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Comparison of alternative media from organic waste as a growth media for the biological agent bacteria *Pseudomonas* spp

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ABSTRACT

Introduction: *Pseudomonas* spp is one of the biological agents that has many abilities. With the high cost of synthetic media used as a medium for the propagation and growth of this bacteria, it is necessary to have alternative media that are cheap and environmentally friendly, one of which is by utilizing organic waste. This study aims to determine the comparison of the growth of *Pseudomonas* spp on alternative media from organic waste. **Methods:** The design used was a non-factorial Completely Randomized Design (CRD) with 3 (three) treatments using types of bacteria propagation media, namely P0 = selective media formulation (*Pseudomonas* Isolation Agar), P1 = soybean water stew waste, and P2 = coconut water waste and repeated 6 times. **Results:** The results showed that the bacteria tested were *Pseudomonas* spp based on the Gram staining test and the pendar fluorescence test. The highest number of colonies on day 5 was found in coconut water media as well as the results of the absorbance test and the *Pseudomonas* spp had antagonistic properties against *Xanthomonas sp*. **Conclusion:** Alternative media from organic waste coconut water are the best media for the growth of *Pseudomonas* spp which have antagonistic properties against *Xanthomonas sp*.

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INTRODUCTION

Pseudomonas spp is a biological agent that has many abilities, such as suppressing disease growth, increasing root uptake, and increasing plant growth. (Cummings, 2009). *Pseudomonas spp* is also a type of bacteria that can decompose organic waste. Apart from that, this bacterium can act as a biological agent in controlling leaf blight caused by the pathogen *Xanthomonas sp* (Zahidah & Shovitri, 2013). The presence of disease attacks caused by bacteria in agricultural cultivation is also a challenge for farmers. Sumarni *et al.*, (2015) explained that *Pseudomonas* spp has a good impact on increasing the productivity of agricultural products, not only in controlling plant disease pathogens but also on nutrient uptake. Therefore, the use of this biological agent is recommended for application in agricultural cultivation considering its natural and environmentally friendly nature, safe for ecosystem balance, and its application does not leave residue (Adiathy *et al.*, 2017).

For the propagation of *Pseudomonas spp* bacteria, a growth medium is needed for the bacteria. Media commonly used in the propagation and growth of *Pseudomonas spp* bacteria are synthetic media such as Nutrient Agar, Nutrient Broth, and *Pseudomonas Isolation Agar* (Rossita *et al.*, 2017). However, the use of synthetic media is quite expensive, so alternative media are needed that can be used as a growth medium for *Pseudomonas spp* bacteria at a low cost and are easy to obtain. One alternative medium that can be used as an alternative medium for bacterial growth can come from organic waste. Organic waste is residual material obtained from activity processes that are easily decomposed through natural processes and have no selling value. Examples of organic waste that can be used as an alternative medium for the growth of *Pseudomonas spp* bacteria are wastewater from boiled soybeans and coconut water which are included as liquid organic waste.

Sari & Rahmawati (2020) explained that soybean cooking water contains water, carbohydrates, protein, and ash. Meanwhile, coconut water waste contains ingredients needed by bacteria such as carbohydrates, fat, calcium, phosphorus, protein, vitamins C and B complex, and mineral salts. The content of coconut water is believed to be able to produce the growth of good bacteria (Mayaserli & Renowati, 2015). These contents are nutrients needed by bacteria for survival so they can be used as alternative media for cultivating *Pseudomonas spp* bacteria. *Pseudomonas*

spp bacteria can inhibit growth and have compound properties that are effective in killing pathogens (bacteria) that cause disease such as *Xanthomonas sp* bacteria which are pathogens that cause leaf blight in rice. This indicates that this bacterium is capable of being antagonistic to other bacteria that cause disease. This is one of the reasons that *Pseudomonas spp* has the potential to be developed en masse (Diarta *et al.*, 2016). Therefore, this research was conducted to determine the comparison of the growth of *Pseudomonas spp* bacteria on alternative media made from organic waste.

METHODS

Materials

The tools used in this research include refrigerators, laminar airflow (LAFC), autoclaves, vortexes, microscopes, glass slides, hot plates, stirrers, test tubes, bottles, filters, micropipettes, measuring cups, dropper pipettes, bunsens, cameras., Erlenmeyer, type, Eppendorf tube, oven, stirrer, falcon, filter cloth, pH paper, stationery, colony counter, digital scales, L glass, 360 nm UV lamp, and spectrophotometer.

The materials used in this research included *Pseudomonas* bacterial isolates, *Xanthomonas sp* bacterial isolates, soybean boiled water, coconut water, glycerol, potassium phosphate, magnesium chloride, granulated sugar, filter paper, distilled water, water, 70% and 96% alcohol, Kings B, sterile tissue, cotton, Gram paint such as crystal violet safranin, iodine, 96% alcohol, agar, label paper, aluminum foil.

the place where the research is carried out

This research was carried out for two months from September to November 2022 at the Politeknik Negeri Jember Bioscience Laboratory.

Selective media creation

Making selective media begins with sterilizing tools and materials using an autoclave at a temperature of 121^{o}C at a pressure of 1 atm for 30 minutes (Ihsan & Retnaningrum, 2017). Making selective media begins with sterilizing tools and materials. Making this media is done by mixing PIA (*Pseudomonas* Isolation Agar) with a composition of 25 grams of gelatin, 2.1 grams of Magnesium Chloride (MgCl), 1.5 grams of Potassium Phosphate (K3PO4), 17 grams of agar. and 13.5 grams of glycerol then mixed with 1000 ml of distilled water and autoclaved at 121^{o}C at 1 atm pressure for 30 minutes (Ryan Withers *et al.*, 2013). The solution was heated using an autoclave at 121^{o}C for 30 minutes. After that, incubation can be done for 1 week.

Four glow test

1 colony of suspected *Pseudomonas* fluoresces on kings B media was taken and then incubated for 24 hours. This test was carried out to ensure that the bacterial isolate present was fluorescent by observing it under UV light with a wavelength of 365 nm.

Selective media

Liquid alternative media is made using organic waste from boiled soybean water and coconut water with a composition of 250 ml each. Put the two ingredients in an Erlenmeyer flask, then add 2.7g of granulated sugar and plain 4 agar then stir until homogeneous. After the solution is homogeneous, the mouth of the Erlenmeyer is closed using aluminum foil and rubber and then sterilized (Nurwahidah & Alif, 2022). Then bacterial optimization is carried out which aims to minimize other unwanted microorganisms.

Bacterial inoculation

Bacterial isolates were inoculated on alternative media by adding the suspension 6 times, namely 10-1, 10-2, 10-3, 10-4, 10-5, and 10-6 (comparison of 1 ml of bacterial suspension with 9 ml of 0.85 NaCl % at dilutions 10-1 to 10-6). Each suspension was poured into a test tube and then vortexed for approximately 1 minute. Then 0.1 ml of the suspension was taken with a micropipette at 10-6 and put into a petri dish containing selective media, namely *Pseudomonas* Isolation Agar, boiled soybean water media, and coconut water media using a syringe. After that, the media was incubated at 37oC and stored with all the plates wrapped in brown paper.

Number of colonies

Counting the number of colonies begins on days 1–5 after inoculation, then further observations and calculations are carried out again from week 1 to week 3 and continued on the 1st and 2nd months. The number of colonies is calculated using the following formula:

$$\text{Number of colonies/ml} = \text{Number of colonies/plate} \times \frac{\text{Inoculated sample volume}}{\text{Dilution}}$$

Experimental design

The design used in this research was a non-factorial Completely Randomized Design (CRD) with 3 (three) treatments using types of bacterial multiplication media, namely P0 = selective media formulation (*Pseudomonas* Isolation Agar), P1 = wastewater from boiling soybeans, and P2 = waste water coconut. Each treatment was repeated 6 times to obtain 18 experimental units.

Data analysis

Data collection was carried out qualitatively and quantitatively. Qualitative data included color observations (gram staining and fluorescence test results) which were compared with the book Bergey's Manual of Determination Bacteriology 9th (1986). Meanwhile, quantitative data includes the number of colonies on day 5, bacterial growth phase, *Pseudomonas* Pendar-Flour Bacterial Antagonism Test with *Xanthomonas sp*, absorbance test, and media pH measurements analyzed using variance (ANOVA). If there is a real difference then it will be continued with the Dunnet Test at 1% level.

RESULTS AND DISCUSSION

Gram staining

Gram staining is carried out to determine the morphology of bacteria and their gram characteristics (Nurhidayati *et al.*, 2015). Based on the results of the gram stain analysis which was identified using a microscope with a magnification of 400 times, it showed that the gram stain produced was red. The red color indicates that the bacteria are gram-negative (Mahmudah *et al.*, 2016). This coloring is determined by the composition of the cell walls of the bacteria. These results also show pink bacterial colonies in the form of rods and bacilli. This is by the characteristics of *Pseudomonas* spp bacteria in the book *Bergey's Manual Of Determination* (1994) which explains that *Pseudomonas spp* bacteria are gram-negative and bacillus-shaped.

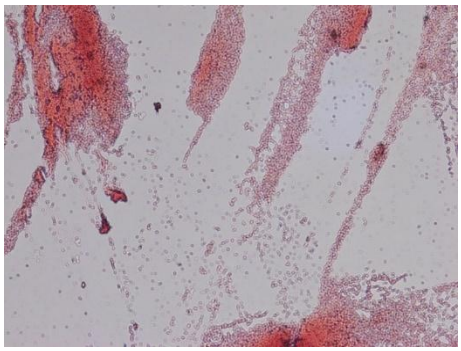


Figure 1. Gram staining results

Nurhidayati *et al.*, (2015) explained that gram-negative bacteria cannot retain the crystal violet dye complex because the complex dissolves during the gram-staining process so that the bacteria show a red safranin color. When viewed through a microscope, these bacteria will tend to appear pink. In contrast to gram-negative bacteria, gram-positive bacteria can maintain the crystal violet dye complex so that when alcohol is given in the gram-staining process, the bacteria will show a purplish-blue color. This shows that gram-negative bacteria have a low affinity for crystal violet (Purwaningsih & Wulandari, 2021). The difference in color in the gram staining process shows that the difference between gram-positive and gram-negative bacteria is also shown by differences in the cell wall structure of the bacteria. The cell wall structure of gram-negative bacteria has a high lipid content but with a low peptidoglycan layer so that the crystal violet dye is easily released and can absorb the safranin dye. (Fitri & Yasmin, 2011). Rahmadian *et al.*, (2018) in his research, it was stated that the peptidoglycan layer in gram-negative cell walls only has a thickness of 10% of the total composition of the bacterial cell wall.

Fluorescence test results

Based on the results of the fluoride test, it can be seen that the bacteria inoculated on Kings'B media have a yellowish-green color. This observation was carried out by looking at the results of inoculation under a UV lamp using a wavelength of 365 nm which was observed for 24 and 48 hours. Adiathy *et al.*, (2017), stated in his research that the *Pseudomonas* group can produce greenish pigment which is one of the criteria for selecting useful *Pseudomonas*. Fluorescent bacteria also have an important role as biological control agents because of their ability to produce secondary metabolite compounds such as siderophores and other antibiotic compounds which can poison other microbes and show bacteriotic and fungiotic effects in vitro.

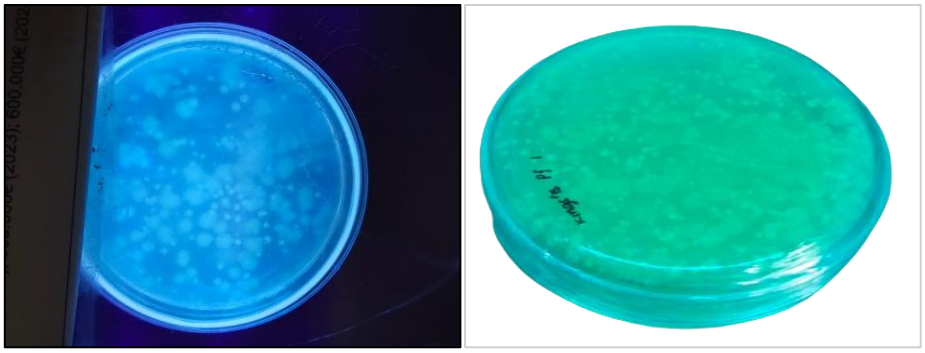


Figure 2. Luminescence Test Results under UV Lamp

The bacteria that have been observed in Figure 2 above belong to the fluorescent *Pseudomonas* group because they have the characteristics of producing antibiotic pigments, siderophores, phytohormones, and volatile compounds. The *Pseudomonas* fluorescent group of bacteria has its ability to inhibit the development of pathogenic microorganisms (Sanjaya *et al.*, 2016). This siderophore compound is beneficial to plants because it can suppress pathogens by binding the available Fe so that it cannot be available to the pathogen (Prihatiningsih *et al.*, 2017). Several species that are included in the *Pseudomonas* fluorescein group and can inhibit pathogens include *P. fluorescens, P. aeruginosa, P. ovalis,* and *P. calciprecipitans.*

Number of bacterial colonies on day 5

One way to determine the quality of a medium for the propagation and growth of bacteria is to count the number of bacterial colonies that grow. In this study, the calculation of the number of *Pseudomonas* spp bacterial colonies was carried out on day 5. The observation variable for the number of colonies on day 5 showed that there was a significant difference between the number of bacterial colonies that grew on the multiplication media tested. These results can be seen in Table 1.

Table 1. Number of *Pseudomonas spp* bacterial colonies on day 5

Treatment	μ	μm-μk	LSD	Notation
Selective Media	19.2	0 ≤	5.34	B
Soybean Water Media	23.5	4.3 ≤	5.34	B
Coconut Water Media	30.5	11.3 ≥	5.34	A

Note: Treatments that show different notation from the control show a significant difference based on the results of the Dunnet test at the 1% level.

Table 1 shows that the number of bacterial colonies that grew the most on day 5 was the bacterial colonies in the coconut water media and was significantly different from the soybean-boiled water media and the control. This proves that coconut water media is considered better as a propagation or growth medium for *Pseudomonas* spp bacteria. The growth of bacteria in a media is influenced by the availability of nutrients contained in the media which can be utilized by the bacteria. The higher number of colonies on the alternative media is thought to be due to the vegetable protein content in the organic waste material. This vegetable protein allows microorganisms to adapt more easily and quickly to environmental conditions. Under these conditions, alternative media influence the number of colonies and metabolism of bacterial growth (Katrin *et al.*, 2015; Nurwahidah & Alif, 2022).

The results of this research are in line with research conducted by Mayaserli & Renowati (2015) which explains that coconut water is the best organic waste material to support the growth of *Pseudomonas* fluorescens. Apart from that, it was also explained that there is good potential in using organic waste as an alternative medium for mass cultivation of *Pseudomonas flourescens* bacteria. Apart from conditions for bacterial growth such as temperature, pH, water content, aeration and agitation, the nutritional content in the propagation medium is a determining factor in determining the number of colonies.

Bacterial growth phase

The bacterial growth phase consists of 4 phases, namely the lag phase, log phase, stationary phase and death phase. In the lag phase, the number of bacteria growing is still very slow because in this phase the bacteria are in the adaptation stage or adjusting to environmental conditions (growth media). Several factors influence the adaptability of these bacteria, such as media composition, temperature, and number of cells in the initial inoculum. Usually, the lag phase lasts up to several hours at a temperature of 37oC, covered with straw paper. Sasdika *et al.*, (2022) states that the number of bacteria in the initial phase will grow very slowly because the bacteria are still adapting to the new environment, especially the temperature, pH, and nutrients needed by the bacteria.

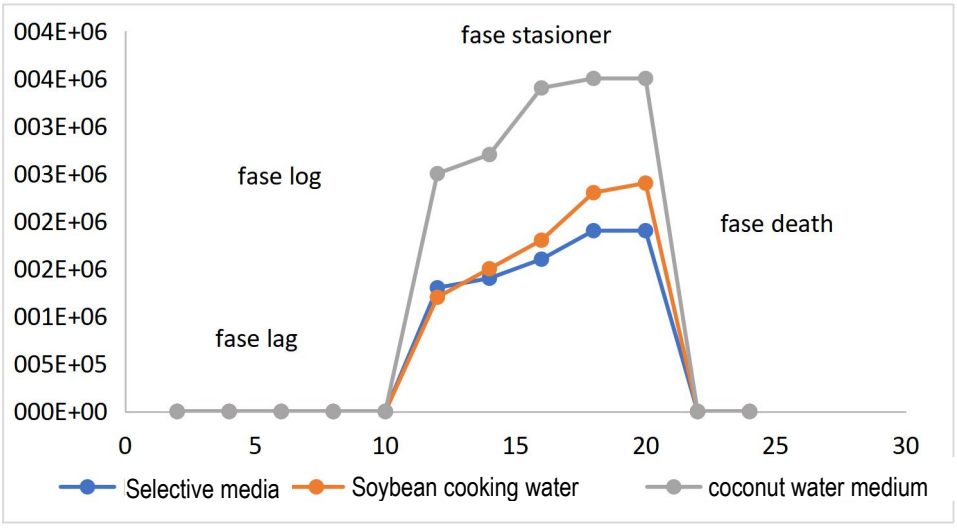


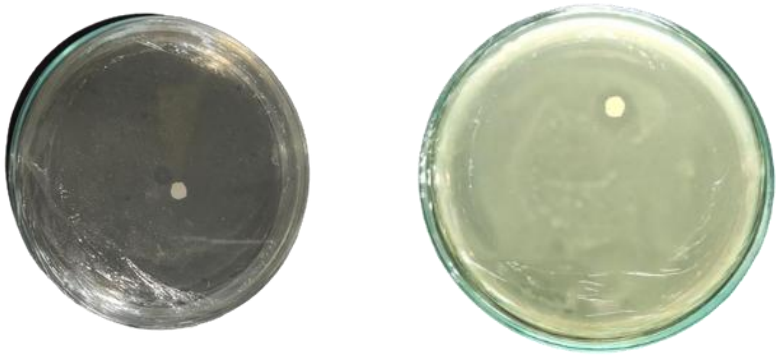
Figure 3. Graph of the growth of *Pseudomonas* spp bacteria on various test media

In the log or exponential phase, a second growth phase occurs. In this phase, bacterial growth occurs very quickly. Conditions of temperature, pH, nutrients in the media, and genetic characteristics of bacteria influence bacterial growth in this phase. The log phase is the phase where microbes divide or multiply cells. Entering the stationary phase, the growth rate is the same as the bacterial death rate so the overall number of bacteria will remain the same. The final phase or death phase is the phase where there is a significant death rate that exceeds the bacterial growth rate (Risna *et al.*, 2022).

The growth rate of *Pseudomonas* spp bacteria cultured on the test media can be seen in Figure 3. The results show that *Pseudomonas* spp bacteria propagated using coconut water media had the highest growth rate compared to selective media and soybean boiled water media. However, both media made from organic waste were able to provide better growth rates compared to selective media (control). This shows that the condition of media made from organic waste, especially coconut water, is very suitable for the needs of bacteria.

Antagonism test of *Pseudomonas* Pender-Flour bacteria against *Xanthomonas sp*

The results of the antagonist test which was carried out using the Kirby-Bauer method showed that *Pseudomonas* spp bacteria can inhibit the development of bacteria from the genus *Xanthomonas sp* as indicated by the average diameter of the inhibition zone. The average inhibition zone for *Pseudomonas* spp bacteria against *Xanthomonas sp* is 8 mm. Based on this research, the average diameter of the inhibition zone at 48 hours of incubation was greater than 24 hours. This is because *Pseudomonas* spp bacteria have bacteriostatic properties. If the area around the barrier no longer looks clear and is growing with bacteria, then this indicates that the chemical content that has antibacterial properties is bacteriostatic because it is only able to inhibit the bacteria (Larasaty *et al.*, 2021).



(a) (b)

Figure 4. *Pseudomonas* spp bacterial antagonist test against *Xanthomonas sp*
(a) before incubation (b) after incubation

Based on the research results, the antagonist test showed that *Pseudomonas* spp bacteria were able to inhibit the pathogenic bacteria *Xanthomonas sp*. The formation of the inhibitory zone is due to a release produced by *Pseudomonas* spp bacteria so that it can enter the media which has an impact on containing pathogens. This agrees with the research results (Soegianto *et al.*, 2013) which state that *Pseudomonas* spp bacteria can form an inhibitory zone against the pathogenic bacteria *Xanthomonas sp* in an in vitro antagonist test. Larasaty *et al.*, (2021) explained that toxic compounds in the antibiosis group such as pyrrolnitrin and pyoluteorin have toxic characteristics to pathogenic bacteria. This compound is produced by the bacterial genus Pseudomonas. This toxic compound is an antibiosis compound that can destroy cell walls so that the fluid in the cell walls will decrease and achieve plasmolysis. Thus, metabolic reactions will be restrained (Laraswati *et al.*, 2021).

Absorbance test

Viability testing on *Pseudomonas* spp bacterial colonies was carried out using a spectrophotometer. The way the spectrophotometer system works is that monochromatic light is passed through a transparent medium, which will produce an increasing and decreasing light intensity, then transmitted according to the thickness and sensitivity of the medium used. This kind of system falls under the Lambert-Beer law (Yanlinastuti & Fatimah, 2016). The absorbance test results can be seen in the table below:

Table 2. Results of the absorbance test for *Pseudomonas* spp bacteria in boiled soybean water and coconut water before and after incubation

Treatment	Before Incubation	After Incubation	Difference
Soybean Water Media	0,953	1,189	0,236
Coconut Water Media	1,849	2,039	1,798

Based on Table 2, the results of the absorbance test for *Pseudomonas* spp bacteria in media made from organic waste, namely boiled soybean water and coconut water, show the results of the turbidity test before incubation and after incubation for 17 hours at a temperature of 37oC and the equality between the media and the number of isolates is 1:10. In 50 ml of media, either boiled soybean water or coconut water and 0.5 ml is a suspension. The starters that have been tested show that the two starters have different difference values. The difference value of the coconut water media starter is 1.798. This can be interpreted as that the absorbance test results are positively correlated with the number of colonies within 5 days. The nutritional content in the coconut water starter medium is more turbid than boiled soybean water, which means the number of colonies in the starter medium is greater. This is in line with the statement by Liyazana *et al.*, (2016), There is a correlation between the number of bacterial colonies and the absorbance value of liquid waste. The higher the absorbance value, the greater the number of bacteria. This indicates that the higher the number of bacteria, the lighter the bacteria will absorb, so that very little light is passed through. The more turbid the media (liquid starter), the more sediment there will be which functions as a source of nutrients for bacterial growth.

Measuring the pH of the growth media for *Pseudomonas spp*

Table 3. pH in each growth medium for *Pseudomonas spp*

	Media type		
	Selective media	Soybean water media	Coconut water media
Before sterilization	7	5,8	6,5
After sterilization	6,8	5	5

Based on the media pH measurement data shown in Table 3 above, each media experienced a decrease in pH after the sterilization process. The highest decrease in pH occurred in coconut water media which was thought to be due to heating of the media during the sterilization process using an autoclave which caused the carbon bonds to break, thus changing the condition of the media to become more acidic. Meanwhile, selective media produces the highest media pH both before and after sterilization compared to alternative media. Media pH that is too high will affect the growth of bacteria so the number of colonies will decrease. This shows that microorganisms such as bacteria have an optimal pH range to grow more quickly. From this research, it is assumed that a media pH value that is too high will inhibit the growth of microorganisms because pH affects the activity of bacterial enzymes. This is in line with the research conducted by Suriani *et al.*, (2013), There are bacterial isolates that grow faster at a media pH of 5. If the media pH is not optimal, it will interfere with the performance of enzymes in the bacteria which will impact their growth. Apart from that, nutritional materials such as organic compounds contained in the media can also affect the pH of the media.

CONCLUSION

Alternative media made from coconut water is considered better than selective media or boiled soybean water media as seen in the variable number of colonies on day 5, bacterial growth phase, and absorbance test results, and is considered to have the best conditions for the growth of *Pseudomonas* spp bacteria. *Pseudomonas* spp bacteria are also antagonistic to *Xanthomonas sp* bacteria due to their ability to inhibit the growth of pathogens.

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