

Screening and Quantification of Antiquorum-Sensing Activity of Actinobacteria Isolates Against Gram-Positive and Gram-Negative Biofilm Associated Bacteria

Marco Wijaya

Universitas Katolik Indonesia Atma Jaya

Dea Delicia

Universitas Katolik Indonesia Atma Jaya

Diana Elizabeth Waturangi (✉ diana.waturangi@atmajaya.ac.id)

Universitas Katolik Indonesia Atma Jaya

Research note

Keywords: Actinobacteria, Antibiofilm, *Chromobacterium violaceum*, Quorum sensing, Quorum quenching

Posted Date: February 25th, 2020

DOI: <https://doi.org/10.21203/rs.2.24412/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Objectives: The purpose of the study is to screen anti-quorum sensing activity compound from actinobacteria using *Chromobacterium violaceum* as indicator and quantify the antibiofilm activity against several biofilm-forming pathogenic bacteria, such as *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 33186, *Vibrio cholerae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* ATCC 27853.

Results: Crude extracts from marine actinobacteria isolated from Indonesia were promising for treating biofilm-related diseases. Out of 40 isolates 10 were found to have great quorum quenching activity against *C. violaceum*. Moreover, these isolates were able to inhibit biofilm formation by Gram positive and Gram negative pathogenic bacteria. Quorum quenching agents in extracts of 1AC, 14PM, 16PM, 18PM, and CW17 isolates were predicted as proteins or enzymes.

Introduction

Bacteria can communicate with each other using unique chemical compounds called autoinducers. Communication activity of bacteria is known as quorum sensing. This mechanism is referring to the capacity of bacteria to monitor their cell density. Quorum sensing also affects the genes expression which influences microbial behaviors, such as regulating virulence factors and biofilm formation [1,2].

Biofilm formation is a process when flocked bacteria develop extracellular matrix that facilitate attachment. It gives rise to serious problems for public health because biofilm-associated organisms are more resistant to chemical agents and physical treatments. In this case, the probability of successful infections will increase [2]. Every activity to inhibit bacterial quorum sensing is known as quorum quenching. It has been proposed as novel therapeutics for treating biofilm-based diseases.

The biofilm-based diseases are usually treated by antibiotics. However, the use of antibiotics is not an effective solution and might lead to antibiotic resistance. Actinobacteria are fungi-like Gram-positive bacteria, well known as a supreme producer of secondary metabolite compounds [3], but research around anti-quorum sensing and antibiofilm from these bacteria yet have not been much explored. The objectives of this research are to screen anti-quorum sensing activity compound from actinobacteria using *C. violaceum* as indicator and quantify the antibiofilm activity against several biofilm-forming pathogenic bacteria.

Methods

Bacterial Cultivation

Fourty actinobacteria isolates used in this research were obtained from the previous study [4,5] that isolated from aquatic environments in Indonesia. Actinobacteria were cultured onto Yeast Malt Extract Agar (YMEA) incubated at 28°C for 7 days. Indicator strains of quorum sensing activity were *C. violaceum*

wild type and *C. violaceum* CV026 cultivated onto Luria Agar (LA) and incubated at 28°C for 24 h. While six pathogenic bacteria which were *B. cereus* ATCC 14579, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 33186, *P. aeruginosa* ATCC 27853, *S. typhimurium*, *V. cholerae* cultivated onto LA and incubated at 37°C for 24 h.

Primary Screening of Antiquorum Sensing Activity

The assay was conducted using the overlay agar method [6] with modification. Actinobacteria isolates were inoculated into YMEA then incubated at 28°C for 3 days. *C. violaceum* were grown separately in Luria Broth (LB) and incubated at 28°C for 24 h. The indicator strain diluted within semisolid agar (0.75% LA) until $OD_{600}=0.132$ and poured onto YMEA plates. These plates were incubated at 28°C for 24 h. Positive result indicating antiquorum sensing can be seen through the absence of violacein pigment production around actinobacteria isolates.

Preparation of Crude Extract

Each isolate was inoculated into Tryptic Soy Broth (TSB) supplemented with glucose (1% w/v). The culture was incubated at 28°C for 7 days at 125 rpm then centrifuged at 7,800xg for 15 minutes. The supernatant was mixed with ethyl acetate in a 1:1 ratio and shaken at 150 rpm overnight. The solvent phase was evaporated using rotary evaporator then dried using vacuum oven and resuspended in Dimethyl Sulphoxide (DMSO). Crude extracts were stored at -20°C for a month [7,8,9]

Antimicrobial Assay

Assay is conducted using agar well diffusion method [10]. Tested bacteria ($OD_{600}=0.132$) were streaked onto Brain Heart Infusion Agar (BHIA). The crude extract (50 μ L) of actinobacteria with various concentration (10 and 20 mg/mL) was added. DMSO (1% v/v) was used as the negative control, while streptomycin (10 mg/mL) was used as the positive control. The plates were incubated at 37°C for 24 h.

Detection of Antiquorum Sensing Activity

This assay was conducted based on [11] with modification. *C. violaceum* ($OD_{600}=0.132$) used as indicator strain streaked onto BHIA. The crude extract (50 μ L) of actinobacteria with various concentration (10 and 20 mg/mL) was added. DMSO (1% v/v) was used as a control. The plates were incubated at 28°C for 24 h. Antiquorum sensing activity was determined qualitatively through the inhibition of violacein production around the wells [12]

Quantification of Antibiofilm Activity

Quantification of antibiofilm activity was conducted based [8] with modification. The antibiofilm activity was divided into inhibition and destruction activity. For inhibition activity assay, each pathogen was inoculated into Brain Heart Infusion Broth (BHIB) supplemented with glucose (2% w/v) and incubated at 37°C, 150 rpm for 24 h. Then 100 μ L of bacterial culture ($OD_{600}=0.132$) and 100 μ L of crude extract was

transferred to the 96-wells microplate to quantify the biofilm inhibition activity. For destruction activity assay, another 96-wells plate was filled with diluted culture and re-incubated at 37°C for 24 h until mature biofilm attached to the well. Crude extract (100 µL) was added to each well to quantify the biofilm destruction activity. Overnight culture of pathogens without any treatment was used as a positive control, while sterile BHIB was used as the negative control.

After incubation, planktonic cells and media were discarded. Adherent cells were rinsed twice with sterile water and stained using crystal violet (0.4% w/v) for 30 minutes. 96-wells were rinsed twice and air-dried for 5 minutes. About 200 µL of ethanol was added and resuspended. Absorbance was determined at 595 nm with a microplate reader (TECAN M200 PRO). This assay was performed in triplicates. Percentage of inhibition or destruction activity was calculated by the equation below:

$$\% \text{ Activity} = \frac{\text{OD Control} - \text{OD Treated}}{\text{OD Control}} \times 100\%$$

Validation of Quorum sensing inhibition

Validation assay was conducted based on [11]. The culture of *C. violaceum* CV026 ($\text{OD}_{600}=0.132$) was mixed with 100 µL of the crude extract and 1 µmol/mL of Hexanoyl-L-Homoserine-Lactone (HHL). The culture was incubated at 28°C for 24 h. Positive control contained culture and 1 µmol/mL of HHL without crude extract. The tested tubes were centrifuged at 1000rpm for 15 minutes. About 1 mL of DMSO (1% v/v) mixed and centrifuged. The absorbance of the supernatant was measured at 540 nm. Furthermore, the determination of protein compounds in the crude extract was performed. Each crude extract was treated with proteinase-K (100 µg/mL) for 2 h incubation in 37°C and heat loss method with 95°C for 1 h. The treated crude extract was used to do the validation assay. Total violacein produced by *C. violaceum* CV026 was compared [13]

Examination of Biofilm formation by Scanning Electron Microscope (SEM)

The effect of crude extract was investigated using SEM at Dexa Laboratory of Biomolecular and Science (DLBS), Cikarang, Indonesia. *B. cereus* and *S. typhimurium* were grown in BHIB supplemented with glucose (2% w/v) and incubated at 37°C for 24 h. Bacterial culture (100 µL) were spotted to sterile cover glass kept in sterile petri dish and incubated at 37°C for 24 h to form a mature biofilm. After the incubation period, 100 µL of crude extract with the highest destruction activity was added into the formed biofilm and incubated at 37°C for 24 h. Mature biofilm without crude extract was used as control [14].

Statistical Analysis

Statistical analysis was conducted by using IBM SPSS Statistics 23. The results were analyzed using the analysis of variances (ANOVA) and the Tukey test for significant difference were determined at $P < 0.05$ and standard error was determined using Microsoft Excel 2016.

Results

Bacterial Characteristic

Actinobacteria isolates have specific characteristics, such as calcareous-like, opaque big colonies, embedded or strongly attached to the agar medium, and produce earthy odor compounds called geosmin.

Screening for Antiquorum Sensing Activity

Inhibition of violacein pigment was determined as a positive result. According to this assay, The results showed that 10 out of 40 actinobacteria isolates had antiquorum sensing activity without inhibiting *C.violaceum* growth.

Antibacterial Activity Assay

There were found 3 out of the 10 actinobacteria crude extracts showed antibacterial activity against *V. cholerae* and non-antibacterial activity against other pathogens, while positive control inhibited the growth of tested pathogens.

Detection of Antiquorum Sensing Activity

All 10 isolates also performed great quorum quenching activity against *C. violaceum* shown with absence of violacein pigment observed around the wells. Presence of quorum quenching activity would inhibit violacein production.

Validation Test Antiquorum Sensing Activity and Protein Characterization

The results shown in Table 1 indicate that all 10 isolates showed quorum quenching activity compared with the control. Quorum quenching agents in extracts 1AC, 14PM, 16PM, 18PM, and CW17 showed a significant increase in absorbance value after Proteinase-K treatment, while extracts from 11AC, 15PM, 20PM, CW01, CW17, and TB12 showed lower absorbance value (Table 1).

Biofilm Inhibition and Destruction Assay

From this assay, all crude extracts were capable of inhibiting and destructing biofilm formation (Table 2). Antibiofilm activity performed were broad spectrum. Antibiofilm against Gram positive bacteria was correlated with the concentration while results against Gram negative bacteria was varied.

Microscopic Observation using Scanning Electron Microscope

Figure 1a and 1b showed the topographical biofilm formation of *B. cereus* before and after treated with crude extract of 20PM isolate. While Figure 1c and 1d showed biofilm of *S. typhimurium*. Drastic morphological change observed after treatment of actinobacteria crude extract.

Discussion

Actinobacteria live in an environment that leads them to do some kind of competitions for combating other microorganisms in various situations. Actinobacteria produce several metabolites such as antimicrobial, anti-quorum sensing, antibiofilm, and several other supporting compounds.

From the first screening of anti-quorum sensing activity, only 10 out of 40 actinobacteria isolates showed anti-quorum sensing activity. Screening of antimicrobial activity is needed because it might lead to false-positive result in further assays. There was no clear zone observed, except in *V. cholerae*. Isolates showing quorum quenching activity are validated. All 10 isolates showed great activity in inhibiting *C. violaceum* quorum sensing activity as seen from lower pigment production.

Quorum quenching agents in extracts of 1AC, 14PM, 16PM, 18PM, and CW17 isolates were predicted as proteins or enzymes because they showed a significant increase in absorbance value after proteinase-K treatment. The treatment might affect protein-based anti-quorum sensing compounds produced by actinobacteria, such as AHL lactonase, acylase, decarboxylase, oxidoreductase, and deaminase [15]. Those enzymes were degraded by the treatment. In the absence of anti-quorum sensing compounds, HHL (C6) signal will diffuse into bacterial cells and binds with LuxR receptor formed a protein-ligand complex. This complex will activate violacein production. The more violacein produced, the higher absorbance will be detected [16].

Crude extract from isolate 15PM, 20PM, CW01, and TB12 treated with proteinase-K showed better activity. It might indicate that proteinase-K degrades the competitive proteins which share similar receptor or degrades proteins that can inhibit the quorum quenching activity [17]. Other probabilities, the anti-quorum sensing compounds were produced in inactive form hence reaction with Proteinase-K will activate the anti-quorum sensing compound [18].

Antibiofilm activity usually correlates with concentration, but few isolates did not show any significant difference. It might be caused by several factors. For instance, lower concentration of crude extracts might degrade all the ECM. Crude extracts might also contain some inhibitors or analog components that match competitively to the receptor. According to [19] undesirable component might affect the effectiveness of the crude extracts. Some components in the crude extracts might work antagonistically or synergistically that can decrease or increase the activity [18, 20].

Topographical observation using SEM showed the presence of biofilm. *S. typhimurium* biofilm before treated with crude extracts showed a rough surface, which means there is a presence of ECM. After the treatment, Fig. 1d showed the crude extracts contained matrix-degrading enzymes due to the depletion and erosion across the surfaces. On the other hand, *B. cereus* showed thick biofilm formation. Treatment with crude extract of 20PM isolate is competent to degrade ECM and Biofilm formation [21].

Conclusion

There were 10 isolates performed great quorum quenching and antibiofilm both destruction and inhibition activity against *P. aeruginosa* ATCC 27853, *S. typhimurium*, *V. cholerae*, *B. cereus* ATCC 14579, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 33186. Quorum quenching compound in extracts 1AC, 14PM, 16PM, 18PM, and CW17 were predicted as proteins. Further research is required to purify the bioactive compounds, characterize the bioactive compounds in each crude extract, and identify the potential isolates.

Limitation

This research did not comprehend the specific compound in the crude extract that competent to perform antibiofilm and antiquorum sensing activity. species of actinobacteria have not identified, characterization of crude extract limited to protein compounds, and the specific mechanism of the quorum quenching activity of each isolate did not determine.

Abbreviations

HHL: Hexanoyl-L-Homoserine-Lactone

AI: Autoinducer

AHL: Acyl Homoserine Lactone

SEM: Scanning Electron Microscope

ECM: Extracellular Matrix

Declarations

Ethics approval and consent to participate. Not applicable

Consent for publication. Not applicable

Availability of data and materials. The data of this study is available with the corresponding author up on request.

Competing interests. The authors declare that they have no competing interests

Funding. This research had funded by Ministry of Research, Technology and Higher Education of the Republic of Indonesia 2019 and Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia 2019. The funder has no contribution in design, collection, writing, and interpreting data in this research.

Authors' Contribution. MW and DD: Proposal Writing, Conducted research, Data Analysis, and manuscript under advisory of DEW. DEW: Provide the materials, methods and proposal design, and bacteria used in this research. being a final investigator in this research.

Acknowledgement. The authors thankfully acknowledge the microscopy facility that provided by the PT Dexa Laboratories of Biomolecular Sciences (DLBS)

Author details. ¹Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia. Jenderal Sudirman 51 Street, South Jakarta, DKI Jakarta, 12930, Indonesia.

References

1. Das S, Dash HR. Microbial-biotechnology: a laboratory manual for bacterial systems. New Delhi: Springer, 2015. p203
2. Rabin N, Zheng Y, Opoku TC, Du Y, Bonsu E, Sintim HO. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Med. Chem.* 2015; doi: 10.4155/FMC.15.6.
3. Li Q, Chen X, Jiang Y, Jiang C. 2016. *Actinobacteria* - Basics and Biotechnological Applications. doi:10.5772/61461
4. Screening of actinomycetes from marine sediments to inhibit *Vibrio cholerae* biofilm formation. Biotechnology, Honours [Thesis]. Jakarta (ID): Atma Jaya Catholic University of Indonesia; 2011.
5. Vidyawan V. Screening of actinomycetes from various environment sediments to inhibit biofilm formation of *Vibrio cholerae*. Biotechnology, Honours [Thesis]. Jakarta (ID): Atma Jaya Catholic University of Indonesia; 2013.
6. Abudoleh SM, Mahasneh AM. Anti-quorum sensing activity of substance isolated from wild berry associated bacteria. *Avicenna J Medical Biotechnol.* 2017;9(1):1-8.
7. Rajivgandhi G, Senthil R, Ramachandran G, Manoharan N. Antibiofilm activity of marine endophytic actinomycetes compound isolated from mangrove plant *Rhizophora mucronata*, Muthupet Mangrove Region, Tamil Nadu, India. *J Terr Mar Res.* 2018; 2(4):1-7.
8. Waturangi DE, Rahayu BS, Lalu KY, Michael, Mulyono N. Characterization of bioactive compound from actinomycetes from antibiofilm activity against Gram-negative and Gram-positive bacteria. *Malays J Microbiol.* 2016; doi :10.21161/mjm.80915.
9. Nithya C, Aravindraja C and Pandian SK. 2010. *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. *Res. Microbiol.* 2010; 161 293–304.
10. Nathan P, Law EJ, Murphy DF. A laboratory method for the selection of topical antimicrobial agents to treat infected burn wounds. *Burn.* 1978; 4:177-17.
11. Rajivgandhi G, Vijayan R, Maruthupandy M, Vaseeharan B, Manoharan N. Antibiofilm effect of *Nocardiosis* GRG 1 (KT235640) compound against biofilm-forming Gram-negative bacteria on UTIs. *Microbial Pathogenesis.* 2018; doi: 10.1016/j.micpath.2018.03.011
12. Khan MSA, Zahin M, Hasan S, Husain FM, Ahmad I. Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. *Lett Appl Microbiol.* 2009; doi:10.1111/j.1472-765x.2009.02666.x.

13. Thenmozhi, R. P., Nithya, J. R. & Pandian, S. K. Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. FEMS Immunol Med Microbiol. 57, 284-294 (2009).
14. Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, Lei D, Chen Y, Kong J, Chen Y. Baicalin 263 inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances 264 *Pseudomonas aeruginosa* clearance in a mouse peritoneal implant infection model. PLoS ONE. 2017;12(4): e0176883.
15. Chen F, Gao Y, Chen X, Yu Z, Li X. Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection. Intl. J. Mol. Sci. 2013; doi:10.3990/IJMS140917477
16. Stauff DL, Bassler BL. Quorum Sensing in *Chromobacterium violaceum*: DNA Recognition and Gene Regulation by the CviR Receptor. J Bacteriol. 2011; doi:10.1128/jb.05125-11.
17. Omran R, Kadhem MF. Production, purification, characterization of bioactive metabolites produced from rare *Actinobacteria psudonocardia* Asian J Pharm Clin res. 2016; 9(3):264-272.
18. LaSarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. Microbiol Mol Biol Rev. 2013; doi:10.1128/mubr.00046-12.
19. Rodriguez-Saona LE, MM. Giusti, W.D. Robert and W.E. Ronald. Development and process optimization of red radish concentration extract as potential natural red colorant. J Food Process Preserv. 2001; 25: 165–182.
20. Giguère, S, Lee EA, Guldbach KM, Berghaus LJ. *In vitro* synergy, pharmacodynamics, and postantibiotic effect of 11 antimicrobial agents against *Rhodococcus equi*. Vet Microbiol. 2012; 160(1-2): 207-213.
21. Li, X. H. & Lee, J. H. Antibiofilm agents: a new perspective for antimicrobial strategy. J Microbiol; 10.1007/s12275-017-7274-x (2017).

Tables

Table 1 Absorbances of total violacein produced by *C. violaceum* CV026

Isolate	CV026 + Extract	CV026 + Extract + proteinase-K + 95 °C
1AC	0.9770±0.0092	1.0310±0.0035
11AC	1.0460±0.0029	1.0260±0.0069
14PM	0.8050±0.046	1.0300±0.0029
15PM	0.8565±0.0020	0.7505±0.0234
16PM	0.8625±0.0026	1.1090±0.0012
18PM	0.9190±0.0058	1.1320±0.0035
20PM	1.1760±0.0003	0.8650±0.0115
CW01	1.1230±0.0012	0.8865±0.0009
CW17	0.6945±0.0430	0.9070±0.0046
TB12	1.1690±0.0018	1.0945±0.0141
Control	1.18515±0.0031	1.1845±0.0032

CV026 = *C. violaceum* CV026

Table 2 Antibiofilm activity of actinobacteria crude extracts against pathogenic biofilm associated bacteria

Pathogen	Antibiofilm Activity	Crude Extract (%)	% Activity									
			1AC	11AC	14PM	15PM	16PM	18PM	20PM	CW01	CW17	TB12
SA		5	65	87*	78	43	64	46	55	76	74	44
		10	90*	90*	79	66	75	74	76	77	75	62
BC		5	89*	85	76	78	84	57	86	84	85	81
		10	90*	87	76	83	87	69	88	87	85	84
EF	Inhibition	5	49	70	12	36	43	15	27	76	83*	33
		10	82	71	34	71	77	40	50	83	84*	81
VC		5	93	89	90	X	93	X	X	9	92	94*
		10	76	79	84*	X	82	X	X	83	70	58
ST		5	76	83	84	80	81	81	85*	80	81	81
		10	56	63	50	54	27	76*	59	63	66	25
PA		5	29	70	49	38	50	39	37	47	54*	42
		10	83	82	86	91	85	81	89	93*	87	37
SA		5	62*	32	41	38	40	38	42	45	50	37
		10	77*	49	54	74	59	65	62	60	59	61
BC		5	61	67	64	67	65	67	75*	72	73	67
		10	84	80	78	68	84	75	85*	83	74	79
EF	Destruction	5	43	44	50	47	47	45	46	50	52*	43
		10	58	63	55	70	72*	70	65	52	63	45
VC		5	-	52	-	X	71	X	X	73*	71	70
		10	11	48	62*	X	13	X	X	43	52	43
ST		5	57	43	54	34	29	58	51	66	73*	64
		10	56	20	72	54	47	82*	64	79	78	42
PA		5	11	53	29	54	63*	17	59	38	26	58
		10	43	45	41	57	42	50	55*	50	55*	50

Staphylococcus aureus ATCC 29213; *Bacillus cereus* ATCC 14579; *Enterococcus faecalis* ATCC 33186, *Vibrio cholerae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* ATCC 27853.

*Highest activity, X Have antimicrobial activity, - No activity

Figures

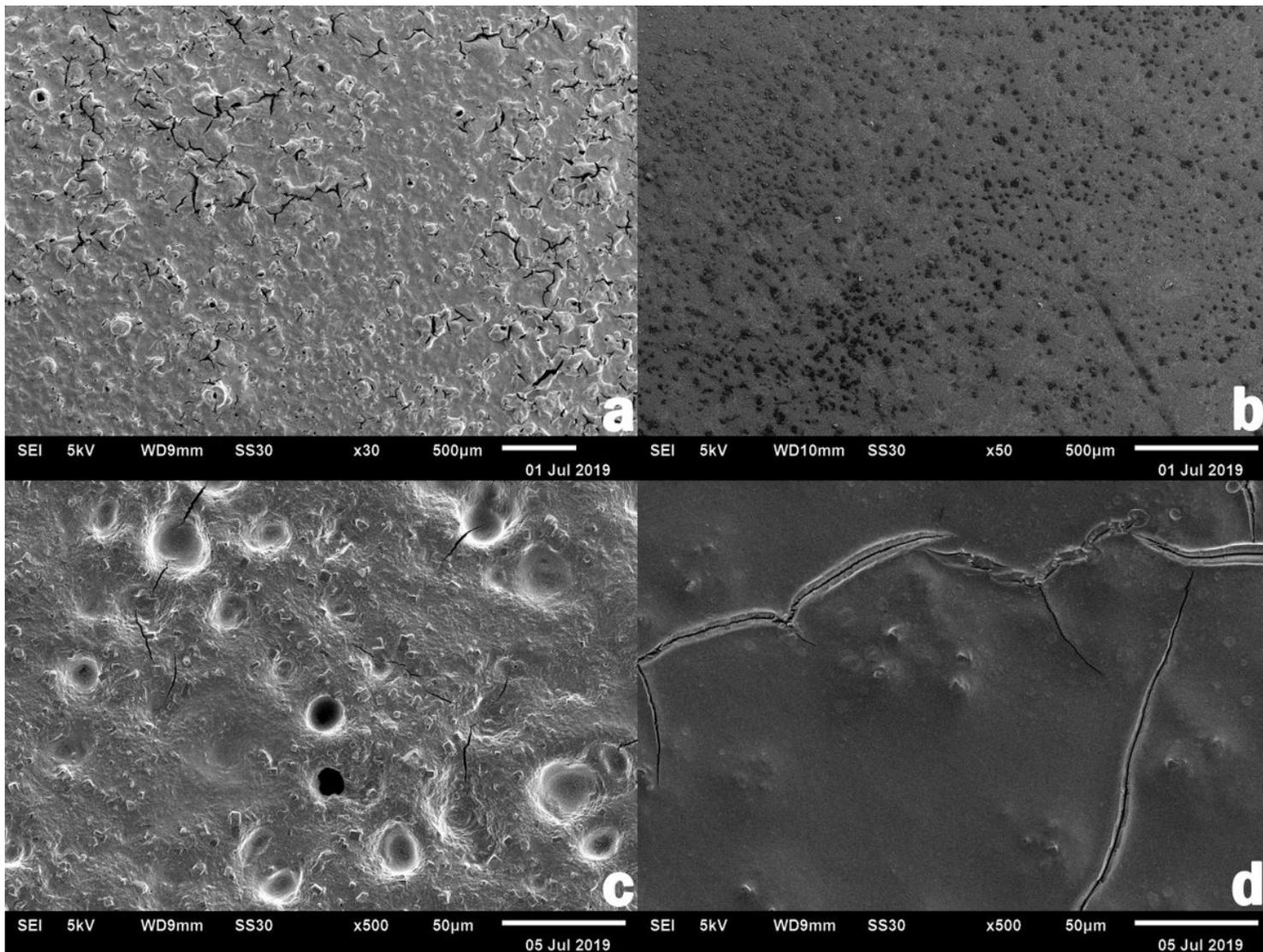


Figure 1

Biofilm of *B. cereus* ATCC 14579. (a) without treatment (b) with addition crude extract of 20PM isolate. Biofilm of *S. typhimurium*. (c) without treatment (d) with addition crude extract of CW01 isolate