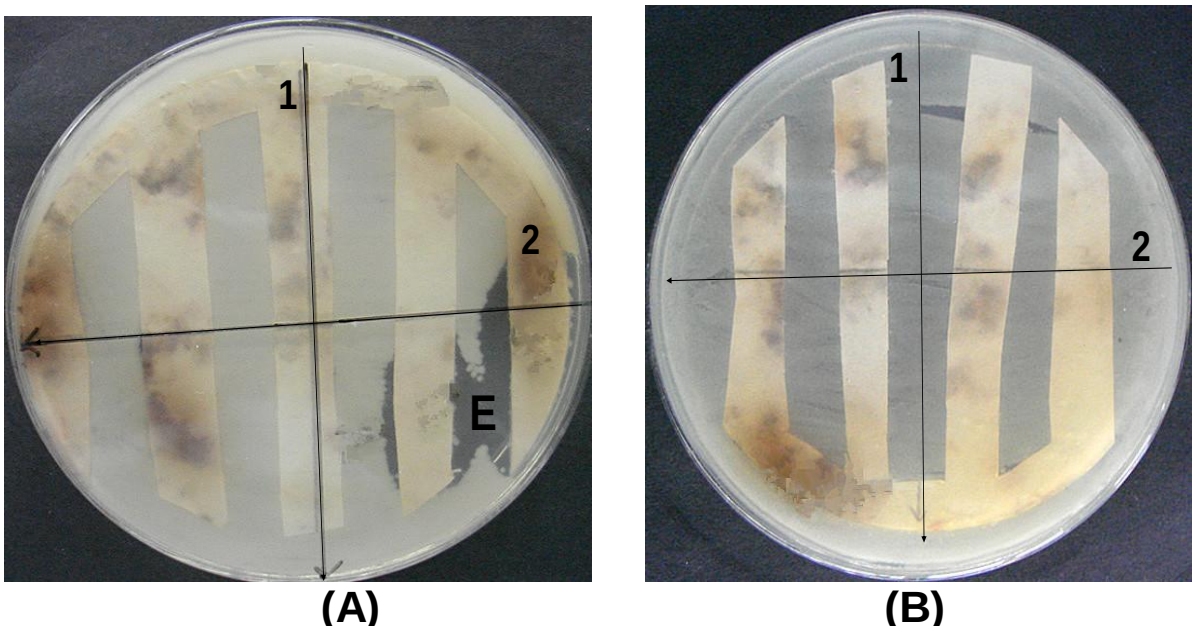

Figure 2. Antibacterial activity of comb-like papers from MAPC. Area **E** showed inhibition against *B. subtilis* in plate A. Plate B did not show any antibacterial activity against *E. coli*.

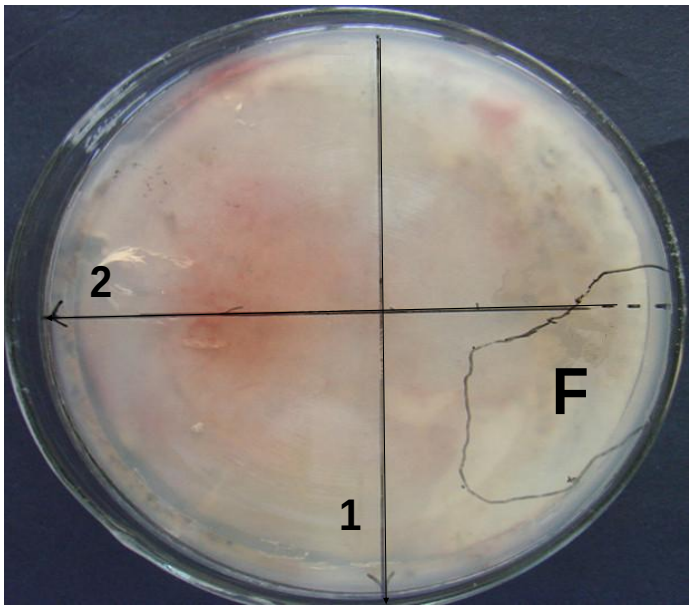

Figure 3. Area F in MAPC plate corresponded to the relevant one (Area E in Figure 2A) expressing antibacterial activity.

colonies in the active areas were isolated following the method described by Zhu et al. (2008). Different types of fungi produce different-looking colonies, so different fungal colonies in the active areas were transferred to separate PSA plates. When hyphae grew into the medium, hyphal tips were transferred and serially transferred until a preliminarily pure culture was obtained. Using this process, antibacterial activity was analyzed with *B. subtilis* and *E. coli* for each pure fungus taken from active areas. After the test bacterium was streaked on the TSA surface, several small pieces of agar media from pure culture plates were carefully put on the TSA surface. Inhibition zones were examined after incubating 18 h at 37°C (Figure 4). Using this technique, the fungi producing antibacterial agents were identified. When a colony of preliminarily pure fungus displayed high antibacterial activity, it was isolated continually to obtain pure fungus. This pure fungus was selected as a candidate for further study against a drug-resistant bacteria pathogen, example *Staphylococcus aureus* ATCC 43300 (MRSA, Methicillin-resistant *S. aureus*) or *E. coli* NDM-1(NDM-1, New Delhi

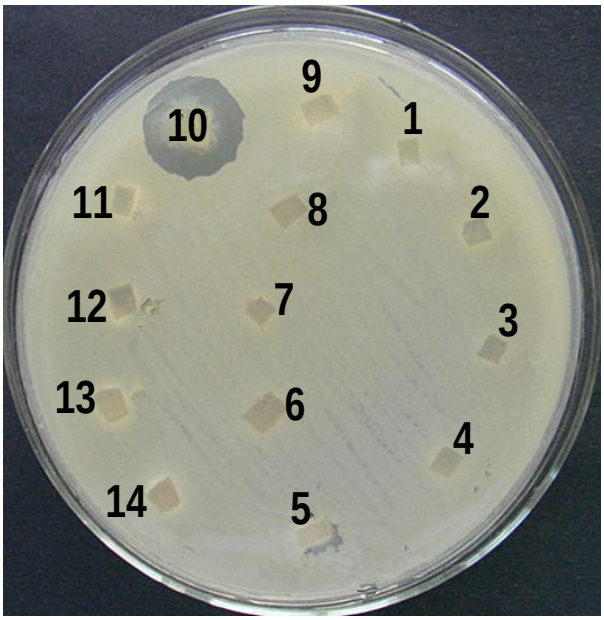

Figure 4. Antibacterial activities of fungi after preliminary purification. The tenth colony in this plate showed inhibition against *B*. *subtilis*.

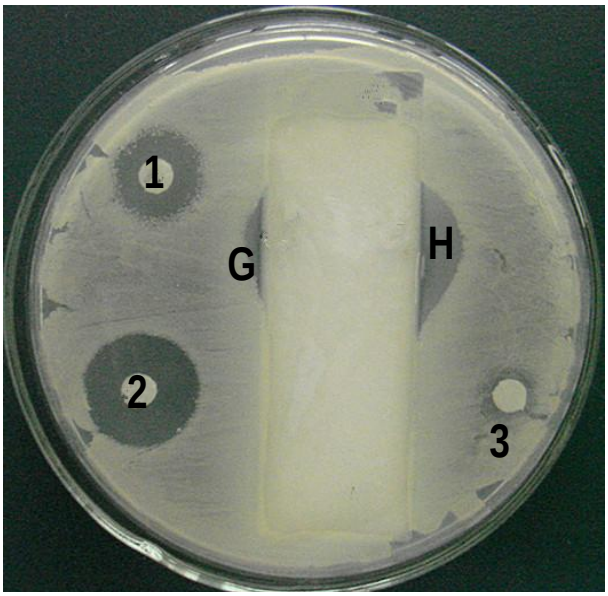

Figure 5. Antibacterial activity against *S. aureus* ATCC 43300**. 1, 5 µg BSI, inhibition zone 13 mm; 2, 5 µg THC, inhibition zone 18 mm; 3, 0.5 µg THC, no clear inhibition zone; G, 0.5 µg THC loaded on TLC; H, 5 µg THC loaded on TLC. **Disk diameter is 6 mm.

metallo-ß- lactamase-1).

Testing antibacterial activity of compounds from filamentous fungi

Using the MAPC method, fungal strains displaying high antibacterial activity were isolated. In this paper, one of these strains was cultured by liquid surface fermentation with potato sucrose (20% potato, 3% sucrose) at 28°C for 7 days. The fermentation broth was separated by filtering and the filtered broth was extracted with ethyl acetate. The ethyl acetate extract was fractionated by silica gel column chromatography and the second fraction eluted with petroleum ether - ethyl acetate (1:1) was re-crystallized and dissolved in methanol for antibacterial testing (all chemical reagents were from Sinopharm Chemical Reagent Co., Ltd., China; silica gel from Qingdao Haiyang Chemical Co., Ltd., China). The crystal called THC, was assayed for antibacterial activity against *S*. *aureus* ATCC 43300 (MRSA) by disk and thin-layer chromatography (TLC) bioactivity assays (Figure 5). Sodium benzylpenicillin (benzylpenicillin sodium for injection (BSI), North China Pharmaceutical Group Corp., China), the most widely used antibiotic in the world, was dissolved in sterile physiological saline and used as the control. In order to increase antibacterial susceptibility, NaCl was added into the TSA to reach a final concentration of 4%.

Identification of the fungus using gene sequencing

Fungal mycelia were picked from the liquid culture for DNA extraction, ground to a fine powder, and then transferred to a 2.0 ml tube. The sample (about 200 - 300 mg of mycelial powder) received 800 µL of extraction buffer (100 mM Tris-Cl, pH8.0, 20 mM ethylenediaminetetraacetic acid (EDTA), 200 mM NaCl, 1% sodium dodecyl sulphate (SDS)) and was incubated for 30 min at 65°C.

After cooling to room temperature, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tube was shaken vigorously to form an emulsion, followed by centrifugation for 10 min at 10000 rpm in a microfuge. The supernatant was transferred to a clean tube and mixed with an equal volume of isopropanol and left in the freezer at -20°C for 1 h. The precipitated DNA was centrifuged at 10000 rpm for 10 min and the pellet was washed twice with 70% ethanol. The dried DNA was dissolved in 500 µL of sterile double distilled H_2O. The DNA samples were sent to Shanghai Gene Star Biotech Co. Ltd and Sangon Biotech (Shanghai) Co., Ltd. for polymerase chian reaction (PCR) and DNA sequencing. Five DNA sequences (18S rDNA, cal gene, act gene, Tef1 gene and rpb2 gene) were analyzed.

RESULTS

Antibacterial testing

For the first stage of the investigation, the aim was to study antibacterial activity displayed by filamentous fungi in mixed culture. Fungi from soil samples were incubated in a Petri dish at 28°C for 8 days. The hyphae of filamentous fungi can grow into their media and secret products into them. Small molecules can easily diffuse into the filter papers between the two layers of agar media, which can later be used to detect antibacterial activity. Fungal colonies form randomly in a plate, leading to unequal distributions of antibacterial agents in the paper, and the filter paper was cut into a comb-like shape to resolve this problem (Figure 2). The upper filter paper helped with the removal of the lower paper. In Figure 2A, the test bacterium *B. subtilis* was inhibited in area E, which implied that some fungal strains colonizing the plate produced antimicrobials against *B. subtilis*. In

Figure 2B, the test bacterium *E. coli,* did not display antibacterial activity, although its comb-like paper was cut from the same lower paper as Figure 2A, suggesting that the antibiotics detected would be specific for Gram positive bacteria. These results demonstrate the value of the MAPC method as a screening tool for discovering new antibiotics produced by filamentous fungi.

Identifying fungi producing antibacterial agents

In order to recover the fungi producing the observed antibacterial activity, the active areas in the Figure 2A plate were marked in the original plate (MAPC plate) and the fungal colonies on these areas were isolated (Figure 3, area marked as F is the active zone).

After colonies of different appearance in the active area "F" were transferred to PSA plates, the colonies formed were used to assess the activity of isolated fungi. Fourteen small pieces of PSA media with fungal growth were placed on the TSA surface streaked with bacterium in advance. There was only one inhibition zone against *B. subtilis* (Figure 4). The ninth and tenth colonies were isolated from the active area F in the MAPC plate, while the others were isolated from inactive areas in the same plate. These results implied that the fungi expressing antibacterial activity can be isolated only from active area, but not from inactive area. The isolation process continued to obtain pure strains from this colony and its antibacterial activity was determined over the entire process of isolation. The other colonies did not exhibit activity against *B. subtilis* and were excluded from further analysis.

Analyzing the potential effectiveness of compounds produced by fungi

Several filamentous fungi producing antibacterial activities were isolated by MAPC method and their antibacterial activities remained. One of them was further studied and its products were extracted and purified for preliminary characterization. In order to evaluate the potential value of these substances, their antibacterial activities were assayed against *S. aureus* ATCC 43300 (MRSA) by disk and thin layer chromatography (TLC) bioactivity assays. As shown in Figure 5, the fraction of the fungal extract THC showed higher activity than BSI with a larger and clearer inhibition zone at a load of 5 µg each disk, but the inhibition zone of 0.5 µg THC on disk was not clear. THC can be developed on a TLC plate (TLC eluent, petroleum ether: ethyl acetate: methanol = 1:1:1) to show that only one spot expressed antibacterial activity at a load of 5 µg and 0.5 µg. THC is stable at room temperature for 2 years, but its activity will lose after incubation for 30 min at 50°C. These results implied that THC was worthy of further study for the potential function.

Identifying the fungus by gene sequencing

Five DNA sequences (18S rDNA, cal gene, act gene, Tef1 gene and rpb2 gene) were sequenced. From the information of a blast search against GenBank (GenBank accession number: 18S rDNA, HM000037; cal gene, HM016865; act gene, HM016866; Tef1 gene, HM016867; rpb2 gene, HM016868), 18S rDNA of this strain had maximum sequence identity (99%) with 30 strains of *Trichoderma viride* strain HS-F9 et al., but it had maximum sequence identity (< 97%) with all strains of *Hypocrea virens* species. However, all of the other genes (cal, act, Tef1 and rpb2) had maximum sequence identity (99%) with *Hypocrea virens*. It was deduced that this fungal strain belongs to genus *Trichoderma* and has not been reported previously. It was given the preliminary name, *T. sp*. H09.

DISCUSSION

A number of fungal metabolites such as penicillins and cephalothin are clearly an important source of useful secondary metabolites. However, most of them obtained from fungi have been encountered by application of random screening methods which often leads to re-isolation of known metabolites in the search for new bioactive compounds. Therefore, screening of new fungi expressing activities from various ecological niches is the first step to isolate new fungal metabolites (Lee and Oh, 2006).

In this paper, a new method, called MAPC is described that can be a valuable tool in the search for novel and more efficacious antibacterial agents derived from filamentous fungi. The method can select promising candidates more efficiently by eliminating less promising samples at the very first stage of screening. By using mixed cultures at the beginning of screening process, screening efficiency increases and a larger number of filamentous fungi taken from their natural habitats can be screened in a given period of time. The traditional method of screening antibacterial from fungi is that samples are suspended in sterile water and vortexed, and the dilutions are used for cultivation, and then pure fungi are obtained to detect their antibacterial activities (Takahashi et al., 2008; Jones, 2010; Kumar et al., 2010). In these processes, a long time will be spent from many samples to many pure fungi, but what is worse, most of them do not produce any antibiotics, which decrease the screening efficiency. Generally, in our screening experiments, we just only isolated one or two fungal strains producing antibacterial compounds directly from about 20 samples, while there are more strains not expressing activity in one sample (Figure 4). This MAPC

method can help us to abandon many unpromising candidates at the first stage and avoid the time- and labor-consuming procedure as before. Another advantage of this method is that the lower paper can be cut into two parts, so that one part is used to test activity against Gram-positive bacteria and another against Gram-negative bacteria in the meantime. Therefore, the screening efficiency is increased by the MAPC method. Also, the compounds discovered with this technique using whole-cell screening give new hope for discovering antibiotics from natural products (Lam, 2007; Suqimoto et al., 2008).

Many microorganisms cannot be cultured using traditional methods. It has been reported that the majority (99%) of microorganisms from the environment resist cultivation in the laboratory, but new techniques are rapidly improving cultivability (Kaeberlein et al. 2002). Suqimoto et al. (2008) established a new simple method using an anthracycline antibiotic that increased success in isolating rare funga genera. Zhu et al. (2008) described a technique for isolating mycorrhizal fungi from roots of orchids that increased the isolation efficiency of previously uncultivable fungi. Given the appropriate culture conditions, the MAPC technique can be employed to discover novel antibiotics produced by common, rare and “uncultivable” species from different ecological niches of soil, water, plants, etc (Warcup, 1950; Haque et al., 2005; Hageskal et al., 2009). Therefore, this method can systematically screen potential antibacterial produced by filamentous fungi from various ecological niches, if their metabolites reach a certain amount being up to the standard required for further research.

Now that genome sequencing, new target identification and combinatorial chemistry have not had notable successes to discover effective antibacterial agents (Fernandes, 2006), the MAPC method, holding the advantages of whole-cell and systematic and efficient screening will help people to discover novel antibiotics to meet clinical needs, as more and more new fungal strains will be cultured under laboratory conditions with new culture techniques.

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