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Potential secondary metabolite analysis of soil *Streptomyces* sp. GMR22 and antibacterial assay on *Porphyromonas gingivalis* ATCC 33277

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ABSTRACT

Infectious diseases caused by oral pathogenic bacteria are currently a serious problem due to the increasing incidence of antimicrobial resistance. *Streptomyces* sp. GMR22, a soil actinobacterium which has large-genome size. In previous studies, it was known to have antifungal, and antibiofilm activity on *Candida albicans*. However, its antibacterial activity on oral pathogenic bacterium, *Porphyromonas gingivalis* is not clear. This study aimed to identify potential active compound based on genome mining analysis and to evaluate the antibacterial activity of GMR22 extract on *P. gingivalis* ATCC 33277. Potential active compounds and biosynthesis gene clusters were analysis using antiSMASH version 5. Antibacterial activity assay was carried out by the microdilution method on *P. gingivalis* ATCC 33277. Based on genome mining analysis polyketide synthase (PKS), the *Streptomyces sp.* GMR22 is the abundant BGCs (35%) and has large-predicted compounds which have antibiotic-antibacterial activity (22.9%). On antibacterial assay, chloroform extract of GMR22 at 7.8 – 62.5 µg/mL has high antibacterial activity on *P. gingivalis* compared to other extracts. Soil *Streptomyces* sp. GMR22 bacterium has biotechnological potential to produce active compounds for antibacterial.

Keywords:
actinobacteria;
antibacterial;
Streptomyces;
Porphyromonas gingivalis

ABSTRACT

Penyakit infeksi yang disebabkan oleh bakteri patogen rongga mulut hingga saat ini masih menjadi masalah serius yang disebabkan oleh peningkatan kejadian resistensi antimikroba. *Streptomyces* sp. GMR22 merupakan aktinobakteri tanah yang memiliki ukuran genom besar. Penelitian sebelumnya, bakteri mempunyai aktivitas antijamur dan antibiofilm pada *Candida albicans*. Namun, belum diketahui aktivitasnya terhadap bakteri patogen oral seperti *Porphyromonas gingivalis*. Penelitian ini bertujuan untuk mengidentifikasi senyawa aktif potensial berdasarkan analisis penambangan genom dan mengevaluasi aktivitas antibakteri dari ekstrak GMR22 terhadap *P. gingivalis* ATCC 33277. Senyawa aktif potensial dan klaster gen – gen biosintesis dianalisis dengan menggunakan antiSMASH version 5. Uji aktivitas antibakteri dilakukan dengan metode mikrodilusi terhadap bakteri *P. gingivalis* ATCC 33277. Berdasarkan analisis penambangan genom *polyketide synthase* (PKS), *Streptomyces sp.* mempunyai klaster gen – gen biosintesis dominan (33%) dan senyawa yang diprediksi memiliki kemampuan sebagai antibiotik – antibakteri (22,9%). Pada uji antibakteri, ekstrak kloroform GMR22 pada konsentrasi 7,8 – 62,5 µg/mL memiliki aktivitas antibakteri tertinggi pada *P. gingivalis* dibandingkan ekstrak lainnya. Bakteri tanah *Streptomyces* sp. GMRR22 memiliki potensi bioteknologi untuk menghasilkan senyawa aktif antibakteri.

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INTRODUCTION

Porphyromonas gingivalis is an opportunistic pathogenic bacterium, commonly found in the human body and especially in the oral cavity, where it is associated with periodontal diseases.[1] Several clinical strains of *P. gingivalis* show moderate susceptibility or resistance to amoxicillin and metronidazole.[1] The other study showed that the *P. gingivalis* samples isolated from periodontitis patients showed relatively similar rates of resistance to amoxicillin (24.6%), azithromycin (21.3%) and metronidazole (24.6%).[2] Some antibiotics maybe does not reach the bacteria in the biofilm, so it requires a higher dose to kill.[3] Efforts are needed to find new sources of antibiotics that are effective against *P. gingivalis*.

Actinobacteria especially genus *Streptomyces* have produced bioactive compounds for more than 10,000 of the 18,000 known bacterial bioactive compounds.[4] These bacteria have a huge genome size, between 6.2 and 12.7 Mb and 5% of their genome is devoted to the synthesis of secondary metabolites.[5] The discovery of new active compounds from Actinobacteria especially genus *Streptomyces* has become an important research because of the resulting novelty in chemical diversity and promising natural products for new drugs.[6]

In previous research, *Streptomyces* sp. GMR22, a soil bacterium[7] demonstrated promising antifungal activity.[8] Based on bioassay studies, this bacterium has the biotechnological potential for drug discovery. In recent years, the drug discovery from *Streptomyces* bacteria is not only based on bioassay procedure but also based on genome analysis and metabolite profiling. The genome mining techniques are currently genome analysis as the solution for accelerating the discovery of new drug candidates.[9] With this technique, biosynthetic gene clusters (BGCs) could be identified from genome analysis and used for the chemical core structures prediction.[10] Over the last 10 years, several new active compounds from *Streptomyces* have been revealed using genome mining approaches.[11]

The huge biotechnology potential of the soil bacteria *Streptomyces* sp. GMR22 drives exploratory research on this bacterium as a source of new antibiotics. This study aimed to identify the potential active compounds of Streptomyces sp. GMR22 using a genome mining approach and to evaluate its activity against *P. gingivalis*. This research is expected to provide information on the potential of *Streptomyces* sp. GMR22 as a source of antibiotics to treat *P. gingivalis* infection.

MATERIALS AND METHODS

Biological material

Streptomyces sp. GMR22, a soil bacterium isolated from the rhizosphere of the Cajuput medicinal plant, *Melaleuca leucadendra* (L), Myrtaceae, in Wanagama Forest, Gunung Kidul, Yogyakarta, Indonesia.[7] The GMR22 isolate has been deposited at InaCC A148, Indonesian Institute of Sciences, and NBRC Japan (NBRC 110112). GMR22 16S RNA sequence has been submitted in the National Center for Biotechnology Information (NCBI), accession code MN922646. The whole-genome shotgun projects have been deposited in Data Bank of Japan/ European Nucleotide Archive/GenBank with accession number JACGSQ000000000 for *Streptomyces* sp. GMR22. *Porphyromonas gingivalis* ATCC® 33277 (Culti-loops™) purchased from Thermo Scientific, Lenexa, USA. *P. gingivalis* was maintained in Brain Hearth Infusion (BHI) Broth at the Microbiology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Morphological analysis

The bacterial cell is grown at 28°C for 14 d on International *Streptomyces* Project-2 (ISP-2) medium (Difco, Spark, USA) were observed by scanning electron microscopy (SEM) (Hitachi SU3500, Japan). The SEM analysis in this study was performed without any chemical fixative.[12] One loop of bacterial biomass was added evenly to the surface of the carbon tape-covered stub. The sample was air-dried and coated with Au (Hitachi MC1000 Au ion sputter, Japan) with a setting of 10 mA for 60 sec and observed using SEM. The SEM settings operated in high vacuum mode, an accelerating voltage of 5 kV, a spot intensity of 30%, and magnifications of 5,000 and 10,000 x. The SEM analysis was conducted at Research Center for Food Technology and Processing, National Research, and Innovation Agency, Gunung Kidul, Indonesia.

Genome mining analysis

The identification, annotation, and analysis of gene clusters involved in the biosynthesis of secondary metabolites and predictions of the core structures produced were conducted by using antiSMASH 5.0,[13]genome mining has seen broad applications in identifying and characterizing new compounds as well as in metabolic engineering. Since 2011, the 'antibiotics and secondary metabolite analysis shell-antiSMASH' (https://antismash.secondarymetabolites.org available at http://antismash.secondarymetabolites.org.

Fermentation and extraction

Streptomyces sp. GMR22 was maintained on ISP-2 Agar medium (Difco, Sparks, USA). *Streptomyces* spp. was cultured at 28 °C with 180 rpm agitation for 2 - 3 d in a 250 mL Erlenmeyer flask containing 100 mL of tryptic soy broth (TSB) (Difco, Sparks, USA) as the seed medium. Then, the cells were transferred into four 1000 mL flasks containing 500 mL of starch nitrate broth (SNB) as the production medium and incubated for 8 d at 28 – 29 °C with 180 rpm agitation in a shaker incubator (Stuart S1500). The SNB medium contained 0.5 g of NaCl, 1 g of KNO_3, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$ and 20 g of soluble starch in 1000 mL of distilled water.[14] All media was sterilized using autoclave machine (Tommy) at 121 °C, 2 atm, for 15 min.

Secondary metabolites were obtained by separating the cell biomass from the liquid using refrigerated centrifugation at 4137 × g at 4 °C for 15 min. The supernatant was extracted two times with an equal volume of ethyl acetate, and evaporated using a rotary vacuum evaporator (Buchi, Switzerland) to obtain the crude extract. The crude ethyl acetate extract was fractionated using chloroform, *n*-hexane, ethyl acetate and methanol to separate the polar and nonpolar fractions. All extracts were then evaporated using a vacuum evaporator (Buchi, Germany). The crude extracts were weighed for yield extract analysis and stored in the refrigerator (Gea). All chemical reagents and solvents were purchased from Merck KGaA, Darmstadt, Germany.

Antibacterial assay

The antibacterial assay against *P. gingivalis* ATCC® 33277 was conducted using the microdilution protocol described in a previous study with minor modification.[15] The extract was prepared in six levels of concentration (7.8 – 62 µg/mL in 0.1% of dimethyl sulfoxide (DMSO)) as solvent and re-diluted using Brain Hearth Infusion (BHI) medium (Merck, Germany). The initial bacterial suspension used for inoculation for assay was adjusted to 5 x 10^5 CFU/mL in BHI medium using

McFarland standard. An untreated growth control (without extract) was included. The plates were incubated at 37°C for 24 h, anerobic condition (AnaeroGen™). Growth inhibition was determined by a spectrophotometer at 0 and 24 h. Optical densities at 600 nm (OD_{600} nm) were measured using a multi-scan reader (Thermo Scientific). All experiments were performed in triplicate. Growth inhibition (%) was determined by change in OD (ΔOD) from the start of incubation to the final time point (24 hours). Growth inhibition was calculated with the following formula: [(ΔOD control –ΔOD test)/ΔOD control] x 100. The IC_{50} was defined as the lowest concentration at which 50% of growth was inhibited. The IC_{50} were determined by nonlinear regression analysis of log_{10} concentrations of the extract against percent *P. gingivalis* inhibition. Statistical analysis was performed using One-way Anova followed by Dunnett's multiple comparison test for analysis of treatment. Nonlinear regression and statistical analysis were done by using GraphPad Prism 9.0.1 software.

Targeted liquid chromatography high-resolution mass spectrometry (LC-HRMS)

Metabolite analysis of the active extract was carried out using ultra-high-performance liquid chromatography (TS Vanquish UHPLC) coupled to targeted high-performance mass spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC paired with Thermo Scientific Q Exactive) (Thermo Fisher Scientific, Bremen, Germany). Targeted LC-HRMS was based on a predicted compound formula which was obtained from the genome mining analysis of *Streptomyces* sp. GMR22 whole-genome sequence using antiSMASH version 5[13] available online at https://antismash.secondarymetabolites.org/. HRMS was carried out with mobile phases A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). The column used was an Accucore™ Phenyl Hexyl analytical column (2.1 mm × 2.6 µm) (Thermo Fisher Scientific) with a flow rate of 40 µL/min, an injection volume of 5 µL, and a gradient with an analysis time of 25 min. The gradient was programmed as follows: 2 min, 5% B; 15 min, 60% B; 22 min, 95% B; 25 min, 95% B; 25.1 min, 5% B; and 30 min, 5% B. Experiments were carried out in full MS data dependent MSMS at 70,000 the full width at half maximum (FWHF) resolution, heated electrospray ionization, positive ionization, and data processing with Thermo Scientific XCalibur and Compound Discover 3.2. The data was analysis using procedures for targeted processing workflows for expected compounds. The mol file of expected compounds was selected and was input in analysis system based on manual procedures of Compound Discoverer version 3.2, user guide for LC studies (ThermoFisher Scientific, 2020).

Ethical clearance

This study has been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (ref. No. KE/0927/09/2020).

RESULT

The GMR22 bacterial strain was observed to be Gram-positive and aerobic, grew well on ISP-2 Agar medium, ISP-4 Agar medium, and TSA after 5 – 7 d at 28 - 29 °C. Morphological observations of the 7-day-old culture grown on ISP2 medium showed a smooth spore surface with aerial and vegetative hyphae. Soil bacterium GMR22 has white aerial spores at young culture and changed into gray spores at old culture. *Streptomyces* sp.

GMR22 spores have a round shape and form a spirals chain (FIGURE 1).

Based on genome mining analysis (FIGURE 2), *Streptomyces* sp. GMR22 had the highest number of BGCs among the large genome-sized *Streptomyces* group. GMR22 has the highest BGCs (65 of BGCs) with PKS as dominant BGCs. The other BGCs, hybrid NRPS – PKS, NRPS, and terpene also as major BGCs.

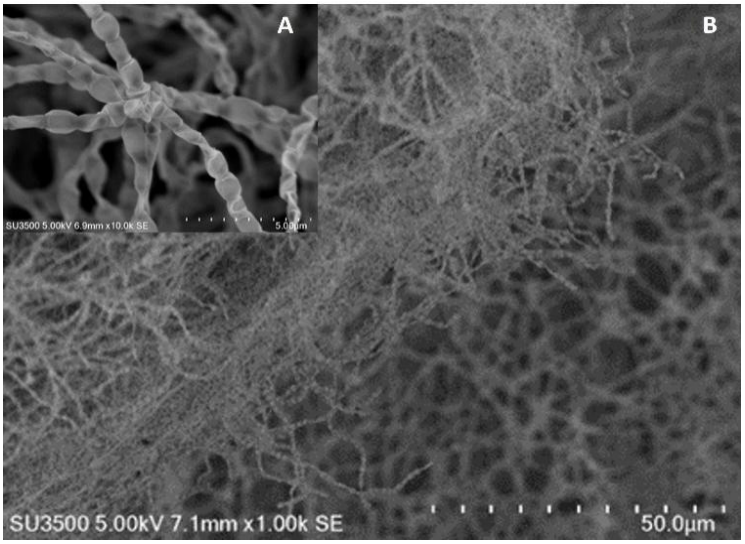

FIGURE 1. Morphology of *Streptomyces* sp. GMR22 using scanning electron microscope with 10,000x of magnification (A) and 1,000x of magnification (B)

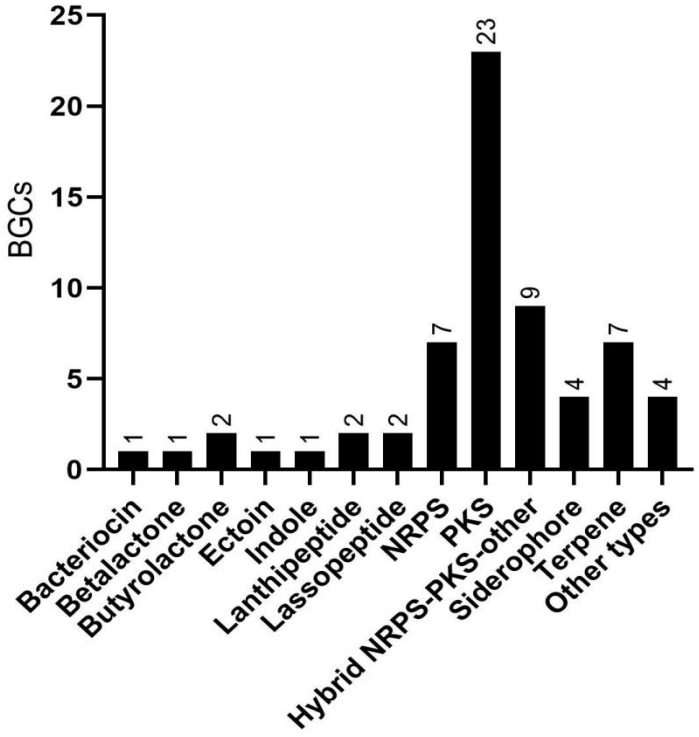

FIGURE 2. Biosynthesis gene clusters of secondary metabolites distribution of *Streptomyces* sp. GMR22 based on genome mining using AntiSMASH 5[13]

Based on their biological activities, most of the compounds had known biological activities, while some compounds had no known specific activity. The biological activities possessed by the compounds predicted to be produced by this bacterium was shown in FIGURE 3. Based on this FIGURE 3, it is dominated by antibiotic compounds that have antibacterial activities.

FIGURE 4 showed that the chloroform extract (CE) at 7.8 – 62.5 µg/mL has the significant antibacterial activity against *P. gingivalis*. This extract has the higher antibacterial activity than *n*-hexane, ethyl acetate and methanol extracts. The fungal growth was 56.16 – 72.65% and it was lower than bacterial growth in the control (100 %). The statistical analysis showed that there was significant difference at 7.8 – 15.62 µg/mL ($p = 0.001$) and at 31.25 – 62.5 µg/mL ($p<0.0001$) with control without CE ($p<0.05$).

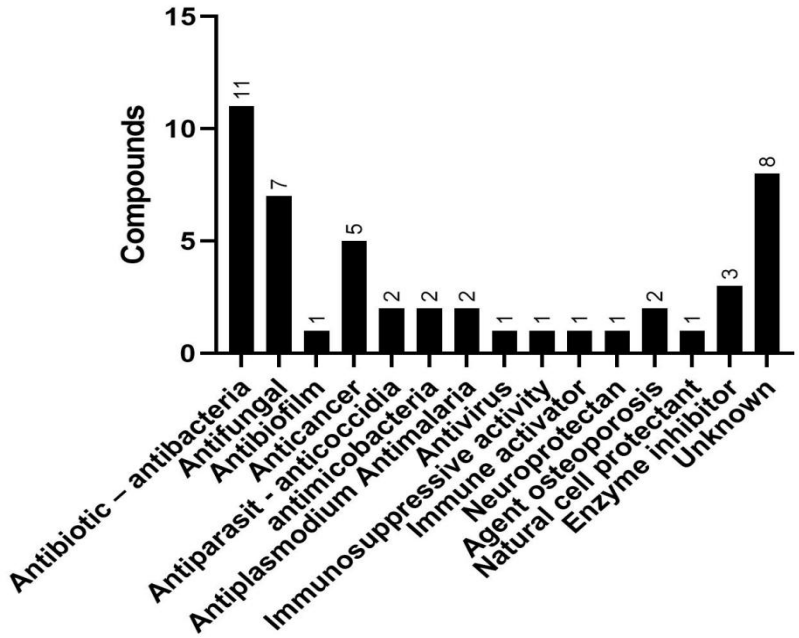

FIGURE 3. The biological activities of known compounds of *Streptomyces* sp. GMR22 based on genome mining analysis

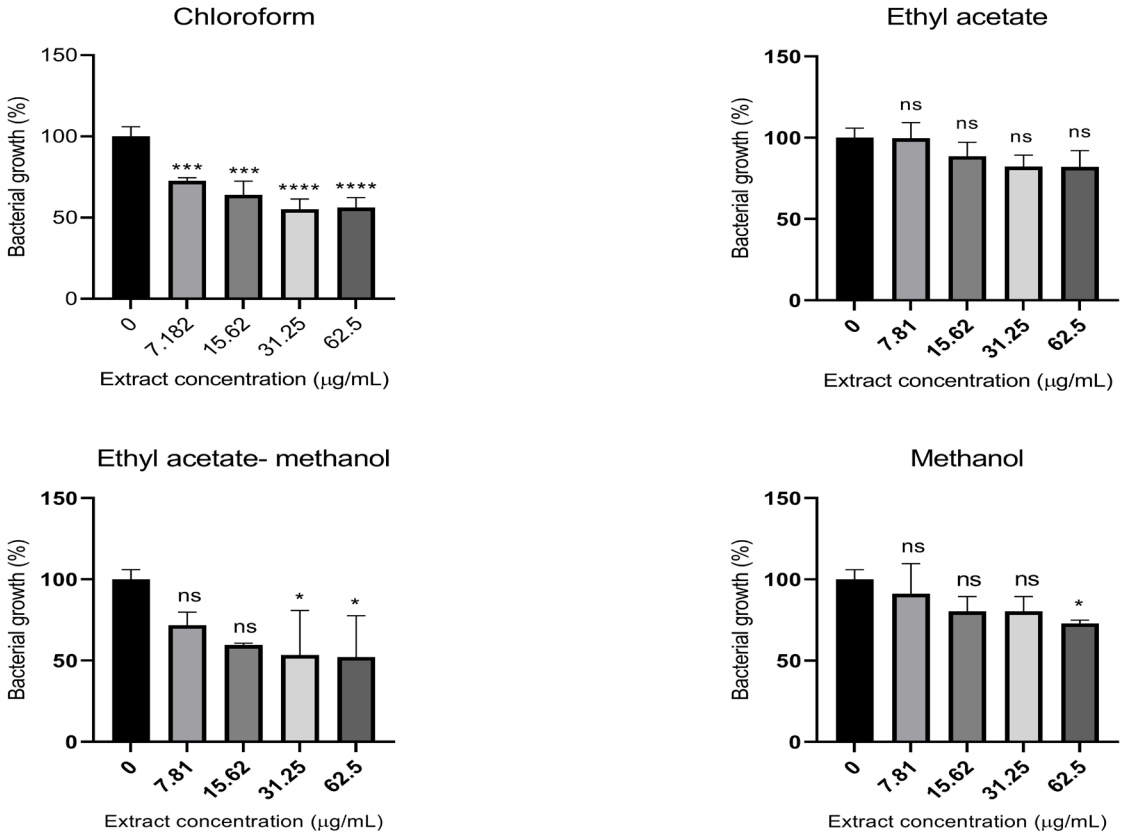

FIGURE 4. Antibacterial activity of soil *Streptomyces* sp. GMR22 extract on *Porphyromonas gingivalis*. Value are expressed as mean ± SD ($^{ns}p>0.5$, $^{*}p<0.1$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$) compared to control without any extract.

Prediction of the content of active compounds from chloroform extract was carried out using targeted LC-HRMS. TABLE 1 shows that the five selected targeted compounds had undergone a transformation and had changes in both formula and molecular weight.

TABLE 1. Targeted LC-HRMS of *Streptomyces* sp. GMRR chloroform extract

Parent compound	Formula	Molecular weight	Transformations	Composition change	RT (min)	Area (Max)
Hygrocin A	$C_{28}H_{31}NO_6$	477.21514	Dehydration, nitro reduction	-(O2)	17.426	23582396.96531
Amipurimycin	$C_{20}H_{35}N_7O_5$	453.26997	Hydration, nitro reduction, nitro reduction	-(O3) +(H6)	23.35	259957945.55138
Geldamycin	$C_{29}H_{42}N_2O_{11}$	594.27886	Hydration, oxidation	+(H2 O2)	23.82	42389047.14245
Bicyclomycin	$C_{12}H_{20}N_2O_2$	224.15248	Dehydration, nitro reduction, nitro reduction	-(O5) +(H2)	25.24	23911337.10273
Nigericin	$C_{40}H_{74}O_{10}$	714.5282	Hydration, nitro reduction, reduction	-(O) +(H6)	25.34	48124828.60041

DISCUSSION

The *Streptomyces* sp. GMR22 isolated from rhizopshere soil has the closest relationship to *S. lactacystinicus* strains OM-6519^T. The *S. lactacystinicus* strains OM-6519^T was isolated from soil samples near Lake Inba, Chiba, Japan.[16]respectively.Here, the taxonomic positions of these two strains were determined. The morphological and chemical features of strains OM-6519T and K04-0144T indicated that they belonged to the genus Streptomyces. Strain OM-6519T showed the highest 16S rRNA gene sequence similarities with Streptomyces xanthocidicus NBRC 13469T (99.7% GMR22 has high BGCs with PKS as dominant BGCs (FIGURE 2). This result was similar to the other previous studies. Genome mining analysis using antiSMASH version 3 revealed that the genome of *Streptomyces* sp. GMR22 harbored at least 63 BGCs with polyketide synthetase (PKS) as the major group of the identified gene cluster products.[17] GMR22 also exhibited the presence of PKS-I and NRPS genes were amplified by PCR[7] These genes were predicted to have a correlation with the antifungal activity of GMR22.[7]

Streptomyces rapamycinicus NRRL 5491 (12.7 Mbp) (accession number QYCY00000000)[18] has 53 BGCs, and *S. bingchenggensis* BCW-1 (11.9 Mbp) (accession number CP002047)[19] has 49 BGCs. The considerable genome potential of GMR22 suggest that this Indonesian *Streptomyces* species may be a promising source of new drugs.

The most antibiotic compounds produced by GMR22 bacteria such as meilingmycin,[20] daptomycin,[21] feglymycin as anti-HIV,[22] antinomycin,[23] glycinocin,[24] bafilomycin B1,[25] chlorothricinc,[26] medermycin,[27] paromomycin,[28] cadaside,[29] and azalomycin F3a,[30] GMR22 soil bacteria are also known to produce the most antifungal compounds such as, ECO-02301,[31] selvamicin,[32] mediomycin A,[33] natamycin,[34] This is in line with the previous studies demonstrating that GMR22 produces antifungal compounds against molds and yeasts.[7,17,35] GMR22 is also known to be the only bacterium that is predicted to produce antiplasmodial compounds, namely geldamycin[36,37] and salinomycin.[38]

The antibacterial activity in this study was similar with previous studies that showed chloroform extract has the highest inhibition against *C. albicans* (>20 mm of clear zone diameter) within n-hexane, benzene, and ethyl acetate extracts.[35] The IC_{50} value of antifungal against *C. albicans* was 62.5 µg/mL.[17]

Therefore, the active compound belonging Streptomyces sp. GMR22 could be predicted to be a new compound and has nothing in common with the predicted compound based on genome mining analysis. Further research related to the isolation of active compounds from the active fraction of chloroform extract is very important. In addition, the mechanism of antibacterial action of the active compounds found is also important to know.

CONCLUSION

Streptomyces sp. GMR22 has abundant biosynthetic cluster genes and has the potential to produce new types of antibiotics. Chloroform extract of GMR22 fermentation product has the potential to produce new compounds as antibacterial against *P. gingivalis*.

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