21. Hidayati_2021_IOP_Conf._Ser.__Earth_Environ._Sci._707_012008

by 21. Bu Amin Samiasih

Submission date: 11-Jan-2023 05:27PM (UTC+0700)
Submission ID: 1991171188
File name: Hidayati_2021_IOP_Conf._Ser.__Earth_Environ._Sci._707_012008.pdf (742.9K)
Word count: 3371
Character count: 19105

IOP Conference Series: Earth and Environmental Science

PAPER • **OPEN ACCESS**

Bacillus tequilensis Isolated from Fermented Intestine of *Holothuria Scabra* Produces Fibrinolytic Protease with Thrombolysis Activity

To cite this article: Nur Hidayati *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **707** 012008

View the article online for updates and enhancements.

You may also like

- Green synthesized novel silver nanoparticles and their application as anticoagulant and thrombolytic agents: A perspective
M A Azeez, F A Durodola, A Lateef et al.
- *In vitro* thrombolytic efficacy of echogenic liposomes loaded with tissue plasminogen activator and octafluoropropane gas
Himanshu Shekhar, Kenneth B Bader, Shenwen Huang et al.
- Synthesis, bioactivities and cytogenotoxicity of animal fur-mediated silver nanoparticles
G O Akintayo, A Lateef, M A Azeez et al.

This content was downloaded from IP address 182.2.37.202 on 23/11/2022 at 03:32

Bacillus tequilensis Isolated from Fermented Intestine of *Holothuria Scabra* Produces Fibrinolytic Protease with Thrombolysis Activity

Nur Hidayati[1], Nurrahman Nurrahman[2], Hayatun Fuad[1], Hendra Munandar[3], Dewi Seswita Zilda[4], Aditya Rahman Ernanto[5], Amin Samiasih[5], Oedjijono Oedjijono[6], Stalis Norma Ethica[1*]

[1]Magister Study Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang, 50273, Indonesia
[2]Food Technology Study Program, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, 50273 Indonesia
[3]Balai Bio Industri Laut - LIPI (Lembaga Ilmu Penelitian Indonesia) Lombok, Nusa Tenggara Barat, 83352, Indonesia
[4]Research and Development Center for Marine and Fishery Product Processing and Biotechnology, Ministry of Maritime Affairs and Fisheries of Indonesia, Jakarta, 10260, Indonesia
[5]Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, 50273 Indonesia
[6]Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, 53122, Indonesia

[*]Corresponding author: norma@unimus.ac.id

Abstract. Among essential treatment of cardiovascular disorders are fibrinolytic proteases. Most thrombolysis agents are fibrinolytic enzymes from group of bacterial proteases. This work reports a potent bacterium isolated from fermented intestine of *H. scabra*, which could produce fibrinolytic protease with high thrombolysis activity. Bacterial selection was conducted based on proteolytic and fibrinolytic activities indicated as clear zone on skim milk and fibrin agar media, respectively. Crude proteases from the selected bacterial isolates were subjected to thrombolytic activity test based on gravimetric method, which results were confirmed after 7 repetitions. As result, 4 fibrinolytic protease-producing bacterial isolates HFSI-3, HFSI-4, HFSI-5 and HFSI-8 were obtained. Among them, HFSI-5 isolate identified as *Bacillus tequilensis* on the basis of the 16S rRNA gene sequencing and morphological properties produced crude protease with the highest thrombolytic activity. The thrombolytic activity of crude protease produced by *B. tequilensis* HFSI-5 is worthy of comparing to that of standard fibrinolytic enzyme Nattokinase showing its potential as thrombolysis agent.

1. Introduction

Cardiovascular diseases (CVDs) have been known as one of the main causes of deaths, which account for 31% of mortality all over the world [1]. It has been predicted by The American Heart Association that by 2030, mortality by CVDs shall go over 23.6 million [2]. The imbalance fibrin formation and fibrinolysis in the body is the underlining problem in CVDs [3]. Such imbalance leads to thrombosis or intravascular clotting in blood vessels [4-5]. Thrombus limits the circulation of blood by blocking it

Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.
Published under licence by IOP Publishing Ltd

from passing through veins and arteries. This medical condition could lead to cardiac abnormalities such as heart stroke, myocardial infarction, embolism, etc. [6-9]. For both the prevention and treatment of thrombosis, thrombolytic agents play important role [8]. In fact, thrombolysis is the only therapy that can re-establish blood flow quickly and easily [7].

Among essential treatment of cardiovascular disorders functioning as thrombolysis agent are fibrinolytic enzymes, mostly from group of proteases of bacteria [4-6]. Since ancient times microorganism has contributed to the use and production the fibrinolytic enzymes. In the last decades, researchers have intensively paid reported production of fibrinolytic enzymes by bacteria, particularly those from the food sources. A number of fibrinolytic enzymes have been reported to have anti-thrombosis activities, particularly from the genus Bacillus [10-13].

Holothuria scabra is among sand sea cucumbers commonly found in Indonesia and has been favourite sea invertebrate captured by local fishermen. The holothurian had been reported to have high protein content giving it a possibility to be a rich substrate for protease producing bacteria [14]. Some of bacterial proteases, particularly those with serine active site have been reported to be able to degrade fibrins. Degradation of fibrins by these fibrinolytic proteases is among the key step of thrombolysis. This makes serine proteases from bacteria are potential to be used as thrombolysis agent.

To date, the applications of fibrinolytic enzymes to manage heart diseases are still costly. Therefore, the search for new sources of fibrinolytic proteases is essential allowing the use of large quantities of the enzymes as the could be produced conveniently and efficiently [11]. This work aimed to report identification of bacterium isolated from fermented intestine of *H. scabra* capable of producing fibrinolytic protease with thrombolysis activity.

1.1. Material

Samples used in this study were 12 bacterial isolates from sea cucumber (*H. scabra*) intestine obtained from its captivity located at Lembaga Ilmu Pengetahuan Indonesia Institute for Bio Industry (BBIL LIPI), Kodek Gulf Village, Lombok, West Nusa Tenggara. Culture media included Nutrient Agar (NA) and Skim Milk Agar (SMA) (both from Oxoid, UK), fresh blood from volunteers, fibrin agar media (freshly prepared from the fresh blood), (Oxoid, UK), and Gram-staining reagent. Identification of bacterium required optical microscope, Presto^{TM} gDNA Bacteria Kit (Geneaid, Taiwan). The equipment used were analytical balance (Mettler Toledo), autoclave (Hirayama HVE-50, Japan), Laminar Cabinet (Dalton, Japan), spectrophotometer Maestro Nano-Pro (Maestro-Gen, Taiwan), Micro-centrifuge (Bechman Counter, USA), incubator (Mammert, Germany), micropipette (Socorex Calibra 822, French), Vortex (Genie 2TM 12-82, USA), calliper, microtubes, and pipette tips.

2. Method

2.1. Isolation and Selection of Proteolytic Bacteria

According to previous work, after colony selection and purification on NA media, 12 bacterial isolates were obtained on bacteria isolated from fermented intestine of *H. scabra*. After sub-culturing and colony purification, Gram staining was performed on bacterial cells and the result was observed under optic microscope. Next, each of these bacterial isolates was tested for proteolytic activity on SMA and the presence of clear (proteolytic) zone was observed within a-week of cultivation [15]. Bacterial isolates which show growing proteolytic zones within 7 days were then screened for fibrinolytic activity using fibrin media [16].

2.2. Selection of Fibrinolytic Protease Producing Bacteria on Fibrin Media

Fibrinolytic activity test on proteolytic bacterial isolates was performed on fibrin agar medium. Fibrin solid medium was prepared from fresh human blood plasma following previously reported method by Zhang et al. (2012) [17]. Well diffusion method was applied on solid fibrin medium to cultivate bacterial colonies. A total of 50 µL of rejuvenated proteolytic bacterial inoculum for each sample and and another 50 µL Nattokinase for control was inserted into each of well. The culture was then incubated at 37°C at pH =7 for 5 days. Every day within 5 days the plates were observed for the presence of a clear zone around the well as indication of fibrinolytic activity [18-19].

IOP Conf. Series: Earth and Environmental Science **707** (2021) 012008 doi:10.1088/1755-1315/707/1/012008

2.3. Clot Lysis Activities of Bacterial Crude Proteases

For the gravimetric thrombolysis test, the empty micro-centrifuge tube was weighed and each weight recorded. Blood was taken from healthy volunteers as much as 500 µl and put in a micro centrifugation tube and incubated at 37 °C for 45 min. After clotting, serum was taken so as not to interfere with the blood clot. Weight of the clot was determined as follows: weight of clot = weight of tube with clot - weight of empty tube [20].

2.4. Bacterial DNA Isolation

The DNA isolation process was carried out according to the steps instructed by the DNA kit manufacturer. Sample preparation for Gram-positive was started by dilution bacterial sample 1 x 10^{-9} then following kit procedures including extraction, lysis, DNA binding to the column filter, washing and elution. Measurement of DNA concentration and purity was done using spectrophotometer Maestro Nano-Pro.

2.5. Amplification 16S rRNA and Gel Agarose Electrophoresis

The amplification process used Go Tag Green Master Mix (Promega). Primers used are universal primers 27-F (5'-AGA GTT TGATCC TGG CTC AG-3') and 1492-R (5'- GGT TAC CTT GTT ACG ACT T-3'). The PCR steps were initial denaturation at 95 °C for 4 minutes, then followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 35 s, extension at 72 °C for 45 s. Electrophoresis used 2% agarose in 100 ml TAE and used Cybersafe as DNA dye. Vivantis 1 Kbp marker was used to check the amplicon size [21].

2.6. DNA Sequencing

The 16S rRNA DNA fragment of bacterium obtained as PCR product or amplicon was then sequenced. The DNA sequencing was based on Sanger method [22] was carried out by PT. Genetika Science Indonesia.

2.7. Bioinformatics Analysis

The results of 16S rRNA DNA sequencing were analysed using bioinformatics tools. Assembly consensus sequence was carried out on forward and reverse sequences of the 16S rRNA gene with the DNABaser Assembler Program v3x [23]. The consensus was then processed and matched with data at www.ncbi.nih.gov through the BLAST (Basic Local Alignment, Search Tool - for nucleotide) program to determine the species of isolate from the Genbank database [24-26].

3. Results and Discussion

This study aimed to obtain a potent bacterium isolated from fermented intestine of *H. scabra,* which could produce fibrinolytic protease with high thrombolysis activity and to identify it using PCR method. Several tests were conducted to analyze the capacity of bacterial isolates to produce protease after 7-day incubation and ability of the isolates to degrade fibrin and blood clot as model of thrombus. The selection of proteolytic bacteria isolates was carried out based on ability to produce protease using Skim Milk Agar (SMA) media. SMA media contains casein, a protease substrate. Activity of bacterial protease may break the peptide bonds of casein in skim milk [15]. Formation of a clear zone indicates the occurrence of casein hydrolysis by bacterial protease.

Table 1 showed the capacity of bacterial isolates to produce protein on SMA media observed on the 1st and 7th day of incubation. As seen on Table 1, proteolytic zones of the colonies of Isolate HSFI-2 to -6 and HFSI-8 to -12 (HSFI refers to *Holothuria scabra* Fermented Intestine) were all greater after 7-day incubation.

Expanded clear zone on SMA medium confirmed capacity of these isolates to produce proteases. Such positive results allow the next selection process to determine which proteolytic bacteria produces fibrinolytic proteases. Fibrinolytic test was then conducted on proteolytic isolates, and the results are displayed by Figure 1. Nattokinase was used as standard positive control. Nattokinase is well established as promising enzyme produced by *Bacillus natto* in fermented soybean in the Japanese diet

providing them with the lowest rate of thrombosis disorders all over the world. Each fibrinolytic enzyme usually has a special structure and mechanism of action [27].

Table 1. Proteolytic zone of bacteria isolates from fermented intestine of *H. scabra* after 7-day incubation on SMA media.

Isolate code	1-Day Proteolytic (clear) zone	7-Day Proteolytic (clear) zone	Isolate code	1-Day Proteolytic (clear) zone	7-Day Proteolytic (clear) zone
HSFI-2			HFSI-8		
HSFI-3			HFSI-9		
HSFI-4			HFSI-10		
HSFI-5			HFSI-11		
HSFI-6			HFSI-12		

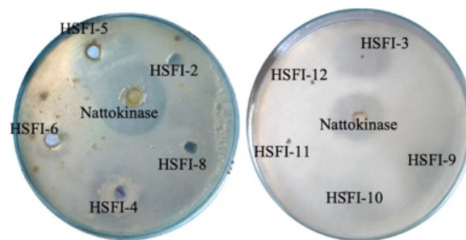

Figure 1. Clear zones indicating positive fibrinolytic test results of isolate HSFI-3, HSFI-4, HSFI-5 and HSFI-6 on fibrin agar media using Nattokinase as positive control

Fibrinolytic activity was determined by the ability of the isolate's enzyme to hydrolyze the fibrin substrate, which is indicated by the formation of a clear zone around the bacterial colony. The greater the fibrinolytic activity, the larger the clear zone formed, indicating the more fibrin is hydrolyzed by fibrinolytic enzymes [28]. Fibrinolytic ability test was performed on selective media containing 3% w/v fibrin. The fibrin used was obtained from healthy human plasma [29]. Results in **Figure 1** also showed that only 4 of 10 proteolytic bacterial isolates, HSFI-3, HSFI-4, HSFI-5 and HSFI-6, could degrade fibrin substrates indicated by their ability to form clear zone around the colony on fibrin agar

media. Based on this, only crude fibrinolytic proteases of the 4 isolates were subjected to thrombolysis test.

In this study, crude fibrinolytic protease from bacteria was tested for its thrombolysis activity. The crude enzyme was isolated by centrifuging the bacterial culture in broth media to obtain supernatant. The supernatant containing crude enzyme could be then tested for its activity in lysing blood clots in *vitro* by gravimetry method as previously reported [30]. Figure 2 and 3 showed centrifugation step and the obtained crude enzyme from the thrombolysis test, respectively, based on gravimetric method.

Figure 2. Centrifugation of bacterial culture in skim milk broth to obtain supernatant considered as crude protease

Figure 3. Crude protease from HSFI-3, -4, -5, and -6 isolates obtained after centrifugation

Results of thrombolytic activity screening test conducted on crude proteases of HSFI-3 to -6 against fresh blood clot using Gravimetric method were displayed in Table 2.

Table 2. Results of the thrombolysis activity test using the Gravimetric method using fresh blood clot

Isolate Code	Average weight of initial blood clot (g)*	Average weight of final blood clot (g)*	Percentage (%)
HSFI-3	0.2326	0.2204	26.06
HSFI-4	0.2388	0.1927	5.24
HSFI-5	0.4128	0.1567	62.03
HSFI-6	0.2486	0.1667	32.94
Aquadest	0.2668	0.2650	0.29
Nattokinase	0.2462	0.1082	56.05

Note: Measurement in duplo

Table 3. Thrombolysis activity test results using the Gravimetric method on crude fibrinolytic protease of HSFI-5 and Nattokinase as standard.

Measurement	HSFI-5 crude enzyme	Nattokinase (standard)
1	60.44	48.39
2	81.13	63.16
3	74.44	78.39
4	70.32	64.41
5	64.41	63.15
6	64.93	60.36
7	62.04	64.26
Average	68.24	63.16

Based on data of Table 2, crude fibrinolytic protease from HSFI-5 isolate showed the highest thrombolytic activity. To confirm this, the gravimetric measurement was carried out with 7 repetitions

in particular for crude fibrinolytic enzyme of HSFI-5 isolate and Nattokinase as standard of thrombolysis agent. The result is shown by Table 3.
The crude enzyme from bacteria previously selected based on proteolytic and fibrinolytic activities were tested for thrombolysis activity to determine whether they had the ability to lyse blood clots. *In vitro* thrombolysis test used Gravimetric method, the weight of clots before lysis and after lysis is considered the right determinant for calculating the percentage of clot lysis [20]. As seen on **Table 3**, the gravimetric test results showed that in average crude protease of isolate HFSI-5 could lyse 68.24% clot lysis isolate HSFI-5. As comparison, commercial fibrinolytic enzyme Nattokinase in average could lyse clot in slightly lower percentage by 63.16%. This shows that crude protease from HFSI-5 has potential to be used as a competitive thrombolysis agent. It is therefore important to identify the species of HFSI-5 isolate to determine optimum condition for larger production scale of the bacterial crude protease.
Part of bacterial identification was determining bacterial morphologic characteristics. The morphological characteristics of colonies and cells of bacterial isolate HSFI-5 are described in Figure 2 and Table 4. Based on Figure 2 and Table 4, colonies and cells of HFSI-5 are uniform showing colony purity of the bacterium. Next molecular identification on HFSI-5 isolate could be conducted based on DNA sequence of 16S rRNA gene of the bacterium [31].

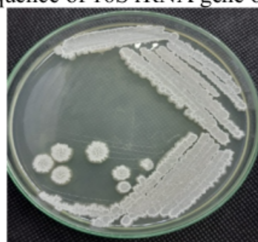

Figure 4. Colony morphology of HFSI-5 isolate

Figure 5. Cell morphology of isolate HSFI-5

Table 4. Colony characteristics (A) and Gram staining and cell characteristics (B) of isolate HSFI-5

Isolate Code	**Colony Characteristics**				
	Form	***Margin***	***Color***	***Elevation***	***Consistency***
HSFI-5	filamentous	filiform	white	crateriform	rough
	Cell Characteristics				
	Shape			*Gram staining*	
	Bacilli			Gram-Positive	

Molecular identification of bacterial isolate HFSI-5 was based on sequence 16S rRNA gene and compared to the database in Genbank [32]. PCR product using bacterial genomic as template was visualized as 16S rRNA DNA band sized about 1500 bp on electrophoresis agarose gel (Figure 6).
The results of BLAST analysis of the HSFI-5 isolate based on the sequence of 16S rRNA gene fragment showed a homology level of 100% with that of *Bacillus tequilensis* JLS-12 (Figure 7). Based on the result, HFSI- bacterial isolate was named *Bacillus tequilensis* HSFI-5. This molecular identification result was confirmed by morphology of colonies and cells of HSFI-5 isolate, which resemble those of *B. tequilensis* previously reported by Li et al. in 2018 [33].

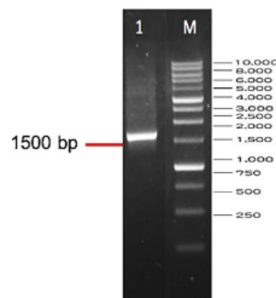

Figure 6. Visualization DNA fragment of 16S rRNA from PCR products

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Bacillus tequilensis strain JLS12 16S ribosomal RNA gene, partial sequence	2535	2535	100%	0.0	100.00%	MT501810.1
☑	Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2535	25264	100%	0.0	100.00%	CP053102.1
☑	Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2535	25264	100%	0.0	100.00%	CP052842.1
☑	Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2535	25264	100%	0.0	100.00%	CP051860.1
☑	Bacillus subtilis subsp. subtilis strain UCMB5121 chromosome, complete genome	2535	25273	100%	0.0	100.00%	CP051465.1
☑	Bacillus subtilis subsp. subtilis str. SMY chromosome, complete genome	2535	25256	100%	0.0	100.00%	CP050532.1
☑	Bacillus subtilis strain NFAA 16S ribosomal RNA gene, partial sequence	2535	2535	100%	0.0	100.00%	MT192659.1
☑	Bacillus subtilis strain H1 chromosome, complete genome	2535	25209	100%	0.0	100.00%	CP026662.1
☑	Bacillus subtilis strain LSRBMoFPIKRGCFTRI33 16S ribosomal RNA gene, partial sequence	2535	2535	100%	0.0	100.00%	MT133340.1
☑	Bacillus subtilis strain LSRBMoFPIKRGCFTRI32 16S ribosomal RNA gene, partial sequence	2535	2535	100%	0.0	100.00%	MT133339.1

Figure 7. Result of 16S rRNA gene fragment sequence alignment from BLAST analysis tool [24]

Bacillus tequilensis was first reported by Gatson *et al*. 2006 from a two-thousand-year-old Mexican shaft tomb near the city of Tequila [34]. *B. tequilensis*, first identified and described in 2006, has the ability to degrade ammonia nitrogen (NH_3-N) in its cells [35]. *B. tequilensis*, Gram-positive spore forming bacteria was reported as a new species [34]. *B. tequilensis* can grow in anaerobic, motile, positive oxidase and catalase tests, has the ability to degrade tryptophan and starch but does not degrade urea, and can utilize citrate as a carbon source, physiologically, bacteria are able to withstand salt concentrations as high as 8% [35].
B. tequilensis isolated from marine sediment samples could produce surfactin, while. *B. tequilensis* from the intestinal content of earthworms that have high collagenase activity [36-37]. The bacterium has also previously been reported to have the ability as a starch hydrolyzer [38]. Based on the 16S rRNA gene sequence, there is a 99% similarity between B. tequilensis and B. subtilis [34;36]. This explains BLAST results shown in Figure 7 displaying very high similarity (100%) shared between *B. tequilensis B. subtilis*.
Based on literature study, *B. tequlensis* with the ability to produce fibrinolytic protease enzymes capable of lysing blood clots (thrombus) has not been reported. Thus, this study is the first to report potential application of *B. tequilensis* in the health sector, particularly in relation to its ability to lyse thrombus competing with a commercial thrombolysis agent, Nattokinase.

4. Conclusion
Bacillus tequilensis HFSI-5, a potent fibrinolytic protease-producing bacterium from fermented intestine of *H. scabra*. The thrombolytic activity of the bacterium's fibrinolytic protease is worthy of comparing to that of standard fibrinolytic enzyme Nattokinase.

5. Statement
This study was approved by the Ethics Committee of Komisi Etik Penelitian Kesehatan (KEPK) of Faculty of Public Health, Universitas Muhammadiyah Semarang, Indonesia (approval no. 377/2020)

6. Acknowledgments

This study was financially supported by Kemenristek/BRIN or Ministry of Research and Technology/ National Research and Innovation Agency of the Republic of Indonesia through Research Program of Thesis Magister 2020 [Grant number 0004/UNIMUS.L/PG/PJ/2020]. Sea cucumber samples were obtained from its captivity at Balai Bio Industry Laut, Lembaga Ilmu Pengetahuan Indonesia (BBIL LIPI/ Indonesian Institute of Science), Kodek Gulf Village, Lombok, West Nusa Tenggara, Indonesia.

21. Hidayati_2021_IOP_Conf._Ser.__Earth_Environ._Sci._707_012...

ORIGINALITY REPORT

14%	12%	12%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	dspace.brunel.ac.uk Internet Source	1%
2	www.wjgnet.com Internet Source	1%
3	link.springer.com Internet Source	1%
4	www.bio-conferences.org Internet Source	1%
5	idus.us.es Internet Source	1%
6	www.frontiersin.org Internet Source	1%
7	www.scilit.net Internet Source	1%
8	Jeffrey M Ede, Richard Beanland. "Adaptive learning rate clipping stabilizes learning", Machine Learning: Science and Technology, 2020 Publication	1%

9	core.ac.uk Internet Source	1 %
10	Asma Shah, Kafeel Ahmad, Ali Talha Khalil, Farhat Amin, Ghosia Lutfullah, Khalid Khan, Ghazala Shah, Aftab Ahmad. " mediated synthesis of gold nanoparticles and its diverse biomedical applications ", Materials Research Express, 2019 Publication	1 %
11	id.123dok.com Internet Source	1 %
12	www.ijesd.org Internet Source	1 %
13	bbrc.in Internet Source	1 %
14	repository.ubaya.ac.id Internet Source	1 %
15	L C R P da Silva, V Todaro, F A do Carmo, F S Frattani, V P de Sousa, C R Rodrigues, P C Sathler, L M Cabral. "A promising oral fucoidan-based antithrombotic nanosystem: development, activity and safety", Nanotechnology, 2018 Publication	<1 %
16	iopscience.iop.org Internet Source	<1 %

17	"Encyclopedia of Marine Biotechnology", Wiley, 2020 Publication	<1 %
18	Sri Darmawati, Afiana Rohmani, Laela Hayu Nurani, Muhammad Evy Prastiyanto et al. "When plasma jet is effective for chronic wound bacteria inactivation, is it also effective for wound healing?", Clinical Plasma Medicine, 2019 Publication	<1 %
19	ejurnal.unisri.ac.id Internet Source	<1 %
20	hqlo.biomedcentral.com Internet Source	<1 %
21	Sri Darmawati, Nasruddin Nasruddin, Gela Setya Ayu Putri, Arya Iswara et al. "Accelerated Healing of Chronic Wounds under a Combinatorial Therapeutic Regimen Based on Cold Atmospheric Plasma Jet Using Contact and Noncontact Styles", Plasma Medicine, 2021 Publication	<1 %

Exclude quotes Off
Exclude bibliography Off
Exclude matches Off