Growth medium condition for antibiotic production in novel Micromonospora species

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Abstract

The objective of this study was to investigate the condition of growth medium suitable for antibiotic production of *Micromonospora* sp. strain SMC253 against ESKAPE pathogens, which was observed at 28°C. Antibacterial metabolite production of SMC253 was evaluated on twenty-six different growth solid media using the agar plug diffusion method. Eighteen of growth media exhibited a wide variety of antimicrobial activities against tested ESKAPE pathogens. A wide range of antibacterial activity against all pathogens was observed only in SMC253 growing on two types of growth agar media (B1 and J1), and the corresponding antibiotic was subjected to ethyl acetate extraction and retested against ESKAPE. It is clear that among the 26 designed growth media SMC253 growing on the B1-agar medium produced the highest yield of the broad-spectrum antibiotic.

Keywords: *Micromonospora* / Growth medium / Antibacterial

Introduction

Bacterial infections have still become a major problem of global healthcare in the twenty-first century. There are due to high occurrence in antibiotic resistance of pathogens, and the emerging in novel strains of infectious agents (Alanis, 2005). Infectious disease, especially hospital-acquired infections (HAIs), has become a major public health problem due to the emergence of antibiotic resistance especially multidrug resistance (MDR) in microbes. The majority causative pathogens composed of *Enterococcus* species, *Staphylococcus aureus*, *Klebsialla pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, are collectively grouped under the acronym ESKAPE (Boucher et al., 2009). Report from ECDC in 2009 at least 25,000 patients in the EU were died per year from infected antibiotic-resistant bacteria. The CDC report in 2013 estimated antibiotic-resistant pathogens causing over 2 million illnesses and 23,000 deaths per year in the United Stated (Laxminarayan et al., 2013). In Thailand, patients at least 30,000 died

from infected ESKAPE pathogens, which were resistant to first-line drugs, lead to a prolonged illness and treatment (Thamlikitkul, 2014). Until now, it is convincing that microbial natural products are still crucial source of novel antibiotic to treat infectious diseases. Actinomycetes are widely accepted as a dominant producer for natural bioactive compounds. Of the 22,500 microbial metabolites obtained from microbes, 45% are produced by actinomycetes, 38% by fungi, and 17% by other bacteria. Over 10,000 bioactive compounds from actinomycetes, 74% (7,600 compounds) are derived from *Streptomyces* and 26% (2,500 compounds) from rare actinomycetes (Tiwari and Gupta, 2012). The advanced genetic and isolation techniques have been developed to isolate rare actinomycetes. It has been reported that rare actinomycetes are capable of producing several novel antimicrobials, e.g. erythromycin, rifampicin and gentamicin, which are produced by *Saccharopolyspora erythraea*, *Amycolatopsis mediterranei* and *Micromonospora purpurea,* respectively (Tiwari and Gupta, 2014). Afterwards, the genus *Micromonospora* became an important source in the drug discovery. Over 740 antibiotics have been isolated from *Micromonospora* species, including rosamicin, everninomicins and neomacquarimicin (Boumehira et at., 2016). It is considered as a potential source of bioactive compounds which have been useful for human medicines such as antibacterial, antiviral, anticancer, antiparasitic and immunosuppressant.

In general, most of the antibiotics in use today are secondary metabolites, which their biosynthesis are regulated by carbon, nitrogen, phosphate, trace elements, precursors, catabolic repression, feedback inhibition, and autoregulators. In addition, the production of secondary metabolites by actinomycetes is strain-specific and dependent upon various parameters, such as incubation period, temperature, and initial pH. Use of appropriate nutrients for fermentation is usually related to improve antibiotic production (Bunch et al., 1986). One of the most crucial factors influencing the antibiotic production includes medium composition. Under different culture conditions, ability of rare actinomycetes to produce antimicrobials can be dramatically increased or decreased. Thus, a particular growth medium composition has significant impact on antibiotic production in individual actinomycetes that is a prerequisite before optimization of antibiotic production and characterization (Goodfellow, 2012). Recently, a new species of rare actinomycetes *Micromonospora* sp. has been isolated in our lab and considered as a potent source for searching novel antimicrobials. In this study, we attempts to investigate the growth medium condition suitable for antibiotic production in *Micromonospora* sp., and to study antimicrobial activity against ESKAPE pathogens.

Methodology

Cultivation and characterization of *Micromonospora* sp. SMC253

Micromonospora sp. SMC253 was previously isolated from soil in Thailand (unpublished data, Associate Professor Dr. Manee Chanama, Department of Microbiology, Faculty of Public Health, Mahidol University). The pure culture was stored in 20% glycerol at -80°C as spore suspension stocks. The colony morphology and color of *Micromonospora* sp. was observed on the International Streptomyces Project (ISP) No. 2, No. 3 and No. 4 agar media after 14 days of incubation at 28°C (Centore, 2016).

Bioassay for antibacterial activity

The antibacterial activity was tested against ESKAPE pathogens, including *Enterococcus* species, *Staphylococcus aureus*, *Klebsialla pneumoniae*, *Acinetobacter baumannii*, *Pseudomonar aeruginosa*, and *Enterobacter* species. The test ESKAPE pathogen was isolated as a single colony on tryptic soy agar (TSA) and subsequently cultivated in tryptic soy broth (TSB) at 37°C for 24 hour. A sterile cotton swab was then dipped into the culture broth and rubbed on the surface of Muller Hinton agar (MHA) plate. The plate was ready-to-use in the antibacterial activity assay. The antibacterial activity of the SMC253 against the ESKAPE was carried out as stated by the agar plug diffusion or disc diffusion method (see below).

Growth media for antibiotic production in *Micromonospora* sp. SMC253

To make a seed culture of *Micromonospora* sp., the SMC253 was streaked and grown on an entire surface of the ISP3 agar at 28°C for 14 days. To screen an appropriate antibiotic production media, 26 types of growth medium recipes with different carbon and nitrogen sources were designed (Table 1), and streaked with a loopful of bacterial seed culture, then incubated at 28°C for 21 days. Antibacterial activity in the designed growth agar medium was measured at 7-day intervals (day 7, 14 and 21) using the agar plug diffusion method (Arasu et al, 2014). Briefly, an agar containing SMC253 mycelia was cut out by using a sterile pipette tip and placed over the surface of MHA plate previously spread with the test pathogen (ESKAPE). A negative control was prepared from an agar plug without SMC253 mycelia. The antimicrobial activity obtained in each growth medium recipes was monitored by observing an inhibition zone surrounding the agar plug after 37ºC-incubation for 24 hour. A diameter of clear zone was recorded in millimetres.

Extraction of the corresponding antibacterial potential from the growth agar medium

An agar extraction method as described by Williams et al., 2017 was used to extract an antibacterial agent from the growth solid medium. The SMC253 was cultured at 28°C on selected growth agar medium that supported antimicrobial production, then antibacterial agent accumulated in agar was extracted twice by ethyl acetate at 7-day intervals (day 7, 14 and 21). The ethyl acetate extract was concentrated under vacuum. The dried residue was dissolved in a small amount of ethyl acetate and stored at -20°C until use. To determine antibacterial activity the extract was subjected to test against the ESKAPE pathogens using the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI). Briefly, the 6-mm paper disc soaked with the ethyl acetate extract was placed on the surface of MHA plate that was inoculated with the bacterial pathogens as mentioned earlier. After incubation at 37°C for 24 hour, the antibacterial activity was measured and recorded as mentioned previously.

Results

Micromonospora sp. SMC253 had ability to grow on all types of designed growth media, but exhibited significant difference in antibiotic productions. The agar plug assay of agar cultures obtained from 26-types of designed growth media demonstrated that only eighteen types of the media supported the production of antibacterial against the ESKAPE pathogens with difference in activity strengths and production times (Table 2). Moreover, SMC253 growing on B1- and J1- growth media exhibited the inhibitory activity on all ESKAPE pathogens with a long production period of 21 days. Therefore, B1- and J1- growth media were selected and used in production of this antibacterial.

From results given in Figure 1 it is clear that the highest yield of antibacterial production corresponding to antibacterial activity was observed in the ethyl acetate extracts from B1- and J1- agar media which the SMC253 was cultured on for 21 days. However, the magnitude of antibiotic production appeared to be higher in B1- growth medium. Thus B1 might be an ideal growth medium suitable for the SMC253 to produce a broad-spectrum antibacterial against ESKAPE pathogens.

Discussion and conclusion

Cultivation of *Micromonospora* sp. SMC253 on different undefined growth media has an influence over the ability for antibiotic production. It has been reported that changing in sources of carbon, nitrogen, phosphate or trace elements affected the formation of biomass

and production of metabolites. Complex medium is mostly preferred to give higher fermentation yields (Dahod et al, 2010). Twenty-six growth media based on different carbon and nitrogen sources were used in cultivation of *Micromonospora* sp. SMC253 for optimizing the antimicrobial production. Only B1- and J1- types of the culture media were chosen as optimum production media on the basis of a broad range of antimicrobial activities against pathogen tested. Antibacterial activities against the ESKAPE in B1- and J1- cultures became appearance early on Day 7 and much stronger on Day 14 and Day 21. The largest sizes of the inhibition zones against the ESKAPE pathogens (both Gram-positive and Gram-negative) were produced by the extract of B1- culture medium which indicated that this type of growth medium strongly enhance the biosynthesis of a broad spectrum antibiotic. However, at the stage of antibiotic production in different types of culture/growth media, different antibiotics might arise and give synergistic or antagonistic response. This evidence was described by Lancini et al (1982). The production of antibiotics is not rigorously species specific. The same antibiotic activity could be produced by different species of the same genus. On the other hand, one species could produce different antibiotics. Further studies will attempt to isolate, purify and elucidate the chemical structure(s) of the antibacterial compound(s). The pure antibacterial compound will be evaluated as chemotherapeutic agent against a wide variety of clinical and multidrug resistant pathogens in the future.

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