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***DETECTION OF ANTIMICROBIAL COMPOUNDS FROM  
THERMOPHILIC ACTINOMYCETES USING ONE STRAIN MANY  
COMPOUNDS (OSMAC) APPROACH***

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**Abstract**

*Actinomycetes are a group of filamentous bacteria with high biosynthetic potential that can produce secondary metabolites. Actinomycetes are known to produce secondary metabolites which are potential as antimicrobial, antitumor, and others. Actinomycetes can be found abundantly in diverse environments, including environments with extremely high temperatures such as hot springs, deserts, geothermal areas, and hydrothermal vents. They can survive in high temperatures due to their membrane lipids containing straight-chains and more saturated fatty acids that protect the membrane's fluidity to maintain membrane function. Thermophilic actinomycetes are potential producers of thermostable enzymes and bioactive compounds, which are important in the pharmaceutical, health, and industrial fields. Thermophilic actinomycetes are still less explored for novel metabolites and antimicrobial compounds due to the difficulty in isolation, maintenance, and preservation in pure culture. Novel bioactive compounds produced by actinomycetes are conventionally discovered by isolating potential strains and screening the compound bioactivity through various bioassays. A sequence-independent approach, termed the OSMAC (one strain many compounds), has been widely used in natural product research for activating cryptic biosynthetic gene clusters (BGCs) by modifying the growth conditions of a bacterial culture. This approach aims to optimize the number of secondary metabolites produced by one single microorganism. The application of the OSMAC method has been proven successful in revealing the biosynthetic potential of bacteria.*

**Keywords:** Antimicrobial; OSMAC approach; Thermophilic actinomycetes

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## INTRODUCTION

Actinomycetes are Gram-positive bacteria group, non-motile, and mostly aerobic. Actinomycetes have a high G+C DNA content of more than 55%. The name actinomycetes come from the Greek words “aktis” or “aktin” meaning light and “mukes” meaning fungus (Rateb *et al.*, 2018). Actinomycetes are considered to have intermediate forms between fungi and bacteria. This is because actinomycetes formed mycelia and hyphae like true fungi, but their thin hyphae lack membrane-bound cell organelles thus their DNA (deoxyribonucleic acid) and other molecules are freely distributed along the hyphae (Trujillo, 2016). Actinomycetes have a variety of morphologies and complex life cycles (Figure 1). The variety of morphologies includes the presence of substrate or aerial mycelia, the color of mycelia, the production of diffusible melanoid pigments, and the structure and appearance of the spores (Barka *et al.*, 2016). Actinomycetes generally developed two types of mycelia: substrate and aerial mycelia. Vegetative substrate or mycelium grows on the surface of the culture medium to absorb nutrients and develops from the outgrowth of a germinating spore.

Vegetative mycelium develops at a particular stage and will develop aerial hyphae, which grow into the air to produce spores. The production of antibiotic compounds is shown during the formation of aerial hyphae (Hamedi & Poorinmohammad, 2017).

The ability of actinomycetes to survive in diverse ecological niches makes them ubiquitous filamentous microbes which are distributed in both terrestrial and aquatic (fresh and marine waters) ecosystems, and often among the dominant population in their ecosystems (Shivlata & Satyanarayana, 2015; Barka *et al.*, 2016). Most actinomycetes are saprophytic and live as semi-dormant spores in the soil, especially under limited nutrients. In addition, actinomycetes can survive in extreme environments such as acid or alkaline pH, low or high temperatures, high salt content, high radiation, low humidity levels, and nutritional limitations (Zenova *et al.*, 2011). Extremophilic actinomycetes thrive in extreme environments by using adaptive strategies such as the production of specific enzymes and having certain particular proteins known as chaperones which aid in refolding the partially denatured proteins (Singh *et al.*, 2010; Shivlata &

Satyanarayana, 2015; Panda *et al.*, 2018). The extremophile groups include halophiles, thermophiles, acidophiles, barophiles, and psychrophiles (Agarwal *et al.*, 2019). These actinomycetes are the source of various antibiotics, secondary metabolites, and industrial important thermostable enzymes (Solecka *et al.*, 2012; Salwan & Sharma, 2018).

The abundance of actinomycetes attracts researchers to isolate and

identify them. The isolation methods which are commonly used e.g., bacterial isolation methods; selective isolation methods (nutritional selection, selective inhibition); and pre-treatment of the samples (physical, chemical, physical-chemical, enrichment, membrane filter, and integrated methods) (Kumar *et al.*, 2016). Other isolation methods include bacteriophages, molecular techniques, and the metagenomic approach (Tiwari *et al.*, 2013).

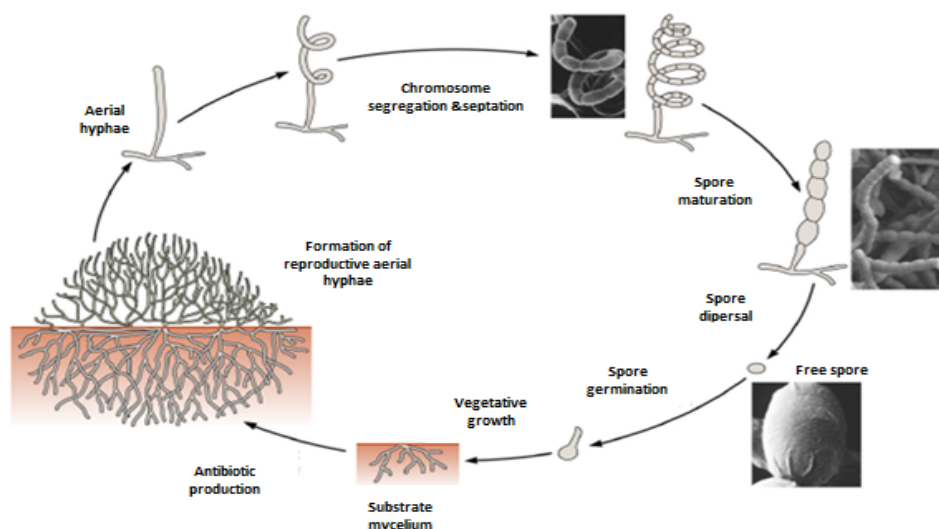


Figure 1. Schematic of the life cycle of sporulating actinomycetes (Barka *et al.*, 2016)

Kurapova *et al.*, (2012) have successfully isolated thermophilic actinomycetes from the Mongolian desert area. The genera *Streptomyces*, *Micromonospora*, *Actinomadura*, and *Streptosporangium* are known to be widely distributed in the Mongolian desert. The genera *Streptomyces* and *Micromonospora* are the most commonly found among others. Moreover, the genus *Micromonospora* represents

thermotolerant and thermophilic actinomycetes. Molecular analysis was carried out by Denaturing Gradient Gel Electrophoresis (DGGE) and fluorescent in situ hybridization (FISH) methods. In soil microcosm research, thermophilic actinomycetes from desert soils are able to grow, develop, and form mycelia with lengths comparable to the mesophilic forms of actinomycetes (Kurapova *et al.*, 2012). The occurrence of the genus

*Streptomyces* was more abundant in soil than in other habitats, especially soils rich in organic matter and alkaline soils.

Rai *et al.*, (2018) conducted a sampling of 30 soil samples from forests, gardens, cultivated land, and riverbanks (Narayani and Rapti Rivers) in Chitwan, Nepal. Six soil samples were taken from each sampling location at a depth of 11 - 15 cm. Bacteria from all samples were isolated and characterized by morphological, microscopic, and biochemical tests. Bacteria from 25 samples were isolated and indicated the actinomycetes group from genera *Streptomyces* and *Micromonospora*. Most of the isolates that were successfully identified originated from forests (9 isolates), riverbanks (4 isolates), gardens (4 isolates), and cultivated land (4 isolates) soils. The genus *Streptomyces* is known to be more dominant (60%; 15 isolates) compared to *Micromonospora* (40%; 10 isolates) (Rai *et al.*, 2018).

The actinomycetes are considered a filamentous bacterial group, with high biosynthetic potential and are capable of producing bioactive metabolites (Schwarz *et al.*,—2018). The genus *Streptomyces* and rare actinomycetes from the order *Actinomycetales* are known as bioactive metabolites producers. The *Streptomyces*

species and rare actinomycetes are currently known to produce 7600 (74%) compounds and 2500 compounds (26%), respectively. About 60% of bioactive compounds are known, which comprise 14.000 metabolites with antimicrobial (antibacterial, antifungal, antiprotozoal), antitumor, and antiviral activities (Berdy *et al.*, 2005). The exploration to discover new metabolites such as antimicrobials from microorganisms is of great interest to researchers. The number of gene clusters identified by bioinformatics analysis using genomic data often shows a discrepancy with the overall results of secondary metabolites from microorganisms based on chemical groups of certain compounds. Many biosynthetic genes remain "cryptic" or "silent", which cannot be expressed under laboratory conditions, and require more effort in finding new metabolites. Therefore, effective methods are needed to activate the expression of these "silent" genes for the possibility of discovering new metabolites for medical and biotechnological purposes (Romano *et al.*, 2018). Several strategies or methods to trigger biosynthetic pathways and produce "cryptic" or "silent" metabolites are known, including the genetic engineering-based approaches. These approaches include promoter exchange, heterologous

expression, and regulatory engineering (ribosome and global regulatory engineering), which are widely used to induce substantial changes in the secondary metabolism of actinomycetes (Kim *et al.*, 2021). In addition, co-culture methods, chromatin remodeling, high throughput elicitor screens (HiTES), and One Strain Many Compounds (OSMAC) are also possible in activating “cryptic” or “silent” metabolites (Okada *et al.*, 2017; Romano *et al.*, 2018).

Ribosome engineering is able to activate “silent” gene clusters by producing mutations in ribosomal components or RNA polymerase (RNAP) using certain antibiotics, resulting in increased production of secondary metabolites (Hosaka *et al.*, 2009). In co-culture techniques, the induction of “silent” gene clusters can result from three modes of interaction such as nutrient supply, competition through the production of antibiotics or signaling molecules, and cell-to-cell contact (Okada *et al.*, 2017). Keller *et al.* (1999) performed chromatin remodeling by supplementing of fungal cultures with histone deacetylase (HDAC) and a DNA methyltransferase inhibitor, which altered the genotype epigenetically. The regulatory pathway of a Sterigmatocystin, a toxin produced by *Aspergillus nidulans*, was investigated and

resulted in suppression of toxin production of several mutants’ strains.

The HiTES method has three important parts, e.g., the selection of microbes, the small molecule library for screens, and the readout. The first appearance of HiTES was done by Nodwell *et al.* (Craney *et al.*, 2012). Among these methods, the simplest method to use is the OSMAC, a culture-based approach that does not need prior knowledge of the biosynthetic gene clusters (BGCs) type and/or the regulatory processes that govern its expression. The OSMAC approach is also an ideal tool that can specifically be used for microbes that are less amenable to genetic manipulation (Romano *et al.*, 2018).

The OSMAC approach can be considered because it can maximize the productivity of silent or cryptic expression of BGCs from microorganisms. The OSMAC approach, through *in vitro* or culture-based variation, includes alteration of physical-chemical factors of culture. The culture condition alterations were as follows: the composition of the media (nutrient content and elicitor chemical), physical parameters (temperature, pH, osmotic pressure, and salinity), ecological factors, biotic and abiotic stressors, and optimization of bioprocesses. These efforts were conducted to selectively activate cryptic

gene clusters, which only be expressed under certain conditions in the natural habitat of these microorganisms (Figure 2) (Bode *et al.*, 2002; Rateb *et al.*, 2011; Pan *et al.*, 2019; Maghembe *et al.*, 2020). Zeeck and colleagues (2002), modified growth parameters such as temperatures, salinity, and aeration, on the fungus *Aspergillus ochraceus*. They later found that the fungus *Aspergillus ochraceus* was able to produce 15 additional metabolites, while previously only known to produce a metabolite called aspinonene (Bode *et al.*,

2002; Romano *et al.*, 2018). The OSMAC approach is carried out under certain growth conditions so that one microorganism strain has the potential to produce several compounds and increase the possibility of discovering new metabolites (Schwarz *et al.*, 2021). Several studies have been carried out using the OSMAC approach to discover new metabolites and assays for the antimicrobial activity from the fungal group (Li *et al.*, 2019; Meng *et al.*, 2017; Ozkaya *et al.*, 2018).

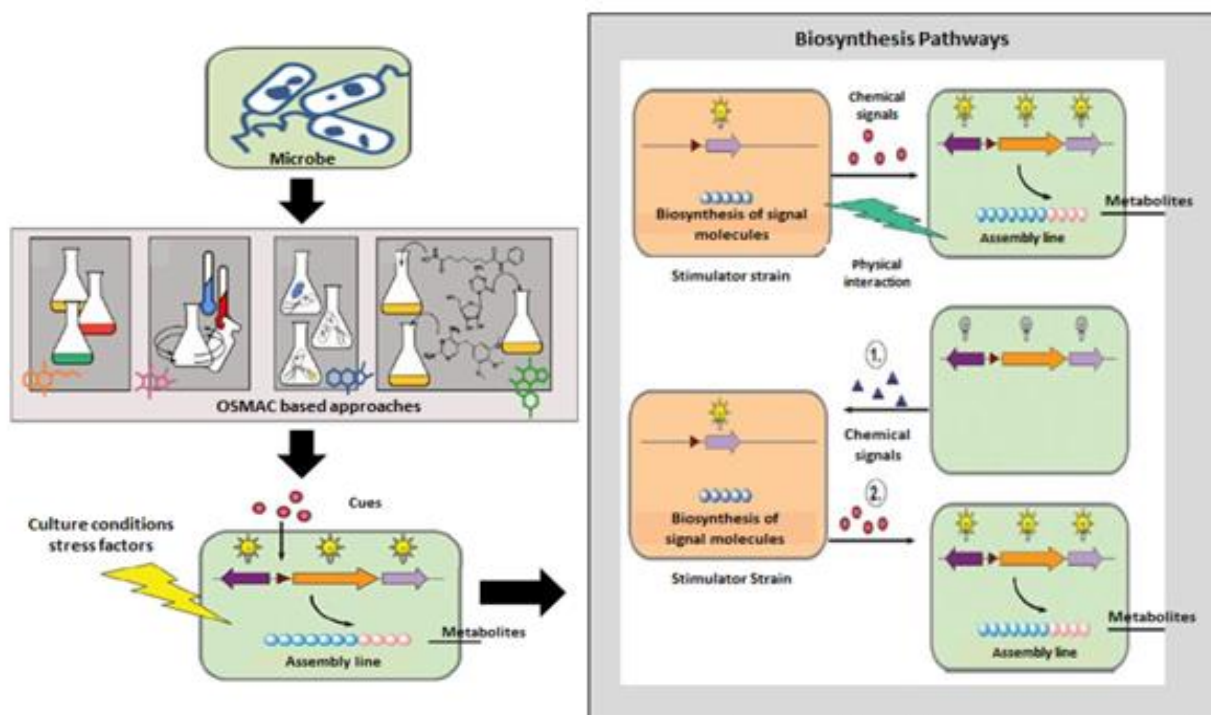


Figure 2. Secondary metabolite production through OSMAC approach and biosynthesis pathways of secondary metabolite (Romano *et al.*, 2018; Scherlach *et al.*, 2009)

Actinomycetes are considered to be the most productive antibiotic producers in the last 40 years. The group

of antimicrobial drugs such as aminoglycosides, macrolides,  $\beta$ -lactams, tetracyclines, glycopeptides, and rifamycin

were successfully introduced to the market and are still used today (Rateb *et al.*, 2018). We have seen microbial resistance to existing antibiotics in the last few decades due to the decline of novel antibiotics discovery. This condition encourages researchers to explore new microorganisms as novel antimicrobial agents with new chemical structures to fight the increasing number of pathogenic microorganism strains resistant to existing antibiotics and for other industrial purposes (Berdy, 2012; Kurtboke, 2012; Tiwari & Gupta, 2012). Exploration of microorganisms in extreme temperature environments has not been widely carried out, especially regarding the exploration of antimicrobial activity and other secondary metabolites so that it can be used as a potential resource to increase the discovery of novel secondary metabolites (Berdy 2012; Tiwari & Gupta 2012). This is due to the difficulty in isolating and maintaining microorganisms as pure cultures (Kikani *et al.*, 2010). Therefore, this article discusses the isolation of thermophilic actinomycetes in relation to their potential as antimicrobial compound producers using the OSMAC approach to discover antimicrobial compounds.

## DISCUSSION

### *Thermophilic Actinomycetes*

Actinomycetes generally have been recorded successfully isolated from normal environments with neutral pH and temperature between 20 and 40°C. However, they are also be found in high temperature range from 40 to 80°C in geothermal area, compost heaps, hydrothermal vents, sea sediment, and hot spring (Shivlata & Satyanaraya, 2015). Actinomycetes can survive at high temperatures due to the membrane lipids containing straight chains and more saturated fatty acids compared to the mesophilic actinomycetes. Thus, the membrane's fluidity at high temperatures is still maintained to carry out the membrane function (Agarwal *et al.*, 2016). In addition, the high temperature can lower the medium's viscosity and prevent contamination (Kikani *et al.*, 2010).

Agarwal *et al.*, (2019) reported the isolation of thermophilic actinomycetes from the Thar desert of Rajasthan and the Heat Generator, Kota. Forty-four soil and sand samples from 11 desert areas and the heat generator were collected for the isolation of thermophilic actinomycetes. Twenty thermophilic actinomycetes isolates were successfully obtained from the samples which were incubated at 65°C. Among the 20 isolates, 18 were known to produce extracellular enzymes (Agarwal *et al.*, 2019).

### **Potential of Thermophilic Actinomycetes as Antimicrobials Producers**

The last 20<sup>th</sup> century, actinomycetes is very beneficial for the health industry as antibacterial, antifungal, antiparasitic, and anticancer agents. There are 45% bioactive compounds of microbial origin, produced by actinomycetes. Moreover, 90% approximately of antibiotics production which produced by actinobacteria from the genus *Streptomyces* (Butler, 2004; Hamaki *et al.*, 2005; Bawazir *et al.*, 2019). The secondary metabolites in actinomycetes, especially the *Streptomyces* group, usually take place at the end of the exponential and the beginning of the stationary growth phase. This period is sometimes referred to as the idiophase (Figure 3), which is related to a tight response due to nutrient limitations (Okada *et al.*, 2017). High temperatures environment, e.g., hot springs which include the extreme environment. They have the potential to produce thermostable enzymes and bioactive compounds which are important in the pharmaceutical, health, and industrial fields (Shivlata & Satyanaraya, 2015). Al-Dhabi *et al.*, (2016) reported

*Streptomyces* sp. Al-Dhabi-1 strain which was isolated at 55°C from soil sediments in Tharban hot springs in Southwest Saudi Arabia. Identification was carried out based on morphological, physiological, and biochemical characteristics as well as the 16S rRNA gene sequence homology with the closely related species. The extract from *Streptomyces* sp. Al-Dhabi-1 strain showed antibacterial activity against *Klebsiella pneumonia* (0.125 mg/ml) and *Streptococcus agalactiae* (<0.039 mg/ml). Antifungal activity was also observed against *Cryptococcus neoformans* (0.078 mg/ml), *Candida albicans* (0.156 mg/ml), *Aspergillus niger* (0.625 mg/ml), and *T. mentagrophytes* (0.156 mg/ml) (Al-Dhabi *et al.*, 2016).

The isolation of thermophilic actinomycetes from geothermal areas in Cisolok, West Java, Indonesia, has been carried out by Yokota *et al.*, (2016) and successfully obtained rare thermophilic actinomycetes isolates from litter sample in the Cisolok geyser. The isolate was identified as *Actinomadura keratinilytica* and has shown antibacterial activity against Gram-positive bacteria, *Kocuria rhizophila* NBRC 12078T, at an incubation temperature of 50°C (Yokota *et al.*, 2016). Nurkanto *et al.*, (2012) successfully obtained 100 actinomycetes isolates from soil and litter from several ecosystems on Batanta and



Salawati islands, West Papua. The non-polar compounds from these actinomycetes isolates were extracted using methanol : ethyl acetate (1:4) and the freeze-drying method, respectively. The compound extraction resulted in 200 extracts which potential for its antimicrobial activities (Nurkanto *et al.*, 2012). The results from the agar diffusion test showed that 43 extracts (21.5%) were active against bacteria and yeasts. The other actinomycetes extracts displayed antibacterial activity against Gram-positive (17%), and Gram-negative (1.5%) bacteria, in addition antifungal activity (17%). Five actinomycetes isolates exhibited the highest antimicrobial activity. These isolates' 16S rRNA gene sequence data were then characterized and identified as genus *Streptomyces*. The isolates displayed the highest homology to *Streptomyces kanamyceticus* (92%), *Streptomyces verne* (92%), *S. narbonensis* (92%), *S. malachitofuscus*

(98%), and *S. hygrosopicus* (96%) (Nurkanto *et al.*, 2012). Actinomycetes isolated from extreme temperature environments are also valuable candidates as thermostable enzyme producers, such as amylase and cellulase (Chaudhary *et al.*, 2016; Jang *et al.*, 2003). Thermophilic actinomycetes strains were also successfully isolated from the soil of the Cisolok geothermal area, West Java, Indonesia, and displayed the ability to degrade soluble starch (Syafitri *et al.*, 2019), carboxymethylcellulose (Setyaningsih *et al.*, 2019), and xylan (Rachmania *et al.*, 2020) at temperatures up to 60°C. Three potential strains were identified as *Actinomadura keratinilytica* based on the 16S rRNA gene data and exhibited amylolytic, cellulolytic, and xylanolytic activities at temperatures of 45-60°C (Syafitri *et al.*, 2019; Setyaningsih *et al.*, 2019; Rachmania *et al.*, 2020). These studies indicated that the geothermal area as one of the extreme temperature habitats has potential for further exploration of thermophilic actinomycetes.

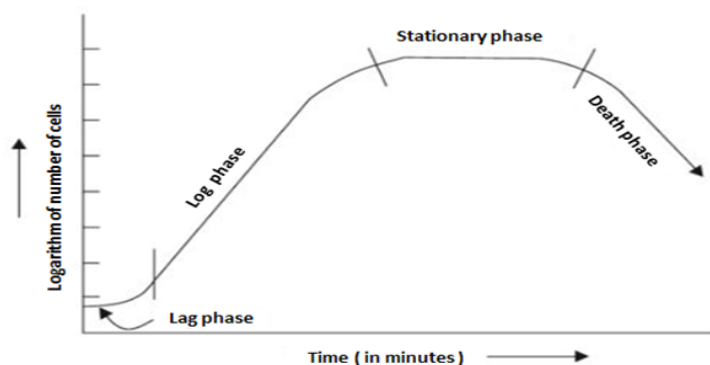


Figure 3. Growth curve of bacteria (Ulhas *et al.*, 2009)

### **OSMAC Approach for Discovery of New Antimicrobials Compounds**

The utilization of actinomycetes for isolation of novel bioactive compounds was discontinued commercially between the years of 1990 and 2000. This situation triggered the rise of antibiotic resistance from pathogenic microbes (Behie *et al.*, 2017). The advanced development of genome sequencing technology and bioinformatics has reinforced the discovery of bioactive metabolites. Various approaches have been used to activate silent or cryptic biosynthetic gene clusters (BGCs) for new metabolites, e.g., co-culture techniques, ribosomal engineering, chromatin remodeling, and HiTES (Okada *et al.*, 2017). Another method that is also considered effective is the one strain many compounds (OSMAC) approach.

The OSMAC approach allowed researchers to induce a single strain to produce more than 20 different metabolites (Bode *et al.*, 2002). The OSMAC approach emphasizes the modification of cultivation parameters, such as medium composition, aeration rate, type of culture container, or the addition of enzyme inhibitors as strategies to activate silent or cryptic BGCs (Bode *et al.*, 2002; Marmann *et al.*, 2004; Meng *et al.*, 2017; & Lima *et al.*, 2018). The OSMAC approach considered factors affecting microbial metabolisms,

e.g., medium composition, pH, temperature, oxygen availability, and light intensity (Rateb *et al.*, 2011). The approach was proven successful in the discovery of new bioactive metabolites. Many researchers have applied this approach in the actinomycetes group as potential producers of active secondary metabolites (Table 1). Sangupta *et al.*, (2015) presented nine out of 54 actinomycetes isolates from the unexplored regions of Sundarbans, which have the potential as bioactive metabolites producers. The isolates were cultivated in four different liquid media (M2, CSM, IM8, and ISP 4) and incubated at 28°C for 8–12 days. Cultivation was also conducted in different incubation periods (24, 96, 192, and 288 hours); temperatures ( $16 \pm 2$ ,  $22 \pm 2$ ,  $28 \pm 2$ , and  $34 \pm 2^\circ\text{C}$ ) for seven days; and salinity concentrations (3, 6, 9, and 12% NaCl) incubated at  $28 \pm 2^\circ\text{C}$  for seven days. Sequence analysis of the 16S rRNA gene revealed that eight isolates showed homology with the genus *Streptomyces*, while one isolate only showed 93,57% homology with *S. albogriseolus* NRRL B-1305T.

Among nine, three strains, SMS\_SU21, SMS\_SU13, and SMS\_7, had high potential for antimicrobial activity. The extract from the filtrate strain was examined for its antimicrobial activity toward bacterial and

fungal strains. The optimum growth and antimicrobial activity were observed in the temperature ranging from 16 to 34°C. The three strains reached optimum growth and highest bioactivity at temperature of 28°C. All isolates were still able to grow and persist its antimicrobial activity at salinity level up to 12%. Isolates SMS\_SU13 and SMS\_SU21 showed declining growth and antimicrobial activity from 3 to 9% salinity level. Meanwhile, isolate SMS\_7 reached the highest biomass at 6% salinity, suggesting halophilic characteristics compared to two other isolates. The crude extracts from isolates SMS\_SU21, SMS\_SU13, and SMS\_7 were then assessed for antimicrobial activity. Extract from isolate SMS\_SU21 demonstrated that it was able to inhibit tested bacteria and fungi, especially the plant pathogenic fungal strains. The highest activity was observed against *Vibrio cholerae* ( $34 \pm 2$  mm) and *Staphylococcus aureus* ( $30 \pm 1$  mm) with a MIC value of 0.05 mg/ml. Meanwhile, isolate SMS\_SU13 was shown active against Gram-negative bacteria, *Pseudomonas aeruginosa* ( $30 \pm 2$  mm, with a minimum inhibitory concentration (MIC) value of 0.05 mg/ml). Isolate SMS\_7 showed moderately active against tested bacteria (MIC values ranged from 0.5-5 mg/ml), but better activity against all fungal strains (MIC values ranged from 0.05-0.5 mg/ml) (Sengupta *et al.*, 2015). The OSMAC

approach was also conducted by Vijayakumar *et al.*, (2012) for antimicrobial activity tests using actinomycetes isolated from marine soil of the Palk Strait area, in the Bay of Bengal, Tamil Nadu, India. The actinomycetes isolates were identified based on 16S rRNA gene sequence and phylogenetic evaluation as strains *Streptomyces* sp. VPTS3-1 and *Streptomyces afghaniensis* VPTS3-1. The growth of strain *S. afghaniensis* VPTS3-1 was observed in various cultivation conditions e.g., culture media, temperature, pH, salinity, incubation period, carbon, and nitrogen sources to induce its secondary metabolites.

The broth media used for cultivation were asparagine-mannitol, ISP 2, ISP 4, ISP 5, starch-nitrate, Kenknight and SC. The isolate was then incubated in a shaker for 7 days. Various growth parameters were also applied on SC broth medium with different temperatures (5, 10, 20, 30, 40, and 50°C), pH (5, 6, 7, 8, and 9), and salinity (1, 2, 4, 8, 16, and 32% NaCl), cultivated for 7 days. The effects of different carbon (maltose, dextrose, glucose, mannitol, starch, and sucrose), and nitrogen (alanine, glycine, phenylalanine, tyrosine, and KNO<sub>3</sub>) sources were also observed in SC broth. Compound extractions were carried out to test the antimicrobial activity against *B. subtilis* (MTCC: 121), *E. coli* (MTCC: 43), *Klebsiella pneumoniae* (MTCC: 39), *Proteus*

*mirabilis* (MTCC: 425), *P. vulgaris* (MTCC: 426), and *Candida albicans* (MTCC: 183).

The results of an antimicrobial test showed that the isolate grown on SC broth medium was active against all tested bacterial & fungal pathogens. The highest antimicrobial activities were observed against *K. pneumoniae* and *P. vulgaris*. The extract from strain VPTS3-1 grown at temperature of 30°C displayed positive antibacterial against *B. subtilis* and *P. vulgaris*, while no activity was observed at temperature of 5 and 10°C. The strain also

showed positive antibacterial against *B. subtilis* and *K. pneumoniae* after incubation for 9-days. The strain's extract which grown at pH 7.0 was also active against *E. coli* and *B. subtilis*. It also active against *P. mirabilis* and *B. subtilis*, when cultivated at 4% salinity. The strain VPTS3-1 did not exhibit antimicrobial activity in glucose-containing medium, while in other carbon sources containing media showed moderate activity towards all the pathogens tested (Vijayakumar *et al.*, 2012).

Table 1. OSMAC approach method in discovering new metabolites and antimicrobial activity.

Strain	Treatment	Secondary Metabolite	Test Bacteria/fungi	Antimicrobial Activity	Reference
<i>Gandjariella thermophila</i> SL3-2-4T, SL3-2-6, SL3-2-7, SL3-2-9, SL3-2-10	Medium modified : -MM1 with gellan gum (7 & 14 days), -MM2 with gellan gum (7 & 14 days), -ISP 3 gellan gum (7 & 14 days)	Indole, Terpene, Siderophore, NRPS-like, Butyrolactone, T2PKS, Ectoine, Nucleoside, Lanthipeptide, Cyanobactin, Bacteriocin, T3PKS, & T1PKS.	- <i>B. subtilis</i> (NBRC 13719) - <i>S. aureus</i> (NBRC 100910) - <i>K. rhizophila</i> (NBRC 12078) - <i>E. coli</i> (NBRC 3301)	-SL3-2-4T and SL3-2-7 (against all Gram-positive on MM2 gellan gum/14 days) -SL3-2-4T and SL3-2-10 (against <i>K. rhizophila</i> NBRC 12078 on ISP 3 gellan gum/ 7 days) -SL3-2-7 (against <i>S. aureus</i> NBRC 100910 and <i>K. rhizophila</i> NBRC 12078 on ISP 3 gellan gum/ 14 days)	(Ningsih <i>et al.</i> , 2020)
<i>Plantactinospora</i> sp. KBS50	ISP 2 medium added with certain biological and chemical elicitors and cultivated at various pH and incubation temperature (ISP 2; ISP 2+NaCl 1%/1,5% ; ISP 2+DMSO 3%; ISP 2+Sc 25 µM / 50 µM; ISP 2+BS cells/ EC cells; ISP 2+AN filtrate; ISP 2+GB filtrate; ISP 2+PPB 5 mM; ISP 2+PPB 30 mM; ISP 2 37°C; ISP 2 pH 9; 1/2 ISP 2; 1/2 ISP 2+GlcNAc 25 mM; ISP 2/GYM; CP100; CP400; NYS100; & MCZ100) *CP = chloramphenicol, NYS = nystatin MCZ100 = miconazole	13 Secondary metabolite compounds (not identified, just namely compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, & 13)	- <i>E. coli</i> (NBRC 3301) - <i>Pseudomonas aeruginosa</i> (NBRC 12689) - <i>S. aureus</i> (NBRC 12732) - <i>B. subtilis</i> (NBRC 3134) <i>Saccharomyces cerevisiae</i> (ATCC 9763) - <i>A. niger</i> (NBRC 4066) - <i>Candida albicans</i> (NBRC 1386)	-ISP 2; ISP 2+NaCl 1%/1,5%; ISP 2+DMSO 3%; ISP 2+Sc 25 µM / 50 µM; ISP 2+BS cells/ EC cells; ISP 2+AN filtrate; ISP2+GB filtrate; ISP 2+PPB 5 mM; ISP 2+PPB 30 mM; ISP 2 37°C; ISP 2 pH 9; 1/2 ISP 2; 1/2 ISP 2+GlcNAc 25 mM; ISP 2/GYM against all test bacteria & fungi, -CP100 against <i>B. subtilis</i> & <i>S. aureus</i> -CP400 against <i>E. coli</i> & <i>P. aeruginosa</i> -NYS100 against <i>A. niger</i> & <i>S. cerevisiae</i> -MCZ100 against <i>C. albicans</i>	(Juboi <i>et al.</i> , 2019)

<i>Streptomyces</i> sp. VPTS3-1 ( <i>Streptomyces</i> <i>afghaniensis</i> VPTS3-1)	-Different medium (Asparagine–mannitol, ISP 2, ISP 4, ISP 5, Kenknight, nutrient medium, starch–nitrate, and SC medium) -SC broth medium with incubated at different carbon sources : dextrose, glucose, maltose, Mannitol, starch and sucrose (10 g/l) -SC broth media with various nitrogen sources : alanine, glycine, phenylalanine, tyrosine, and KNO <sub>3</sub> (2 g/l) -SC media with pH of 5, 6, 7, 8, and 9 with 0.1 N NaOH/0.1 N HCL -SC broth media with various NaCl concentration (1, 2, 4, 8, 16 and 32%), incubated for 28 ± 2°C/ 7- days -SC broth media with various temperatures (5, 10, 20, 30, 40, and 50°C for 7-days)	Identification is not carried out	- <i>Bacillus subtilis</i> (MTCC: 121) - <i>Escherichia coli</i> (MTCC: 43) - <i>Klebsiella pneumoniae</i> (MTCC: 39) - <i>Proteus mirabilis</i> (MTCC: 425) - <i>Proteus vulgaris</i> (MTCC: 426) - <i>Candida albicans</i> (MTCC: 183)	-SC medium against all pathogen test bacteria & fungi - Asparagine–mannitol broth extract had moderate antimicrobial activity - all other medium showed minimum activity - Highest antimicrobial activity at 30°C and practically there was no activity at 5 and 10°C -The highest antimicrobial activity was detected at pH 7.0 and 4% NaCl concentration -The strain did not showed antimicrobial activity in glucose containing antimicrobial activity in glucose containing media and moderate activity in other carbon sources towards all the pathogens tested	(Vijayaku mar <i>et al.</i> ,2012)
<i>Streptomyces</i> sp. strain (SMS_SU21; SMS_SU13 & SMS_7)	-Different media (M2, CSM, IM8, and ISP 4) -Incubation Periods: M2 broth (for SMS_7) & CSM broth (for SMS_SU13 and SMS_SU21) were inoculated and incubated for 24, 96, 192 and 288 hours. -Different temperatures viz. 16 ± 2, 22 ± 2, 28 ± 2, and 34 ± 2°C for 7 days -Different NaCl concentrations (3, 6, 9, and 12%).	2, 2-propyl-N-ethylpiperidine; 4-Dichloromethyl-5 6-epoxy-2-methoxy-4-methyl-2-cyclohexenone; 1,3-cyclopentanedione,2-isopentyl; isoquinoline-1-carbonitrile; & 1,2,4-Triazol 1,5-a pyrimidine,5,7-dimethyl-2-phenyl	- <i>Escherichia coli</i> (ATCC 25922) & (ATCC 8739) - <i>Staphylococcus aureus</i> (ATCC 25923) - <i>Bacillus subtilis</i> (ATCC6633) - <i>Vibrio cholera</i> (MTCC 3906) - <i>Pseudomonas aeruginosa</i> (ATCC 27853) - <i>Enterobacter aerogenes</i> (ATCC 13048) - <i>Salmonella typhi</i> (ATCC 6539) - <i>Salmonella typhimurium</i> (ATCC 14028) - <i>Saccharomyces cerevisiae</i> (ATCC 9763) - <i>Candida albicans</i> (ATCC 0231) - <i>Aspergillus niger</i> (ATCC 16404)	-SMS_SU21 inhibited almost all the bacterial and fungal test strain -SMS_SU13 was active against Gram-negative strain ( <i>P. aeruginosa</i> ) -SMS_7 was active against bacterial and all fungal test strains.	(Sengupta <i>et al.</i> , 2015)

Hussain *et al.*, (2017) employed the OSMAC approach on actinomycetes strain AS 08 isolated from soil in the Thajiwas Glacier, West Himalayas. It was successfully identified as *Lentzea violacea* strain AS08 which exhibited as

sesquiterpenoid compound, a new type of virginiae butanolide and butyl isobutyl phthalate, producer. The OSMAC approach was used by cultivating strain on three kinds of media: CYPS, SCP-1 and SC. The ethyl acetate extracts were obtained and

subjected to HPLC analysis. The results showed that extract from the strain grown on SC medium gave more peaks, and thus was selected for large scale culture. The crude extract was then analyzed using silica gel column chromatography for fractionation and separation of compounds. Three different compounds were successfully obtained and then characterized the structures by NMR analysis. The compounds were identified as eudesmane type novel sesquiterpenoid derivatives (compound 1), new homologue of virginiae butanolide E (compound 2), and butyl isobutyl phthalate (compound 3). Antimicrobial tests displayed that compounds 1 and 2 were moderately active against Gram-negative (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) compared to Gram-positive (*B. subtilis*, *E. faecalis*, *M. luteus*, *S. aureus*, and *S. epidermidis*) bacteria, with MIC values ranging from 32–64 µg/ml and 64–128 µg/ml, respectively (Hussain *et al.*, 2017).

### **Successful OSMAC Approach**

The OSMAC is still considered an effective approach that can induce silent BGCs in microorganisms to produce novel secondary metabolites. The research for the exploration of actinomycetes as one of

potential secondary metabolites producers is further needed (Wu *et al.*, 2015). The alteration of cultivation conditions, including media composition, the addition of enzyme inhibitors, and biosynthetic precursors, mostly contributed to the success of OSMAC approach (Pan *et al.*, 2019). The carbon or nitrogen (C/N) ratio, metal ions, and salinity, on culture media can induce the regulation of gene clusters expression to produce various secondary metabolite products. Carbon and nitrogen sources are significant as energy source and for synthesis of essential proteins or nucleic acids, respectively, in addition to as building blocks of secondary metabolites (Ruiz *et al.*, 2009; Singh *et al.*, 2017, Pan *et al.*, 2019). Salinity can influence the growth and maintains the osmotic pressure of microorganisms resulted in various biochemical reactions (Wang *et al.*, 2011). Metal ions, tensides, precursors, solvent and other small molecule elicitors can affect the function and physiological structure of microorganisms and induce production of secondary metabolite (Schwarz *et al.*, 2021).

The different culture media components affected microorganisms to adjust their metabolism by expressing specific sets of BGCs, resulting in the

biosynthesis of various specific metabolites (Ma *et al.*, 2009). Culture media play significant roles in ensuring the availability of nutrients and affected biochemical reactions. Cultivation conditions such as suitable temperature, pH, oxygen concentration, and solidifying agents are essential for microorganisms' growth and biochemical reactions. Temperature availability affect biochemical reactions, enzyme activation, and activating different functional gene clusters to produce secondary metabolites (Lind *et al.*, 2016; Keller, 2019). Meanwhile, pH is influential during the microbial fermentation process, decomposition, utilization of nutrients or controls the energy and regulation of chemical speciation (Jin & Kirk, 2018; Pan *et al.*, 2019). Enzymes inhibitors such as monooxygenase and hydroxylase play an important role in regulating the biosynthesis of secondary metabolites. These enzymes activity can be inhibited by some chemicals in the biosynthetic pathways and induce the advancement of other metabolic pathways. Biosynthetic precursors are chemicals added to the fermentation media that can change the biosynthetic pathway of secondary metabolisms and produce new compounds (Ramm *et al.*, 2017).

Modification of the media composition using the OSMAC approach

for the discovery of new metabolites from actinomycetes that have potential as antimicrobials has been carried out by Ningsih *et al.*, (2020). The study was conducted using rare thermophilic actinomycete, *Gandjariella thermophila* SL3-2-4<sup>T</sup>, and its closely related strains SL3-2-6, SL3-2-7, SL3-2-9, and SL3-2-10. The five strain were obtained from the forest soil under the bamboo trees in Cisolok geothermal area, West Java, Indonesia. The results of NCBI BLAST displayed that strain *G. thermophila* SL3-2-4<sup>T</sup> was a novel genus and species from the family *Pseudonocardiaceae*. Additionally, another four strains were closely related with 99.7-100% homology to the type strain. The OSMAC method was used to modify the solidifying agent in the culture medium using gellan gum and changes in the incubation period. The media used in the study were MM1, MM2, and ISP 3. The aerial mycelium and spore formation in *Actinobispora yunnanensis* IFO 15681T were stimulated by adding gellan gum as a compacting agent. Additionally, it was also observed in other rare actinomycete genera, e.g., *Planobispora*, *Planomonospora*, and *Sporichthya*. In addition, gellan gum is also used in the isolation media of new bioactive metabolites producing strains. The results of the antibacterial activity test displayed that two strains, SL3-2-4<sup>T</sup> and

SL3-2-7, were active against all Gram-positive bacteria (*Bacillus subtilis* NBRC 13719, *Staphylococcus aureus* NBRC 100910, and *Kocuria rhizophila* NBRC 12078) test strains on MM2 gellan gum media incubated at 45°C for 14 days. Strains SL3-2-4<sup>T</sup> and SL3-2-10 showed positive activity against *K. rhizophila* in ISP 3 gellan gum medium incubated for 7 days. Meanwhile, strain SL3-2-7 was incubated in ISP3 medium for 14 days showed active against *S. aureus* and *K. rhizophila*. All strains did not show activity against Gram-negative *E. coli* NBRC 3301 in the test medium. Strains SL3-2-6 and SL3-2-9 did not show antibacterial activity against all tested bacterial strains in all culture media which incubated for 7 and 14 days. The study showed that *G. thermophila* SL3-2-4<sup>T</sup> and other closely related strains have the ability to produce secondary metabolites. The genome sequence of strain SL3-2-4<sup>T</sup> was also analyzed using the antiSMASH and successfully revealed the presence of low and no-similarity BGCs compared with published known clusters which suggested the high potential of strain SL3-2-4<sup>T</sup> to produce novel bioactive compounds (Ningsih *et al.*, 2020).

Despite the effectiveness, the OSMAC approach can also be time-consuming and challenging since the alterations of many

cultivation parameters were difficult to determine (Okada *et al.*, 2017). Recently, it is known that thermotolerant actinomycetes can be cultured on temperature between 30 to 45°C, and could produce secondary metabolites. Saito *et al.* (2020) reported that actinomycetes cultured at high temperatures could produce as many as 131 secondary metabolites. However, modification of one growth parameter in cultivation conditions can be achieved optimally if supported by other approaches, such as mathematical statistics, to identify more efficient and effective manipulation parameters. A mathematical-statistical approach could reduce the number of tested variables, processing time, and labor costs. Modifying growth parameters such as carbon sources with various concentrations is possible if there is little or no information regarding the ability of microorganisms to produce metabolites (Singh *et al.*, 2017). Previous studies using the OSMAC approach emphasized variation of culture media and cultural conditions (Bode *et al.*, 2002).

## CONCLUSION

Actinomycetes can be found abundantly in extreme environments with



high temperatures condition. Thermophilic actinomycetes were known to produce various metabolites. These metabolites were potential used to antimicrobial for against pathogenic microorganisms. The development of advanced biotechnology and bioinformatics has facilitated the discovery of these metabolites by activating the actinomycetes BGCs, which were previously cryptic or silent under laboratory conditions. The OSMAC approach is one method that has been able to activate cryptic or silent BGCs, especially which produce active metabolites potential in inhibiting the growth of pathogenic microorganisms.

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