Jurnal Profesi Medika : Jurnal Kedokteran dan Kesehatan
DOI: https://doi.org/10.33533/jpm.v17i2.6405
Vol 17 No 2 (2023) ISSN 0216-3438 (print); ISSN 2621-1122 (online)

ARTICLE

THE GENERATION TIME OF *Escherichia coli* BACTERIA BASED ON VARIATION IN EOSIN METHYLENE BLUE AGAR MEDIA PH

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ABSTRACT

Bacteria require optimum environmental conditions to support cell growth and reproduction. States that are less than optimal cause bacteria to adapt and can infect humans for invasion of other organs. The speed of bacteria to infect is influenced by the rate and time of generation of bacteria in multiplying cells. This study aims to determine the effect of variations in pH (4.8; 5.8; 6.8; 7.8; and 8.8) in Eosin Methylene Blue Agar (EMBA) media on the generation time of *Escherichia coli* bacteria. Researchers used the True Experiment Post Only Control Design and One Way ANOVA test. The results showed that at a variation of pH 4.8 there was no growth; pH 5,8 with a generation time of 1.5-24.9; pH 6,8 with a generation time of 1.5-21.7 minutes; pH 7.8 with a generation time of 1.6-22.3 minutes; and pH 8.8 with a generation time of 1.6-22.8 minutes. The study concludes is that there is a significant effect of the variation in pH of the EMBA media on the generation time of *Escherichia coli* bacteria with a p-value of 0.000 < α 0.05, with the fastest generation time at pH 6.8.

Keywords: Eosin Methylene Blue Agar Media, *Escherichia coli*, Generation Time, pH variation

АБСТРАКТ

Для роста и размножения клеток бактериям необходимы оптимальные условия окружающей среды. Состояния, которые меньше оптимальных, заставляют бактерии адаптироваться и могут заражать человека с целью инвазии в другие органы. На скорость инфицирования бактерий влияет скорость и время генерации бактерий в размножающихся клетках. Цель данного исследования - определить влияние изменения pH (4,8; 5,8; 6,8; 7,8; и 8,8) в среде Eosin Methylene Blue Agar (EMBA) на время генерации бактерий Escherichia coli. Исследователи использовали схему "истинный эксперимент - постконтроль" и односторонний тест ANOVA. Результаты показали, что при изменении pH 4,8 рост отсутствует; при pH 5,8 время генерации составляет 1,5-24,9; при pH 6,8 время генерации составляет 1,5-21,7 минуты; при pH 7,8 время генерации составляет 1,6-22,3 минуты; при pH 8,8 время генерации составляет 1,6-22,8 минуты. В результате исследования сделан вывод о значительном влиянии изменения pH среды ЭМБА на время генерации бактерий Escherichia coli при p-значении 0,000 < α 0,05, причем наиболее быстрое время генерации наблюдается при pH 6,8.

Ключевые слова: Эозин-метиленовый синий агаровая среда, Escherichia coli, время генерации, изменение pH

Submitted: 14 Agustus 2023; Received in revised form: 1 November 2023; Accepted: 15 November 2023; Available online: 22 November 2023;
Published regularly: Desember 2023

INTRODUCTION

Escherichia coli is a *coliform* bacterium from the *Enterobacteriaceae* family that lives in the human digestive tract [1]. *E. coli* includes gram-negative rod-shaped bacteria with a size range of 1.0-1.5 µm x 2.0-6.0 µm, does not form spores, is not motile or motile with flagella, can grow with or without oxygen, is facultative aerobic and anaerobic, and is resistant to nutrient-poor media[2]. *E. coli* becomes pathogenic and can cause disease when it enters other organs or tissues [3].

This bacterium is associated with various types of intestinal diseases such as diarrhea in humans, especially in infants and children in developing countries. One of the causes of diarrhea caused by *E. coli* bacteria is the extreme environment, which is less supportive of the life and development of these bacteria. So *E. coli* makes self-defense to protect itself, adapt and survive in this extreme environment [4]. Bacterial growth is influenced by temperature, pH, osmotic pressure, oxygen, and nutrients [5]. Bacteria do not always live in optimal pH. In nature, bacteria may grow much slower than the maximum speed observed in the laboratory. This is because the conditions and sources of nutrients needed for optimal growth in the laboratory may not exist in natural habitats [6]. The pH value is one of the most essential factors in bacterial growth, if the pH is too acidic or too alkaline, bacteria cannot grow [7]. Media that can provide sufficient nutrition for bacteria to grow is Eosin Methylene Blue Agar (EMBA) media which is a selective medium for pathogenic *Enterobactericeae* bacteria, one of which is Escherichia coli [8]. TSB (Trypticase Soy Broth) media is one of the enrichment media used to multiply bacteria and aerobic bacterial cultures. This medium is commonly used for Enterobacter bacteria [9].

Some bacteria have generation times that vary depending on optimal conditions with the appropriate temperature, pH, and nutrients. Bacterial generation time is when bacteria divide from one cell to two cells, two cells to four cells, and so on [10]. The generation time for *E. coli* is 20 minutes at 37ºC [11]. Bacterial generation time can last much longer and is closely related to infection in the host. The faster the generation time of pathogenic bacteria, the faster the bacteria infect the host. An overview of the time of this generation will be the basis for assessing when diarrheal disease due to *E. coli* occurs because the growth rate of *E. coli* bacteria is difficult to predict, especially in environments with extreme conditions The extreme conditions in question are not optimal media for the growth of *E. coli* bacteria, such as conditions of pH, temperature, and other nutrients needed by bacteria to live. These extreme conditions will affect the speed of growth and the speed of bacteria to infect and invade other host organs [5]. The incubation period for bacteria to cause disease occurs for 6 hours, but these extreme conditions can cause bacterial growth to be faster [2]. This can provide information to anticipate preventive measures for diseases caused by *E. coli* bacteria.

In the research by Sariadji and friends, conducted research on the generation time of *Vibrio cholerae* bacteria with optimum temperature and pH [12]. In the research by Hikmah, she has researched pH and temperature on the antibacterial activity of fermented rice bran by *Rhizopus oryzae* with variations in pH and temperature [13]. In the research by Yusuf and friends, he has researched the growth of *E. coli* in an alkaline pH environment [14]. Much is known about the time of generation of *E. coli*, both in environments and mediums where conditions are suitable or not suitable for bacterial growth. A suitable environment can accelerate the growth and division of bacterial cells, which can accelerate the emergence of infection and, worse, it can invade other organs. Vice versa [15]. For this reason, information is needed to know how the influence of variations in the pH of media such as EMBA media on the generation time of *E. coli* bacteria so that the speed of infection in the host can be determined and prevention of invasion to other organs can be carried out.

From the description of the background above, the researcher is interested in

 DOI : https://doi.org/10.33533/jpm.v17i2.6405 Vol 17 No 2 (2023)

conducting further research regarding the effect of variations in the pH of Eosin Methylene Blue Agar (EMBA) media on the generation time of *E. coli* bacteria.

MATERIAL AND METHODS

This research was conducted from February to June 2023 at the Jakarta III Ministry of Health Polytechnic Laboratory. This study uses the True Experiment Post Test Only Control Design.

A suspension of E. coli ATCC 25922 was utilised in this investigation. The bacteria were isolated on 0.56 grammes of EMBA (Oxoid) media that was dissolved in 15 millilitres of distilled water. Additionally, a metallic green colony was obtained, which had a dark hue in the core of the colony, smooth borders, and a convex shape within the colony. A total of 0.09 grammes were cultured in TSB (Merck) enrichment media in 3 millilitres of distilled water for a period of twenty-four hours at a temperature of 37 degrees Celsius. In the event that bacterial growth takes place, the colour will change from clear to hazy. A total of 37.55 grammes of EMBA media (Oxoid) were dissolved in 1000 millilitres of distilled water in order to complete the preparation of EMBA media for each of the different media pH values. The pH was then changed to 4.8, 5.8; 6.8; 7.8; and 8.8 for each of the different media variations using a solution of hydrochloric acid and sodium hydroxide. Additionally, the bacterial suspension is made by progressively adding the E. coli bacterial suspension that develops on the TSB media together with 0.9% sterile NaCl (Merck) (0.027 gramme in 3 mL of distilled water) to 0.5 McFarland. This process is repeated until the desired concentration is reached. Up to a concentration of 10-6, serial dilutions were prepared. After that, the mixture was incubated for a period of up to six hours, with intervals of twenty minutes. After that, one millilitre of the diluted suspension was grown using the pour plate method at dilutions of 10-5 and 10-6 for each modification of pH of the EMBA media. This was done for a period of twenty-four hours at a temperature of 37 degrees Celsius. Each of the experimental groups was carried out in duplicate, and a total of 380 samples were collected to obtain the whole sample. A non-probability sampling technique in conjunction with a purposive sampling strategy was utilised in the process of carrying out the sampling technique. The pH of the factory media was used to determine the positive control, which was maintained at 6.8. On the other hand, the negative control was not implanted with bacteria but rather was planted with 1 mL of physiological sodium chloride. Using the plate count method, the number of bacteria was determined in terms of colony forming units per millilitre (CFU/mL) from colonies that were growing on EMBA media. The plate count method was used under the condition that the growth of the colonies was between 30 and 300 colonies. [16:]

$$\text{Number of colonies/mL} = \frac{\text{Number of colonies x 1}}{\text{dilution factor per plate}}$$

Next, the generation time for each sample is calculated using the formula (17):

$$G = \frac{t}{n}$$

Information:
G = Generation time
t = Time taken from the number of initial cells (N0) to the final cell (Nt)
n = Number of generation

After the results of the calculation of the generation time have been obtained, the next step is to process the data using SPSS software. This is done in order to determine whether or not there is an effect of variations in the pH of the EMBA media on the generation time of Escherichia coli. The data normality test (Kormogorov-Smirnov) and the One-Way ANOVA test are utilised in order to make this determination. In addition to that, the Bonferroni test was utilised in order to determine the significance of the differences in pH.

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Table 1. Frequency distribution of *E. coli* generation time calculations on Variations in the pH of EMBA media

pH Variation	Number of Bacterial Cells (CFU/mL)					Generation Time (minutes)				
	Average	Lowest	Highest	C (+)	C (-)	Average	Fastest	Oldest	C (+)	C (-)
4.8	0	0	0	0	0	0	0	0	0	0
5.8	(<300) 1.5×10^8	(>300) 8.3×10^3	(<300) 1.9×10^8	(>300) 8.3×10^3	0	13.2	1.5	24.9	1.5	0
6.8	(<300) 1.1×10^9	(>300) 2.1×10^4	(<300) 2.0×10^9	(>300) 2.1×10^4	0	12.0	1.5	21.7	1.5	0
7.8	(<300) 4.3×10^9	(>300) 2.0×10^4	(<300) 1.4×10^9	(>300) 2.0×10^4	0	13.1	1.6	22.3	1.6	0
8.8	(<300) 2.7×10^9	(>300) 1.6×10^4	(<300) 1.1×10^9	(>300) 1.6×10^4	0	13.5	1.6	22.8	1.6	0

RESULT

According to the data shown in Table 1, it can be observed that there was no development of bacterial cells within the incubation period ranging from 20 minutes to 360 minutes (equivalent to 6 hours) at a pH level of 4.8. The optimal generation time is characterised by a shorter duration, facilitating the production of a larger number of cells.. The fastest generation time and the highest number of cells were at pH 6.8, with the lowest number of bacterial cells at 2.1×10^4 CFU/mL, the highest at 2.0×10^9 CFU/mL, with the fastest generation time at 1.5 minutes, the longest 21.7 minutes. The average generation time is 12 minutes. The results have been adjusted for positive control and negative control.

The ANOVA test will be employed to examine the data in order to ascertain the impact of the pH of the media (EMBA) on the generation time of E. coli bacteria. In order to do an ANOVA test, it is necessary for the data to meet certain requirements. Firstly, the data should follow a normal distribution. Additionally, the variance of the data should be homogenous. According to the Kolmogorov-Smirnov test, the data has been determined to have normal distribution. Therefore, it is OK to proceed with doing the ANOVA test.

Table 2. Kormogorov-Smirnov Data Normality Test Results

	pH variation	P-*value*
E. coli Generation Time	pH 5.8	0.200
	pH 6.8	0.200
	pH 7.8	0.200
	pH 8.8	0.200

The data distribution is normal based on the data normality test results, p-value> α (0.05). So it was continued with the One Way ANOVA test, and the test results were obtained as follows

Table 3. One-Way ANOVA Test Results

	pH variation	P-*value*
E. coli Generation Time	pH 5.8	0.000
	pH 6.8	
	pH 7.8	
	pH 8.8	

After the One Way ANOVA test, Table 3 indicates sig/p = 0.000. Value of $p < \alpha$ indicates significance (0.00 < 0.05). It appears that the pH of the EMBA media affects E. coli bacteria generation time, hence the Bonferroni test can be used to determine the significance of each pH difference.

 DOI : https://doi.org/10.33533/jpm.v17i2.6405 Vol 17 No 2 (2023)

Table 4. Bonferroni Test Results

Comparison Between EMBA Media pH Variations		P-*value*	Information
4.8	5.8	0.000	There is a significant difference
	6.8	0.000	There is a significant difference
	7.8	0.000	There is a significant difference
	8.8	0.000	There is a significant difference
5.8	4.8	0.000	There is a significant difference
	6.8	1.000	There is no significant difference
	7.8	1.000	There is no significant difference
	8.8	1.000	There is no significant difference
6.8	4.8	0.000	There is a significant difference
	5.8	1.000	There is no significant difference
	7.8	1.000	There is no significant difference
	8.8	1.000	There is no significant difference
7.8	4.8	0.000	There is a significant difference
	5.8	1.000	There is no significant difference
	6.8	1.000	There is no significant difference
	8.8	1.000	There is no significant difference
8.8	4.8	0.000	There is a significant difference
	5.8	1.000	There is no significant difference
	6.8	1.000	There is no significant difference
	7.8	1.000	There is no significant difference

Based on the Bonferroni test analysis, the p-value < α (0.05) means that there is a significant difference. In Table 4, it is known that not all concentrations have a considerable difference. This is because in some variations, there is a decrease in growth which is not too different, and there are variations in pH where there is no growth of bacterial colonies.

DISCUSSION

Based on Table 1, the number of bacterial cells with an incubation time of 20 minutes to 360 minutes (6 hours) at pH 4.8, no colony growth occurred. The best generation time is when the generation time is shorter and can produce many cells. The fastest generation time and produced the highest number of cells was at pH 6.8, with the lowest number of bacterial cells at 2.1 x 10^4 CFU/mL, the highest at 2.0 x 10^9 CFU/mL, with the fastest generation time at 1.5 minutes, the longest 21.7 minutes. The average generation time is 12 minutes. The results have been adjusted for positive control and negative control. Generation time is good if it has a short generation time but produces many bacterial cells. The growth of bacteria or the generation time of *E. coli* bacteria in the initial phase of the new bacteria adapt themselves to the environment so that the cells have not divided or reproduced perfectly. Cells begin to divide completely in the log or growth phase. Cell division is accelerated because *E. coli* bacteria have a sufficient source of nutrients [12]. The fastest growth rate is found at pH 6.8, where the pH is the optimum pH (6.5-7.5) which is suitable for the growth of *E. coli* bacteria so that the growth of these bacteria is faster. The cells produced are many [18]. pH 7.8 has a slower rate than 6.8 because pH 7.8 is still close to the optimum range with the growth pH of Escherichia coli, so there is a slight decrease in bacterial growth activity at this pH. Followed

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by a pH of 8.8 which is in the range above the maximum pH, which will be close to an alkaline pH so that it can cause a decrease in cell growth. At pH 5.8, there was a significant decrease in the growth and speed of bacterial division compared to pH 6.8; 7.8; and 8.8. Furthermore, at pH 4.8 there is no colony growth. The pH is included in a very low pH to cause denaturation, which will reduce enzyme activity. Some bacteria need enzymes to catalyze reactions related to bacterial growth. As a result of a decrease in the activity of these enzymes, cell growth is also disrupted, so the possibility for the bacteria to grow is tiny [7]. Adequate nutritional factors can accelerate bacteria growth, and a good environment can also accelerate cell growth. However, suppose these bacteria contaminate food and drink or are in an environment where they are not supposed to live. In that case, *E. coli* bacteria can survive and adapt to new conditions well and allow them to be pathogenic to survive [19]. The bacteria will multiply and increase in number rapidly, and the faster they can infect the host and invade other organs [12].

Based on the One Way ANOVA statistical test in Table 3, overall, there is an effect on variations in the pH of the EMBA media. However, based on the Bonferroni test, several pH variations did not have a significant difference except for the pH variation of 4.8 with each pH variation. There was no significant difference between the concentrations of these pH variations because at pH 5.8; 6.8; 7.8; and 8.8, cell growth is still formed, which is not too far away because this pH is still close to the optimum pH and can be tolerated by *E. coli* bacteria to grow compared to pH 4.8 which is acidic according to the theory of Rahayu and friends [2]. pH variation 5.8; 6.8; 7.8; and 8.8 correlates with *E. coli*'s pathogenicity, which infects humans based on the growth rate of bacterial cells to divide. The faster bacterial cells divide, the faster the bacteria can infect humans [15].

CONCLUSION

The conclusion in this study is that the fastest generation time for bacterial cell growth is 1.5-21.7 minutes at pH 6.8. Variation of pH 4.8 no growth occurs. There is a significant effect on the variation of the pH of the EMBA media on the generation time of *E. coli* bacteria with p (0.000) < α (0.05). This research has received approval from the ethical commission of the University of Muhamadiyah Purwokerto with No. KEPK/UMP/68N/2023.

ACKNOWLEDGMENT

We want to thank to Health Polytechnic Ministry of Health Jakarta III for helping to facilitate and infrastructure for this research.

DECLARATIONS

The author declare no conflict of interest.

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