The Growth and Bacteriocin Productions of *Enterococcus Faecium* Cultured in Aerobic and Anaerobic Conditions

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Abstract. Bacteriocins are antimicrobial compounds produced by diverse members of lactic acid bacteria (LAB). Bacteriocins can be used as food bio preservatives to increase the shelf life of food naturally by preventing or killing foodborne pathogens. One of the lactic acid bacteria that produce bacteriocin is *Enterococcus faecium*. Some LAB grown on semi-synthetic complex media such as MRS (de Mann Rogosa Sharpe) can make a high population of bacterial cells and relatively large bacteriocins. This study aimed to observe the growth of *E. faecium* on MRS and LB media under aerobic and anaerobic conditions. Cultures were performed for 4.5h, 5h, 5.5h, 6h, 6.5h, and 7h. The amount of bacteriocin produced was investigated by SDS-PAGE. Meanwhile, the inhibitory activity was measured against *Listeria monocytogenes* (LM). The results showed that *E. faecium* grew better in deMan Rogosa Sharp (MRS) medium under anaerobic conditions than in MRS medium under aerobic conditions as well as Luria Bertani (LB) media under aerobic and anaerobic conditions. The SDS-PAGE results showed a protein band measuring about 90 kDa, which was thought to be a bacteriocin. The inhibition test showed a clear zone in the LM culture, which indicated that the bacteria produced bacteriocins that could inhibit the growth of pathogenic bacteria.

1. Introduction
The shelf life of foodstuff including chicken fillet dramatically determines the price and market areas. Foodstuffs that are easily damaged will experience limitations in storage and marketing. Conversely, foods that have a long shelflife can be stored longer to reach broader market areas. Some factors that can affect the damage to foodstuffs include the growth and activity of microbes, especially bacteria, fat, and yeast, the activity of enzymes in food, insects, parasites, and rats, temperature including heating and cooling, water content, the presence of air including oxygen, light and time [1]. To increase the shelf life, the factors that determine the damage above must be considered. For this reason, various methods have been used to prevent damage in order to extend shelf life while still paying attention to food quality and safety, so as not to cause negative impacts for consumers if consumed [1].

One alternative to extend the storage period of a food ingredient naturally and safer is the use of bacteriocins [2]. Bacteriocins are polypeptides that have the activity of inhibiting the growth or killing of spoilage bacteria and pathogens produced by several microbes. The ability of bacteriocins to kill spoilers microorganisms or food-borne pathogenic bacteria have made bacteriocins a natural preservative (biopreservative) in food systems [3]. The use of bacteriocins in food preservation can reduce the addition of chemical preservatives and heating to produce naturally preserved food and enrich
organoleptic and nutritional benefits [4]. So that it becomes a new alternative to meet the demands of safety, fresh flavor, ready to eat, minimize the preservation process. The excess possessed by bacteriocin as a bio preservative is stable, at a temperature of 100°C for 30 minutes, resistant to processing processes that use acids and bases, and hot and cold temperatures can adapt well to the environment is produced, not toxic and easily degraded by enzymes. Proteolytic, does not change the taste and intestinal microflora, is easily digested by digestive tract enzymes and has a small spectrum of microorganism activity [2, 5]. Sidhu and Nehra (2020) add that bacteriocin produced by lactic acid bacteria is the safest and most effective food preservative to control pathogens in food [6].

Enterococcus faecium is one microbe classified into lactic acid bacteria (BAL) that can produce bacteriocin[2]. The bacteriocin produced by BAL E. faecium can be used as a preservative, especially in processing livestock products such as milk and meat. Efek dari pengawetan disebabkan oleh metabolit yang dihasilkan bakteri tersebut [4-7]. To produce bacteriocin on a particular scale, bacterial culture-making bacteriocin and the study of factors that affect growth and its ability to make bacteriocin is necessary. This study has been done to cultivate E. faecium on de Man Rogosa and Sharpe (MRS) media and Luria Bertani (LB) both in aerobic and anaerobic conditions to determine the growth and activity of bacteriocin produced.

2. Material and Method

2.1. Cultivation of E. faecium

Enterococcus Faecium was inoculated into 6 MRS media tubes and 6 LB media tubes and incubated at 37°C with shakers on aerobic treatment and incubators for anaerobic treatment. Cultivation is done for 4.5h, 5h, 5.5h, 6h, 6.5h, and 7h. Bacterial growth in aerobic and anaerobic conditions is measured using a spectrophotometer at a wavelength of 600 nm.

2.2. Production of Bacteriocin

About 15 g ammonium sulfate (NH4)2SO4 was weighed and dissolved with 15 ml of aquades on a falcon tube. Ammonium sulfate solution is then put into a Beker glass containing 25 ml of BAL culture in MRS media that had been shaken for 24 hours. The culture was homogenized using a hot plate stirrer for 4 hours. After 4h, the culture was inserted into the Falcon tube and concentrated for 15 min at a speed of 4,000rpm. Centrifugation pellets and PBS rinse results on Beker glass are stored in the Eppendorf tube before the following method is carried out. To see the presence of bacteriocin, SDS-PAGE was carried out with the previous method [8].

2.3. Bacteriocin Resistance Test Against Pathogenic Bacteria

To test the bacteriocin activity against pathogenic bacteria was carried out using the diffusion method. A total of 100 μl of L. monocytogenes indicator bacteria are inoculated on solid LB media, then made wells with a diameter of approximately 6 mm to solidify. Wells are made using yellow tips that are rotated on solid LB media according to the given label. At each well, as much as 10μl sample (control positive using ampicillin). The solid LB media is then incubated at a temperature of 37°C for 24 hours. Bacteriocin activity is indicated by the presence of clear zones around the well.

3. Result and Discussion

Enterococcus faecium is a gram-positive lactic acid bacterium that can grow well on MRS media since MRS media is specifically made to grow lactic acid bacteria with a pH of about 5.7. Some of the media often used for culturing lactic acid bacteria are MRS media, Tryptone Glucose yeast Extract (TGE), and Glucose, Yeast Pepton (GYP) [9–11]. It is further explained that some of the nutritional components of MRS media are a tryptic digest of casein, beef extract, yeast extract, glucose, sorbitan monooleate, di-potassium hydrogen orthophosphate, magnesium sulfate, manganese (II) sulfate, ammonium citrate, sodium citrate.

The results of measurements of the density of E. faecium bacteria showed differences in the growth density of bacteria from each treatment. In MRS media and anaerobic conditions, the density of bacterial
cells is obtained the highest, followed by MRS media in aerobic conditions. At the same time, with the use of LB media with anaerobic and aerobic conditions, the growth of \textit{E. faecium} bacteria is slower. The results of these measurements can be seen in Figure 1.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{BAL \textit{E. faecium} Growth Curve on MRS aerobic medium, MRS anaerobic, LB aerobic, and anaerobic LB media}
\end{figure}

These results showed that the growth of BAL \textit{E. faecium} was better in MRS media compared to LB media. This is because the nutritional content of MRS media is complete compared to the nutritional content of LB media. When compared between the growth of these bacteria on the same medium, \textit{E. faecium} bacteria grow best in anaerobic conditions. But these bacteria can still grow in aerobic conditions, although slightly slower when compared to anaerobic conditions.

These results indicate that \textit{E. faecium} bacteria are facultatively anaerobic, which can multiply with or without oxygen. Most lactic acid bacteria are anaerobic facultative, although some are anaerobic obligate such as \textit{Bifidobacteria}. The presence of oxygen in bacterial lactic acid cultures will affect cell growth and produce various metabolite compounds [12]. Medium for BAL growth commonly used, MRS was developed by de Man, Rogossa, dan Sharpe. The medium used for BAL genus \textit{Lactobacillus} and other BAL such as \textit{Streptococcus}, \textit{Pediococcus}, and \textit{Leuconostoc} [9-11].

Bacteriocins are generally produced optimally by BAL in the final phase, i.e., logarithmic phase to the beginning of the stationary phase [13]. Synthesis of bacteriocin was occurred during the exponential phase of cell growth follow the classical pattern of protein synthesis. The synthesis is regulated by plasmid extrachromosomal DNA and is affected by some significant factors such as pH. Generally, bacteriocin is synthesized through ribosomal pathways as short peptides then undergo modifications [14].

Based on the SDS-PAGE results of bacteriocin from the four purification results in Figure 2, a band corresponding to bacteriocin with a size of about 95 kDa in 24 h culture has appeared. While the results of PBS rinse on the walls of baker glass cultured 24 hours showed the presence of protein band tires measuring about 90 kDa.
Figure 2. SDS-PAGE result bacteriocin of *E. faecium* after purification using ammonium sulfate. (M) Protein marker, (S1) 24-h culture, (S2) 24-h culture after centrifugation, (S3) 48-h culture, (S4) 48-h culture after centrifugation.

The results of this study are different from those conducted by Stern et al., (2006), but show similarities to those conducted by Albano et al., (2007) which states that the less clear form of tricine SDS-PAGE is also obtained in studies conducted a separation of peptide bacteriocin HA-5692 from *Pediococcus acidilactici* with precipitation using 60% saturated ammonium sulfate [15, 16].

The assay of bacteriocin activity produced by *E. faecium* bacteria was performed on *Listeria monocytogenes*. This antimicrobial test uses the well-Diffusion Method because the diffuse well has the advantage that all metabolites make by BAL can be produced during the assay [17]. Results of testing of growth inhibition activity on *L. monocytogenes* using bacteriocin purification with ammonium sulfate indicate the presence of a clear zone visible around the well (Figure 3). This suggests that the bacteriocin produced by *E. faecium* bacteria can inhibit the pathogenic bacteria *L. monocytogenes*.

Figure 3. Bacteriocin activity of the *E. faecium* against the bacteria pathogen *L. monocytogenes*, (a) culture for 24 h, (b) culture for 48 h, (c) control (ampicillin).

Inhibition of *E. faecium* lactic acid bacteria is less than optimal compared to the clear zones formed on positive control using antibiotics (ampicillin). This can be due to the smaller number of antimicrobial
compounds produced by *E. faecium* bacteria than the number of antibiotic ampicillin used as positive controls[18]. It takes more bacteriocins than the use of antibiotics[6]. In general, bacteriocins have a broad spectrum of inhibition and are stable over a wide temperature and pH range. The broad spectrum of inhibition of bacteriocins is usually tested against pathogenic Gram-positive and Gram-negative bacteria. *Escherichia coli* is a Gram-negative bacterium, parasitic in the intestines of humans and animals, and many of them are pathogenic [19].

4. Conclusion
Lactic acid *bacteria E. faecium* grows best in MRS media in anaerobic conditions. Electrophoresis results in SDS - PAGE showing the presence of bacteriocin with a size of about 90 kDa. The antimicrobial activity assay of bacteria *E. faecium* indicates a clear zone around the *L. monocytogenes*, which indicates that the lactic acid bacteria *E. faecium* can inhibit pathogenic bacteria.

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