

The Bacterial Endospore Stain on Schaeffer Fulton using Variation of Methylene Blue Solution

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Abstract. Endospores staining is the type of staining to recognize the presence spore in bacterial vegetative cells. The bacterial endospores need a staining which can penetrate wall thickness of spore bacteria. A method of endospores staining is Schaeffer Fulton method that used Malachite Green. It is an alkaline substance staining that can staining the spore bacteria. In this research, it have found the alternative staining that can replace Malachite Green solution in spore bacterial stain. The alternative staining used is Methylene Blue solution (0,5 %, 0,7%, and 1% concentration) with pH variation (10, 11, and 12), and varyous heating time (3, 4, and 5 minutes). The all treatments staining have been effect on bacterial spores staining results. The warming time greatly affect the dye to penetrate the walls of bacterial spores, this can be seen in the results with various concentration at pH 10, indicates that the not long warm-up time 3 and 4 minutes, bacterial spores are not stained, while in the longer heating time is 5 minutes bacterial spores stained. This is caused because the longer heating time can make the pores of spore wall is open so that can facilitate the dye to get into the bacterial spores.

1. Introduction

The bacteria is an organism that was the most numerous and widespread compared the others. Bacteria are generally unicellular organisms, prokaryotic, and microscopic. Bacteria also have a chromosome, ribosomes, and some other species have a granular food, gas vacuoles, and magnestom. Some bacteria are able to form themselves into endospores which enable them to survive in extreme environments [1].

Bacterial spores are bacterial mechanism that is intentionally set in an attempt to secure themselves to the adverse effects of the external environment. Bacterial spores have the same functionality as Amoeba cysts, because the bacteria in the form of spores and cysts of Amoeba in the form of a second phase in which the microorganisms that changes shape to protect themselves against unfavorable external factors eksospora [2].

At the bacterial spore staining, there are two methods commonly used, the method of Schaeffer Fulton and Klein methods. The difference of these two methods is the dye used. In the method of Schaeffer Fulton dyes used are Malachite Green and Safranin, while in the Klein method using dye Carbol Fuchsin and Methylene Blue. Schaeffer Fulton method is a method often used by laboratory technicians, due to staining time faster than the method of Klein [2]. So in this study, researchers prefer to use the method of Schaeffer Fulton commonly used in coloring endospores.



In endospores staining method Schaeffer Fulton, that used an alkaline solution, namely Malachite Green and Safranin. Malachite Green used has a pH of 11.2 (it is alkaline). Dye Malachite Green and Safranin can work well in bacteria because of the alkaline (chromophoric component positively charged), while the bacterial cytoplasm is basophilic, it causes the bacteria can absorb dyes well [3].

Methylene Blue is one dye Thiazine frequently used, because they are economical and easy to obtain. It is a basic dye which is important in the process of dyeing leather, cloth and cotton cloth. The use of Methylene Blue may cause some effects, such as gastrointestinal irritation if ingested, cause cyanosis inhalation and skin irritation if touched by the skin. Methylene blue is an aromatic hydrocarbon compounds with molecular formula $C_{16}H_{18}N_3SCl$ has many uses in different fields, such as biology and chemistry. At room temperature appears as a solid, odorless, dark, and green colored powder [4].

Methylene Blue solution was also used as a dye in chromoendoscopy, smear staining bacteria, and is also used to examine RNA or DNA under a microscope. This solution is also an alkaline solution, which has a pH of 11.4, and is soluble in water and alcohol, have the same properties as malachite green so that researchers use it as an alternative to the dye staining method Shcaeffe Fulton endospores. From the results of the preliminary test, a solution of Methylene Blue with a concentration of 0.5%, 0.7%, and 1% can pierce or penetrate the bacterial endospores so endospores appear blue.

2. Experimental Method

Preparation of the solution pH variation begins 250 mL $Na_2B_4O_7$ 0.025 M NaOH is added with 27 ml 0.20 M NaOH, then add up to 500 mL with distilled water (for pH 10). The next solution for pH 11 begins 250 mL of 0.50 M $NaHCO_3$ was added with 57 ml of 0.20 M NaOH, then add up to 500 mL with distilled water. The last solution for pH 12 begins 250 mL 0.20 M KCl was added with 30 mL of 0.20 M NaOH, then add up to 500 mL with distilled water.

Preparation the solution of Methylene Blue 0.5% begins with dilute 0.1 grams of this dye with a pH buffer (10, 11, and 12) 20 mL. Then for Methylene Blue 0.7% begins with dilute 0.14 grams of this dye with a pH buffer (10, 11, and 12) 20 mL. The last step for Methylene Blue 1% begins with dilute 0.2 grams of this dye with a pH buffer (10, 11, and 12) 20 mL. The procedure of Schaeffer Fulton method using Malachite Green 5% begins prepare bacterial smear on the objek glass, fixation over a low heat and Malachite green 5% added over the heat fixed bacterial smear then heated over a steam bath for few minutes. This will soften the hard outer coverings of the spore and the primary stain gets stick to the spore. When taken from the steam bath followed by further cooling hardens the outer layer of the spore, during this stage both the spore and vegetative cells appear as green in color, then added water and H_2SO_4 1% as decolorizing agent for vegetative cells. The last added dye Safranin 0.5%, when counterstained with this dye, vegetative cells are easily stained with Safranin, and the cells appear in red or pink color. Check using microscope 1000x.

The procedure of Schaeffer Fulton method using variation concentration of Methylene Blue (0.5%, 0.7%, and 1%) as the same as standard procedure but the difference is Malachite green replaced by variation concentration of Methylene Blue (0.5%, 0.7%, and 1%). The subject of bacteria in this experiment are *Bacillus subtilis* and *Clostridium tetani* that can produce endospores with the different type.

3. Result and Discussion

3.1. Result

Staining of bacterial spores is staining using Malachite Green solution of 5% and 0.5% Safranin, which results in the coloring will appear green on the spores, as well as red in the vegetative cells. At Schaeffer Fulton method that is widely used in painting endospores, endospore first stained with Malachite Green by a heating process, this solution is a powerful dye to penetrate into endospores. After treatment Malachite Green, the cell culture is washed with water and then covered with paint safranin, this technique will produce endospores and green in color pink in vegetative cells [5].

In endospores staining methods Schaffer Fulton, that used an alkaline solution (Malachite Green and Safranin). Dye Malachite Green and Safranin can work well in bacteria because of the alkaline (chromophore component positively charged), while the bacterial cytoplasm is basophilic so there was the attraction between the components chromophore in a dye with bacterial cells, it causes bacteria can absorb dyes well [6].

In this study, researchers used a dye that is wet is Methylene Blue as an alternative to dye Malachite Green. Researchers varying the solution of Methylene Blue with a concentration of 0.5%, 0.7% and 1%, then varying the pH of a solution of Methylene Blue, and longer heating time at 3-5 minutes. Based on the results of research and observation and then analyzed with a statistical calculation Two-Way ANOVA test done on the variation of Methylene Blue concentration (0.5%, 0.7%, 1%), the pH variation 10, 11, 12, and the variation of time heating 3 minutes, 4 minutes and 5 minutes, showed a statistically significant difference, where $F_{\text{count}} > F_{\text{table}}$. Because of the significant differences in staining results are then followed a further test using LSD test. After further test (LSD), the data obtained from each treatment on a modified staining bacterial spores *Bacillus subtilis* and *Clostridium tetani* is there is a difference in each treatment.

In *Bacillus subtilis* bacterial spores staining with a variety of used give different results. At a concentration of 0.5% (Fig.1a), the same coloring treatment results with the standard is in the variation of pH 10 with variation of time (5 minutes), variations in pH 12 with variation of time (3 minutes, 4 minutes and 5 minutes). While the results of different treatments with standard staining is in the variation of pH 11 with variation of time (3 minutes, 4 minutes and 5 minutes). At a concentration of 0.7% (Fig.1b) results equal treatment with standard staining is in the variation of pH 10 with variation of time (4 minutes and 5 minutes), and variations in pH 11 with a variation of the time (3 minutes and 5 minutes). While the results of treatment of different staining with the standard is in the variation of pH 10 with a variation of the time (3 minutes), variations in pH 11 with variation of time (4 minutes), and at various pH 12 with a variation of the time (3 minutes, 4 minutes and 5 minutes).

At a concentration of 1% (Fig.2a) results the same treatment staining with the standard is in the variation of pH 10 with variations (4 minutes, 5 minutes), and variations in pH 11 with variation of time (4 minutes and 5 minutes). While the results of treatment of different staining with the standard is in the variation of pH 10 with a variation of the time (3 minutes), variations in pH 11 with a variation of the time (3 minutes), and variations in pH 12 with a variation of the time (3 minutes, 4 minutes and 5 minutes).

From observations obtained in the modification staining bacteria *Bacillus subtilis* spores, it can be concluded that all the coloring treatment effect on bacterial spores staining results. As we know that the warming time greatly affect the dye to penetrate the walls of bacterial spores [7], this can be seen in the results of staining with various concentration (0.5%, 0.7% and 1%) at pH 10, indicates that the not long warm-up time 3 minutes and 4 minutes, bacterial spores are not stained, while in the longer heating time is 5 minutes bacterial spores stained. This is caused because the heating time is longer make the pores of the spore wall is open so as to facilitate the dye to get into the bacterial spores.



Figure 1. The endospore staining of *Bacillus subtilis* using Methylene Blue (a) 0,5% at pH 12 and 3 minutes; (b) 0,7% at pH 10 and 5 minutes

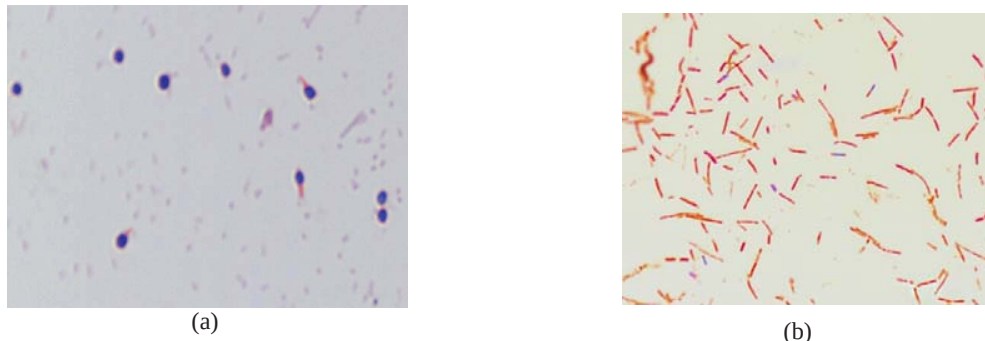


Figure 2. The endospore staining using Methylene Blue (a) *Bacillus subtilis* at 1%, pH 11 and 5 minutes; (b) *Clostridium tetani* at 0.5%, pH 11 and 5 minutes

In *Clostridium tetani* bacterial spores staining with a variety of used give different results. At a concentration of 0.5% (Fig.2b) results equal treatment with standard staining is in the variation of pH 10 with variation of time (4 minutes and 5 minutes), variations in pH 11 with variation (time of 3 minutes, 4 minutes and 5 minutes), and the pH 12 with a variation of the time (3 minutes, 4 minutes and 5 minutes). While the results of different treatments with standard staining is in the variation of pH 10 with a variation of the time (3 minutes), variations in pH 12 with variation of time (3 minutes).

At a concentration of 0.7% (Fig.3a) results equal treatment with standard staining is in the variation of pH 10 with variation of time (4 minutes, and 5 minutes), variations in pH 11 with variation of time (3 minutes, 4 minutes and 5 minutes). While the results of different treatments with standard staining is in the variation of pH 10 with a variation of the time (3 minutes), and variations in pH 12 with variation of time (3 minutes, 4 minutes and 5 minutes). Meanwhile, at a concentration of 1% (Fig.3b) by varying the pH (10, 11, and 12) and the variation of heating time (3 minutes, 4 minutes and 5 minutes), the results obtained do not have the same standards.

From observations obtained in the modification staining the bacteria *Clostridium tetani* spores it can be concluded that, all the coloring treatment effect on bacterial spores staining results. As we know that the warming time greatly affect the dye to penetrate the walls of bacterial spores, this can be seen in the results of staining with various concentration (0.5%, 0.7% and 1%) at pH 10, indicates that the warm-up time is not long (3 minutes) of bacterial spores are not stained, while in the longer heating time (4 minutes and 5 minutes) stained bacterial spores. This is caused because the heating time is longer make the pores of the spore wall is open so as to facilitate the dye to get into the bacterial spores [8].

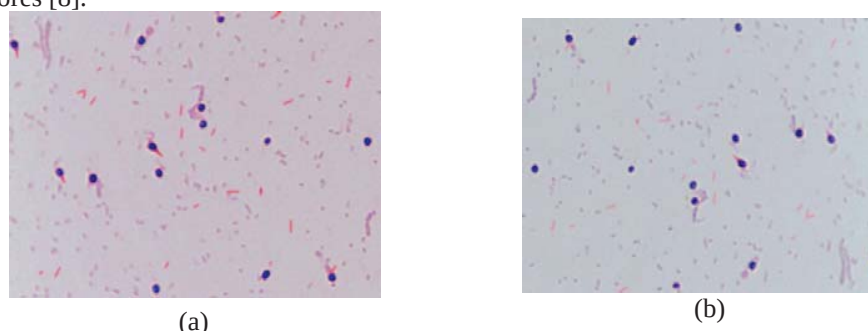


Figure 3. The endospore staining of *Clostridium tetani* using Methylene Blue (a) 0.7% at pH 10 and 3 minutes; (b) 1% at pH 11 and 4 minutes

pH of a solution is very influential in the staining bacteria, where the solution Methylene blue is used in the coloring of bacterial spores must be alkaline, because the nature of the bacterial cytoplasm is basophilic [9], so in this study the researchers varying the pH of the solution Methylene Blue (pH

10, pH 11, and pH 12) to determine the optimum pH which can provide the same results with standard staining. From the observations obtained, variations Methylene Blue solution with a pH (10, 11, and 12) is influenced by the concentration of Methylene Blue solution (0.5%, 0.7% and 1%).

The concentration of the solution is also affected by variations in pH of the solution used. As an example can be seen at a concentration of 0.7%, at pH 10 and 11 look good bacteria spores, whereas at pH 12 of bacterial spores does not look good, because the use of high concentration and pH of the solution is more alkaline (strong bases) then dye methylene blue is more concentrated, so that when given a second dye with Safranin [10], vegetative cells appear red bluish and purplish red. So that the bacterial spores and vegetative cells does not look good.

4. Conclusion

Optimal concentration using Methylene Blue as a coloring alternative to Malachite Green in staining the bacterium *Bacillus subtilis* spores are at a concentration of 0.5% at pH 12 and the heating time for 3 minutes. While in staining the bacterium *Clostridium tetani* spores are at a concentration of 0.5% at pH 11 and the heating time 3 minutes.

5. References

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