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Effect of pH Variations in Eosin Methylene Blue Agar (EMBA) Medium on E. coli Growth

Diah Lestari[1a]*, Eva Nur Haliza[1b], Heru Setiawan[1c]

[1] Medical Laboratory Technology, Health Polytechnic Jakarta III, Bekasi, West Java, Indonesia

[a] Email address: diahtari1411@gmail.com
[b] Email address: evanurhaliza0213@gmail.com
[c] Email address: heru@poltekkesjakarta3.ac.id

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Abstract
The growth of E. coli is influenced by several factors, including environmental pH. Environmental pH unsuitable for bacterial growth conditions will interfere with the enzyme activity and influence bacterial growth. This study aimed to determine the effect of pH variations (5.3, 5.8, 6.3, 7.3, 7.8, 8.3) in EMBA medium on E. coli growth. The research method used was true experiment post-test-only control design. The samples in this study were suspensions with dilutions of 10-4, 10-5, 10-6, and 10-7, inoculated using the duplo test to EMBA with various pH conditions, so that the total sample size is 48. The results showed that the mean number of E. coli bacteria on EMBA with pH 5.3 was 9.1 x 106 CFU/mL; with pH 5.8 was 9.6 x 106 CFU/mL; with pH 6.3 was 1.2 x 107 CFU/mL; with pH 7.3 was 1.1 x 107 CFU/mL; with pH 7.8 was 9.7 x 106 CFU/mL; and with pH 8.3 was 7.1 x 106 CFU/mL. Growth in positive control showed the mean number 1.4 x 107 CFU/mL; negative control showed no growth of E. coli or other microorganisms. Based on the One-Way ANOVA statistical test with a 95% confidence level, there was no difference in the mean number of E. coli bacteria in the six pH variations of EMBA medium (p-value > 0.05). E. coli bacteria grew best at neutral pH. Its growth decreases in slightly acidic and slightly alkaline pH, but it can still be observed. This allows E. coli to survive in extreme pH. Pathogenic E. coli have developed the potential to live inside the human body. They will experience temporary stress in unfavourable conditions before finally adapting. The advice for future researchers is to test the effect of pH on E. coli growth by using other E. coli growth media or with a wider range of pH (more acidic and more basic). It is also recommended to conduct further research about the effect of various environmental conditions such as temperature, nutrients, and others on bacterial growth.

Keywords: E. coli, EMBA, pH Variations.

Corresponding Author:

Diah Lestari
Medical Laboratory Technology, Health Polytechnic Jakarta III, Bekasi, West Java, Indonesia
Email: diahtari1411@gmail.com

1. INTRODUCTION

E. coli are bacteria commonly found in the environment, food, and the intestines of humans and animals. *E. coli* is a large and diverse group of bacteria. Most types of *E. coli* are harmless, but some pathogenic ones can infect humans. These pathogenic *E. coli* can produce and release toxins that cause disease (Rizky et al., 2021). The growth of *E. coli* is influenced by several factors, one of which is physical factors, including pH, temperature, oxygen, humidity, and light (Arivo & Annissatussholeh, 2017). In addition, bacteria also need nutrients to grow. The nutrients needed for *E. coli* growth are carbon, hydrogen, nitrogen, phosphorus, sulfur, vitamins, water, and others. These nutrients can be met and must be present in the media for bacteria growth. Synthetic media for the growth of *E. coli* can be solid or liquid media, such as eosin methylene blue agar (EMBA), mac-Conkey agar (MCA), endo agar (EA), nutrient agar (NA), triple sugar iron agar (TSIA), indol medium, sugars medium, and another medium for *E. coli* growth (Arianda, 2016).

The media used for bacterial growth depends on the purpose or need, whether differential media, enriched media, or selective media. Selective media for *E. coli* growth can be used for endo agar/endo's medium (EA) and eosin methylene blue agar (EMBA). EMBA is a selective medium because the methylene blue contained in it can inhibit the growth of gram-positive bacteria. Sugars contained in the media are lactose and sucrose, which are substrates that can be fermented by most gram-negative bacteria, especially coliform bacteria. The presence of lactose and sucrose also aims to distinguish between coliform bacteria that can ferment sucrose faster than lactose and those that cannot ferment sucrose (Juwita et al., 2014).

Besides nutrients, the pH value of the growth medium is also an essential factor in bacterial growth. pH is the number of Hydrogen ion (H^+) concentrations expressing the acidity level and basicity in a solution (Ngafifuddin et al., 2017). There are three groups of bacteria based on the optimum pH value of their growth, neutrophil bacteria that grow optimally at a pH of neutral range (pH 7), acidophil bacteria that grow at a pH of less than 5.55, and alkaliphilic bacteria that grow optimally at a pH between 8-10.5 (Isnawati & Trimulyono, 2018). *E. coli* can grow at pH 4.5 - 9 (Ratzke & Gore, 2018). These bacteria belong to neutrophilic bacteria that grow optimally at a neutral pH range of 6.5 - 7.5 depending on the temperature (Philip et al., 2018). The optimum pH condition for *E. coli* growth currently widely known is the pH condition in the laboratory. *E. coli* is a bacteria that can survive and proliferate in a wide range of environmental conditions. *E. coli* will undergo brief stress when placed in an environment that does not support its growth but will adapt. Its ability to adapt to pH levels other than its optimum growth pH enables it to invade the human body. Creating slightly acidic to slightly alkaline pH fluctuations in *E. coli* growth medium can reflect different conditions in the human body or the external environment, aiding knowledge of how *E. coli* bacteria adapt to varied pH situations.

This study aimed to determine the effect of pH variations (5.3; 5.8; 6.3; 7.3; 7.8; 8.3) in EMBA selective medium on *E. coli* growth. This research tests the hypothesis that there is a difference in the mean number of *E. coli* bacteria in the six pH variations of the EMBA medium, with a confidence level of 95%.

The benefit of this research is to increase understanding related to environmental conditions that can prevent the growth of *E. coli*, such as in highly acidic and alkaline environments. So that, diseases can be prevented and controlled. The study results can also add information for medical laboratory technologists (ATLM) regarding the determination of pH in *E. coli* growth media and support identifying *E. coli* on agar media. Additionally, the study's findings can be used as reference for the development of new antibiotics.

2. RESEARCH METHOD

The research design used was a true experiment post-test-only control design. This study consisted of two groups: the treatment and control groups. The treatment group consisted of EMBA medium that received a pH intervention. In contrast, the control group consisted of EMBA medium that did not get a pH intervention (the pH utilized was the factory pH of 6.8). The population in this study was a 0.5 McFarland *E. coli* suspension, and the samples used were part of a serially diluted *E. coli* suspension. The samples used were suspensions with dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, inoculated using the duplo test to EMBA with various pH conditions, so that the total sample size is 48. Sampling using purposive sampling technique with inclusion criteria is *E. coli* colonies that grow on EMBA medium with pH variations of 5.3, 5.8, 6.3, 7.3, 7.8, 8.3 and exclusion criteria is *E. Coli* colonies that grow on EMBA medium with six pH variations are accompanied by fungal growth or other contaminants.

From May to June 2023, data were collected in the bacteriology laboratory of the medical laboratory technology department of the Jakarta III of Health Polytechnic, Ministry of Health. Data were collected by preparing the instruments, materials, chemicals, and growth media. Furthermore, preparation of test bacteria such as making *E. coli* stock on NA medium, bacterial rejuvenation, identification of *E. coli* purity with IMViC test, gram staining, making *E. coli* 0.5 McFarland suspension, and serial dilution of *E. coli* 0.5 McFarland suspension. Test materials were prepared by making an EMBA medium with six pH variations. Making EMBA medium pH 5.3, 5.8, 6.3 was performed by adding an acidic solution (HCl) to the initial media. EMBA medium with pH 7.3, 7.8, and 8.3 was made by adding a basic solution (NaOH) to the initial media. *E. coli* inoculation on EMBA with six pH variations was conducted in duplicate (duplo) with the pour plate inoculation technique. The media was then incubated at 37°C for 2x24 hours.

Data analysis using univariate and bivariate analyses. Bivariate analysis was carried out using the One-Way ANOVA statistical test, with the data being normally distributed and homogeneous. Data normality test using The Saphiro-Wilk test.

This study has passed the ethical review with a statement of ethical feasibility of research issued by the Health Research Ethics Commission (KEPK) of Prof. Dr. Hamka Muhammadiyah University on May 24, 2023, with No: 03/23.05/02544.

3. RESULTS AND DISCUSSION

The results of reading the number of *E. coli* growth with variations in pH on EMBA medium can be seen in Table 1.

Table 1. Distribution of *E. coli* number on EMBA medium with pH variations

pH	Dilution	Mean number of *E. coli* (CFU/mL)
5,3	10^{-5}	$9{,}1 \times 10^{6}$
	Media control	0
5,8	10^{-5}	$9{,}6 \times 10^{6}$
	Media control	0
6,3	10^{-5}	$1{,}2 \times 10^{7}$
	Media control	0
Positive control (Factory pH = 6,8)	10^{-5}	$1{,}4 \times 10^{7}$
	Media control	0
7,3	10^{-5}	$1{,}1 \times 10^{7}$
	Media control	0

pH	Dilution	Mean number of *E. coli* (CFU/mL)
7,8	10^{-5}	9,7 x 10^6
	Media control	0
8,3	10^{-5}	7,1 x 10^6
	Media control	0

Table 1 shows that the amount of *E. coli* growth differs in the six pH groups of EMBA medium and the control group. The highest change was in pH 6.3, with a mean of 1.2 x 10^7 CFU/mL, followed by pH 7.3, which had a mean of 1.1 x 10^7 CFU/mL. The amount of growth decreased gradually as the pH increased and decreased from neutral pH. However, the most apparent germs decreased at a slightly alkaline pH (8.3) with a mean number of 7.1 x 10^6 CFU/mL. While at a slightly acidic pH (5.3), the mean number was 9.1 x 10^6 CFU/mL. The positive control group with a factory pH of 6.8 (neutral) showed the most fertile growth among the treatment groups, with a mean of 1.4 x 10^7 CFU/mL.

Table 2. Descriptive Statistics of *E. coli* Colonies Number based on pH Variations

pH	Min	Mean	Max	Range	Std deviation
5.3	0	113	356	356	152
5.8	0	123	396	396	168
6.3	0	144	444	444	189
Positive Control	2	162	504	502	211
7.3	0	134	424	424	180
7.8	0	126	400	400	171
8.3	1	112	376	375	159

Table 2 shows the descriptive statistics of *E. coli* colonies, including mean value, minimum, maximum, range, and standard deviation. The highest number of colony was observed at pH 6.3, while the lowest number of colony was observed at pH 8.3. The positive control shows the best growth of *E. coli*, exhibiting the highest colony count among all pH variations.

Table 3. *One-Way ANOVA* Statistical Analysis

	dF	Mean Square	F	Sig.
Between Groups	6	0,210	0,250	0,957

Table 3 shows the analysis of the number of *E. coli* growth with six pH variations of EMBA medium using the One-Way ANOVA statistical test. The result obtained was the value of P = 0.957 (P> 0.05), which means that at the 95% confidence level, there was no significant difference or there was no difference in the number of *E. coli* bacteria in the six pH variations of the EMBA medium.

a. Frequency Distribution of pH Variation in EMBA Medium on *E. coli* growth

The study found that *E. coli* bacteria grew best at a neutral pH. This can be seen from the high number of *E. coli* growth in the control group. The pH value in the positive control was 6.8 (neutral). The growth decreased gradually at increasingly acidic and increasingly alkaline pH. However, the most negligible change occurs at alkaline pH (8.3). This is in line with Thornton et al. (2018) research which stated that *E. coli* growth is higher at acidic urine pH than alkaline urine, but the highest growth is at neutral pH. *E. coli* is more resistant to slightly

acidic pH than slightly alkaline pH because, in alkaline conditions, bacterial cells require higher energy to maintain the balance of their condition, which results in cells losing protons (AlRabiah et al., 2018).

This study used EMBA as a selective medium for *E. coli* growth. EMBA medium was chosen because it is a selective media that can quickly and accurately differentiate *E. coli* from other gram-negative bacteria (Jamrin et al., 2022). The samples used were *E. coli* suspensions with dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. However, after research, the number of representative colonies was at a dilution of 10^{-5}, with the number of colony growth between 30-300 in line with the standard plate count (SPC). According to Fardiaz (2013), counting colonies and writing germ numbers refers to the standard plate count (SPC), with the Petri dish chosen to have a colony count of 30-300. In dilutions that produced less than 30 colonies, the results were presented as fewer than 30 colonies multiplied by the dilution factor, with the actual number in parenthesis. In dilutions that produced more than 300 colonies, the results were reported as more than 300 colonies multiplied by the dilution factor, with the actual number included in parentheses (Purnamasari et al., 2013).

This study used sterile 0.9% NaCl as media control or negative control. The use of negative control aimed to ensure that the *E. coli* that grows does come from samples produced on EMBA medium with pH variations and not due to contamination or other factors that may be present in EMBA medium. The results on negative control showed no growth of *E. coli* or other microorganisms.

b. Effect of pH Variation in EMBA Medium on E. coli Growth

Based on the One-Way ANOVA statistical test, at the 95% confidence level, there was no significant difference in the number of *E. coli* bacteria in the six pH variations of EMBA medium. According to Isnawati & Trimulyono (2018), most bacteria are neutrophil bacteria, including *E. coli*. They grow well at a pH range of one or two units from neutral pH of 7. Some microorganisms can survive and adapt to small changes in environmental pH (Guan & Liu, 2020). According to research by Martín-Gutiérrez et al. (2016), *E. coli* can grow at pH 5, 6, and 7 in urine. Although urine is acidic such as at pH 5, the growth of *E. coli* is not too disturbed, which indicates that the bacteria can adapt well to slightly acidic conditions. However, compared to Mueller-Hinton (MH) media, the growth in urine is relatively low. This is due to the lack of nutrients in the urine.

In addition to pH value, growth media is a crucial component for bacterial growth since it contains nutrients that can encourage bacterial growth. The dyes eosin and methylene blue in Eosin Methylene Blue Agar (EMBA) can suppress the growth of gram-positive bacteria. As carbon sources, this medium contains sucrose and lactose, which distinguishes gram-negative bacteria based on their ability to ferment lactose. Eosin and methylene blue dyes can identify lactose fermentors from non-lactose fermentor bacteria found in the colour of the colonies generated (Widinugroho & Asri, 2021). Levine modified this medium 1918 by reducing the sucrose content and increasing the lactose concentration (Zimbro, 2009).

Lactose sugar is used as a nutrition and carbon source by *E. coli* in an EMBA medium. *E. coli* can ferment lactose because *E. coli* has the β-galactosidase enzyme, which is triggered by lactose. Lactose in the media can activate the lac operon and produce the enzyme expression. Lactose can be broken down into glucose and galactose by the β-galactosidase enzyme. If lactose concentration in the medium decreases, so will the concentration of β-galactosidase enzyme, which may have a detrimental impact on the growth of *E. coli* (Seager & Slabaugh, 2010).

The findings from this research are consistent with previous research by Arivo & Annissatussholeh (2017) , which used Nutrient Broth (NB) as growth medium with a pH range of 3, 5, 7, and 9. It indicates that *E. coli* grow best at neutral pH (7), while growth decreases at pH 3, 5, and 9. The measurements on NB media with neutral pH (7) showed high absorbance values, indicating a significant growth of *E. coli* at that pH. Meanwhile, low absorbance values were obtained at other pH levels (3, 5, 9), indicating minimal growth of *E. coli*.

No significant differences in bacteria numbers in the six pH variations of EMBA medium could occur because the selection of the pH range was still relatively narrow with many pH variations. Hence, the differences between variations were slight. In addition, the selected pH variations have not reached extreme pH. Small differences in pH in EMBA medium might not significantly affect the amount of *E. coli* growth because the content of this media was focused on identifying gram-negative bacteria based on their ability to ferment lactose seen from the colour produced by colonies. *E. coli*, a bacterium that can ferment lactose quickly, will produce greenish colonies with a metallic sheen when exposed to light, and the colony centre is blue-black. Bacteria that ferment lactose slowly will produce pink colonies with a dark centre and bacteria that cannot ferment lactose will be transparently coloured (Sophian, 2022).

At the time of observation, the effect of pH variation was also seen in the morphology of *E. coli* colonies. At a slightly acidic pH, *E. coli* colonies gave a paler colour when compared to the optimal pH. The colour difference might occur due to decreased enzyme activity, one of which was the β-galactosidase enzyme. The optimum pH range for the β-galactosidase enzyme to work was 6-8 (Sun et al., 2018). The decrease in enzyme activity due to small changes in the environmental pH (slightly below or above its optimal pH) was caused by changes in the ionic state of the enzyme and often also the ionic form of the substrate. At a specific pH, enzyme denaturation might occur, resulting in a steady decline in enzyme activity (Khusniati et al., 2015). A decrease in β-galactosidase enzyme activity can hinder the lactose breakdown, requiring a more extended incubation period for *E. coli* growth in an environment with a pH slightly outside the optimum pH range.

Most *E. coli* are non-pathogenic bacteria that are beneficial to their hosts. However, some specific strains, like bacteria of fecal origin, can be dangerous to health. These strains have developed the potential to live inside the human body. Pathogenic *E. coli* is a major problem for water, food industries, human health, and the general environment. Once *E. coli* is released into the environment through fecal deposition, it is considered a fecal indicator for assessing water quality. Contamination of food and water sources with pathogenic bacteria is a major cause of diseases such as diarrhea, typhoid fever, UTIs, and others. Therefore, monitoring environmental conditions regarding *E. coli* strains is important for disease prevention and control, and also for the development of new antibiotics. Additionally, the interaction between *E. coli* and the environment can be understood in depth, opening up opportunities for further research in microbiology and related fields (Razmi et al., 2023).

This study has limitations, as the pH range used only consists of six types of pH, with the highest and lowest pH values not yet reaching the extremes. Additionally, to collect data on the number of *E. coli*, this study only used EMBA as the growth medium. Meanwhile, it is highly probable that different effects may occur when using other growth media. Therefore, further research is necessary to gain a better understanding of the growth condition of *E. coli*. Future studies could involve a wider pH range and consider the use of either the same or different media. It is also recommended to conduct further research about the effect of temperature, nutrients, or other environmental conditions on bacterial growth.

4. CONCLUSION

Regarding the result of this study, statistically, there was no significant difference in the number of E. coli bacteria between pH variations in EMBA medium, with a p-value of 0.957 (p-value > 0.05). Nevertheless, the differences can still be observed in the morphology of colonies. Future studies could enhance this research by using a wider range of pH, perhaps extending to extreme pH levels. Future researchers could also use more selective media for E. coli growth. To increase the understanding of bacterial interactions with the environment, it is also recommended to conduct further research about the effect of various environmental conditions such as temperature, nutrients, and others on bacterial growth.

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