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## Antibacterial activity of a thermophilic actinobacterium *Streptomyces cellulosa* SL2-2-R-9 on different growth media

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**Abstract.** Thermophilic *Actinobacteria* are known as potential producers of novel antimicrobial compounds. However, the optimum growth medium for antibacterial activity assessment of thermophilic *Actinobacteria* has rarely been reported. This study demonstrated the effects of nine different microbial growth media on antibacterial activity assessment of a thermophilic actinobacterium from the soil in Cisolok geysers, Sukabumi, West Java (Indonesia). The strain SL2-2-R-9 was identified as *Streptomyces cellulosa* based on 16S rRNA gene data (100% similarity). The antibacterial activity was examined by the agar plug diffusion method against five bacterial test strains. The result of antibacterial activity screening showed that SL2-2-R-9 grown on ISP 7 agar and Bennett's gellan gum inhibited the growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *Kocuria rhizophila*. Strain grown on ISP 3 gellan gum inhibited the growth of *B. subtilis* and *S. aureus*, while on 301 agar and TSA, inhibited only *K. rhizophila*. Strain grown on ISP 6 agar and modified Bennett's gellan gum, inhibited only *S. aureus*. Strain grown on ISP 3 agar and SFM agar showed no inhibition zone against all tested bacteria. There was no inhibition observed against Gram-negative bacteria when the strain was grown on all media.

### 1. Introduction

*Actinobacteria* represent the most well-known taxonomic group within the domain *Bacteria*, which produces over 45% of bioactive microbial metabolites [1]. *Actinobacteria* are largely studied for their ability to produce important bioactive compounds such as extracellular enzymes, enzyme inhibitors, and biocontrol agents [2]. Over 80% of known clinical metabolites were produced by the genus *Streptomyces* [3]. *Streptomyces* are the most prolific sources of novel antibiotics and important antimicrobial drug classes such as  $\beta$ -lactams, tetracyclines, macrolides, and glycopeptides [4].

Secondary metabolites in *Streptomyces* are generally produced along with the development of aerial mycelia and sporulation [5]. *Streptomyces* generally produced aerial mycelia and secondary metabolites as responses to the environmental changes and availability of nutrients in their habitat [6].



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In order to optimize the production of aerial mycelia and secondary metabolites, many studies have been conducted to apply different nutrients modifications in growth media [7]. Various media have been developed to induce aerial mycelium production and antimicrobial production of *Actinobacteria* [8]. The solidifying agent also played an important role in inducing aerial mycelia and spore formation in *Actinobacteria* [9].

In the last decades, thermophilic *Actinobacteria* are recognized as valuable sources of important secondary metabolites [10]. Cisolok geothermal area in West Java, Indonesia is one of the high-potential habitats of thermophilic bacteria. In the previous study, Ningsih *et al.* [11] successfully isolated a novel thermophilic *Actinobacteria* genus and species, *Gandjariella thermophila* from forest soil near geysers in Cisolok geothermal area. In another study, Ningsih *et al.* [12] obtained 25 isolates of thermophilic *Actinobacteria* from soil samples in the geyser of Cisolok. Among the 25 isolates, strain SL2-2-R-9 which used in this study, was identified as *Streptomyces cellulosa* based on 16S rRNA gene sequence analysis.

*Streptomyces cellulosa* is known as the producer of fungichromin, a polyene antifungal [13]. Muvva *et al.* [14] reported that *S. cellulosa* VJDS-1 isolated from mangrove ecosystems has antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Lactobacillus casei*, *Xanthomonas campestris*, *Bacillus megaterium*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella entericus*, *Proteus vulgaris*) and fungal isolates (*Aspergillus niger*, *Botrytis cinerea*, *Fusarium solani*, *Fusarium oxysporum*, *Candida albicans*). Zothanpuia *et al.* [15] also reported that *S. cellulosa* DST28, isolated from freshwater sediment, showed great potential as a source of novel bioactive compounds demonstrated by potent antimicrobial activity against five pathogenic bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, *M. luteus*, *B. subtilis*) and yeast (*C. albicans*).

The study on the optimum growth media for antibacterial activity assessment of thermophilic *S. cellulosa* strain SL2-2-R-9 has not yet been conducted. Therefore, this study investigated the effect of nine different media, e.g. International *Streptomyces* Project (ISP) 3 agar; ISP 3 gellan gum; ISP 6 agar; ISP 7 agar; Bennett's agar; modified Bennett's gellan gum; 301 agar; soya flour mannitol (SFM) agar; and tryptone soy agar (TSA), on antibacterial activity assessment of strain SL2-2-R-9. The results of this study will provide information about the effect of each medium on antibacterial activity in order to improve the methods of cultivation and antimicrobial screening of thermophilic *Actinobacteria* from the geothermal area in Indonesia.

## 2. Materials and Methods

### 2.1. Microorganisms

*Streptomyces cellulosa* SL2-2-R-9 used in this study was obtained from the soil sample in the Cisolok geysers, Sukabumi, West Java, Indonesia. This strain was maintained on ISP 1 agar medium at room temperature as stock cultures; stored as agar blocks in 20% (v/v) glycerol at -80°C and as lyophilized cells for long-term preservation.

### 2.2 Preparation of growth media

Nine different media were used for cultivation of strain SL2-2-R-9. ISP 3 agar, ISP 3 gellan gum, ISP 6 agar, and ISP 7 agar media were prepared according to Shirling and Gottlieb [16]. Bennett's agar and modified Bennett's gellan gum were prepared according to Jones [17]. Soya flour mannitol (SFM) agar was prepared according to Hobbs *et al.* [18]. Medium 301 agar was prepared according to Kuncharoen *et al.* [19]. Tryptone soya agar (TSA) (Difco) was also used as a growth medium. The strain grown on each medium was incubated at 45°C for 7 and 14 days.

### 2.3 Screening for antibacterial activity

The antibacterial activity was screened against three strains of Gram-positive bacteria (*Staphylococcus aureus* NBRC 100910, *Bacillus subtilis* NBRC 13719, *Kocuria rhizophila* NBRC 12078) and two strains of Gram-negative bacteria (*Escherichia coli* NBRC 3301 and *Pseudomonas aeruginosa*). All test strains were grown overnight in ISP 1 broth at 30°C and inoculated in Mueller-Hinton agar (MHA, Difco) as overlay agar. The

antibacterial activity was screened using the agar plug diffusion method [20]. The strain grown on each medium was cut using a sterile cork-borer and placed onto the surface of the MHA containing the test strain. Plates were incubated at 30°C, and the inhibition zone (mm) was determined after 20h.

### 3. Results and Discussions

The strain *S. cellulosa* SL2-2-R-9 was grown on nine different media incubated at 45°C for 7 and 14 days. The antibacterial activity screening was conducted using the agar plug diffusion method against three Gram-positive bacteria (*S. aureus*, *B. subtilis*, and *K. rhizophila*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*), then observed after 20 h of incubation. The result of antibacterial screening and inhibition zone diameter is presented in Table 1.

**Table 1.** Antibacterial activities of strain SL2-2-R-9 grown on various media against five test bacterial strains after 20h incubation at 30°C.

No.	Medium	Inhibition zone diameter (mm)									
		<i>S. aureus</i> NBRC 100910		<i>B. subtilis</i> NBRC 13719		<i>E. coli</i> NBRC 3301		<i>K. rhizophila</i> NBRC 12078		<i>P. aeruginosa</i>	
		7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
1	301 agar	-	-	-	-	-	-	6.87	-	-	-
2	ISP 3 agar	-	-	-	-	-	-	-	-	-	-
3	ISP 3 gellan gum	9.63	8.17	7.98	-	-	-	-	-	-	-
4	ISP 6 agar	6.2	-	-	-	-	-	-	-	-	-
5	ISP 7 agar	-	11.2	-	8.8	-	-	-	7.96	-	-
6	Bennett's gellan gum	-	15.8	-	13.3	-	-	-	12.04	-	-
7	Modified Bennett's Agar	-	4.2	-	-	-	-	-	-	-	-
8	SFM agar	-	-	-	-	-	-	-	-	-	-
9	TSA	-	-	-	-	-	-	7.22	-	-	-

(-): no inhibition zone

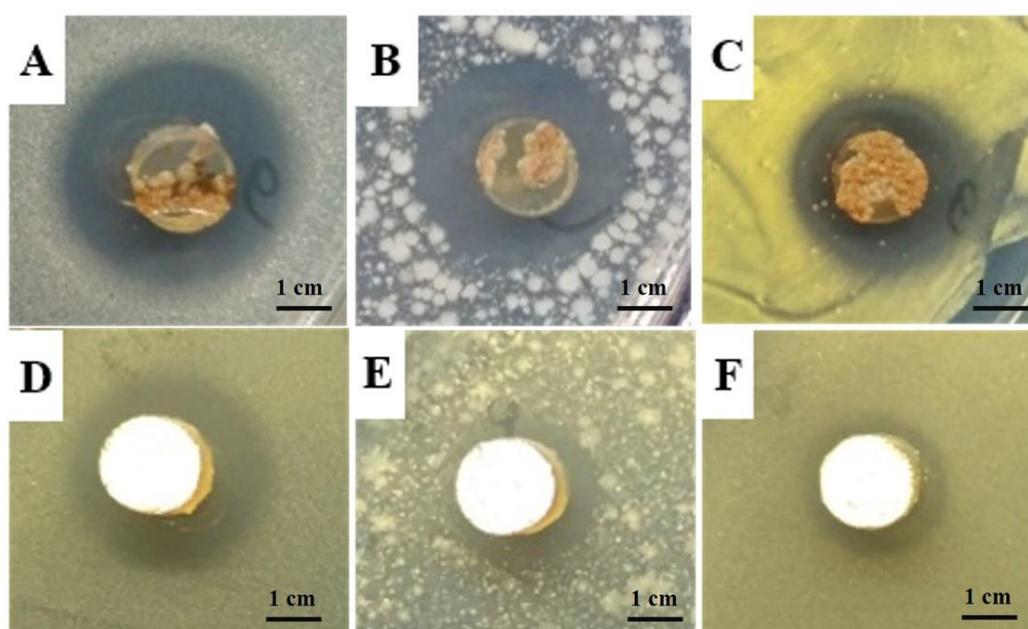
The inhibition zone was observed on strain SL2-2-R-9 grown on seven media: 301 agar, ISP 3 gellan gum, ISP 3 agar, ISP 6 agar, ISP 7 agar, Bennett's gellan gum, modified Bennett's agar, and TSA. The antibacterial activities were observed on strain SL2-2-R-9 grown on ISP 7 agar and Bennett's gellan gum for 14 days. The strain SL2-2-R-9 exhibited an inhibition zone against all Gram-positive bacteria, while no inhibition was observed against Gram-negative bacteria. Strain SL2-2-R-9 grown on ISP 3 gellan gum for seven days of incubation inhibited the growth of *B. subtilis* and *S. aureus*. On 14 days of incubation, this strain inhibited only the growth of *S. aureus*.

Strain SL2-2-R-9 grown on both ISP 6 agar for seven days and modified Bennett's agar for 14 days showed inhibition zones against *S. aureus*. The inhibition zone was also observed on strain grown on 301 agar, and TSA incubated for seven days against *K. rhizophila*. Antibacterial activity was not observed in all tested bacterial strains using ISP 3 agar and SFM agar plugs. The strain SL2-2-R-9 grown on all media did not show antibacterial activity against Gram-negative bacteria *E. coli* and *P. aeruginosa*.

According to Horinouchi and Beppu [21], the ability of actinobacterium to produce antimicrobial compounds depends on nutrient composition in the growth medium. Horinouchi and Beppu [21] stated

that nutrient provided in the substrate affected both morphological development and metabolites production of *Actinobacteria*. Antimicrobial production was influenced by the composition of carbon, nitrogen, and phosphate in the substrate, which initiated complex regulation of secondary metabolites production [4]. The secondary metabolite production is frequently assumed as the result of nutrient limitation and correlates with aerial mycelium [6].

The antibacterial activities of strain SL2-2-R-9 grown on ISP 7 agar and Bennett's gellan gum exhibited strong inhibition against all Gram-positive bacteria (Figure 1). Bennett's medium composition contained yeast extract, beef extract, casein as nitrogen sources, and dextrose as a carbon source [17]. ISP 7 medium contained L-asparagine and L-tyrosine as nitrogen sources [16]. Solidifying agents such as agar and gellan gum also have important roles in the media. Agar and gellan gum are polysaccharides that act as a carbon sources for microorganisms [22, 23]. All other media also contained rich carbon and nitrogen sources but exhibited a small inhibition zone or no inhibition activity.



**Figure 1.** Inhibition zone of strain SL2-2-R-9 grown on Bennett's gellan gum against (A) *S. aureus*; (B) *B. subtilis*; (C) *K. rhizophila*, and SL2-2-R-9 grown on ISP 7 agar against (D) *S. aureus*; (E) *B. subtilis*; (F) *K. rhizophila*

In this preliminary study, we cannot determine which factors that affect the antibacterial activity of strain SL2-2-R-9. According to many scientists, there are many factors that could affect the antibacterial activity of *Actinobacteria*. Filippova and Vinogradova [24] reported that high concentrations of carbon sources such as N-acetylglucosamine in the poor medium could stimulate and accelerate cell development and secondary metabolite production. Conversely, high concentrations of N-acetylglucosamine in the nutrient-rich medium can inhibit the formation of aerial mycelium and secondary metabolites. Liu *et al.* [25] reported that antibiotic production depends on the balance between each nutrient composition as a precursor and repressor on the antibiotic biosynthesis regulation.

The inhibition activity also depends on the type of antibacterial compound produce by the actinobacterial strain. Wanger [26] reported that the inhibition zone's diameter could be an indicator of the sensitivity of the tested microorganisms to antimicrobial compounds. According to Ouchari *et al.* [27] the differences in abilities to produce inhibition zone depend on the metabolites produced by the strain and the test strain resistance to secondary metabolites.

As shown in Table 1, there was no antibacterial activity against all Gram-negative bacteria when *S. cellulosa* SL2-2-R-9 was cultivated on all media. However, Muvva *et al.* [14] reported that *S. cellulosa*

VJDS-1 isolated from mangrove ecosystems has antimicrobial activity against Gram-negative bacteria. Zothanpuia *et al.* [15] also reported that *S. cellulosa* DST28 isolated from freshwater sediment showed antimicrobial activity against Gram-negative bacteria. The different results in this study might due to the difference in their natural habitat condition. According to van der Meij *et al.* [28] many specialized metabolites of *Actinobacteria* are most likely produced specifically in response to ecological demands of both biotic and abiotic factors in their natural environment. The gene cluster that regulates antimicrobial production is tied to the preferred nutrient source, symbiotic interaction with other organisms, and competition with other microorganisms. Thus, many of these specific biosynthetic gene clusters for antibiotics are poorly expressed due to the inability to mimic the environmental triggers under laboratory conditions.

#### 4. Conclusions

The results in this preliminary study showed that the thermophilic actinobacterium strain *S. cellulosa* SL2-2-R-9 grown on ISP 7 agar and Bennett's gellan gum inhibited the growth of all Gram-positive bacteria. The strain grown on ISP 3 gellan gum showed growth inhibition of two tested bacteria, while on ISP 6 agar, modified Bennett's gellan gum, 301 agar, and TSA, inhibited only one tested bacteria. Strain SL2-2-R-9 grown on ISP 3 agar and SFM agar showed no inhibition zone against all tested bacteria. Antibacterial activity was also not observed on all tested media against the Gram-negative bacteria. At present, we cannot determine which factors affect the antibacterial activity of strain SL2-2-R-9. Further studies on the variation of nutrient composition for growth media, solidifying agent, and incubation period for antibacterial screening needed to be clarified.

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