

Antimicrobial activity of extracellular liquid obtained from molasses fermentation by *Nocardia* sp strain V1

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Abstract. Molasses is one of agricultural wastes that could be utilized as a substrate for microbial fermentation. The utilization of molasses as a fermentation medium is expected to increase its added value. In this study, molasses served as a carbon source for bolstering the growth and the production of extracellular liquid having antimicrobial activity produced by *Nocardia* sp strain V1. The six types of fermentation media containing three carbon sources namely molasses, molasses-starch, and molasses-glucose were prepared; wherein molasses used were the ones with and without the pre-treatment using $K_4Fe(CN)_6 \cdot 3H_2O$. This chemical was used to precipitate heavy metals and other substances in molasses that might inhibit the growth of the bacteria. The antimicrobial activity of extracellular liquid produced by *Nocardia* sp strain V1 was evaluated against 10 microbes isolated from infected strawberries and pseudostem of banana plant. The result showed that the antimicrobial activities were demonstrated by the extracellular liquid derived from the bacteria grown in the media containing pre-treated molasses-starch (M2 medium), pre-treated molasses-glucose (M3 medium), and glucose-starch (synthetic medium) as the carbon sources. The extracellular liquid from those fermented media was able to inhibit 2 microbes from a total of 10 microbes identified as *Rhodotorula* sp and *Fusarium graminearum* with the inhibition diameter ranged from 10-21 mm.

1. Introduction

The attempts to harness byproducts from the sugar industry continue to expand along with the emergence of various products with high added value such as alcohol, artificial sweeteners, vitamins, amino acids, and so forth. The products converted from these by-products are widely used by the public in the field of food, pharmaceutical, and bioenergy [1]. One of the most common by-products produced in sugar industry is molasses. This compound is produced in the final stage of the sugar crystallization process and could potentially cause serious environmental problems. Molasses is known as one of the major sources of contaminants in the aquatic environment since it is brownish, has strong acid properties and high COD and BOD level [2,3]. Meanwhile, the production of molasses in Indonesia has been increasing since 1964 with a total of 1.36 million tons in 2013 [4]. This increasing number has encouraged the researchers to continue their efforts in utilizing and converting molasses into different products with high added value.

One of the applications of molasses utilization in the field of biotechnology is its use as a fermentation medium. This usage could reduce the production costs since the synthetic medium accounts for 30% of the total cost in the fermentation process [5]. The use of synthetic carbon sources such as



glucose, sucrose, mannitol, fructose, and arabitol are not economical due to their high price. Therefore, finding the new and inexpensive carbon sources is an important aspect to obtain more cost-effective cultures [6,7,8]. Besides being affordable, molasses contains various nutrients including carbohydrates, vitamins, minerals, and other micronutrients needed for microbial growth. Carbohydrate contained in molasses comprises glucose and fructose with the fermentable total sugar ranges from 45-55% [1,4].

The use of molasses as a medium in various fermentation processes has been widely studied. Research conducted by Zhu *et al* found that molasses could serve as a fermentation medium for *Trichosporon fermentans* to produce biodiesel [9]. Molasses has also been used as a fermentation medium for producing lactic acid [10], polyhydroxybutyrate [11], ethanol [12], pullulan [13], cellulose [14], and xantan sap [15]. In this study, molasses was used as a fermentation medium for *Nocardia* sp strain V1 to produce extracellular liquid having antimicrobial activity.

Bacteria of the genus *Nocardia* has been shown to produce a variety of secondary metabolites that actively inhibit the microbial growth. For example, *Nocardia* sp strain WW-12651 was known to produce a broad-spectrum of antibiotic compounds from thiazolyl peptide group such as nocrin I, II and III. These compounds had the ability to inhibit the growth of gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Enterococcus faecium* (MREF), and penicillin-resistant *Streptococcus pneumoniae* [16]. *Nocardia* sp strain CS682 also produced an antibiotic compound called nargecin that could inhibit the growth of MRSA. In addition, other antibiotic compounds such as peptidolipin B and peptidolipin E were also produced by *Nocardia araeensis* strain W9705 and proven to inhibit the growth of MRSA [17].

As regard to the potential of *Nocardia* as a source of antibiotic compounds, this study aimed to investigate whether *Nocardia* sp strain V1 was capable of producing extracellular liquid having antimicrobial activity. Extracellular liquid was obtained through fermentation with a variety of media composition containing molasses. The antimicrobial activity of this extracellular liquid was intended as a biocontrol agent that could be used in the fruits plantation. Biocontrol agents in the form of microorganism could serve as the alternative tools to replace chemical pesticides and fertilizers which in turn reduce the deleterious environmental impact of agriculture. Hence, the antimicrobial activity was evaluated against 11 microbial species isolated from infected strawberries and pseudostem of banana plant.

2. Methods

2.1. Microorganism and reagents

Nocardia sp strain V1 was the collection of Research Unit for Clean Technology, Indonesian Institute of Sciences. Molasses was obtained from the local sugar industry. Nutrient agar (NA), potato dextrose agar (PDA), glucose, starch, triptone, CaCO₃, PDA, and K₄Fe(CN)₆·3H₂O were purchased from Merck (Darmstadt, Germany). Yeast extract was purchased from Laboratorios conda S.A (Madrid, Spain). The analytical grade of Nelson reagents was purchased from Merck (Darmstadt, Germany) and used for determination of total sugar concentration using Nelson-Somogyi method.

2.2. The isolation and identification of allegedly phytopathogenic microorganisms

The infected strawberries and pseudostem of banana plant were sampled aseptically from the local farm. Each sample was chopped, mixed with distilled water, and vortexed for 5 min prior to serial dilution. The samples were serially diluted in the ten-fold dilution method until the 10⁻⁵ dilution. Each serially diluted sample of 0.1 mL was spread on NA and PDA. The plates were incubated at 30°C for 48 h. The microorganisms were isolated and stored at 4°C. Some of the isolates were identified based on 18s rRNA in the School of Life Sciences, Bandung Institute of Technology.

2.3. Molasses pre-treatment

Molasses of 500 g was mixed with 500 mL distilled water and centrifuged at 7000 rpm for 15 min. The supernatant was collected and mixed with 3 mL of K₄Fe(CN)₆·3H₂O to remove the heavy metals and

other inhibitory substances. The mixture was heated at 70°C for 30 min and stored overnight. The mixture was centrifuged at 7000 rpm for 15 min and the supernatant was collected [18].

2.4. Molasses fermentation by *Nocardia* sp strain V1

The inoculum was prepared by inoculating *Nocardia* sp strain V1 into 250 mL medium containing glucose (10 g/L), starch (10 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L) at pH 7. The medium was incubated in a shaker incubator at 30°C with agitation velocity of 150 rpm for 48 h. The inoculum of 10% (v/v) as inoculated into 250 mL fermentation media containing different compositions. The seven types of fermentation media were prepared as follows:

- a. M1 (pre-treated molasses (20 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L);
- b. M2 (pre-treated molasses (20 g/L), starch (5 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L);
- c. M3 (pre-treated molasses (20 g/L), glucose (5 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L);
- d. M1a (untreated molasses (20 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L);
- e. M2a (untreated molasses (20 g/L), starch (5 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L);
- f. M3a (untreated molasses (20 g/L), glucose (5 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L).
- g. Synthetic Medium (glucose (10 g/L), starch (10 g/L), triptone (5 g/L), yeast extract (2.5 g/L), and CaCO₃ (1 g/L).

The pH of each medium was 7.0. The inoculated media were shaken at 150 rpm and incubated at 30°C for 7 days. The fermented medium of 10 mL was sampled each day and centrifuged at 7000 rpm for 15 min to collect the supernatant.

2.5. Antimicrobial activity test

The collected supernatant from each fermented medium was subjected to antimicrobial activity test using agar diffusion method. The solid media containing PDA which previously mixed with 1 mL of each microbial suspension were prepared in a sterile petri dish. The supernatant of 20 µL was dropped into a sterile paper disc previously put in the media and incubated for 72 h at 30°C. The observations were made by measuring the diameter of the inhibition zone in millimeters.

2.6. Determination of total sugar consumption rate and pH measurement [19]

The determination of total sugar concentration in the sample was determined as follows: the supernatant of 1 mL was collected after 7 days of incubation. The sample was subjected to strong acid hydrolysis by mixing with 0.5 mL of concentrated H₂SO₄. The mixture was incubated at 100°C for 1 h. Afterwards, the mixture was diluted to 10 mL distilled water. The diluted sample of 1 mL was added into the test tube and mixed with 1 mL Nelson reagent. The mixture was incubated for 20 min in a 100°C water bath. The sample was cooled down, mixed with 1 mL arsenomolybdate solution, and added with 7 mL distilled water. The solution mixture was mixed by vortex and subjected to absorbance measurement using a spectrophotometer at 520 nm. The absorbance value was plotted into the standard curve to obtain the total sugar concentration on each sample. The percentage of total sugar consumption was calculated by comparing with the initial sugar concentration from each medium before inoculation obtained through the same method. The pH of each fermented medium was measured every day by a pH meter to monitor the acidity of the medium during fermentation.

3. Results and Discussion

3.1. The isolation and identification of allegedly phytopathogenic microbes

The microbes were isolated from infected strawberries and pseudostem of banana plant in order to obtain allegedly phytopathogenic microbes that could be inhibited by the extracellular liquid being produced.

The result showed different colonies of microbes appeared on PDA in the dilution of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} . However, the microbial colonies did not appear on NA. This indicated that the microbes infecting strawberries and banana's pseudostem were fungi, yeasts, and molds. These microorganisms prefer PDA to NA since media containing high carbohydrate and nitrogen source are required for the growth of fungi [20]. PDA contains the infusion of potato and dextrose that foster luxuriant fungal growth whereas NA offers the cultivation of microbes supporting the growth of a wide range of non-fastidious microorganisms and contains many nutrients needed for the bacterial growth [21]. The result showed six microbes suspected as fungi were obtained from the infected banana's pseudostem and coded as Cador, Caue, Cadung, Cabe1, Cadom, Caug and 1 fungus was identified as *Fusarium graminearum* based on 18s rRNA analysis (Figure 1).

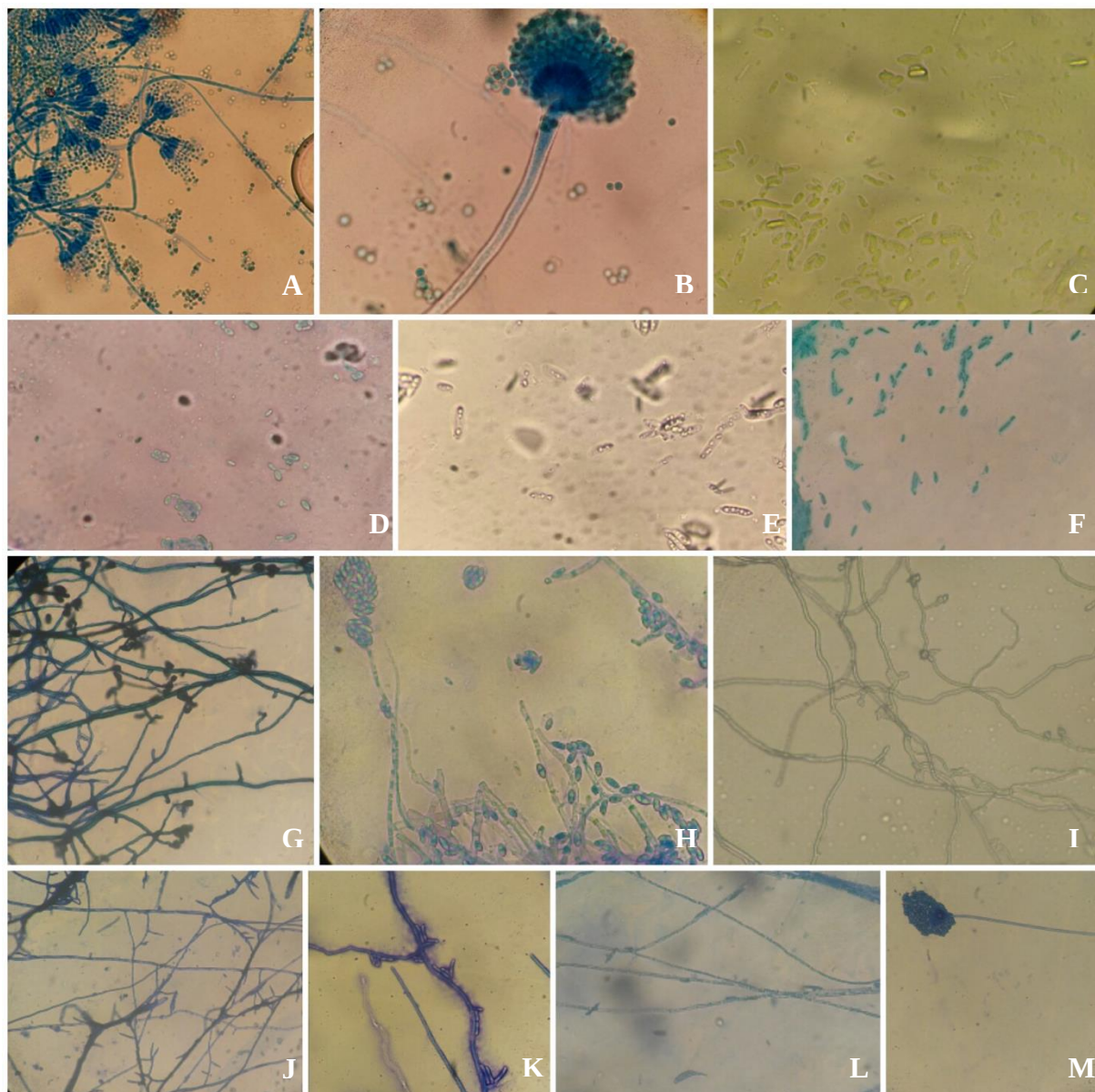


Figure 1. The appearance of microbes isolated from infected strawberries and banana's pseudostem under a microscope with magnification of 40x10 (A: *Aspergillus* sp, B: *Penicillium* sp, C: *Rhodotorula* sp, D: *Candida psudotropicalis*, E: *Kluyveromyces* sp, F: *Candida krusei*, G: *Fusarium graminearum*, H: Cador, I: Caue, J: Cadung, K: Cabe1, L: Cadom, and M: Caug).

Fusarium graminearum has been identified as a pathogen that causes devastating disease in banana and head blight of small grain cereals including wheat and barley [22,23]. This fungus spreads the spores on or inside flowering spikelets [24]. The infection of this fungus in crops causes great economic loss due to the reduced quantity and quality as well as to the contamination by diverse mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEA), which are harmful for humans and animals [24]. DON is an effector molecule that inhibits protein synthesis through the activation of activating critical cellular kinases involved in signal transduction and binding to the ribosome disrupting growth, immune function, and reproduction [25]. Meanwhile, ZEA has hepatotoxic, haematotoxic, immunotoxic and genotoxic properties due to the formation of two metabolites a-zearalenol (a-ZEA) and b-zearalenol (b-ZEA) [26].

In addition, two fungi and four yeasts were successfully isolated from infected strawberries and identified based on 18s rRNA analysis as *Aspergillus* sp, *Penicillium* sp, *Rhodotorula* sp, *Candida pseudotropicalis*, *Kluyveromyces* sp, and *Candida krusei* (see Figure 1) [27]. Along with microbes isolated from infected banana's pseudostem, the microbes having significant influence to cause strawberry diseases were subjected to antimicrobial activity test. They were *Aspergillus* sp, *Penicillium* sp, and *Rhodotorula* sp. *Aspergillus* species are known to be responsible for several disorders in various plants and plant products including strawberries. They contaminate agricultural products during pre-harvest, harvest, processing and handling stages [28]. *Aspergillus* causes root diseases in strawberry and responsible for its post-harvest decay. This microbe also produces mycotoxins such as aflatoxins and ochratoxin A that are responsible for carcinogenicity and immunotoxicity [29]. Aside from *Aspergillus*, *Penicillium* is also one of the important microbes causing diseases in strawberries. *Penicillium* species are one of the main pathogenic fungi that decay strawberry fruit and cause fruit blotch disease [30]. Among the fungi isolated from strawberries, *Penicillium* spp are also known as potential mycotoxin producers [31, 32]. They are the most common fungal genus on the surface of the strawberries and the most frequently isolated genus growing from both conventional and organic grown berries [33]. In addition to *Aspergillus* and *Penicillium*, *Rhodotorula* is also found as the most common yeast isolated from strawberries [30]. *Rhodotorula* spp could cause skin, ocular, meningeal, prosthetic joint, and peritoneal infections in human [34]. A study conducted by Tomsíková showed the contamination of *Rhodotorula* sp in food that was provided for immunocompromised patients in hospitals [35]. Due to its severity and pathogenicity, these three microbes along with other seven microbes isolated from infected banana's pseudostem were tested against extracellular fluid produced by *Nocardia* sp strain V1 through molasses fermentation.

3.2. Antimicrobial activity

The medium composition was optimized to determine which medium favored *Nocardia* sp strain V1 to produce the highest antimicrobial activity. The molasses pre-treatment through the addition of $K_4Fe(CN)_6 \cdot 3H_2O$ was also investigated to know whether this treatment had significant influence on the production of antimicrobial extracellular liquid. The pre-treatment of molasses is required since it contains a lot of suspended particles and complex structures which cause heterogeneity in the medium and affect the cell growth rate. Therefore, many types of molasses treatment have been proposed in order to remove undesirable substances such as coloring substances, heavy metals, and unknown compounds that may act as growth inhibitors [36]. This could be done by heat treatment, acidification treatment, and metal complexing agent treatment [37].

The result exhibited *Nocardia* sp strain V1 grown in the media containing pre-treated molasses-starch (M2) and pre-treated molasses-glucose (M3) produced extracellular liquid having antimicrobial activity (see Table 1). Meanwhile, *Nocardia* sp strain V1 grown in all media containing untreated molasses did not show any inhibition on the growth of allegedly phytopathogenic microbes. $K_4Fe(CN)_6 \cdot 3H_2O$ acted to reduce the load of various fungal growth inhibitory substances in molasses like Ca, Cu, and Mn. The chemical served as metal complexing agent that reacted with those heavy metals, causing their precipitation. This compound was proven capable of complexing 18 out of 21 microelements found in molasses [38]. Hence, the growth of *Nocardia* sp strain V1 in the media containing pre-treated molasses was better since the inhibitory substances were removed by

$K_4Fe(CN)_6 \cdot 3H_2O$. This led to better production of extracellular liquid that could inhibit the growth of allegedly phytopathogenic microbes. Some research papers have shown that the molasses pre-treatment using $K_4Fe(CN)_6 \cdot 3H_2O$ also helped to increase the production of inulinase [39], pullulan [40], and succinic acid [41]. In contrast, pre-treated molasses did not increase the production of lactic acid indicating that molasses contained desirable substances which significantly affected lactic acid production [10].

Table 1. The antimicrobial activity of extracellular liquid produced by *Nocardia* sp strain V1 in different fermentation medium.

Allegedly phytopathogenic microbes	Medium type	Diameter of inhibition zone (mm)			
		Day-4	Day-5	Day-6	Day-7
<i>Fusarium graminearum</i>	M1	-	-	-	-
	M2	-	-	21	20
	M3	-	-	20	17
	M1a	-	-	-	-
	M2a	-	-	-	-
	M3a	-	-	-	-
	Synthetic medium	15	18	-	-
<i>Rhodotorulla</i> sp	M1	-	-	-	-
	M2	-	10	14	10
	M3	-	10	14	12
	M1a	-	-	-	-
	M2a	-	-	-	-
	M3a	-	-	-	-
	Synthetic medium	15	20	-	-
Cador, Caue, Cadung, Cabe1, Cadom, Caug, <i>Aspergillus</i> sp, <i>Penicillium</i> sp	M1	-	-	-	-
	M2	-	-	-	-
	M3	-	-	-	-
	M1a	-	-	-	-
	M2a	-	-	-	-
	M3a	-	-	-	-
	Synthetic medium	-	-	-	-

Nocardia sp strain V1 grown on the synthetic, M2, and M3 media produced extracellular liquid that was active to inhibit 2 out of 10 allegedly phytopathogenic microbes identified as *Fusarium graminearum* and *Rhodotorulla* sp (see Table 1). The inhibition zone diameter varied depending on the fermentation time, medium type, and allegedly phytopathogenic microbes being inhibited. When the extracellular liquid was tested against *Fusarium graminearum*, the highest antimicrobial activity was shown by M2 and M3 media on the sixth day of fermentation, displaying the widest inhibition zone diameters of 21 mm and 20 mm respectively. Meanwhile, the extracellular liquid from the synthetic medium showed less ability to inhibit *Fusarium graminearum* with inhibition zone diameters

of 15 mm and 18 mm on the fourth day and the fifth day of fermentation respectively. However, the highest antimicrobial activity against *Rhodotorulla* sp was shown by extracellular liquid obtained from the synthetic medium. This was also appeared on the fifth day of fermentation with the inhibition zone diameter of 20 mm. Meanwhile, the M2 and M3 media formed their widest inhibition zone with a diameter of 14 mm each when they were tested against *Rhodotorulla* sp. This appeared on the sixth day of fermentation. Nonetheless, the bacteria grown in the medium containing molasses alone as a carbon source did not show any antimicrobial activity. This might be due to the absence of glucose and starch as carbon sources used in the starter culture, incommoding the bacteria to adapt in a new environment and hampering them from producing antimicrobial metabolites. Similar result was shown by Kotzamanidis *et al* [10] who revealed that the highest lactic acid concentration was produced by the bacteria grown in the medium containing molasses mixed with sucrose and yeast extract. They suggested that sucrose and yeast extract played a key role to boost up the concentration of lactic acid in the culture whereas molasses did not.

The result also showed that *Nocardia* sp strain V1 produced their maximum inhibition zone in different fermentation time depending on the medium composition. In the synthetic medium, the bacterium could utilize the nutrients faster since they were less complex compared to the ones containing molasses. Molasses contains various complex chemicals in addition to sugars such as waxes, sterols, phospholipids, non-nitrogenous compounds, and ash contents [33]. This made the bacteria grew longer due to slow adaptation process in the presence of those chemicals and confined the early production of antimicrobial metabolites than those grown in the synthetic medium. Therefore, *Nocardia* sp strain V1 grown in the synthetic medium was able to inhibit the allegedly phytopathogenic microbes earlier on the fourth day of fermentation whereas the bacteria grown in the medium containing molasses inhibited later. The antimicrobial activity of extracellular liquids in the M2 and M3 media decreased on the seventh day of fermentation while the ones in synthetic medium weakened on day sixth. This declining trend was possibly caused by the reduction of available nutrients inside the medium leading to the drop of bacterial growth which in turn slowed down their metabolism and decreased the production of antimicrobial metabolites.

3.3. Total sugar concentration and pH measurement

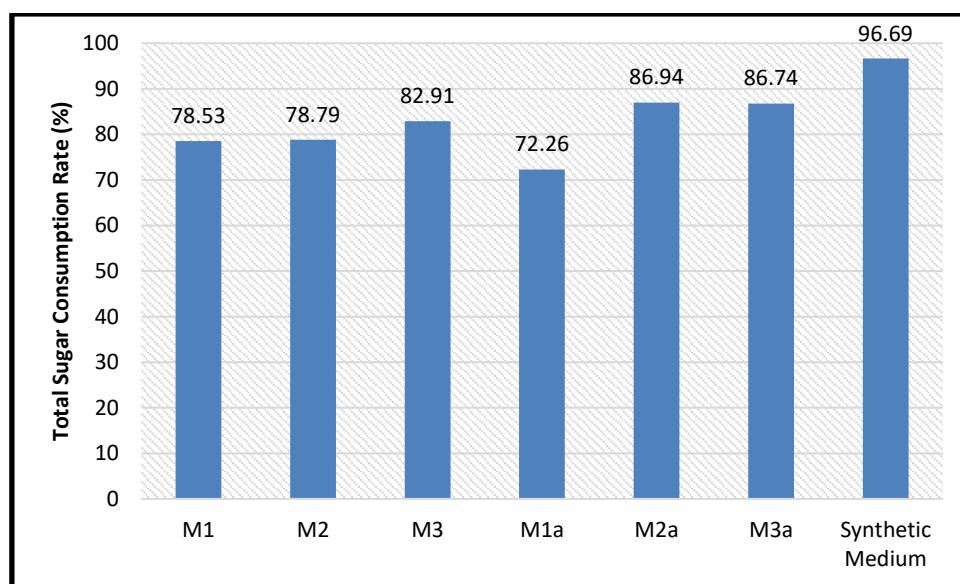


Figure 2. Total sugar consumption of the bacteria grown on various media.

The total sugar concentration was measured during fermentation time to determine the sugar consumption rate on each medium. The data could imply the ability of the bacteria grown on each

medium to produce the extracellular metabolites having antimicrobial activity. The high sugar consumption rate indicated the high nutrient utilization by the bacteria to undergo their metabolism. The result showed the highest sugar consumption rate of 96.68% belonged to the bacteria grown in the synthetic medium (see Figure 2). This data indicated that the bacteria grown in the synthetic medium had the highest efficiency to utilize the sugar for their metabolism which was confirmed by the higher antimicrobial activity to inhibit *Rhodotorula* sp compared to the others.

The bacteria grown in the media containing molasses-starch and molasses-glucose showed higher sugar consumption rate compared to the ones containing molasses alone regardless the pre-treatment of molasses (see Figure 2). The bacteria grown on M2 and M3 media had sugar consumption rate of 78.80% and 82.91% respectively, being higher than the media containing pre-treated molasses alone which was 78.50%. This was also confirmed by the fact that the antimicrobial activity of the bacteria grown in those media was higher when they were tested against *Fusarium graminearum* compared to the others. This trend was similar to the media containing untreated molasses (see Table 1). The sugar consumption rate of the bacteria grown on M2a and M3a media were 86.94% and 86.73% respectively, being higher compared to the one containing untreated molasses alone which was 72.26%. However, they did not show any antimicrobial activity since the inhibitory compounds in molasses were not removed.

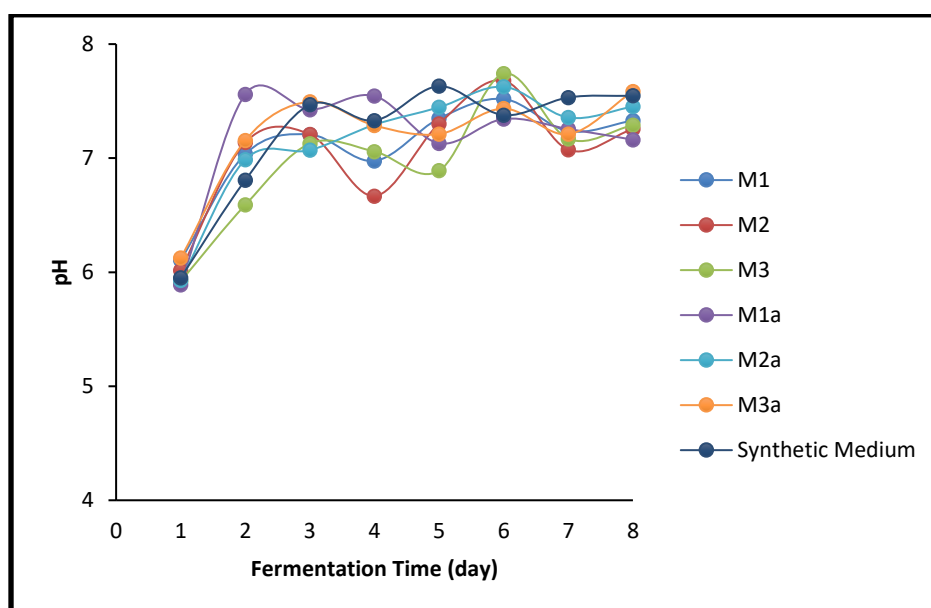


Figure 3. The change of pH on each medium during fermentation.

The pH of each fermented medium increased as fermentation time took longer. *Nocardia* sp strain V1 employed in this study actively fermented all the media and increased the pH to as high as 7.58 which belonged to those grown in the synthetic medium. As shown in Figure 3, the pH of each fermented medium was similar to each other since the acidity of the resulting metabolites were not that different. The increasing pH indicated that the bacteria might have the ability to metabolize amino acids and proteins in the medium and produce NH_3 which had basic property.

4. Conclusion

The molasses employed in this study could be used by *Nocardia* sp strain V1 as a carbon source in the fermentation medium to produce extracellular liquid having antimicrobial activity. The pre-treatment through the addition of $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ was proven effective to remove inhibitory substances from molasses that could confine the production of antimicrobial metabolites. The bacteria grown in the synthetic, M2, and M3 media were able to produce extracellular liquid that could inhibit the growth of

Rhodotorulla sp and *Fusarium graminearum* with inhibition diameter ranges from 10-20 mm. The total sugar concentration in each medium decreased as fermentation time increased indicating that the bacteria grown in all media utilized the carbon sources inside the medium. The highest total sugar consumption rate of 96.69% was shown by the bacteria grown in the synthetic medium followed by the media containing pre-treated molasses starch and pre-treated molasses-glucose. The pH measurement showed an increasing trend during fermentation time with the highest pH of 7.58 belonged to the culture in the synthetic medium. To the best of our knowledge, this is the first report of the extracellular liquid production by *Nocardia* sp strain V1 having antimicrobial activity obtained from molasses fermentation. Further research should be focused on the biomass study and optimization process in terms of the pH and temperature to yield optimum antimicrobial activity.

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