Research article

Soil-derived *Streptomyces* sp. GMR22 producing antibiofilm activity against *Candida albicans*: bioassay, untargeted LC-HRMS, and gene cluster analysis

Hera Nirwati [a,*], Ema Damayanti [b], Eti Nurwening Sholikhah [c], Mustofa Mutofa [c], Jaka Widada [d]

[a] Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
[b] Research Center for Food Technology and Processing, National Research and Innovation Agency, Gunungkidul, 55861, Indonesia
[c] Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
[d] Department of Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia

ABSTRACT

Biofilm-forming fungi, *Candida albicans*, are currently a serious problem in infectious disease cases. Soil bacteria *Streptomyces* sp. GMR22 have a large genome size and antifungal metabolites against *C. albicans*, but its potential antibiofilm activity is not clearly defined. The aims of this study were to determine the antibiofilm activity of GMR22 against *C. albicans*, identify the main constituents of active extracts, and investigate the biosynthesis gene clusters encoding the enzymes related to metabolism pathways. Antifungal and antibiofilm measurements were performed using in vitro assays on *C. albicans* ATCC 10231. Main constituents of active extracts were analyzed using untargeted Liquid Chromatography tandem High-Resolution Mass Spectrometry (LC-HRMS). RAST software was applied to investigate the gene clusters of the biosynthesis pathways based on whole genome sequences. Chloroform extract of GMR22 has antifungal and antibiofilm properties at 13–420 μg/mL with palmitic acid ($C_{16}H_{32}O_2$, 273.27028 Da), a saturated fatty acid as a major constituent (42.74). *Streptomyces* sp. GMR22 has 53 subsystems related to fatty acids biosynthesis (Fab) FAS II. The Kyoto Encyclopedia of Gene and Genome map of Fab revealed 10 of 21 (47.6%) gene clusters encode enzymes related to Fab. There were six gene clusters encoding the enzymes related to the hexadecenoic acid (palmitic acid) biosynthesis pathways: 6.4.12; FabD, FabH, FabF, FabG, FabI and 1.14.192. Each enzyme was encoded by 3–14 genes. These results confirmed that soil *Streptomyces* sp. GMR22 bacterium has remarkable biotechnological potential by producing fatty acids which are mostly palmitic acid as an active antibiofilm agent against *C. albicans*.

1. Introduction

Infectious diseases, especially those caused by bacteria and fungi, are still a major health problem in a high population country such as Indonesia [1]. The increasing population at risk of opportunistic infections and the massive spread of multi-resistant microorganisms are the main factors for the high prevalence of infectious diseases, especially in Indonesia [2]. The emergence of antibiotic resistance in microbials that are no longer sensitive to treatment which is not followed by the discovery of new antibiotics causes the availability of antibiotics to be increasingly limited. To protect the public health from this imminent threat, it is imperative that new antibiotic agents are identified and developed.

As one of the most commonly found and adaptable commensal human fungi, *Candida albicans* (*C. albicans*) contributes to a wide range of infections since its many virulence factors permit it to become both a mucosal and a systemic pathogen [3]. *C. albicans* biofilms protect themselves from external factors such as antifungal drugs. Causing many treatments to fail, *C. albicans* can survive because it has become multi-drug resistant in its pathogenesis [4]. Attributed to its ability to develop communities on hosts and medical devices, by switching morphology and forming biofilms, this pathogen is recognized for its high morbidity and mortality [3]. With the biofilms' resistance to antimicrobials, *C. albicans* continues to have low susceptibility to present-day treatments [5].

Many microorganisms have been found that produce secondary metabolites which have antibiotic effects. In particular, Actinomycetes have been widely applied in various fields, especially medicine [6]. A total of 18,000 active compounds have been isolated from Actinomycetes, with 10,000 of them from *Streptomyces* [7]. As many as 50% of the antibiotics currently available in clinics are from the genus *Streptomyces* [8], which

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* Corresponding author.
E-mail address: hera.nirwati@mail.ugm.ac.id (H. Nirwati).

is one of the gram-positive bacteria that has been known to have many medicinal benefits such as antibiotics, antifungals, antivirals, and anti-cancer [9, 10, 11, 12].

Indonesia as a mega biodiversity country is abundant in microflora, both in terrestrial and marine habitats [13] especially as a source of *Streptomyces* bacteria. In our previous study, we obtained a large genome size *Streptomyces* sp. GMR22 bacterium from Cajuput rhizosphere soil in Wanagama Forest, Gunungkidul, Indonesia [14]. This bacterium showed inhibition activities with clear zone diameter (mm) in fungi including *Fusarium oxysporum* (14.22), *Saccharomyces cerevisiae* (19.83), *Aspergillus flavus* (14.5), and *C. albicans* (19.15) [15]. The genes encoding secondary metabolites analysis showed that *Streptomyces* sp. GMR22 has a diversity of polyketide synthase (*PKS*) genes [16, 17]. However, it is not certain which metabolites are responsible for the antifungal and antibiofilm activity. The objective of this study was to determine the antibiofilm activity and to identify the main constituents of the antibiofilm metabolites. Another aim was to investigate the gene clusters encoding the enzymes related to the biosynthesis pathways of active metabolites produced by the GMR22 bacterium.

2. Materials and methods

2.1. Biological material

Streptomyces sp. GMR22 was isolated from Cajuput rhizosphere soil at Wanagama I Forest, Gunungkidul, Indonesia [14]. GMR22 isolate was deposited at the Indonesian Culture Collection (InaCC A 148), Indonesian Institute of Sciences, and NITE Biological Research Center (NBRC), Japan (NBRC 110112). GMR22 was maintained in International *Streptomyces* Project-2 (ISP2) medium. *Candida albicans* ATCC 10230 was maintained in Sabouraud Dextrose Agar (SDA) at the Microbiology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

2.2. Fermentation and extraction

The fermentation process for secondary metabolite production followed the methods of Alimuddin *et al.* (2011) with minor modification. GMR22 was cultured at 28 °C with 180 rpm agitation for 3 days in a 250 mL Erlenmeyer flask containing 100 mL Tryptic Soy Broth (Difco, Sparks, USA) as the seed medium. Next, cells were transferred into four 1000 mL flasks containing 500 mL Starch Nitrate Broth (SNB) [(20 g soluble starch, 1 g KNO_3, 0.5 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.5 g $K_2HPO_4.3H_2O$, 0.01 g $FeSO_4.7H_2O$ in 1000 mL distilled water (pH 7.4)] as the production medium and incubated for 8 days at 28 °C with 180 rpm agitation in a shaking incubator [15]. Refrigerated centrifugation at 4.137 × *g* at 4 °C for 15 min was used to separate the secondary metabolites in the cell biomass from the liquid as described in a previous study [14]. The supernatant was extracted twice with an equal volume of *n*-hexane and chloroform, sequentially. In a previous study, chloroform extract showed the highest antifungal activity against *C. albicans* [16]. The liquid extract was evaporated to obtain the crude extract. All chemical reagents and solvents were purchased from Merck KGaA, Darmstadt, Germany.

2.3. Antifungal, antibiofilm and biofilm quantification assay

The antifungal activity, antibiofilm formation and biofilm quantification were performed using the microdilution protocol described in a previous study [18] with minor modification. The extract was prepared in six levels of concentration (13.125–420 μg/mL in 0.1% of DMSO as solvent and re-diluted using Roswell Park Memorial Institute 1640 (RPMI 1640) medium. For inoculation in the antifungal assay, the first fungal suspension was cultured in 10^6 CFU/mL in RPMI 1640 medium. The plates were incubated at 37 °C for 24 h. A spectrophotometer was used to

measure the growth inhibition. A multi-scan reader (Thermo Scientific) was used to measure the cell densities at 540 nm (OD_{540}). For antibiofilm formation, MTT reduction assay was used. A 100 μL of 10^6 cells/mL cell suspension in RPMI 1640 medium was grown in a 96-well microplate (Nunc, Merck) and incubated anaerobically at 37 °C for 1.5 h for initial adhesion. Non-adherent cells were removed by removing the supernatant. New RPMI 1640 media with or without the chloroform extract added in the microplate followed by the next incubation for 24 h. For detection of chloroform extract on mature (24 h) biofilms, various concentrations of chloroform extract were used to treat biofilms pre-grown and incubated for 24 h at 37 °C. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) was added to the prewashed biofilms after incubation. The MTT reaction was left for 2–3 h in the dark condition at 37 °C. A total of 80 μL aliquots of the supernatant from each well were transferred to a new microplate. The antibiofilm effect was read with a microplate reader at 490 nm (OD_{490}).

The biofilm concentration was measured using an indirect method using crystal violet (CV) (Merck, Germany) staining. The nonadherent cells on biofilm after 24 h incubation was removed by washing three times with sterile phosphate buffered saline (PBS) (Merck, Germany). The microplate was air dried, and the cell fixation were carried out by adding 200 μL of 99% methanol for 15 min. The methanol was removed and plate air dried. A total of 200 μL of CV stain (1%, v/v) was added to each well and allowed to stained for 5 min. The excess CV was removed by washing the microplate three times using sterile distilled water and then drying at room temperature. A total of 200 μL of acetic acid (Merck, Germany) (33%, v/v) was added to each well to dissolve the CV stain of the biofilm. A total of 100 μL of the resulting solution was transferred to a new microplate, and the absorbance was read at 570 nm (OD_{570}).

The group without the chloroform extract was used as a control. Fungal growth (%) and biofilm formation (%) calculated as follows: (OD after extract treatment)/OD of control) x 100. All experiments were performed in triplicate, and the mean absorbance, fungal growth (%), and biofilm formation (%) of each well was plotted against the chloroform concentration. One-way ANOVA followed by Dunnett's multiple comparison test for analysis of treatment was done by using GraphPad Prism 9.0.1 software.

2.4. Scanning electron microscope (SEM)

SEM observation used the method according to the previous study with modification [18]. *C. albicans* ATCC 10231 biofilms of were grown at 35 °C for 48 h on sterile coverslips (a diameter of 12 mm, SPL life sciences) in a 24-wells microplate (NEST Scientific) in the presence of inhibitory concentration of GMR22 extract. The biofilm which not exposed to the chloroform extract used as control. After incubation, the coverslip was washed twice with sterile PBS and dehydrated in an ethanol series (70% for 10 min, 95% for 10 min and 100% for 20 min) and air dried overnight. The coverslip was coated with with Au (Hitachi MC1000 Au ion sputter, Japan) with a setting of 10 mA for 60 s 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 500; 1,500; and 2,500× (Hitachi SU3500, Japan).

2.5. Untargeted liquid chromatography tandem High-Resolution Mass Spectrometry (LC-HRMS)

Metabolomic analysis of the chloroform fraction was done using an ultra-high-performance liquid chromatography coupled to untargeted high-performance mass spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC paired with Thermo Scientific Q Extractive (Thermo Fisher Scientific, Massachusetts, USA). High-resolution mass spectrometry (HRMS) was performed with mobile phase A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). The column

used was a Hypersil Gold aQ analytical column (50 mm × 1 mm × 1.9 μm) (Thermo Fisher Scientific, USA) with a flow rate of 40 μL/min, an injection volume of 5 μL, and a gradient with an analysis time of 30 min. The gradient was programed as following: 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 done in parallel reaction monitoring at 35,000 the full width at half maximum (FWHM) resolution, heated electrospray ionization, positive ionization, and data processing with Thermo Scientific XCalibur.

2.6. Ethical clearance

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

2.7. Genome annotation analysis for fatty acid metabolism pathway

Genome annotation was performed with Rapid Annotation using Subsystem Technology (RAST) version 2.0 [19] and organism overview of *Streptomyces* sp. GMR22 was analyzed using the SEED Viewer version 2.0 [20]. Additionally, KEGG map of fatty acid metabolism was analyzed to investigate the gene cluster encoding enzymes related to fatty acid biosynthesis. The Whole Genome Shotgun project of GMR22 has been deposited at DDBJ/ENA/GenBank under the accession JACGSQ 000000000.

3. Results

3.1. Antifungal and antibiofilm activity

Figure 1a shows that the chloroform extract (CE) at 25.25–420 μg/mL has antifungal activity against *C. albicans*. The fungal growth was 79.93 ± 1.9–$95.24 \pm 10.6\%$ and it was lower than fungal growth in the control ($103.6 \pm 8.9\%$). The statistical analysis (one way ANOVA, p value = 0.05) showed that there was significant difference only at 420 μg/mL with control without CE ($p < 0.05$). This antifungal activity result was similar with the result of biofilm concentration reduction (Figure 1b). In all concentration levels of CE (13.12–420 μg/mL), there were biofilm concentration reductions ($OD_{570\ nm}$) (0.042 ± 0.00–0.079 ± 0.00) lower than in control without CE (0.109 ± 0.02). The one way ANOVA analysis showed that there were significant differences only at 25.25 and 420 μg/mL with control without CE ($p < 0.05$). The antifungal and biofilm concentration reduction results were similar with the results of biofilm maturation analysis at 1 and 24 h of biofilm formation. At 1 h maturation showed that the reductions of biofilm formation in all concentration levels of CE (69.23 ± 2.34–$86.40 \pm 1.57\%$) were lower than in control without CE ($97.23 \pm 0.05\%$). The one way ANOVA indicated all concentration levels of CE showed significant difference in biofilm formation
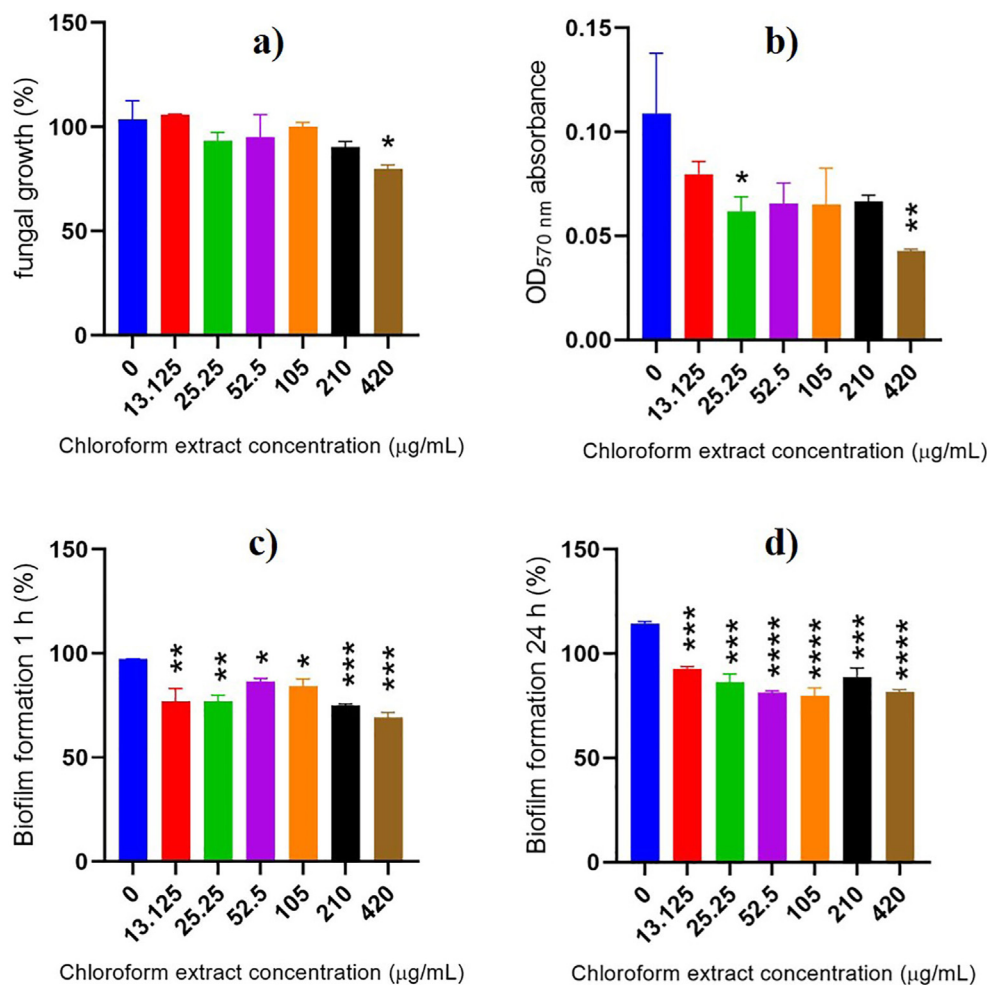

Figure 1. Antifungal activity (a), biofilm concentration at 24 h of incubation times (b), biofilm formation at 1 h incubation times (c), and biofilm formation at 24 h of incubation times of chloroform extracted from *Streptomyces* sp. GMR22 on *Candida albicans* ATCC 10230. Value are expressed as mean $\pm$ SD (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

at 1 h with control without CE ($p < 0.05$). The same phenomenon was seen in biofilm formation at 24 h. All concentration levels of CE were very significantly different in biofilm formation at 1 h with control without CE ($p < 0.0005$). The very significant difference ($p < 0.0001$) of biofilm formation at 24 h was seen starting from 52.5 μg/mL. This concentration level of CE was used for biofilm formation analysis using structural equation modeling (SEM).

3.2. Inhibition of biofilm formation

Biofilm formation of *C. albicans* ATCC 10230 treated by CE of GMR22 compared with control (without extract) is showed in Figure 2. The addition of CE at 50 μg/mL inhibited the biofilm formation of *C. albicans*. In the control, it was seen that *C. albicans* grew normally with abundant and dense cells, while in the CE treatment, the cells grew minimally and were separated. This finding indicates that there was no biofilm formation in CE treatment because fungal growth was inhibited.

3.3. Main constituents of active metabolites

The main constituent analysis of CE using untargeted LC-HRMS detected 114 compounds. The twenty-highest compounds which have >0.49% relative abundance shows in Table 1. The highest detected compound was palmitic acid which was four-fold more abundant than the second-highest compound. This study indicated that palmitic acid as a fatty acid resulted in *Streptomyces* sp. GMR22 and has remarkable potential as an antifungal and antibiofilm agent against *C. albicans*.

3.4. Gene clusters related to fatty acid biosynthesis

In this study, we analyzed the subsystem distribution of the whole genome GMR22 with RAST version 2.0 [19] and SEED viewer tools version 2.0 [20]. This analysis showed that there were 132 subsystems related to fatty acids (Figure 3). There were 53 gene clusters related to fatty acids biosynthesis FAS II. The further analysis showed the gene cluster encoding enzymes were related to the hexadecenoic acid (palmitic acid) biosynthesis in *Streptomyces* sp. GMR22. Based on KEGG map fatty acid biosynthesis using RAST (Figure 4), there were six gene clusters encoding the enzymes related to the hexadecenoic acid biosynthesis pathway (Table 2). The enzymes are indicated with red circles and the

hexadecenoic acid products are indicated with blue boxes (Figure 3.). Each enzyme was encoded by 3–14 genes.

4. Discussion

Based on the previous study, the chloroform fraction of supernatant GMR22 has the highest antifungal activity against *C. albicans* [15]. The highest antifungal activity was at 420 μg/mL (Figure 1a) similar with the lowest biofilm concentration (Figure 1b), biofilm formation in 1 h incubation (Figure 1c) and mature biofilm formation in 24 h incubation times (Figure 1d). This result 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 [16]. The IC_{50} value of antifungal against *C. albicans* was 62.5 μg/mL [17]. This concentration was similar with the biofilm formation analysis using SEM (Figure 2). There was biofilm formation inhibition by 50 μg/mL CE addition (Figure 2A).

Biofilm formation is the main indication of *C. albicans* infection [3]. As three-dimensional structures biofilms consist of a complex collection of cells connected with host tissues or abiotic surfaces and embedded in extracellular polysaccharides (EPS) [5]. The EPS of *C. albicans* biofilm has is complex, where several main polysaccharides including a-mannan and b-1,6-glucan. Although a minor constituent, due to its ability to protect against antibiotic agents, b-1,3 glucan is the main polysaccharide of the biofilm matrix formed to resist treatment with antifungals [5]. This structure is considered a form of protection for microorganisms. The results of in vitro studies indicate that biofilms develop over a period of 24–48 h incrementally [5].

The key step in *C. albicans* developing biofilms is the transition from yeast cells to hyphae [21]. The development of *C. albicans* biofilms is generally divided into four main stages, namely the process of attachment, proliferation, maturation, and dispersion. In the adhesion stage, *Candida* cells attach to the substrate forming a basal layer of cells. At the proliferative stage, the *Candida* cells begin to stretch and develop into filamentous hyphae.

At the maturation stage, there is secretion of EPS as the hyphae are forming [5]. In Figure 2, *C. albicans* cells lost their ability in cell proliferation indicated by the minimal cell formation. As a consequence, *Candida* cells could not continue to the maturation and biofilm formation stages as shown in Figures 1c and 1d. This finding indicated that CE of
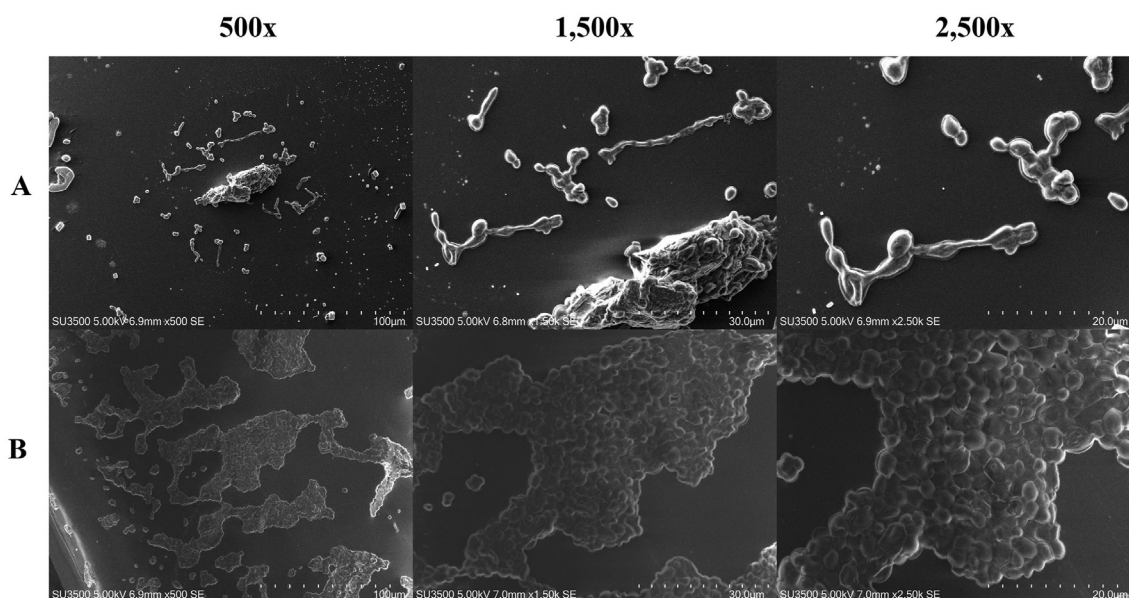

Figure 2. Scanning electron microscope of *Candida albicans* ATCC 10230 biofilm formation treated by 50 μg/mL chloroform extract of *Streptomyces* sp. GMR22 (A) and *C. albicans* ATCC 10230 biofilm formation in control RPMI medium (B).

Table 1. The main constituents detected from chloroform fractions of Streptomyces sp. GMR22 detected using untargeted liquid chromatography tandem high-resolution mass spectrometry (LC-HRMS).

Name	Formula	Molecular Weight	RT [min]	Area (Max.)	% relative abundance
Palmitic Acid	$C_{16}H_{32}O_2$	273.27028	22.66	2.19927E+11	42.74
NP-011220	$C_{11}H_{18}N_2O_2$	210.13708	11.554	51363915160	9.98
Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	244.12406	13.806	50333117870	9.78
Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	244.12403	13.082	25765471575	5.01
Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	210.13708	12.02	20062234275	3.90
NP-011220	$C_{11}H_{18}N_2O_2$	210.1371	11.222	16842455783	3.27
3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{10}H_{16}N_2O_2$	196.1207	8.547	15670947015	3.05
3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{10}H_{16}N_2O_2$	196.1207	8.295	15085842482	2.93
NP-013736	$C_{10}H_{16}N_2O_2$	196.1207	1.155	10914053940	2.12
NP-013736	$C_{10}H_{16}N_2O_2$	155.09415	0.975	10700738495	2.08
NP-011220	$C_{11}H_{18}N_2O_2$	210.1371	1.164	8076454693	1.57
Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15687	23.921	7780756638	1.51
Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	244.12403	1.153	5935004194	1.15
3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_3$	260.11987	8.01	4538639670	0.88
Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	262.13461	0.973	4199836617	0.82
Diisobutylphthalate	$C_{16}H_{22}O_4$	278.15675	23.756	3970303770	0.77
3-amino-2-phenyl-2H-pyrazolo[4,3-c]pyridine-4,6-diol	$C_{12}H_{10}N_4O_2$	242.0833	16.718	2994623288	0.58
Anhydroecgonine methyl ester	$C_{10}H_{15}NO_2$	181.11002	1.026	2641722827	0.51
NP-022474	$C_{16}H_{24}O_3$	246.16465	23.15	2598363703	0.50
NP-008521	$C_{14}H_{18}N_2O_2$	246.13983	16.53	2534489675	0.49
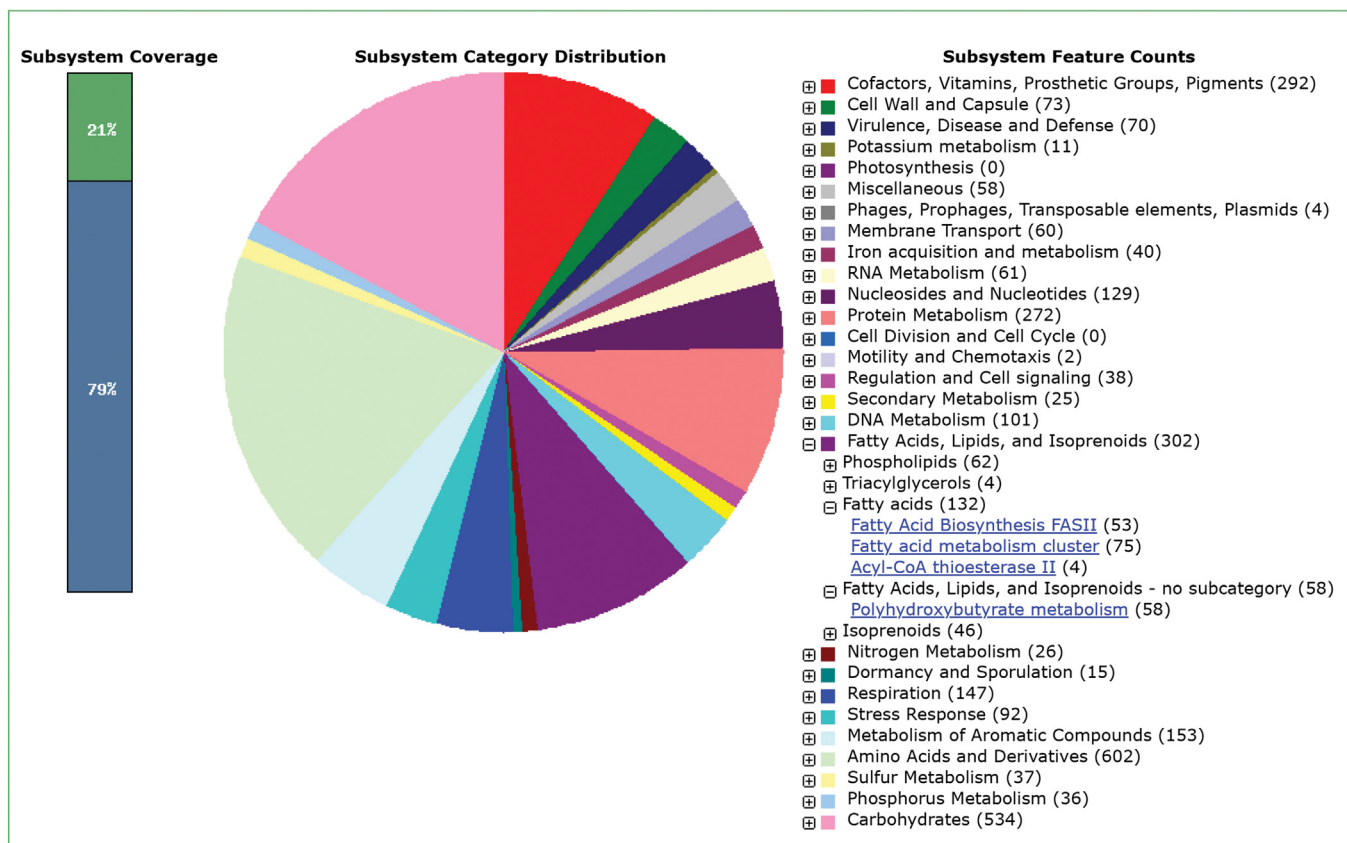
Figure 3. Subsystem category distribution and subsystem feature count of *Streptomyces* sp. GMR22 genome. This image was original downloaded from RAST version 2.0 and SEED viewer version 2.0 [19, 20].

GMR22 has the ability to inhibit biofilm formation by a proliferative stage inhibition mechanism.

Quorum sensing has an important role in the formation of cell-cell communication in biofilms, where the microbial response in biofilm formation is regulated by cell density mediated by secreted signaling molecules [22]. One of the well-known quorum-sensing molecules, farnesol is secreted extracellularly and accumulates in the mature biofilm supernatant [3]. Another important molecule in biofilm formation is
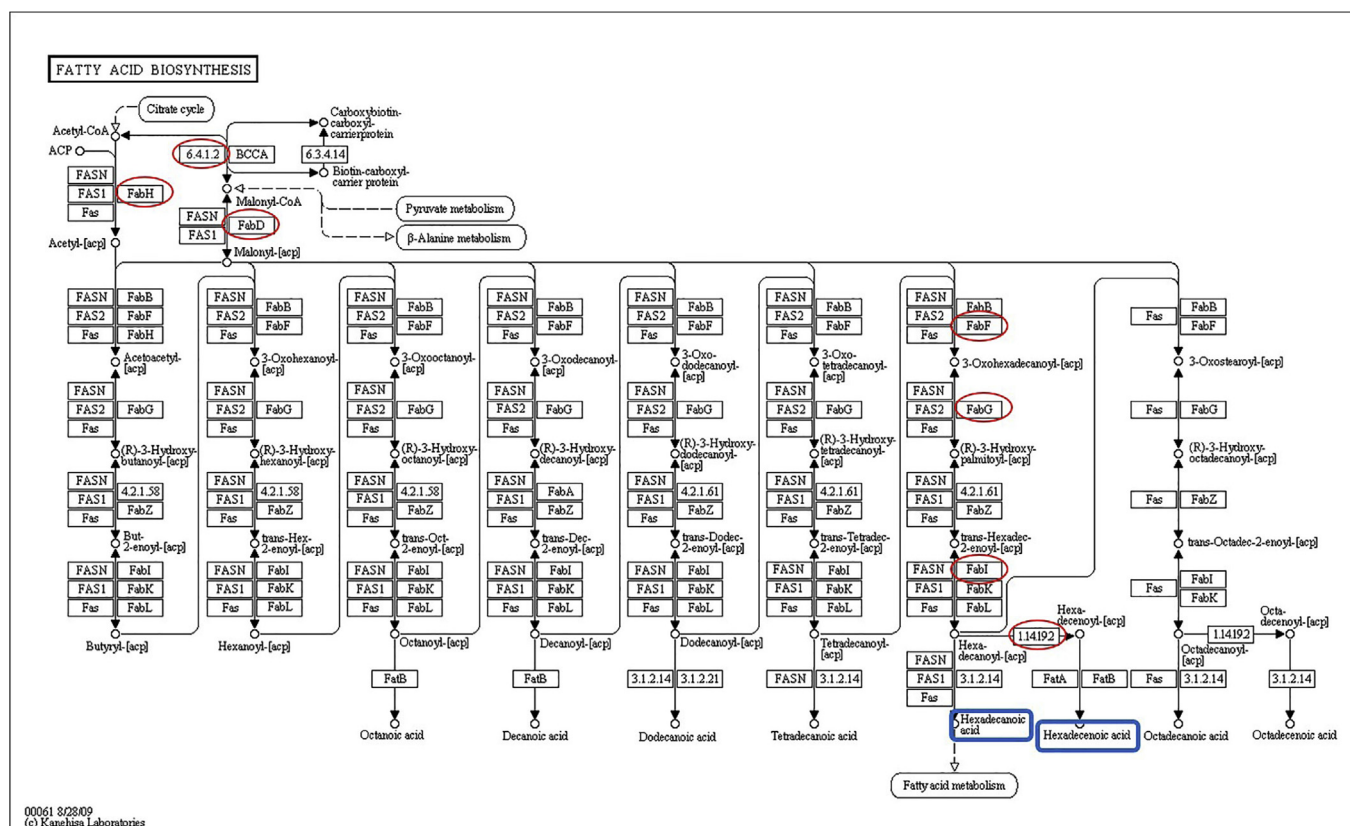
Figure 4. KEGG map fatty acid biosynthesis of *Streptomyces* sp. GMR22. Red circle indicates the gene clusters of GMR22 related with hexadecenoic acid (palmitic acid). Blue box indicates hexadecenoic acid (palmitic acid) as product. KEGG map downloaded from SEED Viewer version 2.0 [20] with additional marks.

Table 2. Gene cluster encoded enzymes related to hexadecenoic acid (palmitic acid) biosynthesis in *Streptomyces* sp. GMR22 based on KEGG map fatty acid biosynthesis with Rapid Annotation using Subsystem Technology (RAST) version 2.0.

Gene clusters	Enzyme code	Enzyme	Features of genes on GMR22
6.4.12	EC: 6.4.1.2	Acetyl-coenzyme A carboxyl transferase alpha chain	5
FabD	EC: 2.3.1.39	Malonyl CoA-acyl carrier protein transacylase	7
FabH	EC: 2.3.1.180	3-oxoacyl-[acyl-carrier-protein] synthase, KASIII	3
FabF	EC: 2.3.1.179	3-oxoacyl-[acyl-carrier-protein] synthase, KASII	14
FabG	EC: 1.1.1.100	3-oxoacyl-[acyl-carrier protein] reductase	12
FabI	EC: 1.3.1.9	Enoyl-[acyl-carrier-protein] reductase	3
1.14.192	EC 1.14.19.2	Probable acyl-ACP desaturase, Stearoyl-ACP desaturase	1

cyclic-di-AMP [23]. As a nucleotide second messenger, C-di-AMP aids in regulating stress responses and forming biofilm in a variety of microbial species [24]. The accumulation of C-di-AMP influences a myriad of gene expression including the two major operons integral in biofilm formation [25]. This also indicates that the CE acid of GMR22 could inhibit biofilms through inhibition of molecule secretion and molecule accumulation related to quorum sensing. However, this justification needs to be proven by further research.

The result of constituent analysis in CE revealed that palmitic acid as a fatty acid was the main constituent in CE GMR22. Hexadecanoic (palmitic) acid is a typical bacterial fatty acid which is a very abundant saturated fatty acid found in bacteria [26]. Potential alternatives to conventional antibiotics include saturated fatty acids such as C8 to C18 since they can control biofilms [27]. Mimicking farnesol, they might prevent *C. albicans* from forming hyphae and biofilm matrices [21]. Medium-chain saturated fatty acids are known to inhibit *C. albicans* biofilm formation by more than 75% at 2 g/mL with MIC in the range of 100–200 g/mL. These six fatty acids: heptanoic acid, octanoic acid,

nonanoic acid, decanoic acid, undecanoic acid, and lauric acid, at 2 g/mL and farnesol at 100 g/mL inhibited cell aggregation and growth of hyphae [21]. Fatty acids not only act as antibiofilm agents against *C. albicans* but also in various microbial pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Burkholderia cenocepacia*, and *Vibrio* spp [27].

In this study, 50 μg/mL of CE GMR22 containing 42.74% palmitic acid inhibited the formation of *C. albicans* biofilm. This finding means that the palmitic acid concentration of 21.37 μg/mL of GMR22 was able to inhibit *C. albicans* from forming biofilms. In the previous study, the effect of palmitic acid at 100 μg/mL identified from *Synechococcus elongatus* was assessed for its effect in inhibiting biofilm formation. In this concentration, palmitic acid exhibited a significant inhibition of quorum sensing regulated biofilm formation in *Vibrio* spp. including *Vibrio parahaemolyticus* ATCC 17802, *V. vulnificus* MTCC 1145, *V. harveyi* MTCC 3438, and *V. alginolyticus* ATCC 17749 without inhibiting their planktonic growth [28]. In other studies, palmitic acid combined with myristic acid demonstrated anti-candidiasis activity against *C. albicans* and

C. tropicalis [29]. The six new palmitic acid-based neoglycolipids related to Papulacandin D also have antifungal agency against *Candida* spp [30]. Palmitic acid was also found as a dominant fatty acids constituent (56.02%) in leaves of *Excoecaria agallocha* extract and has antibacterial and antifungal activity including against *C. albicans* [31]. Palmitic acid salt combined with ultra-pure soft water has antifungal effect on skin fungal disease, *Scedosporium apiospermum* [32].

The possibility of palmitic acid playing a role in the inhibition mechanism of quorum sensing (QS) has been demonstrated in several previous studies. After decreasing bioluminescence production and positively interfering with the initial adhesion stages of biofilm formation, transcriptomic analysis of palmitic acid showed *Vibrio harveyi* to undergo down-regulation of QS mediated response regulator genes expression [28]. In addition to its antibiofilm activity against *P. aeruginosa* and *E. coli*, with an IC$_{50}$ of 32.2 and 7.1 μM, respectively, in cultures of *Vibrio* spp., treatment with palmitic acid caused the down-regulation of QS-related genes (*luxS* and *luxR*) and impaired biofilm formation and EPS production [27].

Fatty acid biosynthetic pathways in bacteria have been extensively studied. Based on Figure 3, there were three subsystems related to fatty acid metabolism in *Streptomyces* sp. GMR22. Previous studies revealed that the type II FAS pathway is the most common fatty acid biosynthetic pathway in bacteria [33] This been extensively studied in *E. coli* and has been the basis for type II FAS in other bacteria [34]. Fatty acid biosynthesis is more diverse in the phylum Actinobacteria but the *Streptomyces* spp. have only the multienzyme FAS II system. In the most relevant Streptomycetes genomes, the fatty acid biosynthetic genes revealed various putative orthologs to several of the classical FAS II components [34]. The genes encoding the main enzymes involved in the biosynthesis of saturated fatty acids consist of the following genes: *fabD*, *fabF*, *fabH*, and *acpP* which are in the fatty acid biosynthesis cluster (fab). The genomic regions of Actinomycetes group which possessing this universal gene set show a high degree of conservation in the evolution of this important biosynthetic pathway [34].

Based on Table 2, there were six gene clusters which encode enzymes related with fatty acid biosynthesis in *Streptomyces* sp. GMR22. The acetyl-CoA group is a precursor for fatty acid biosynthesis. All elongation steps require malonyl-CoA and are formed in the first step in fatty acid biosynthesis where acetyl-CoA carboxylase acts as a catalyst. In fatty acid biosynthesis, malonyl-CoA is converted to malonyl-ACP by malonyl-CoA:ACP transacylase. In the initiation step, malonyl-CoA is converted to malonyl-ACP by malonyl trasacylase (FabD) (Figure 4). Fatty acid synthesis is initiated by FabH, which condenses malonyl-ACP with acetyl-CoA.

Fatty acid elongation cycle consists of four stages. Condensation of acyl-ACP and malonyl-ACP by FabB or FabF which is one of the elongation condensation enzymes initiates each new cycle of 2 carbon elongation. The next step is the reduction of 3-ketoacyl-ACP by NADPH-dependent FabG. This 3-hydroxylacyl-ACP is dehydrated to trans-2-acyl-ACP by FabZ. The NADH-dependent reduction of enoyl-ACP to acyl-ACP by FabI is the final step in the fatty acid elongation process [26].

This study has revealed a novel potential for palmitic acid produced by soil bacteria *Streptomyces* sp. GMR22 as antifungal. Palmitic acid as the main constituent of the active fraction has the potential to be an antifungal and antibiofilm alternative against biofilm-forming fungus *C. albicans*. Although demonstrated in the in vitro assays and analyses of its genetic potential, the mechanism of action of palmitic acid specifically on the quorum sensing phenomenon of biofilm formation needs further research to determine the effective dose as an antifungal agent.

5. Conclusions

This initial research provides information about the remarkable potential of fatty acids produced by *Streptomyces* sp. GMR22 as antibiofilm agents. Palmitic acid (C$_{16}$H$_{32}$O$_2$, 273.27028 Da) as a major compound in the chloroform extract of GMR22 showed antifungal and antibiofilm activity against *C. albicans*. This finding is related to the cluster of genes encoding enzymes associated with fatty acid biosynthesis. This result confirmed that soil *Streptomyces* sp. GMR22 bacterium has excellent biotechnological potential by producing the most common fatty acid, palmitic acid as an antibiofilm agent against *C. albicans*. Furthermore, palmitic acid could be utilized for further exploration as an antifungal and antibiofilm alternative.

Declarations

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