Viability of biofertilizer bacteria Rhizobium spp based on household waste

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Abstract. The research was carried out from May 2021 to August 2021. The research was carried out at the Jember State Polytechnic Plant Protection Laboratory. This study used four types of household waste media, namely rice washing water, rice bran water, soybean dregs water, and Yeast Mannitol Agar media as controls. Each treatment was repeated 5 times to obtain 20 experimental units. The data obtained were then analyzed qualitatively and quantitatively. Quantitative data analysis by calculating the number of colonies with TPC (total plate count) and T-test techniques, qualitative data obtained by observing bacterial purity and gram staining. The results of this study indicate that alternative media for bran water waste conventionally produced the highest bacterial population with a colony number of 6,80 x 10^{24} CFU and the lowest bacterial population came from rice alternative media with a colony number of 1.28 x 10^{24} CFU. Bacterial viability observed for 4 months between aseptic media and conventional media showed the results of the t-test with a count of 1.49 x 10^{-1} CFU. shows the results (ns) are not significantly different.

1. Intoduction

Viability is the ability to live a living thing which is indicated by the presence of a growth or biomass. Knowledge of the factors that affect the viability of a microbe is very important to know to treat a microbe. Preservation of *Rhizobium spp.* for long-term purposes is a crucial stage in studying microbiology to maintain and maintain its metabolic activity so that it does not change [1]. [2] states that, "viability is the possibility of a living being to be able to live". [3] Viability is the activity of life, or the possibility of life as indicated by the presence of growth in bacteria. *Rhizobium* is one of the bacteria that can bind nutrients in the form of N when in symbiosis with legume plants [4] in conducting a *Rhizobium* viability test requires a suitable medium as a place for growth and storage. A suitable medium for growth and storage must meet the requirements, namely the adequate intake of nutrients needed by bacteria [5].

YMB (Yeast Mannitol Broth) is a liquid medium that is very effective in the growth and storage of Rhizobium spp. for a long time because YMB (Yeast Mannitol Broth) is a specific medium for the growth of Rhizobium spp. bacteria, but the price of YMB (Yeast Mannitol Broth) instant media is very expensive and also very difficult to obtain among Indonesian farmers, therefore it takes an alternative media that can grow *Rhizobium spp.* quickly, cheaply and have materials that are easily obtained and

available among farmers. This study aims to obtain the best alternative liquid media to restrain the rate of bacterial metabolic activity so that viability or growth power can be maintained to maintain microbial isolates so that they have good growth power and high survival with minimum character changes.

2. Materials and Methods

The research was carried out from February to May 2021, the research was carried out at the Jember State Polytechnic Plant Protection Laboratory. This study used four types of household waste media, namely rice washing water, rice bran water, soybean dregs water, and Yeast Mannitol Agar media as controls. Each treatment was repeated 5 times to obtain 20 experimental units. The data obtained were then analyzed qualitatively and quantitatively. Quantitative data analysis by calculating the number of colonies with TPC (total plate count) and T-test techniques, qualitative data obtained by observing bacterial purity and gram staining.

2.1. Liquid Selective Media Production

YMB is a liquid medium that has specifications (selective media) in growing Rhizobium spp. The following is the composition of YMB (Yeast Mannitol Broth) media aseptically in 1000 ml of distilled water: 10 grams of Mannitol, 0.5 grams of K2HPO2, 0.4 grams of MgSO4.7H20, 0.2 grams of NaCl, 1 gram of Yeast extract. All ingredients were put into an Erlenmeyer containing 1000 ml of aqua dest. When all the ingredients have been mixed in aqua dest, the ingredients are stirred with a stirrer on a hot plate until homogeneous. After all the ingredients are completely dissolved. Erlenmeyer's mouth was closed with aluminum foil and rubber, then sterilized using an autoclave at 121oC with a pressure of 17.5 psi for 15 minutes.

2.2. Liquid Alternative Media Production

The ingredients used as alternative liquid media are rice, bean, and soybean, then rice, bran, tempeh is washed each in a separate container with a ratio of 1 kg of material: 3 liters of water, each jar contains 1 liter of alternative media. Soybean water, rice water, and bran water are each placed in an Erlenmeyer then added a solution of brown sugar as much as 10g/liter and shrimp paste 2.5/liter then filtered, then the ingredients are stirred with a stirrer on a hot plate until homogeneous. After all the ingredients are completely dissolved. Erlenmeyer's mouth was closed with aluminum foil and rubber then sterilized using an autoclave at 121oC with a pressure of 17.5 psi for 15 minutes.

2.3. Bacterial Inoculation

The isolate used was isolated [6] which had the highest adaptation rate of 6.88 x 1030 CFU/g of soil from Ajung District with a depth of 15 cm from the soil surface. Rhizobium spp. which will be inoculated into alternative media and selective media first cultured using a shaker for 4 days using YMB (Yeast Mannitol Broth) media. After 4 days, the inoculant culture was inoculated into a jar containing media.

2.4. Bacterial Purity and Preparation of YEMA + Congo Red Media Media

The materials that will be used in the manufacture of YEMA + Congo Red media are weighed using a digital Ohaus balance according to the composition of each ingredient. The materials used were 3 g Yeast Extract, 10 g Sucrose, 0.2 g MgSO4 (Magnesium Sulfate), 0.1 g NaCl, 0.5 g K2HPO4, 3 g CaCO3, 20 g Agar and 0.025 g Congo Red were weighed. using a digital Ohaus balance for each treatment. then the solution was homogenized using a hot plate stirrer equipped with a magnetic stirrer, then the pH was checked using a pH meter. The desired pH by Rhizobium is a pH of 6.8. after the solution is homogeneous and then cooked at a temperature of 1000C. After boiling, it was placed in an Erlenmeyer and autoclaved for 15 minutes at a temperature of 121 C and a pressure of 17.5 psi.

2.5. Gram Staining

Gram staining is used to determine the bacteria *Rhizobium spp*. this is a type of gram-negative bacteria or gram-positive bacteria by first sterilizing the preparation by spraying it with 96% alcohol and then rinsing with distilled water, dividing the solution into 4 kinds, namely crystal violet solution A, B iod solution, 96% alcohol C solution, D safranin solution After that, take 1 ose of bacterial isolate and scratch it on the surface of the preparation until it is evenly distributed and then fixation is carried out. Take 1 drop of solution A (crystal violet) to add on the surface of the preparation that has been coated with bacteria and then let stand for 1 minute. Then the preparations were rinsed with distilled water and added 1 drop of B (iod) solution on the surface of the preparations and allowed to stand for 1 minute. The preparations were rinsed with aqua dest and then dripped with solution C (96% alcohol) and allowed to stand for 30 seconds, after that, added 1 drop of D solution (safranin) on the surface of the preparations and allowed to stand for 1 minute then the preparations were rinsed with distilled water and waited until dry.

3. Results and Discussion

Rhizobium spp. which has been stored at room temperature, viability was observed by calculating the number of bacterial colonies that grew. Calculations were carried out using the plate count method and dilution was carried out before being grown on YEMMA medium so that after incubation several colonies would be formed on the plate in countable amounts. The petri dish is used to count the number of bacterial colonies in a petri dish containing 30-300 colonies [7]. The effectiveness of *Rhizobium spp.* can survive if the microorganism can be active in a variety of different environmental conditions and survive in various forms. These bacteria must meet several criteria, including: (1) can be mass-produced; (2) remain stable and viable for a long time in storage conditions; (3) can survive if symbiotic with legume plants; and (4) when applied it has a beneficial effect on the host. After storage at room temperature according to treatment time and grown in YEMMA media, the number of colonies was calculated after 24 hours of incubation. Colony growth of Rhizobium spp. in the viability test can be seen in Figure 1. Based on Figure 1. it is known that different time treatments produce different variations in the number of populations. Changes in the number of populations are caused by the growth of bacteria in the media [8]. Based on the graph, there are 3 forms of growth rate, namely increasing growth rate, optimum growth rate, and decreasing growth rate.

Table 1. Bacterial Viability Rate for 4 Months

Treatment (Conventional/Aseptic)	Rhizobium Spp. Bacteria Population/Sample	The Average Population of Rhizobium Spp. / Samplel
R0 (YMB Conventional 1)	3,53X 10^{24} CFU/ml	3,44X 10^{24} CFU/ml
R0 (YMB Conventional 2)	3,58X 10^{24} CFU/ml	
R0 (YMB Conventional 3)	3,30X 10^{24} CFU/ml	
R0 (YMB Conventional 4)	3,34X 10^{24} CFU/ml	
R1 (Soybean Dregs Water 1)	5,00X 10^{24} CFU/ml	6,31X 10^{24} CFU/ml
R1 (Soybean Dregs Water 2)	6,81X 10^{24} CFU/ml	
R1 (Soybean Dregs Water 3)	6,94X 10^{24} CFU/ml	
R1 (Soybean Dregs Water 4)	6,48X 10^{24} CFU/ml	
R2 (Rice Washing Water 1)	1,05X 10^{24} CFU/ml	1,22X 10^{24} CFU/ml
R2 (Rice Washing Water 2)	1,45X 10^{24} CFU/ml	
R2 (Rice Washing Water 3)	1,28X 10^{24} CFU/ml	
R2 (Rice Washing Water 4)	1,10X 10^{24} CFU/ml	
R3 (Rice Bran Water 1)	6,53X 10^{24} CFU/ml	6,80X 10^{24} CFU/ml
R3 (Rice Bran Water 2)	6,93X 10^{24} CFU/ml	

R3 (Rice Bran Water 3)	6,75X 10^{24} CFU/ml	
R3 (Rice Bran Water 4)	6,99X 10^{24} CFU/ml	
S0 (YMB Aseptic 1)	3,00X 10^{24} CFU/ml	3,07X 10^{24} CFU/ml
S0 (YMB Aseptic 2)	3,26X 10^{24} CFU/ml	
S0 (YMB Aseptic 3)	3,08X 10^{24} CFU/ml	
S0 (YMB Aseptic 4)	2,93X 10^{24} CFU/ml	
S1 (Soybean Dregs Water 1)	4,35X 10^{24} CFU/ml	5,34X 10^{24} CFU/ml
S1 (Soybean Dregs Water 2)	5,55X 10^{24} CFU/ml	
S1 (Soybean Dregs Water 3)	5,78X 10^{24} CFU/ml	
S1 (Soybean Dregs Water 4)	5,66X 10^{24} CFU/ml	
S2 (Rice Washing Water 1)	1,24X 10^{24} CFU/ml	1,28X 10^{24} CFU/ml
S2 (Rice Washing Water 2)	1,29X 10^{24} CFU/ml	
S2 (Rice Washing Water 3)	1,33X 10^{24} CFU/ml	
S2 (Rice Washing Water 4)	1,24X 10^{24} CFU/ml	
S3 (Rice Bran Water 1)	6,08X 10^{24} CFU/ml	6,59X 10^{24} CFU/ml
S3 (Rice Bran Water 2)	6,91X 10^{24} CFU/ml	
S3 (Rice Bran Water 3)	6,59X 10^{24} CFU/ml	
S3 (Rice Bran Water 4)	6,79X 10^{24} CFU/ml	

Note : CFU/ml = Jumlah koloni per unit dalam 1 ml media
6,80 X 0^{24} = The highest bacterial population came from aseptic bran alternative media
1,28 X 10^{24} = The lowest bacterial population comes from aseptic rice alternative media

The growth rate increased in two alternative media, namely wastewater bran, and soybean water waste with a storage time of 4 months, on alternative media for wastewater bran and soybeans, the results obtained were conventional bran water waste from the first month of 6,53 x 10^{24} CFU menjadi 6,99 x 10^{24} CFU in the fourth month so that it increased by 7%, alternative media for aseptic bran water waste in the first month was 6.08 x 10^{24} CFU to 6.79 x 10^{24} in the fourth month so that it increased by 13%, alternative media for soybean water waste conventional method in the first month 5,00 x 10^{24} CFU to 6,48 x 10^{24} CFU in the fourth month so that it increased by 36%, alternative media for aseptic soybean wastewater in the first month of 4,35 x 10^{24} CFU to 5,66 x 10^{24} CFU in the fourth month so that it increased by 30%.

According to [9], at the time of growth, the speed of division is high, the metabolic activity of cells is high, and the generation time is short. The high increase was due to the bacteria synthesizing substances contained in the bran and soybean media which could trigger the bacteria to secrete their cell metabolites for optimal cell growth. The high protein content in bran and soybean media can be used as a source of nutrition for bacteria during storage.

The optimum growth rate occurred in alternative media for conventional and aseptic rice with a storage time of 4 months, on alternative media for conventional rice water waste the results obtained were 1,05 x 10^{24} CFU to 1,10 x 10^{24} CFU in the fourth month so that it increased by 4%, the increase of only 4% is still in the category of stagnant growth so that the growth rate is optimum. In the alternative media of aseptic rice water waste, the results obtained were from the first month of 1,24 x 10^{24} CFU to 1,24 x 10^{24} CFU in the fourth month so that there was an increase of 0% so that in this alternative media the optimum bacterial growth did not decrease and increase in the fourth month but increased in the second and third months.

Optimum growth of bacteria is caused by the substrate in the bran carrier medium that is still able to provide nutrition or is still supportive for the life of the growing bacterial population. In line with

[10], that the climax point in the number of bacterial populations depends on the amount of food/nutrients, maximum cleavage speed, and high metabolic activity in synthesizing substances contained in the rice carrier medium. The high growth of bacteria in a medium indicates the high viability of bacteria in rice carrier media.

The growth rate decreased in conventional and aseptic YMB (Yeast Mannitol Broth) selective media with a storage time of 4 months, on conventional YMB (Yeast Mannitol Broth) selective media the results obtained in the first month were 3,53 x 10^{24} CFU to 3,34 x 10^{24} CFU in the fourth month so that it increased by 5.3%. On aseptic YMB (Yeast Mannitol Broth) selective media the results obtained were from the first month of 3,00 x 10^{24} CFU to 2,93 x 10^{24} CFU in the fourth month increasing by 2.5%. The population decline was due to a lack of growth factors such as a decrease in the supply of nutrients contained in YMB (Yeast Mannitol Broth) media.

According to [11], the decline in the number of populations in an organism is due to the reduced supply of food contained in a medium, with a reduced supply of limited food, the population of organisms will decrease drastically. This decrease in population is due to the formation of dead cells faster than the formation of cells. -new cells. In line with [10] that decreased growth rate can occur due to increased mortality or decreased reproduction of an organism or both can occur simultaneously. According to [9], such a situation would indicate the existence of competition among organisms for nutrients and space and was also caused by the materials released by cells into the medium.

According to [12], the length of storage time affects the growth and survival (viability) of bacteria with the occurrence of cell lysis which causes turbidity and the number of cells counted directly will decrease. According to [13] almost all the bacteria in their viability will begin to decrease when the storage time of bacteria exceeds the 3-month storage time limit.

The existence of three forms of bacterial growth rate indicates that there are factors that affect the growth of bacteria in a medium. According to [14] the factors that influence bacterial growth, in general, include storage time, nutrient availability, water density, temperature, dissolved oxygen, pH. Bacteria will do binary fission every 20 minutes and bacteria that do fission need nutrients for their division. If nutrition is inadequate, then growth will decline. Water is needed by bacteria because water plays a role in metabolic reactions in cells.

Bacteria generally grow and reproduce in media with high aw (water activity) (0.91), therefore a medium that is too concentrated causes cells to lack water and dies because the growth of the bacterial population is affected by the length of storage time. According to [15], population growth is a function of time (Nt = f(t)) meaning that bacterial population growth is strongly influenced by the length of time the bacteria live in a medium. So, if the bacteria's lifetime is not too long in a medium, the growth of the bacterial population will be small.

Based on Table 1 the results of the viability test by observing and counting the number of colonies from the eight media used in bacterial propagation, on media treated aseptically and conventionally showed that the highest mean bacterial population observed for 4 months was in conventional bran wastewater media (R3), which is 6,80 x 10^{24} CFU and the lowest bacterial population is found in aseptic rice water waste media (S3), which is 1,28 x 10^{24}CFU.

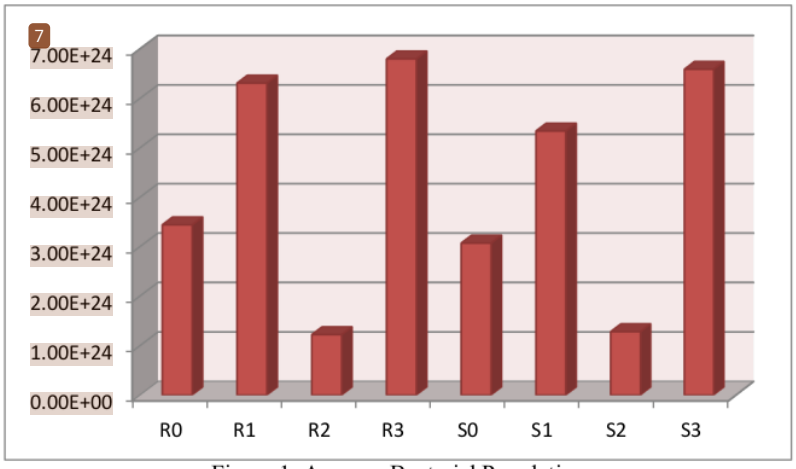

Figure 1. Average Bacterial Population

The growth of aseptic soybean liquid alternative media (S1) was not as high as the results of conventional soybean liquid alternative media (R1) which experienced a significant increase in population. The comparison between the first month to the second month is 36% of the total population in the first month 5,00 x 10^{24} CFU and in the second month 6,81 x 10^{24} CFU until it reaches the peak of the population in the third month which is 6,94 x 10^{24} CFU and back decreased in the fourth month, namely 66,48 x 10^{24} CFU, so that the average can be taken which is 6,31 x 10^{24} CFU so that the difference in results between alternative media for soybean water waste and the highest bacterial population is in alternative media for bran water waste which has a total population 6,80 x 10^{24} CFU is just a 7% difference.

Based on the growth pattern in Figure 1. then the growth of Rhizobium spp. The alternative media for soybean wastewater (R1) found third place after alternative media for aseptic (S4) and conventional (R4) bran, which experienced a growth rate of 36% from the first month of storage. This is because the bacteria experienced regrowth after experiencing a decline in the growth graph. [16] The ability of Rhizobium spp. bacteria to grow again is due to the presence of a source of nutrients originating from other bacterial cells that have died, so that the new nutrient source can be utilized by bacteria as a metabolic material for these bacterial cells. Bacterial bodies undergo decomposition so that they can be used as a source of energy for the remaining living bacteria to grow again.

Based on the results of the t-test analysis of the number of bacterial populations between the two treatments of aseptic media and conventional media during the viability test, which was carried out for 4 months, the results were 1.49 x 10^{-1} CFU. This shows that the effect of aseptic media and conventional media in bacterial propagation was not significantly different. The comparison between aseptic and conventional treatment media is said to be not significantly different considering that aseptic treatment is so complicated because it requires many installations such as electrical installations, hoses and requires expensive equipment such as autoclaves and ovens in the sterilization process which differs greatly from conventional treatment which is so simple. so that conventional treatment is easier to reach by farmers who will propagate *Rhizobium spp* [17].

4. Conclusion

Alternative media for bran water waste conventionally produced the highest bacterial population with a colony number of 6.80 x 10^{24} CFU and the lowest bacterial population came from aseptic rice alternative media with a colony number of 1.28 x 10^{24} CFU. Viability of bacteria observed for 4 months between aseptic media and conventional media showed the results of the t-test with t count 1.49 x 10^{-1} CFU showed the results (ns) were not significantly different.

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