

# Pathogenicity Scoring System for Selection of Bacterial Consortium Formulated as Bioremediation Agent of Hospital Wastewater in Central Java

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## 6 Pathogenicity Scoring System for Selection of Bacterial Consortium Formulated as Bioremediation Agent of Hospital Wastewater in Central Java

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**Abstract.** The search of cost-effective bioremediation agent of hospital wastewater is critical since current methods to treat biomedical waste worldwide are still costly and not environmentally friendly. Use of hydrolytic bacteria as bioremediation agent has been known, yet it is important to ensure that they fit safety requirement. This study aimed to establish and implement a simple plate-based pathogenicity selection scoring method to determine the pathogenicity levels of 26 indigenous hydrolytic bacteria isolated from the untreated wastewater of two hospitals in Semarang, Central Java, Indonesia. Bacterial cultivations were carried out in parallel on MacConkey Agar Plate (MAP), Blood Agar Plate (BAP) and Chocolate Agar Plate (CAP) followed by molecular identification. Next, a scoring system was set based on the ability of isolates to produce violet colour on the MA and hemolysis characteristics of the bacteria on both the BAP and CAP media. Based on the scoring system, 6 out of 26 bacterial isolates mostly belong to the members of *Bacillus velezensis*, *B. amyloliquefaciens* and *B. licheniformis* were identified having low pathogenicity, which make them a suitable bioremediation agent of the studied hospital wastewater. As conclusion, the set plate-based pathogenicity scoring system could be a simple, yet useful and reliable tool for selecting non-pathogenic indigenous hydrolytic bacterial strains potential as a bioremediation agent.

### 1. Introduction

The importance of bioremediation as biological remediation involving the use of living organisms including bacteria to reduce or to eliminate pollutants in polluted area has been acknowledged. Such remediation is expected to result in the restoration of the contaminated are to its original or natural

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state without further disruption to the local environment [1,2]. Bioremediation is considered as more environmentally friendly and cost-effective way to degrade waste as compared to other traditional technique such as incineration commonly practiced in developing countries. However, the implementation of bioremediation is often discouraged by a lack of information about the factors controlling the growth and metabolism of microorganisms in the polluted environments. In bioremediation, there is challenge in the selection of bacterial consortium used as tool to remediate polluted environment [3-5].

Most treatments for hospital liquid biomedical waste all over the world in the last decade used the non-bioremediation methods. Meanwhile, in Indonesia all studies reporting hydrolytic bacteria as bioremediation agents in the last 11 years had been applied for wastes other than biomedical ones. Hence, known as inexpensive and environmentally friendly, bioremediation of hospital liquid biomedical waste using hydrolytic bacteria offers both necessity, yet novelty. The development method of bioremediation of hospital liquid biomedical waste using hydrolytic bacteria to obtain bioremediation agents for such waste is quite promising because many feasible facilities are available to test their pathogenicity and ability to decrease parameter values of liquid organic waste. The method could be a breakthrough for Indonesian government to meet World Health Organization's recommendation on promoting non-incineration technology for handling biomedical wastes [6].

In regard with the hospital biomedical waste problem in developing countries, hydrolytic bacterial bioremediation appeared to be a potentially economical way to overcome the increased amount of biomedical wastes. Outlines of steps for acquiring new bioremediation agent from group of indigenous, non-pathogenic, hydrolytic bacteria isolated from hospital biomedical waste reservoir include pathogenicity level-based selection. The steps should be designed to hamper the proliferation of pathogens as well as to remove other pollutants contained in hospital liquid biomedical wastes since they are toxic to human and its surrounding [2,7-8].

Hydrolytic bacteria are bacteria capable of secreting hydrolytic enzymes to catabolize major components of biomass such as polysaccharides, proteins and fats (9,10). Examples of hydrolytic enzymes are amylase, protease, lipase, DNase, and xylanase. They play important role in bioremediation [11,12,13,14,15]. Screening of bacteria capable of producing extracellular hydrolytic enzymes could be performed by plate tests, while the identification could be done based on *Bergey's Manual of Systematic Bacteriology* and analysis of the 16S rRNA genes [16].

Bacteria are considered suitable to be used in bioremediation including wastewater treatment for being able to use a variety of carbon sources or electron acceptor [17]. Bacterial hydrolytic enzymes are important degraders of organic pollutants since they could break the main chemical bonds of toxic molecules in wastes, thus playing key role in bioremediation (18,19). Hydrolytic bacteria have been known for their ability to improve water pollution parameters such as COD (Chemical Oxygen Demand), BOD (Biological Oxygen Demand),  $\text{NH}_4$ , and  $\text{PO}_4$  of organic waste. They also play key role in accelerating degradation of organic wastes by limiting available nutrients required for the proliferation of pathogenic microorganisms [20-22]. However, for safety reason on public use, only the non-pathogenic ones should be developed as bioremediation agent [23-24].

Hydrolytic bacteria, particularly the non-pathogenic ones, which could metabolize organic wastes play important role in accelerating biomedical waste degradation process. They are believed as capable to limit the proliferation of pathogenic microorganisms. Hence, they could help in reducing the danger of infection and contamination they may cause [25]. A study on isolation of bacteria producing hydrolytic lipase, *Alcaligenes* sp. JG3, which is capable of degrading fat as well as glycerol and thereby potential to become effective agents of biodegradation of organic waste was carried out (26). Unfortunately, the obtained strain is known to be pathogenic. Pathogenic bacteria are not safe to be used as bioremediation agent considering public safety [2]. It is therefore important to set simple, yet effective and affordable pathogenicity test aiming to select the non-pathogenic group of indigenous hydrolytic bacteria, so they could be used as bioremediation agent suitable for developing countries [2, 23].

Bioremediation study aiming to obtain bioremediation agent had been started by sampling bacteria from primary reservoir of liquid biomedical waste from two hospitals in Semarang City, Central Java Province (25). This step shall be followed by purification of bacterial colonies. After being selected by

groups of enzymes produced using tributyrin, skim milk, starch, and carboxymethyl cellulose agar media, 26 bacterial isolates could be determined as indigenous, hydrolytic bacteria capable of producing lipase, protease, amylase and cellulase [6]. Meanwhile, various media are available to select potential pathogenic bacteria [27].

As summarized from previous studies reporting feasible facilities to develop bacterial bioremediation system, the initial step should include selection of non-pathogenic group of hydrolytic bacteria to be used as bioremediation agent [2]. This paper aimed to report scoring pathogenicity level system used to develop bioremediation agent from the reported 26 isolates of bacteria, relying on the bacterial growth characteristics on MacConkey Agar Plate [MAP], Blood Agar Plate (BAP) and Chocolate Agar Plate (CAP) media. The selected and tested isolates are expected to be field tested as bioremediation agent and then produced on an industrial scale for larger application. It is expected to become solutions for handling liquid biomedical waste effectively and efficiently in a wide range of hospitals, not only in Central Java, but also in other regions of developing countries.

## <sup>27</sup> 2. Materials and Method

### 2.1. Materials

Materials used in this study were 26 indigenous hydrolytic bacterial isolates originated from reservoirs of biomedical waste of two hospitals in Semarang, obtained from previous experiment [6]. Three types of media commonly used to isolate pathogenic bacteria were used, i.e. MacConkey agar (cat. no. Catalog no. R061322), Blood agar (cat. no. CM0055B) enriched with fresh human blood (from medical faculty laboratory of Universitas Diponegoro, Semarang) and Chocolate agar (cat. no. R01302) (Thermo Scientific, UK), while Nutrient agar (Sigma, Germany), sterile aquadest and pepton was used for sub-culturing the isolates. Equipment required for this study included anti-infection (self-protection) equipment (Dupont), petri dish, reaction tubes, laminar UV, autoclave, bunsen, shaker, incubator, SEM (Scanning Electron Microscope) microscope (JEOL, Japan).

### 2.2. Method

<sup>11</sup>  
2.2.1. *Pathogenicity Test on MAP.* MacConkey agar plate, which is designed to selectively isolate Gram-negative and enteric bacilli based on lactose fermentation, and blood agar, used to culture almost all microorganisms [28]. Preparation of the media was done according to the manufacturer's instruction (Thermo Scientific, UK). Observation was performed based on the formation of violet coloured media on bacterial plates after 24-h incubation.

2.2.2. *Pathogenicity Test on BAP.* The characterization of hemolysis properties of hydrolytic bacterial isolates was carried out by planting a loop-full of each single colony on the surface of the sheep blood agar plate, then incubating at 37 °C for 18-24 hours (extended for another 24-h when  $\alpha$  hemolysis is observed). Preparation of BAP media was done based on the manufacturer's instruction (Thermo Scientific, UK). Considering a technique first reported by Buxton [29], to sterile blood agar base which has been melted and cooled to 45 to 50°C, sterile defibrinated blood warmed to room temperature was added by 5% (v/v). The flask was swirled to homogenize the mixture thoroughly while avoiding formation of bubbles. After that, the mixture was dispensed into sterile plates carefully to avoid formation bubbles and froth on solid media's surface. The character of hemolysis was observed after 24-36-h of incubation based on color changes caused by hemolysis zone around the bacterial colonies. According to Skala<sup>19</sup> [30], cooling the agar and warming the blood are essential steps in BAP preparation procedure. Hot agar can damage red blood cells, and cold blood can cause the agar to gel before pouring.

2.2.3. *Pathogenicity Test on CAP.* Preparation of the CAP media was carried out by following the manufacturer's instruction (Thermo Scientific, UK). Observation on the media was carried out after 24-h incubation for specific colony morphology and hemolysis. It was carried out after 24 hour of incubation for the presence of colony characteristics matched to those of very highly pathogenic



species: *Streptococcus pneumoniae*, *Neisseria meningitides*, *N. gonorrhoeae*, and *Haemophilus influenzae* bacteria [31-32].

**2.2.4. Pathogenicity Scoring System.** Plate based pathogenicity scoring system was set in this study based on observation techniques previously reported on MAP, BAP and CAP [29, 30]. In principle, non-fermenting bacteria, which could not show violet media color on MAP is labelled “-”, while that of the opposites were labelled “+”. Bacterial colonies on BAP with  $\beta$  hemolysis is labelled “ $\beta$ ”, while those with  $\alpha$  and  $\gamma$  hemolyses were labelled “ $\alpha$ ” and “ $\gamma$ ”, respectively. Levels of bacterial pathogenicity was scored extremely (extra) high if the bacteria could show signs of colony characteristics of any fastidious bacteria commonly grow on CAP, regardless their colony characteristics on MAP and BAP. The fastidious species commonly grow on CAP are *Streptococcus pneumoniae*, *Neisseria meningitides*, *N. gonorrhoeae*, and *Haemophilus influenzae* bacteria [32-33]. Pathogenicity levels could then be determined using pathogenicity scoring table shown in **Table 1**.

**Table 1.** Plate-based pathogenicity scoring set in this study

No	MacConkey Agar Plate (MAP) Result	Blood Agar Plate (BAP) Result	Chocolate Agar Plate (CAP) Result	Pathogenicity Score
1.	+	$\beta$	-	High
2.	+	$\alpha$	-	Medium
3.	+	$\gamma$	-	Low
4.	-	$\beta$	-	Very high
5.	-	$\alpha$	-	High
6.	-	$\gamma$	-	Medium
7.	Any	Any	+	Extra high

**2.2.5. 16S rRNA Gene Sequence Analysis and Phylogenetic Tree Construction.** The analysis of 16S rRNA gene sequences of 26 bacterial isolates obtained from previous work was carried out based on Genbank access codes submitted in Genbank [33]. A phylogenetic tree highlighting bacterial strains with their pathogenicity levels was constructed using a freely available web tool <http://www.phylogeny.fr/>[34].

**2.2.6. SEM (Scanning Electron Microscope) Observation.** The observation of cells of non-pathogenic bacterial strains selected by the previously set scoring system was conducted using SEM at Laboratorium of Lembaga Ilmu Pengetahuan Indonesia (LIPI), Citibong, Indonesia. In a phosphate-buffered salt solution fresh bacterial cells were suspended. The cells were then fixed with 0.5% glutaraldehyde. After being washed several times they were dehydrated in a series of ethanol concentrations. The cells they were observed with a SEM (model JSM 6300 F; JEOL, Japan) at 3 kV after they were sputter coated with gold-palladium [35].

### 3. Results and Discussion

In developing countries including Indonesia, bioremediation is among the recommended ways by the World Health Organization to handle biomedical waste. Indigenous hydrolytic bacteria from hospital wastes could be an option for potent, yet inexpensive bioremediation agent. However, to be widely applied, group of indigenous hydrolytic bacteria proposed formulated bioremediation agent for hospitals in developing countries should be safe for public use. This indirectly infers that the selection of non-pathogenic bacterial consortium used to remediate biomedical wastewater is a crucial step, where the technique should consequently be simple, cost-effective, yet adequately accurate.

In this study, a plate-based scoring system based on observation on 3 “key” agar media, Macconkey, Blood and Chocolate Agar. The media were used to determine pathogenicity levels of indigenous hydrolytic bacteria from 2 hospitals in Central Java, i.e. Roemani Muhammadiyah and RSUD KRT Wongsonegoro. The selection of low- or non-pathogenic strains among 26 indigenous hydrolytic bacterial isolates obtained using the scoring system is important to determine the fate of these isolates as bioremediation agent of biomedical wastewater.

3.1. Observation of Indigenous Hydrolytic Bacterial Isolates on MAP

Pathogenicity test carried out with the MAP showed most all of hydrolytic bacterial isolates obtained were able to grow on the media (Table 1). It shows the possibility that all of these isolates were a group of Gram-negative bacteria. This is because McConkey media inhibits the growth of Gram-positive bacteria in the presence of bile salts which will form crystal violet.



The result observations (Table 2) showed that hydrolytic bacterial isolates which could grow but did not show violet or yellow in MAP were R1. (4, 5, 7, 8, and 10) and R2 (1, 2, 4 and 8). The rest, namely R1 isolates (1, 2, 3, 6, 9, 11, 12, 13, 14, 15, 16 and 17) and R2 (5,6,7, and 9), show violet colors as evidence of crystal violet formation from lactose fermentation. Of 26 hydrolytic bacterial isolates obtained from 2 hospitals, 16 of them were Gram-negative hydrolytic bacteria groups able to ferment lactose. These 16 isolates had shown the possibility of nonpathogenic properties. On the other hand, the remaining 10 isolates were unable to demonstrate the ability to ferment lactose. So, based on MAP test, the 10 isolates belong to pathogenic group of Gram-negative bacteria.

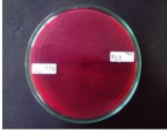
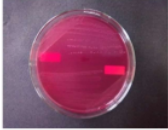




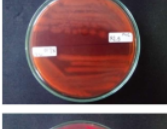
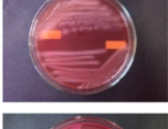
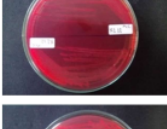
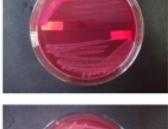
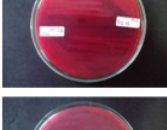
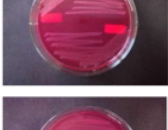
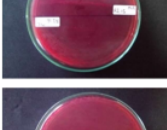
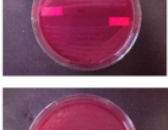
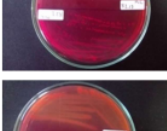
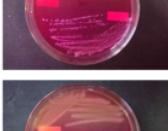


In Table 2, the only one isolate which could not show growth in MAP was isolate R2.3. Isolate R2.3, which later identified as *Bacillus amyloliquefaciens*, theoretically should be able to grow in MacConkey agar (MA) because it is a Gram-negative type of species. This phenomenon could happen due reasons, which could reveal limitation of MA media uses. First, it is known that some strains (regardless Gram-negative type) may be encountered that grow poorly or fail to grow on this medium. Second, incubation of MAPs under increased CO<sub>2</sub> reduces growth and recovery of a number of strains of Gram-negative bacilli [36].

3.2. Observation of Indigenous Hydrolytic Bacterial Isolates on BAP

After tests on MA media, a further pathogenicity test was carried out with a selective blood agar differential media, Blood Agar (BA) with the aim to see the potential of isolates to produce hemolysin-class toxins. The results of the pathogenicity test of hydrolytic bacterial isolates obtained using BAP media are shown in Table 3. As seen in Table 3, observations on BA media showed that all bacterial isolates could grow with hemolysis patterns α (10 isolates), β (6 isolates) and γ (10 isolates). Beta hemolysis (β) is defined as complete or true lysis of red blood cells. A clear zone approaching the color and transparency of the base medium, surrounds the colony. Many species of bacteria produce toxic by-products capable of destroying red blood cells resulting transparent medium. Alpha hemolysis(α) is the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in brown discoloration in the medium. The colour can be equated with "bruising" the cells [29].

Table 2. Pathogenicity tests using McConkey Agar Plate (MAP)

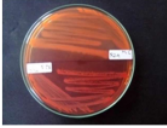
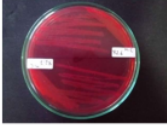
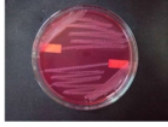

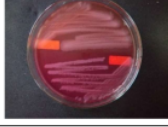
Observation from bottom of plate	Observation from top of plate	Sample code and medium color change
		R1.1 Violet R1.2 Violet

		R1.3 Violet R1.4 Yellow
		R1.5 Yellow R1.6 Violet
		R1.7 Yellow R1.8 Yellow
		R1.9 Violet R1.10 Yellow
		R1.11 Violet R1.12 Violet
		R1.13 Violet R1.14 Violet
		R1.15 Violet R1.16 Violet
		R1.17 Violet R2.1 Yellow
		R2.2 Yellow R2.3 No growth

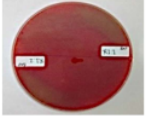

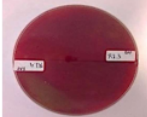

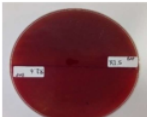

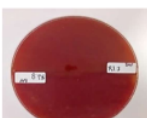
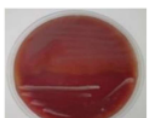
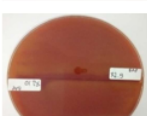
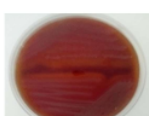
**Table 2.** ... Continued

Observation from bottom of plate	Observation from top of plate	Sample code and medium color change
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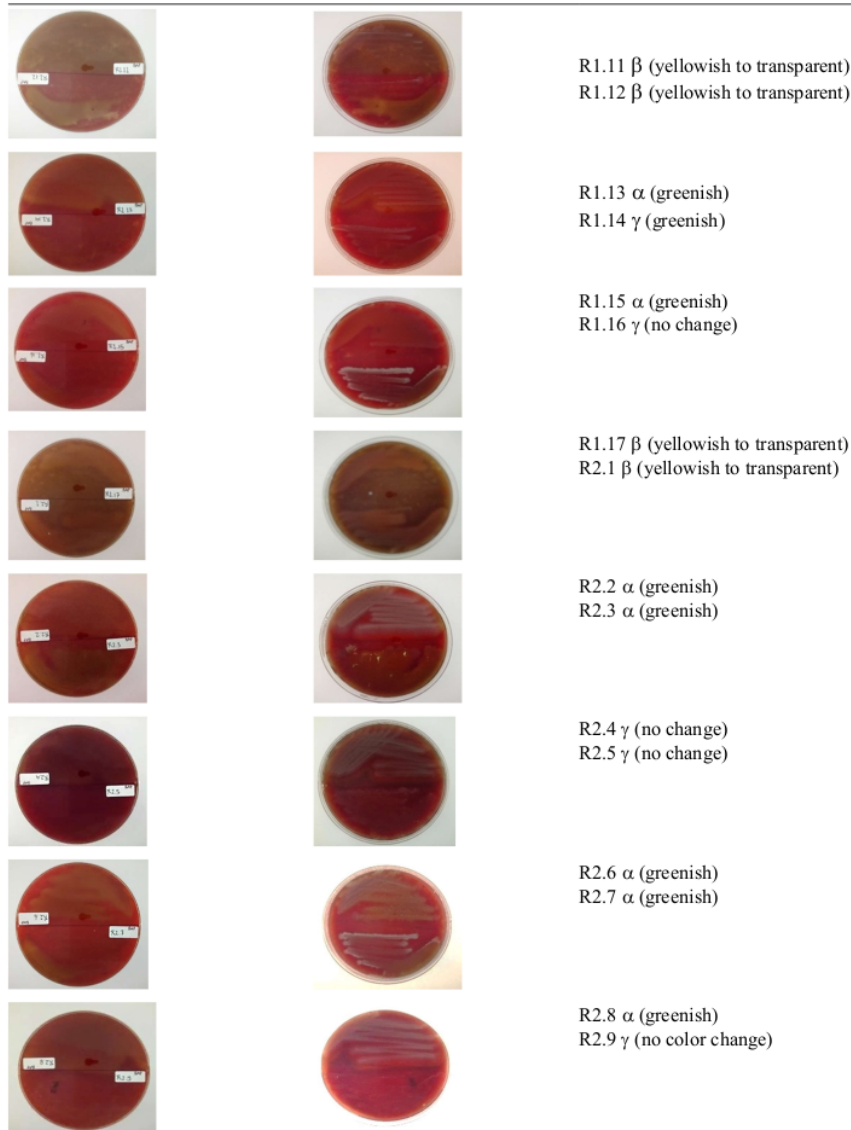
		R2.4 Yellow R2.5 Violet
		R2.6 Violet R2.7 Violet
		R2.8 Yellow R2.9 Violet

**Table 3.** Pathogenicity tests using Blood Agar Plate (BAP)

Observation from bottom of plate	Observation from top of plate	Sample code and hemolysis type (medium color change)
		R1.1 $\alpha$ (greenish) R1.2 $\alpha$ (greenish)
		R1.3 $\gamma$ (no change) R1.4 $\alpha$ (greenish)
		R1.5 $\gamma$ (no change) R1.6 $\gamma$ (no change)
		R1.7 $\gamma$ (no change) R1.8 $\gamma$ (no change)
		R1.9 $\beta$ (yellowish to transparent) R1.10 $\beta$ (yellowish to transparent)

**Table 3.** ... Continued

Observation from bottom of plate	Observation from top of plate	Sample code and hemolysis type (medium color change)
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Microscopic inspection of alpha-hemolyzed red blood cells shows that the cell membrane is intact, so it is not, in fact, true lysis. Some text book authors refer to alpha as “partial hemolysis,” which might be confusing. It is most important to not confuse this “partial” or “incomplete” hemolysis with the “weak” or “subtle” lysis of *Streptococcus agalactiae* or *Listeria monocytogenes*, as seen above. The  $\beta$  hemolysis will never include the brown or green discoloration of the cells in the surrounding medium. On prolonged incubation, many alpha hemolytic organisms will begin to appear clearer, but if the surrounding medium contains any shades of brown or green the “hemolysis” is still considered “alpha”. Gamma hemolysis ( $\gamma$ ) is somewhat self-contradictory. Gamma indicates the lack of hemolysis. There should be no reaction in the surrounding medium. The hemolytic activity, more widely known as the Kanagawa phenomenon, is among significant virulence markers. Red blood cell of the host organism is lysed due to the presence of hemolysin gene

in turn helping in the spread of the pathogen in the host blood [37]. Applications of MA and BA media include pathogenicity determination of *Escherichia coli* and *Streptococcus pneumoniae*. On MA and BA media, *E. coli* colonies appear as large, circular, pink, flat, moist, non-mucoid and lactose fermenting; flat, transparent,  $\beta$ -haemolytic. On the other hand, *S. pneumoniae* could not grow on MA due to the absence of blood and also the presence of high concentration of bile in the medium inhibiting the growth of *S. pneumoniae*. The pathogen however, could grow and showing  $\beta$ -haemolytic pattern on blood agar medium [38].

In the stage II pathogenicity test using the BAP selective medium showed that in general all isolates showed growth even at different levels. A total of 8 isolates showed a pattern of hemolysis which means that it can produce hemolysin which can cause total blood hemoglobin damage. The other 10 isolates showed a pattern of hemolysis so that it was associated with the potential for moderate to high pathogenicity because it can produce hemolysin which is partially damaging to the blood. There are 6 remaining isolates that show low hemolysis production ability so that it does not cause changes in the medium of growth.

A total of 16 bacterial isolates that tended to be nonpathogenic based on MA media test, after testing with BA media only remained only 6 which did not produce hemolysin toxin. This means that there are 6 isolates that can be used as candidates for further testing. Later, from the 6 candidates who have the ability to reduce the parameters of liquid waste pollution, namely COD, BOD, phosphate and TSS, it will have the potential to become a bioremediation agent. The number of candidates is good to increase the number, so that it can be considered to do one more sampling to get a number of new bacterial isolates from the waste water storage tank in one other hospital.

On the other hand, 20 isolates which are thought to have moderate to very high levels of pathogenicity also still have the potential to be studied further regarding their ability to produce one to several types of hydrolytic enzymes. In addition, all isolates need to be identified to provide a description of the genetic diversity of hydrolytic bacteria from liquid hospital biomedical waste that can be cultured.

BA is a general purpose enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties [29]. Hemolysin is a toxic enzyme causing lyses of red blood cells. It plays a role in increasing cell permeability, making cells more susceptible to infectious agents. According to Park et al. [40] hemolysin in a pathogenic bacterial species, *S. aureus*, acts as a pathogenicity factor and is among important toxins formed by the species. According to Jawetz et al. [41],  $\alpha$ -hemolysin is able to dissolve rabbit erythrocytes and damage platelets, and further could also damage the circulatory system as well as various tissues including muscle and renal cortex tissues. According to Buxton [29] "*Gamma streptococcus*" are usually non-hemolytic after 24-h of incubation, but many eventually display weak alpha hemolysis.

### 3.3. Observation of Indigenous Hydrolytic Bacterial Isolates on CAP

In this study, the use of CAP was part the use of 3 key media for simple plate-based pathogenicity scoring system. The idea is to find out if any of the selected bacterial isolates show colony characteristics of *Streptococcus pneumoniae*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenzae* bacteria. Based on data from <https://microbeonline.com/chocolate-agar-composition-uses-colony-characteristics/>, colonies of *N. meningitidis* and *H. influenzae* on CAP = large, round, smooth, convex, colorless-to grey, opaque colonies with no discoloration of the medium. Characteristics of colonies *S. pneumoniae* = small green colonies with a zone of  $\alpha$ -hemolysis (only slightly green). *N. gonorrhoeae* colonies = pinkish-brown and translucent, exhibit smooth consistency and defined margins, and are typically 0.5-1 mm in diameter. Any signs of colony morphology and hemolysis similarity to the mentioned characteristics were carefully checked. The results showed that none of the studied bacterial isolates exhibit large diameter, and or  $\alpha$ -hemolysis, and or pinkish-brown/ green/ translucent color. Therefore, as seen in Table 4, results from CAP were stated as (-). It means none of the indigenous, hydrolytic bacterial isolates studied categorized in "extra high pathogenic level" group.

**Table 4.** Summary of pathogenicity test results of bacterial isolates with selective media.

Sample code*	Hydrolytic Enzyme Production Plate Test					Pathogenity & Identification Test				Pathogenicity Score
	Prote-ase	Amy-lase	Cellu-lase	Lipase	Hydro-lytic	MAP	BAP	CAP	Identity based on 16S rRNA gene	
R1.1	++	+	++	++	√	+	α	-	<i>B. velezensis</i> R1.1	Medium
R1.2	++	++	++	++	√	+	α	-	<i>B. amyloliquefaciens</i> R1.2	Medim
R1.3	+	++	++	++	√	+	γ	-	<i>B. velezensis</i> R1.3	Low
R1.4	++	++	++	+	√	-	α	-	<i>B. amyloliquefaciens</i> R1.4	High
R1.5	++	-	-	+	√	-	γ	-	<i>B. cereus</i> R1.5	Medium
R1.6	++	-	-	++	√	+	γ	-	<i>B. amyloliquefaciens</i> R1.6	Low
R1.7	++	++	++	++	√	-	γ	-	<i>B. cereus</i> R1.7	Medium
R1.8	++	++	+	++	√	-	γ	-	<i>B. amyloliquefaciens</i> R1.8	Medium
R1.9	++	-	-	++	√	+	β	-	<i>B. cereus</i> R1.9	High
R1.10	++	-	-	++	√	-	β	-	<i>B. cereus</i> R1.10	Very high
R1.11	++	+	++	+	√	+	β	-	<i>V. marismortui</i> R1.11	High
R1.12	++	-	-	++	√	+	β	-	<i>B. velezensis</i> R1.12	High
R1.13	++	-	-	-	√	+	α	-	<i>B. aerius</i> R1.13	Medium
R1.14	+	++	+	++	√	+	γ	-	<i>B. amyloliquefaciens</i> R1.14	Low
R1.15	++	+	-	++	√	+	α	-	<i>B. subtilis</i> R1.15	Medium
R1.16	++	-	-	++	√	+	γ	-	<i>B. velezensis</i> R1.16	Low
R1.17	+	++	+	-	√	+	β	-	<i>V. marismortui</i> R1.17	High
R2.1	++	++	-	-	√	-	β	-	<i>V. salarius</i> R2.1	Very high
R2.2	++	-	-	++	√	-	α	-	<i>B. cereus</i> R2.2	High
R2.3	++	++	++	++	√	0	α	-	<i>B. amyloliquefaciens</i> R2.3	Medium
R2.4	++	-	+	+	√	-	γ	-	<i>B. cereus</i> R2.4	Medium
R2.5	++	+	-	+	√	+	γ	-	<i>B. licheniformis</i> R2.5	Low
R2.6	+	+	+	++	√	+	α	-	<i>B. subtilis</i> R2.6	Medium
R2.7	++	-	+	+	√	+	α	-	<i>B. velezensis</i> R2.7	Medium
R2.8	++	-	-	+	√	-	α	-	<i>B. cereus</i> R2.8	Medium
R2.9	++	-	-	++	√	+	γ	-	<i>B. amyloliquefaciens</i> R2.9	Low
Σ (+)	10	26	14	13	24	26	16	10	N/A	

Note: \* R1 = Roemani Hospital, R2 = RSUD KRT Wongsonegoro Hospital, \*\* in duplo (25).

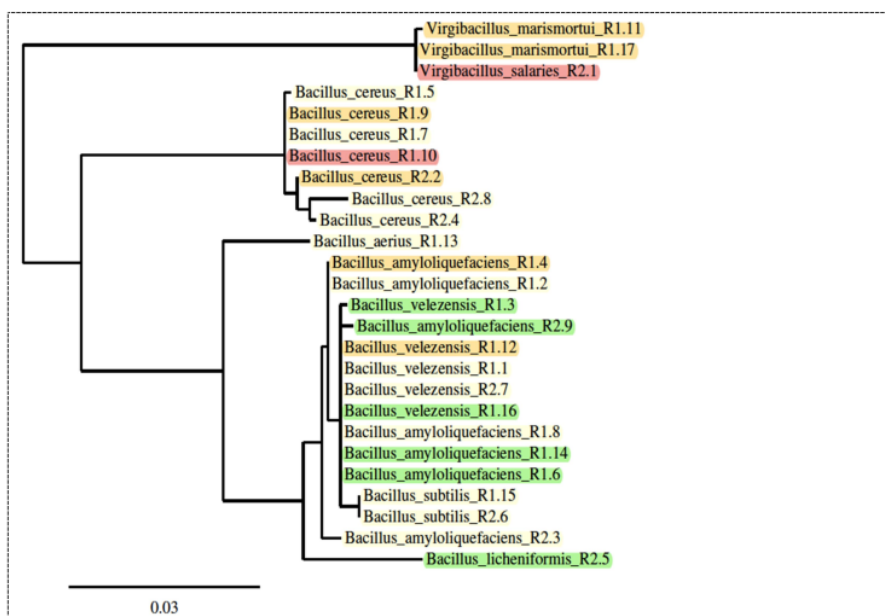
### 3.4. Pathogenicity Scoring on Indigenous Hydrolytic Bacterial Isolates and Their Molecular Identity

It is also revealed by this study (Table 4) that none of all strains studied that later identified as *B. cereus* belong to pathogenic because although they exhibit various types of  $\gamma$  hemolysis to no hemolysis, all of them did not show ability to ferment lactose on MAP. This result is in line to the fact that *B. cereus* is a pathogenic organism known as serious food poisoning agent. It could produce 4 different types of toxins, which therefore exposes high risk to public health if used as bioremediation agent. Interestingly, though group on non- to low-pathogenic bacteria are occupied by *B. velezensis*, *B. amyloliquefaciens* and *B. licheniformis*, a bacterial strain, pathogenicity of *B. amyloliquefaciens* R1.4, differently scored as high. Based on data from the literature, such result is actually not surprising. A bacterial species name should mean the same organism, but within a species, strains could differ by the disease they produce, their environmental habitat, and many other characteristics including genetic properties and plasmids. It is possible that within similar species, some strains are more pathogenic than the others [42].

### 3.5. The 16S rRNA Sequence and Morphology Analysis on Selected Non-Pathogenic Strains

The 16S rRNA sequence analysis was conducted to all of 26 indigenous hydrolytic bacteria studied based on data already submitted on National Center for Biotechnology Information (NCBI) website [32]. A new phylogenetic tree was constructed highlighting pathogenicity level of each bacterial strain (Figure 1).





**Figure 1.** Phylogenetic tree of 26 indigenous hydrolytic bacteria studied highlighting their pathogenicity levels. Note: Pathogenicity levels based on colors, green = low, yellow = medium, orange = high, red = very high.

As seen in the phylogenetic tree, the green highlighted bacterial strains are the 6 non-pathogenic selected strains based on plate-based scoring system: *Bacillus velezensis* R1.3, *B. amyloliquefaciens* R1.6, *B. amyloliquefaciens* R1.14, *B. velezensis* R1.16, *B. licheniformis* R2.5, and *B. amyloliquefaciens* R2.9. The SEM test results (Table 5) were conducted only on these 6 non-pathogenic selected isolates to confirm their morphology identity. Based on data in Table 5, all of these non-pathogenic isolates determined using plate-based scoring system in this study were short rod-shaped bacteria. These cellular morphology test results supported results from molecular identification that the selected non-pathogenic strains belong to Bacillus groups.

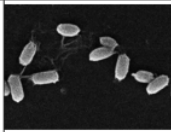
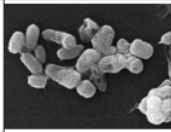
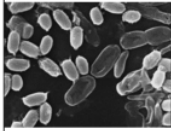
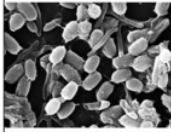
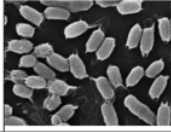
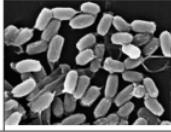
Bioremediation as biological remediation involving the use of bacteria is beneficial to reduce or to eliminate organic matters as main pollutants in hospital wastewater. However, pathogenic bacteria should be avoided to be used as bioremediation agent due to public safety [2]. Pathogenicity test using molecular method is not always available in low income hospitals. Therefore, a simple, yet effective and affordable pathogenicity scoring system aiming to select the non-pathogenic group of indigenous hydrolytic bacteria had been set and applied in this study. The aim was to determine if a group of isolated hydrolytic bacteria could be used (in consortium) as bioremediation agent suitable to treat hospital wastewater in developing countries, or low income countries where molecular methods might be non-economical.

Results from plate-based pathogenicity scoring system set in this study based on observation on 3 “key” agar media plates, MAP, BAP and CAP, found that 6 out of 26 indigenous hydrolytic bacterial isolates studied are low- or non-pathogenic strains. The results were in line with their molecular identity confirming that the isolates belong to non-pathogenic species of Bacillus, i.e. *Bacillus velezensis*, *B. amyloliquefaciens* and *B. licheniformis*. The results demonstrated that using the proposed plate-based pathogenicity scoring system, it is possible to practically set aside non-pathogenic indigenous hydrolytic bacterial strains from the pathogenic ones in wastewater of hospitals studied. When molecular method is not available, the plate-based pathogenicity scoring system could be a simple,



low cost, yet useful and reliable step to develop bacterial bioremediation agent for hospital wastewater in developing countries.

**Table 5.** Pathogenicity tests using McConkey Agar Plate (MAP)

No	Sampel Code	Cellular morphology
1.	R1 3	
2.	R1 6	
3.	R1 14	
4.	R1 16	
5.	R2 5	
6.	R2 9	

Note: \* R1 = Roeman Hospital, R2 = RSUD KRT Wongsonegoro Hospital

#### 4. Conclusion

Results from plate-based pathogenicity scoring system set in this study based on observation on 3 “key” agar media, MA, BAP and CAP could determine low- or non-pathogenic bacterial strains potential as bioremediation agent of hospital wastewater supported by their 16S rRNA molecular identity. The results inferred that the set plate-based pathogenicity scoring system could be a simple, yet useful and reliable tool for selecting non-pathogenic indigenous hydrolytic bacterial strains potential as bioremediation agent for hospital wastewater in developing countries.

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