Characterization of bacteria from Liquid Clinical Laboratory Waste with Potential as Bioremediation Agent

by Stalis Norma Ethica

Submission date: 06-Sep-2021 10:23AM (UTC+0700)
Submission ID: 1642170106
File name: 2021_09_JURN_SNE_WJPLS_Ellyka_et_al.pdf (636.74K)
Word count: 5733
Character count: 31101

wjpls, 2021, Vol. 7, Issue 9, 16 – 26.

Research Article

ISSN 2454-2229

World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org

SJIF Impact Factor: 6.129

CHARACTERIZATION OF BACTERIA FROM LIQUID CLINICAL LABORATORY WASTE WITH POTENTIAL AS BIOREMEDIATION AGENT

Ellyka Purwaningrum[1], Siti Thomas Zulaikhah[2] and Stalis Norma Ethica[1]*

[1]Magister Study Program of Clinical Laboratory Science, Universitas Muhammadiyah Semarang, Indonesia.
[2]Medical Faculty, Universitas Sultan Agung, Indonesia.

Corresponding Author: Stalis Norma Ethica
Magister Study Program of Clinical Laboratory Science, Universitas Muhammadiyah Semarang, Indonesia.

Article Received on 10/07/2021 | Article Revised on 31/07/2021 | Article Accepted on 20/08/2021

ABSTRACTS

Background Liquid biomedical waste, though generated in small quantity, exposes great risk to public health because aside of being infectious, it could easily contaminate other wastes. World Health Organization (WHO) has recommended bioremediation method for handling biomedical waste as it is considered economical and environmentally friendly. Indigenous hydrolytic bacteria with low level of pathogenicity have been acknowledged for their potential to act as bioremediation agents of liquid biomedical waste. This research aimed to obtain indigenous, hydrolytic bacterial isolates with low pathogenicity level, so to fulfill the characteristics as a bioremediation agent for liquid clinical laboratory waste. **Methods:** To obtain bacterial isolates with potential as bioremediation agents for liquid biomedical, procedures of isolation, selection and molecular identification using PCR (Polymerase Chain Reaction) targeting bacterial 16S rRNA gene were conducted. Indigenous bacteria were first isolated from liquid clinical waste on Nutrient Agar (NA) media and then selected through hydrolysis test on Skim Milk Agar (SMA) and tributyrin media as well as pathogenicity test on Blood Agar Plate (BAP) and McConkey (MC) media. Finally, the selected bacteria were subjected to molecular identification. **Results:** Six isolates of indigenous bacteria, IBLS-1 to IBLS-6, could be obtained from liquid waste of IBL clinical laboratory. Two of them molecularly identified as *Bacillus paramycodes IBLS-1* and *B. firmus* IBLS-4 were hydrolytic and lactose fermenters, yet had no ability to cause hemolysis on BAP media. **Conclusion/Conclusions**: Two indigenous *B. paramycodes* IBLS-1 and *B. firmus* IBLS-4, fulfill characteristics as bioremediation agent of liquid biomedical waste of IBL clinical laboratory at Semarang.

KEYWORDS: Clinical laboratory waste, bioremediation agent, bacterial isolation, bacterial identification, Polymerase Chain Reaction (PCR).

INTRODUCTION

Clinical laboratory is a health facility carrying out tests on materials or specimens of human origin. Specimens include serum, plasma, blood (whole blood), urine, stool, sputum, pus, sperm, throat swab, rectum swab, secret (urethra, vagina, ear, nose, eye), pleural fluid, bronchial fluid, acid fluid, brain fluids, gastric rinses, bone marrow, nails, hair, skin scrapings, and vomit.[1]

Liquid biomedical waste produced by clinical laboratories poses a great risk to public health. Biomedical waste is usually generated in smaller quantity compared to other wastes, but it is infectious and can contaminate other wastes, so that the amount is multiplied. This is because biomedical waste does not only contain toxic chemicals, but also parasites and pathogenic microorganisms from fungi, bacteria and viruses that can cause infection and could easily replicates.[2-4]

Inadequate handling of medical waste is a serious problem in developing countries such as Nepal, Algeria, Nigeria and Indonesia because of the risks it poses to human health and the environment. Biomedical waste contains various chemicals, parasites and pathogenic microorganisms from the fungi, bacteria and viruses that can cause infection.[3-5] This problem causes medical waste to be handled properly so as not to endanger human and environment.[6]

In 2017, the World Health Organization (WHO) specifically recommended non-incineration methods for handling biomedical waste in Indonesia. One of the non-incineration methods that are considered economical and environmentally friendly is the bioremediation method.[6] Bioremediation is a process that involves biological mechanisms to reduce or decrease, detoxify, change the concentration of pollutants into a harmless state.[7-8] The

main biological agents in the bioremediation process are microorganisms and enzymes.[9-11]

Hydrolytic bacteria having the potential as bioremediation agents could be obtained from indigenous bacteria which have low to non-pathogenic levels of pathogenicity. Hydrolytic bacteria could produce enzymes that degrade the main ingredients of liquid biomedical waste including fats and proteins. Non-pathogenic characteristics of bacteria are an important attribute for bioremediation agent, so they could be applied safely in the field.[5-12]

The discovery of bacterial isolates that have the potential as bioremediation agents from biomedical waste was obtained by isolation, selection and molecular identification.[6] Isolation of bacteria is the process of taking bacteria from the medium or environment of origin, and growing it on an artificial medium so that a pure culture or culture is obtained from the isolation. Isolation was carried out by cultivating and purifying bacterial colonies from wastewater samples on Nutrient Agar (NA) media. Pathogenicity selection used MacConkey (MC) and Blood Agar Plate (BAP) media.[6,13,14] Lipolytic selection was carried out then using Tributyrin 1% medium.[15] Molecular bacterial identification by detecting genomic elements (DNA and RNA) is necessary to confirm the phenotypic identification results including morphological identification, as well as biochemical identification.[16-17]

Molecular technology allows the isolation of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) directly from samples obtained from the environment that can be described as a whole for a community. This method is mainly based on polymerase chain reaction (PCR) amplification of the nucleotide sequence of the 16S rRNA gene. The 16S rDNA sequence (the gene encoding 16S rRNA) in living things has a conserved sequence so it can be used as a basis for kinship analysis. The application of the 16S rRNA gene molecular technique using the PCR technique is one of the relatively easy, fast and inexpensive methods of bacterial molecular identification that can be done.[18-20]

Data from 16S rRNA DNA sequencing can be traced through the Basic Local Alignment Search Tool (BLAST) database at the National Center for Biotechnology Information, National Institute for Health, USA (www.ncbi.nlm.nih.gov) and then deposited to GenBank for access numbers. Although there are contamination disturbances and insufficient sensitivity issues, this 16S rRNA sequencing method is not affected by phenotypic variations or technological bias, and has the potential to reduce errors.[21-22]

Several studies on bacteria as bioremediation agents from biomedical waste have been reported. Ethica and Sabdono (2017) stated that in hospital liquid biomedical waste, six isolates of non-pathogenic hydrolytic bacteria from the genus *Bacillus* synergized but interacted antagonistically with pathogenic hydrolytic bacteria, which were able to improve the parameters of hospital liquid biomedical waste.[4] According to various studies, *Bacillus* sp. common bacteria found in biomedical waste.[23-24]

Bacillus sp, which produces lipase and protease enzymes, can degrade complex materials so that they can be used in the management of liquid biomedical waste.[25] Research by Sabrina (2018) and Arifiani (2018) on biomedical waste from different health centers came to the conclusion that the discovery of indigenous bacterial isolates that produce protease and lipase enzymes.[12,16]

The number of clinical laboratories in the city of Semarang, Indonesia is increasing. Meanwhile, clinical laboratories produce biomedical waste which is infectious and harmful to the environment. Thus, studies to obtain indigenized bacterial isolates with low pathogenicity levels so that they fulfill the characteristics as bioremediation agents of clinical laboratory waste is still very much needed. This study has objective to obtain indigenous, hydrolytic bacterial isolates with low pathogenicity level, so to fulfill the characteristics as a bioremediation agent for liquid clinical laboratory waste.

METHODS

A. Research Design

This study used exploratory research design with the object of research on indigenized bacterial isolates of clinical laboratory waste of IBL (Imam Bonjol Laboratory) in Semarang City, Central Java Province. Indigenous bacteria were isolated from 8 points of location using grab technique. The research was conducted during August-October 2020.

B. Biomedical waste sampling

Sampling permit was requested early before the study was addressed to the director of IBL Clinical Laboratory. During sampling, personal protective equipment (PPE), and sampling equipment was prepared according to ISO/IEC 17025 standards. Samples were taken from the primary tank of biomedical waste from the IBL Clinic Laboratory by taking 100 mL of liquid biomedical waste 8 points of location. The tubes containing sample were put into a plastic zip in triplicate and labeled before stored in an ice box and sent to the laboratory. In the laboratory, samples were stored a refrigerator at 4 °C.[26]

2. Media preparation

a) Nutrient agar (NA) media

The NA powder (20 g) was dissolved in 1-L of aquadest. The solution was boiled using a hot plate then homogenized with a magnetic stirrer. The solution was then poured into a 1000 mL Erlenmeyer, and closed tightly then sterilized using an autoclave at 121°C for 15 min. The solution was cooled to a temperature of 45-50 °C, then poured into a petri dish.[12]

b) MacConkey Agar media

The MacConkey powder (20 g) was dissolved in 1-L of aquadest and then boiled using a hot plate and homogenized with a magnetic stirrer. The solution was poured into 1000 mL Erlenmeyer, and closed tightly then sterilized using an autoclave at 121°C for 15 min. The solution was cooled to a temperature of 45-50°C, then poured into a petri dish.[12,16]

c) Blood Agar Plate (BAP) media

The BAP powder (40 g) was dissolved in 1-L of aquadest and then boiled using a hot plate and homogenized with a magnetic stirrer. The solution was poured into a 1000-mL Erlenmeyer, and closed tightly then sterilized using an autoclave at 121°C for 15 min. After cooled to a temperature of 45-50°C, then 5% lamb blood was added, and poured into a petri dish.[12,16]

d) Tributyrin Agar Media

As much as 51.5 g of tributyrin powder was dissolve in 1000 mL of aquadest. The solution is boiled using a hot plate and homogenized with a magnetic stirrer. The solution was poured into a 1000 mL Erlenmeyer, closed tightly then sterilized by autoclave at 121°C for 15 min. It was then cooled to a temperature of 45-50 °C, then spread into a petri dish.[12]

e) Skim Milk Agar (SMA) media

The SMA powder (51.5 g) was dissolved in 1000 mL aquadest and then boiled using a hot plate and homogenized with a magnetic stirrer. The solution was poured into a 1000 mL Erlenmeyer, then closed tightly and sterilized using an autoclave at 121°C for 15 min. The solution was cooled to a temperature of 45-50 °C, then poured into a petri dish.[16]

3. Isolation of Bacteria in Liquid Biomedical Waste

Six test tubes were prepared and filled with 9 mL physiological NaCl and sample of each followed by serial dilutions. Starter was then inoculated on NA medium, and incubated for 24 h at 37 °C. After incubation, each unique colony obtained was purified 3 times on Nutrient Agar (NA) media. Morphology of growing colonies growth was observed and recorded. Purification was carried out three times until one pure colony type was obtained. One type of colony was selected from the unique NA media, then Gram-stained.[12]

4. Morphology observation of bacterial cells by Gram-staining.

Object glasses are cleaned using 70% alcohol. Sterile loop was heated until smoldering and let it cool again. Physiological NaCl is dropped on a glass object and bacterial colonies are added from NA media taken by sterile loop. NaCl and the colony are flattened and made smears, waiting to dry. After drying, the glass object was fixed to attach cells. The glass was flooded with Gram A stain for 3 min, then rinsed under running water. Gram B stain was added to the glass object for 1 minute, rinsed with running water. Gram C stain was added to the glass object for 0.5 min, rinsed under running water. Gram D stain is added to the glass object for 2 min, rinsed with running water. The glass object was air dried, then observed under a microscope with a 100x magnification of the objective lens.

5. Test the pathogenicity of protease and lipase producing bacteria

The pathogenicity test was carried out by testing each colony purified from NA medium grown on McConkey Agar and BAP media and then incubated for 24 h at 37°C. Hydrolytic bacteria that could not grow on selective media for pathogenicity tests are considered as relatively low-pathogenic bacteria.

6. Selection of protease and lipase producing (hydrolytic) bacteria

The selection of bacterial colony isolates that produced protease and lipase enzymes was carried out on tributyrin and SMA media. The purified inoculum was taken by sterile loop, streaked on tributyrin and SMA using the quadrant method, and incubated for 24 h at 37 °C. The colony of bacteria that could grow and produce a clear zone around the colony on tributyrin media were classified as lipase producers. Bacteria that can grow and produce clear zones around colonies in skim milk media are grouped as protease producers.

7. Molecular Identification of 16S rRNA by PCR Method

a. Amplification of 16S rRNA gene by PCR method

The amplification process uses Go Tag Green Master Mix (Promega). The primers used are the universal primers 27-F (5 '-AGA GTT TGA TCC TGG CTC AG-3') and 1492-R (5 '-GGTTACCTTGTTACGACTT-3'). Formulation of reagents used for DNA amplification using PCR can be seen in Table 1. PCR results were examined using 2% agarose gel electrophoresis based on the presence of a single band at 1500 bp. Visualization of amplicons using the Major Science UV-Transluminator.[27-28]

b. Preparation of 2% Agarose Gel Electrophoresis

The steps for conducting electrophoresis are as follows

i) Electrophoresis Preparation

Agarose gel was prepared in a concentration of 2% agarose weighed as much as 2 g and then dissolved in 100 ml TAE buffer. Agarose is heated in the microwave. The obtained liquid was promptly added with Syber safe as much as 4µl and homogenized, then poured onto the plate. The comb sheet was stuck while it was still hot and not yet mandated. The comb sheet was removed from the agarose gel when the agarose gel solidified to form a well to enter the PCR product sample. The solid agarose gel was put into a tank containing the TAE buffer solution.

ii) Gel Electrophoresis analysis

The results of DNA amplification and markers were inserted into each well. The tank is closed and the running electrophoresis process begins by connecting the electrophoresis device to the power source. The side containing the amplification is given a negative current. The magnitude of the electrophoretic current is 100 volts for 50 min. In the process of electrophoresis the amplification product is used markers 1-kb DNA Ladder (Geneaid) consisting of fragments ranging from 100 to 3,000. PCR amplification used 16S rRNA primers which had an amplification target area of ±1,500 bp.[27]

c. 16S rRNA coding gene sequencing

The 16S rRNA gene product as a result of the esterification was then sequenced. The 16S rRNA encoding gene sequencing using Sanger method was then carried out.[29]

d. Bioinformatics Analysis

The results of DNA sequencing were analyzed using a bioinformatic device, then manually processed and matched with data at www.ncbi.nih.gov through the BLAST-n (Basic Local Alignment, Search Tool-for nucleotide) program to determine the type of these isolates from the Genbank database.[30]

RESULTS

The sample in this study was liquid biomedical waste was obtained from the primary tank at the IBL Semarang clinical laboratory. Sampling was carried out at 8 points with a sample volume of 100 mL.[25] After being packaged in sterile threaded bottles that have been labeled, the results of the sampling process are identified using a permanent marker. Series of steps were carried out to obtain indigenized, hydrolytic bacterial isolates with a low pathogenicity level that met the characteristics of the bioremediation agent. The first step was sampling process, followed by sample dilution, bacterial colony purification, hydrolytic enzyme production tests, pathogenicity test, and completed by molecular identification as well as bioinformatics analysis. The steps were summarized in Figure 1.

1. Isolation of Indigenous Bacteria

Isolation is the process of moving organisms from their original habitat into new habitats for breeding. In this study, the isolated bacteria were bacteria from biomedical waste from the clinical laboratory of IBL Semarang, so they could be called indigenous bacteria. The results were 6 pure isolates labelled with codes IBLS-1, IBLS-2, IBLS-3, IBLS-4, IBLS-5, and IBLS (IBLS is short for Imam Bonjol Semarang Laboratory). Microscopic observation aims to determine the shape of cells with Gram-staining. Macroscopic observation aims to determine colony morphology by observing on NA media. The results can be seen in Table 1 and Table 2. Based on Table 2, on NA media, 6 isolates had round colonies and were white in color, but had different sizes. The observation result of colony edge is dominated by flat edge. The results of the elevation observations were dominated by flat elevation with dry consistency.

Results of the Gram-staining on bacterial cells are presented in Table 3 and Figure 2. Table 3 shows that of the 6 isolates obtained as a result of purification, there were 5 isolates in the form of bacilli and 1 isolate cocci. The observations also showed that 6 isolates were Gram-positive. The results of microscopic observations on Gram-staining showed that the 6 bacterial isolates were lined up and clustered. All isolates showed different characteristics so that they were considered as different bacterial species. Uniform cells of the six isolates were indication of no contamination, they could be screened for their pathogenicity level.

2. Selection of hydrolytic bacteria with low pathogenicity based on hemolysis characteristics

The bacterial isolates were then tested for their pathogenicity level by cultivation on BAP media and then incubated for 24 h at 37˚C. Colony observations on BAP media carried out after the incubation process was completed are presented in Table 4 and Figure 3. Based on Table 4 and Figure 4 the results on BAP media show that the isolates IBLS-1 and IBLS-4 did not cause hemolysis, so it is concluded that these isolates have low pathogenicity. So, it can be determined that the IBLS-1 and IBLS-4 isolates have a low pathogenicity level so that they are in accordance with the characteristics required as bioremediation agents.[6]

The next step required is to test the ability to produce protease and lipase enzymes. The goal is to find out whether bacteria could degrade the main components of biomedical waste, organic matter which can be protein and fat.[31] IBLS-1 and IBLS-4 isolates were grown on SMA media and Tributyrin agar, incubated for 24 h at 37˚C. Figure 4 shows that both isolates formed clear zones around their colonies on SMA and Tributyrin media. Based on these observations, the IBLS-1 and IBLS-4 isolates were bacteria that produced lipase and protease.

2. Molecular identification based on the 16S rRNA gene.

Isolation of bacterial DNA is the initial step of selected bacterial isolates as a result of the isolation examination and identification of bacteria whose genomic DNA was isolated and cleaned using the Zymo-SpinTM Reagent Kit to obtain pure DNA.[32] The results of the isolation were then measured the quantity of bacterial DNA using a Nanodrop spectrophotometer.[32]

a. DNA Extract Purity Test

DNA extract purity test to quantitatively measure the purity level of extract DNA against contaminants was carried out using a Nanodrop spectrophotometer with a wavelength of 260 and 280 nm. The maximum value of DNA can be 260 nm, while the maximum value of residue or protein can be absorbed with a wavelength of

280 nm. DNA is said to be pure if it has a wavelength ratio of 260/280 between 1.8-2.0 ng / μl so that when visualized the DNA bands on the electrophoresis gel can be read.[33]

Table 5 shows the concentration data ratio of the mean purity of the template DNA extract from the IBLS-1 and IBLS-4 isolates. Based on the data, the IBLS-4 isolate had a higher concentration than IBLS-1, which was 326.3 ng/ μL, while the IBLS.1 isolate had a concentration of 199.5 ng/ μL. With the data from the purity of the template DNA which was quite good, the two isolates of bacterial genomic DNA obtained were used in the next step, PCR. PCR is a technique of enzymatic DNA amplification or propagation using primers from pieces of certain base sequences.[34]

b. PCR amplification of 16S rRNA gene and its visualization

Amplification of DNA strand multiplication was carried out using the PCR 16S rRNA method. Analysis of DNA molecules using PCR was then continued by separating the DNA fragments using agarose gel electrophoresis with the help of electric currents.[35] The PCR product was inserted into the well contained in the agarose gel at the negative pole. Then the electrophoresis results were visualized using UV Transluminator.[27] The results of 16S rRNA gene amplification gel electrophoresis of the IBLS-1 and IBLS-4 isolates along with the DNA markers used can be seen in Figure 6. Figure 6 shows the results of the 16S rRNA gene amplification of the two bacterial isolates based on the size of the marker band showing a single band from each isolate with slightly different sizes, but around 1500 bp. From various literature studies, the amplicon size of the PCR 16S rRNA gene using the primers used in this study was ~ 1500 bp.[20,27] This indicates that the PCR product obtained is most likely a 16S rRNA DNA fragment, and to ensure this it is necessary to carry out a DNA sequencing step for the PCR product.

c. DNA sequencing and Basic Local Alignment Search Tools (BLAST)

The PCR 16S rRNA products of the two selected isolates were subjected to Sanger sequencing analysis.[20-26] Then the 16S rRNA gene sequences from both forward and reserve primers obtained from the sequencing results are aligned.[22] The analysis of this sequence was carried out using MEGA X software.[30] DNA sequencing is one way to identify or see the identity of a gene whose sequence is known to be determined by comparing the sequence data found in Genbank.[31] In the identification of bacterial molecules, the results of the 16S rRNA gene sequence analysis need to be traced to the same 16S rRNA gene sequences belonging to other bacteria on Genbank through the BLAST program at www.ncbi.nih.gov.blast. Comparison of this similarity was carried out with all comparative bacterial sequence data in the database to make a relationship between the bacterial kinship in multiple sequence alignments with the ClustalW program and then stored in the form of MEGA (*.mega) or FASTA (*.fasta).[30]

Bioinformatics analysis results using BLAST-n show that IBLS-1 isolate has 99.91% similarities to *Bacillus paramycodes*, while IBLS-4 isolate was 100.00% similar to *Bacillus firmus*. Based on the results of the molecular identification, the IBLS-1 isolate was named *B. paramycodes* IBLS-1 and IBLS-4 was named *B. firmus* IBLS-4. Results from the isolation, selection and molecular identification of bacteria with the characteristics of bioremediation agents from clinical laboratory waste is presented in Figure 9.

Table 1. Formula of reagents for the PCR amplification of 16S rRNA gene.

No	Reagent	Volume (μl)
1	Go Taq Green Master Mix	12,5
2	primer 27 F	2
3	primer 1492 R	2
4	DNA template	3
5	ddH_2O	5,5
	Total	25

Table 2: Observation result of bacterial colony morphology on NA media (24-h).

Code Sample	Shape	Color	Ukuran (mm)	Edge	Elevation	Consistency
IBLS-1	round	white	2,3	even	flat	dry
IBLS-2	round	white	4,0	serrated	flat	dry
IBLS-3	round	white	2,5	even	convex	moist
IBLS-4	round	white	2,0	even	convex	dry
IBLS-5	round	white	5,0	serrated	flat	moist
IBLS-6	round	white	3,5	serrated	convex	dry

Table 3: Bacterial cell morphology observation result by Gram-staining.

Name Isolat	Shape	Arrangement	Gram-staining result
IBLS-1	rod	chain	Gram-positive
IBLS-2	rod	chain	Gram-positive
IBLS-3	coccus	cluster	Gram-positive
IBLS-4	rod	chain	Gram-positive
IBLS-5	rod	chain	Gram-positive
IBLS-6	rod	Chain	Gram-positive

Table 4: Colony observation result on BAP media (24-h).

	Media BAP					
	IBLS1	IBLS2	IBLS3	IBLS4	IBLS5	IBLS6
Shape	round	round	round	round	round	round
Color	white	white	white	white	white	white
Diameter (mm)	3	2	2	3,5	2.5	3.5
Edge	even	even	even	even	even	even
Elevation	flat	flat	flat	convex	flat	flat
Consistency	dry	moist	dry	dry	dry	dry
Hemolysis type	γ	β	β	γ	β	β

Table 5: Purity test on DNA extract.

Code Sample	Concentration (ng/μL)	A260	A280
IBLS-1	199,5	1,89	1,39
IBLS-4	326,3	1,87	1,83

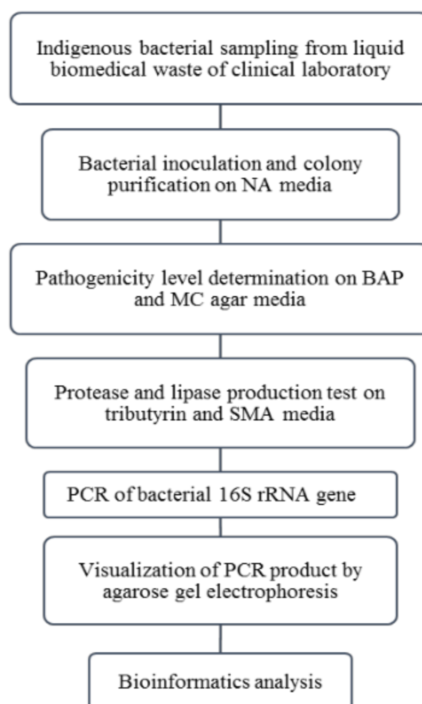

Figure 1: Summarized steps conducted in this study.

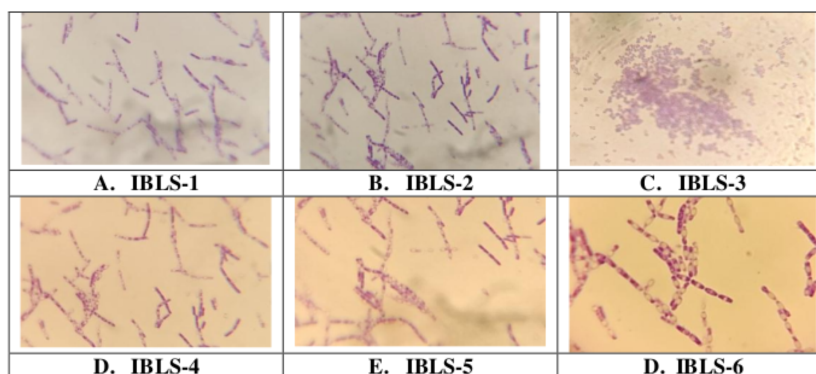

Figure 2: Gram-staining result on IBLS-1 –IBLS-6 isolates under optic microscope with 100 x magnification.

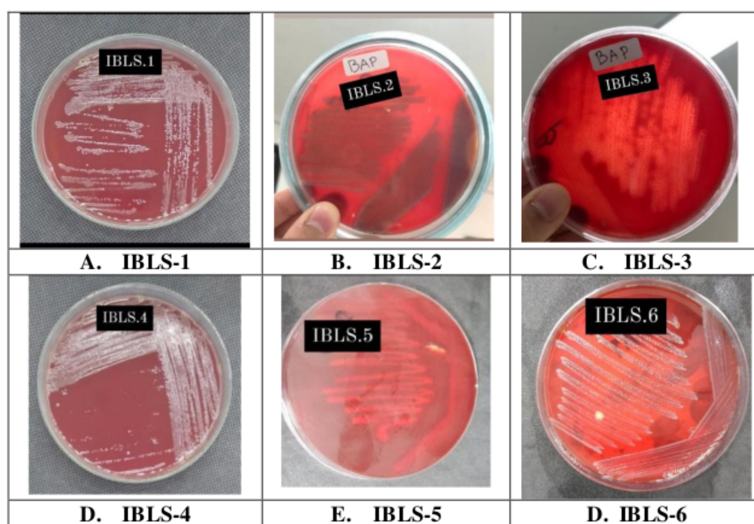

Figure 3: Hemolysis observation result of IBLS-1 to IBLS-6 islates on BAP (24 h).

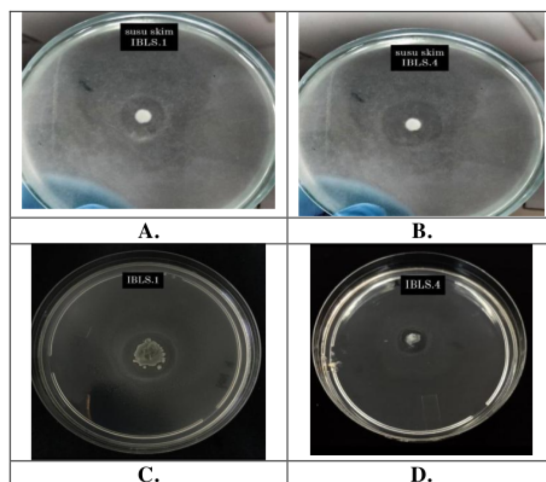

Figure 4. Proteolytic zone observation on SMA plate A. Isolate IBLS-1. B. IBLS-4 and Lipolytic zone observation on Tributyrin plate C. Isolate IBLS-1. D. IBLS-4

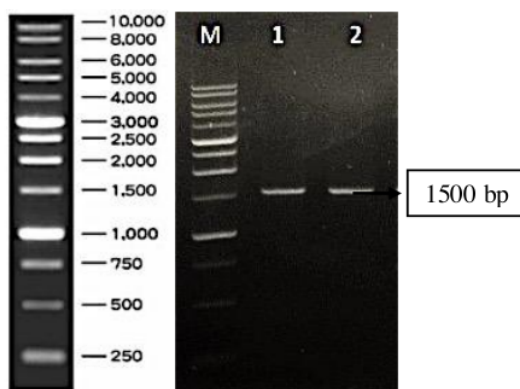

Figure 5: Result of PCR amplification of bacterial 16S rRNA gene visualized on 2% agarose gel electrophoresis by UV *Transluminator* 1. Isolate IBLS-1, 2. Isolate IBLS-4.

DISCUSSION

Research on the isolation, selection and molecular identification of bacteria with the characteristics of bioremediation agents from the laboratory waste of the IBL Semarang clinical laboratory has been carried out. Initially, the results obtained 8 bacterial isolates which were then further processed by colony purification, and identification of cell morphology based on Gram stain by microscopy with 100x magnification. After going through the colony purification and Gram staining processes, of the 6 isolates had different colony forms, and only 6 isolates were Gram-positive. The different or unique colony characteristics of 6 indigenous bacterial isolates indicate that it is likely that the 6 bacterial isolates are different bacterial species. Macroscopic characters can be seen from the shape, color, size, edge and elevation of the colony.[30] Each colony with a unique morphology was then purified three times with the final result of 6 bacterial isolates coded IBLS-1, IBLS-2, IBLS-3, IBLS-4, IBLS-5, and IBLS-6.

The selection of six bacterial isolates by pathogenicity test was carried out by planting the isolates on BAP media. Selection on BAP media obtained IBLS-1, and IBLS-4 were bacteria with low pathogenicity because both did not cause hemolysis on the test media. The ability test of IBLS-1 and IBLS-4 in degrading organic matter was carried out using SMA media and Tributyrin agar which was incubated for 24 h at 37 °C. As a result, a clear zone was found around the colonies of the two isolates on SMA medium and agar tributyrin. This shows that IBLS-1 and IBLS-4 isolates are protease and lipase-producing enzymes that show the ability to degrade proteins and lipids.[31] Both of these capabilities are important for biomedical waste bioremediation agents considering that the main components of biomedical waste are organic materials such as proteins and lipids. Based on selection processes that have been carried out, the IBLS-1 and IBLS-4 isolates have 3 main characteristics of a waste bioremediation agent in general. They are being bacteria from waste origin (indigenous), having a low level of pathogenicity, and being able to break down the main components of waste.[6]

The results of molecular identification based on the 16S rRNA gene showed that two bacterial isolates that had the potential as bioremediation agents were *B. paramycodes* IBLS-1 and *B. firmus* IBLS-4. Results of this study are thus in line with various studies reporting that *Bacillus* sp. is an important bacterium in the processing of biomedical waste. *Bacillus* sp. It is widely known as a producer of lipase and protease enzymes which can degrade complex materials so that they can be used in the management of liquid biomedical waste.[23] This is confirmed by the results of previous study on hospital liquid biomedical waste.[4] Research reported by Sabrina (2018) and Arifiani (2018) on liquid biomedical waste also obtained bacterial isolates with low levels of pathogenicity which could be used as bioremediation agents.[12,16]

Bacillus sp. are widely known protease producer, which could be proven by their ability to produce proteolytic (clear) zone around their colonies when grown in SMA media produces a clear zone around the colony.[31] Skim milk in SMA media contains casein milk protein which is a substrate for proteolytic bacteria.[36] In degradation of casein, it is expected that the extracellular protease enzyme secreted by bacteria is able to produce a clear zone around the colony. *Bacillus* sp. also the enzyme producer of lipase works to hydrolyze fats and oils into simpler compounds, namely fatty acids. The results of the isolation of lipolytic bacteria with media containing fat or medium to contain 1% tributyrin, the bacteria produced a clear zone around the colony.[31] As a proteolytic bacterium, *Bacillus* sp. has the ability to degrade protein, producing extracellular protease enzymes.[37,38] Protease catalyzes the breaking of peptide bonds in proteins.[39] Based on the literature review, it is very likely that *B. paramycodes* IBLS-1 and *B. firmus* IBLS-4 isolates obtained from this study may be

potential as a bioremediation agent for IBL liquid clinical waste.

This study has limitations to the fact that characteristics of bioremediation agents were could only be demonstrated *in vitro*. The evaluation step that needs to be done is to prove the effect of *Bacillus* sp. isolate in degrading biomedical waste. This can be done by measuring various parameters of waste pollution before and after applying bacteria on waste. According to previous study, testing the ability of bacterial bioremediation agents needs to be carried out by antagonistic tests.[6,24] The antagonistic test is useful for measuring the ability of bioremediation agents to inhibit growth of pathogenic bacteria. Furthermore, encapsulation is also necessary, namely an effort to provide protection to bacterial cells in dry form against selected bacterial isolates or consortia so that they can be used as prototypes of bioremediation agents that can be protected for longer time.[40]

CONCLUSIONS

Two indigenous *Bacillus paramycodes* IBLS-1 and *B. firmus* IBLS-4 were isolated and fulfill the main characteristics of bioremediation agent for liquid biomedical waste of IBL clinical laboratory at Semarang.

Conflict of interest

"Competing interests: No relevant disclosures".

Acknowledgement

Authors presented gratitude to Imam Bonjol Laboratory (IBL) Semarang for providing samples used in this study.

Contributors

EP, SNE and STZ altogether planned the experiments. EP conducted the laboratory experiments including sample preparation. EP and SNE contributed to the interpretation of the results. SNE led the writing and submission of the manuscript. STZ provided critical feedback on the research. All authors had approved the final version.

REFERENCES

1. Pertiwi OA, Novrikasari N, Lestari M. Analysis of Factors Related to Compliance with the Use of Personal Protective Equipment (PPE) in Clinical Laborator Hospital Dr. Ibnu Sutowo Baturaja. J. Ilmu Kesehatan Masyarakat, 2016 Jun 21; 7(2): 118-23. https://www.neliti.com/publications/58020/analysis-of-factors-related-to-compliance-with-the-use-of-personal-protective-eq
2. Mathur P, Patan S, Shobhawat AS. Need of biomedical waste management system in hospitals-An emerging issue-a review. Curr. World Environ, 2012; 7(1): 117. http://www.cwejournal.org/vol7no1/need-of-biomedical-waste-management-system-in-hospitals-an-emerging-issue-a-review/
3. Sawyerr HO, Adeolu AT, Salami OO, Adejoh PA. Evaluation of biomedical waste in Kogi State University Teaching Hospital, Anyigba, Kogi State, Nigeria. Journal of Toxicology and Environmental Health Sciences, 2017 Apr 30; 9(4): 29-34. https://academicjournals.org/journal/JTEHS/article-full-text-pdf/480F5F563727.
4. Ethica, S.N., Sabdono, A. Bio-Remediation Potential of Hydrolytic Bacteria Isolated from Hospital Liquid Biomedical Waste in Central Java. Proc. World Congr. New Technol, 2017; 10–11. https://pdfs.semanticscholar.org/1c4f/045900b708bf 2df3d2e43aa08622299f0.pdf.
5. Ilyas S, Srivastava RR, Kim H. Disinfection technology and strategies for COVID-19 hospital and bio-medical waste management. Science of the Total Environment, 2020 Dec 20; 749: 141652. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC741 20/.
6. Ethica SN, Saptaningtyas R, Muchlissin SI, Sabdono A. The development method of bioremediation of hospital biomedical waste using hydrolytic bacteria. Health and Technol, 2018 Sep 1; 8(4): 239-54. https://www.springerprofessional.de/the-development-method-of-bioremediation-of-hospital-biomedical-/15743886.
7. Ola-Adisa EO, Mangden EY, Sati YC, Adisa JO. Knowledge, Attitudes, Beliefs and Practices in Medical Waste Management-An Appraisal of Jos North LGA, Plateau State, Nigeria. International Journal, 2015; 43. https://www.ijrhss.org/pdf/v2- /5.pdf.
8. Azubuike CC, Chikere CB, Okpokwasili GC. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J. of Microbiol. and Biotech, 2016 Nov 1; 32(11): 180.
9. Jacob JM, Karthik C, Saratale RG, Kumar SS, Prabakar D, Kadirvelu K, Pugazhendhi A. Biological approaches to tackle heavy metal pollution: a survey of literature. J. of Environ. Man, 2018 Jul 1; 217: 56-70. https://pubmed.ncbi.nlm.nih.gov/29597108/.
10. Kundu D, Dutta D, Mondal S, Haque S, Bhakta JN, Jana BB. Application of Potential Biological Agents in Green Bioremediation Technology: Case Studies. In Waste Management: Concepts, Methodologies, Tools, and Applications, 2020; 1192-1216. IGI Global.
11. Nyika JM. The Use of Microorganism-Derived Enzymes for Bioremediation of Soil Pollutants. InRecent Advancements in Bioremediation of Metal Contaminants, 2020 Jul 10; 54-71. IGI Global. https://www.igi-global.com/chapter/the-use-of-microorganism-derived-enzymes-for-bioremediation-of-soil-pollutants/259566.
12. Arifiani N, Ethica SN. Isolasi Bakteri Penghasil Lipase dan Protease yang Berpotensi sebagaiAgen Bioremediasi dari Limbah Biomedis Cair Puskesmas Halmahera Kota Semarang [Isolation of Potential

Characterization of bacteria from Liquid Clinical Laboratory Waste with Potential as Bioremediation Agent

ORIGINALITY REPORT

18%	12%	12%	8%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

#	Source	%
1	Submitted to Udayana University Student Paper	3%
2	A U Iskandar, S N Ethica, A Sukeksi, A H Mukaromah, A R Sulistyaningtyas, S Darmawati. "Molecular systematic and phylogenetic analysis of indigenous bacterial isolates with potential as bioremediation agent based on 16S rRNA gene analysis", IOP Conference Series: Earth and Environmental Science, 2021 Publication	2%
3	Nur Hidayati, Nurrahman Nurrahman, Hayatun Fuad, Hendra Munandar et al. "Bacillus tequilensis Isolated from Fermented Intestine of Holothuria Scabra Produces Fibrinolytic Protease with Thrombolysis Activity", IOP Conference Series: Earth and Environmental Science, 2021 Publication	1%
4	prosiding.unimus.ac.id Internet Source	

1 %

5 Nur Hidayati, Hayatun Fuad, Hendra Munandar, Dewi Seswita Zilda et al. "Proteolytic and Clot Lysis Activity Screening of Crude Proteases Extracted from Tissues and Bacterial Isolates of Holothuria Scabra", IOP Conference Series: Earth and Environmental Science, 2021
Publication
1 %

6 mafiadoc.com
Internet Source
1 %

7 journal.ugm.ac.id
Internet Source
1 %

8 www.neliti.com
Internet Source
1 %

9 Submitted to University of Al-Qadisiyah
Student Paper
1 %

10 link.springer.com
Internet Source
1 %

11 pinnacle.allenpress.com
Internet Source
1 %

12 Submitted to Universitas Jambi
Student Paper
<1 %

13 www.cwejournal.org
Internet Source

<1 %

14	Submitted to RMIT University Student Paper	<1 %
15	S N Ethica, A Firmansyah, E Purwaningrum, N Hidayati et al. "Degradation performance and microencapsulation of hydrolytic bacterial consortium formulated as bioremediation agent of liquid biomedical waste", IOP Conference Series: Earth and Environmental Science, 2021 Publication	<1 %
16	bmcmicrobiol.biomedcentral.com Internet Source	<1 %
17	ijcmas.com Internet Source	<1 %
18	www.omicsonline.org Internet Source	<1 %
19	ejournal.mrcpp.machung.ac.id Internet Source	<1 %
20	www.bioflux.com.ro Internet Source	<1 %
21	dypatilunikop.org Internet Source	<1 %

#	Source	Type	Similarity
22	Submitted to Higher Education Commission Pakistan	Student Paper	<1 %
23	Submitted to NALSAR University of Law Hyderabad	Student Paper	<1 %
24	ijbmb.org	Internet Source	<1 %
25	www.upo.es	Internet Source	<1 %
26	"Environmental Pollution", Springer Science and Business Media LLC, 2018	Publication	<1 %
27	www.springerprofessional.de	Internet Source	<1 %
28	"Enhancing Halal Sustainability", Springer Science and Business Media LLC, 2021	Publication	<1 %
29	Submitted to Universitas Jember	Student Paper	<1 %
30	Saranya Kuppusamy, Palanisami Thavamani, Mallavarapu Megharaj, Yong Bok Lee, Ravi Naidu. "Kinetics of PAH degradation by a new acid-metal-tolerant Trabulsiella isolated from the MGP site soil and identification of its potential to fix nitrogen and solubilize		<1 %

phosphorous", Journal of Hazardous Materials, 2016
Publication

31	core-cms.prod.aop.cambridge.org Internet Source	<1 %
32	dokumen.pub Internet Source	<1 %
33	searchworks.stanford.edu Internet Source	<1 %
34	www.frontiersin.org Internet Source	<1 %
35	www.tandfonline.com Internet Source	<1 %
36	H. O. Sawyerr, A. T. Adeolu, O. O. Salami, P. A. Adejoh. "Evaluation of biomedical waste in Kogi State University Teaching Hospital, Anyigba, Kogi State, Nigeria", Journal of Toxicology and Environmental Health Sciences, 2017 Publication	<1 %
37	N Hidayati, H Fuad, H Munandar, DS Zilda, AR Sulistyaningtyas, N Nurrahman, S Darmawati, SN Ethica. "Potential of fibrinolytic protease enzyme from tissue of sand sea cucumber (Holothuria scabra) as thrombolysis agent",	<1 %

IOP Conference Series: Earth and Environmental Science, 2021
Publication

38	Stalis Norma Ethica, Sakti Imam Muchlissin, Ragil Saptaningtyas, Agus Sabdono. "Protease Producers Predominate Cultivable Hydrolytic Bacteria Isolated from Liquid Biomedical Waste", Asian Journal of Chemistry, 2018 Publication	<1%

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	Off		