Optimization of the Growth of *Pediococcus pentosaceus* Strain 2397 in Inhibiting Pathogenic *Listeria monocytogenes*

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**Abstract.** *Listeria monocytogenes* (LM) is a pathogenic bacteria that can cause listeriosis in humans, generally transmitted through food. Various food preservatives have been used to prevent contamination from LM in food; one of them is bacteriocin produced by lactic acid bacteria. The purpose of the present study was to determine the optimum growth conditions for *Pediococcus pentosaceus* strain 2397 isolated from dadih in inhibiting LM in vitro assay. The supernatant's antimicrobial activity obtained from *Pediococcus pentosaceus* strain 2397 against LM was determined using a well diffusion method. The results showed that the supernatants from strain R-55 could inhibit LM's growth with various inhibition zones. The optimal growth conditions for *Pediococcus pentosaceus* strain 2397 to perform its antimicrobial activity against LM were at 72 h of incubation time, pH 6.3, and 2.5% of starter concentration with an inhibition zone of 12.3 mm.

1. Introduction

*Listeria monocytogenes* (LM) is a pathogenic Gram-positive bacterium that can grow at temperatures below 5°C and causes food-borne diseases. Diseases caused by these bacteria are, in most cases, mild but can also cause invasive listeriosis with more severe symptoms, high hospitalization, and death in humans. [1]. Invasive infection by LM, like sepsis or meningitis, is most commonly seen in the elderly or patients with underlying diseases that compromise immunity. Listeriosis can also cause abortion, stillbirth, or neonatal infections in pregnant women. The case-fatality rate by LM ranges from 20 to 30% [2][3]. The USA CDC [4] reported that LM infected 11 people, and among them, 10 people were hospitalized, and one person died.

Ready-to-eat meals made from meat products, soft cheeses, and fish, fruit, and vegetable products are often reported as potential vehicles providing a profitable growth matrix for LM [1][3][5][6]. To avoid food-borne diseases, for example, listeriosis, currently many food products that are sold commercially use preservatives, especially chemical preservatives. Natural or synthetic chemical preservatives can be added to food products to prevent unwanted microbial growth or chemical changes. Thus preservatives added to various foods can extend their shelf life. The addition of preservatives to food products is vital to avoid changes and degradation by microorganisms, especially bacteria, yeast, and disease-causing fungi. But keep in mind that chemical preservatives cannot completely keep the product from spoiling but only slow down the damage caused by microorganisms. Frozen and canned foods generally do not need to be added with preservatives because freezing temperatures can inhibit microbial growth. Processed foods are made through a process to kill harmful
bacteria that may be present or contaminate food. This process is expected to prevent and kill microbes in the product, but it can also add harmful substances to the product from the preservatives used [7][8]. Chemical preservatives can cause health problems ranging from allergies and asthma to cancer [9]. Therefore it is necessary to look for safer natural food preservatives, namely bacteriocin or other antimicrobial compounds produced by lactic acid bacteria [10][11][12]. Bacteriocins are generally peptides or ribosomal proteins that are synthesized by bacteria to inhibit or kill certain microorganisms. Thus bacteriocin is a natural preservative that is safe for human consumption. Bacteriocins with a narrow spectrum can only inhibit bacteria closely related, and bacteriocins with a broad spectrum can inhibit various kinds of bacteria and other microbes [13][14].

Dadih is a spontaneously fermented food from West Sumatra, Indonesia, made from buffalo milk [15][16]. Several researchers reported the antimicrobial properties of dadih’s BAL against pathogenic bacteria, namely E. coli, S. aureus, L. monocytogenes, S. typhi, and spoilage bacteria, namely E. carotovora [17][18][19]. This present study aimed to find optimal growth conditions for Pediococcus pentosaceus strain 2397 to inhibit the growth of L. monocytogenes.

2. Materials and Method
2.1. LAB and pathogenic bacteria
LAB used in this study was isolated from dadih and identified as Enterococcus faecalis subsp. liquefaciens R-55 using API CHL 50 test kit [20]. After doing molecular re-identification using DNA sequencing, it was found that this LAB was Pediococcus pentosaceus strain 2397 (LIPI, 2020). The pathogenic bacteria L. monocytogenes was obtained from the Center for Food and Nutrition Research, Universitas Gadjah Mada, Yogyakarta.

2.2. Activation of Pediococcus pentosaceus strain 2397 and pathogenic bacteria
The active culture was made by taking 0.1 mL of the dadih LAB culture in a test tube containing 5 mL of MRS broth ([17]. It was then shaken evenly and incubated at 37°C for 24 hours. The pathogenic bacteria was activated by inoculating 0.1 mL of the test bacteria into 5 mL of the nutrient broth before it was shaken evenly and incubated at 37°C for 24 hours.

2.3. Antimicrobial activity of Pediococcus pentosaceus strain 2397 in the in-vitro test
The supernatant's antimicrobial activity obtained from strain PP2397 was determined using the well diffusion method as described by Melia et al. [22]. Strain PP2397 was grown under various conditions to obtain the optimum growth for this LAB, which could inhibit Listeria monocytogenes. The growth conditions tested included the concentrations of starter strain PP2397, namely 2.5, 5.0, 7.5, and 10.0; variations in the initial pH of the medium, namely control (without setting the pH of the medium) pH 4, 5, 6, 7, 8, 9 and 10.0, as well as variations in the incubation time, namely 12, 24, 36, 48 and 72 hours. The indicator bacterium, L. monocytogenes, was aerobically grown in nutrient broth at 37°C for 24 hours. Following this, 100 μL of pathogenic microorganisms were placed and spread using glass hockey sticks on MRS agar's surface. Cell-free supernatants were obtained from strain PP2397 aerobically grown in MRS Broth as per conditions mentioned above and centrifuged at 10,000 rpm for 5 minutes at 4°C. A 50 μL supernatant was inserted into a well (9 mm) perforated with a blue tip. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of the zone of growth inhibition was measured.

3. Results and Discussion
The incubation time is one of the growth factors determining the biomass and the primary and secondary metabolites formed. The incubation time of P. pentosaceus strain 2397 is presented in Figure 1.
Figure 1. Growth of *Pediococcus pentosaceus* strain 2397 in MRS broth at various incubation times

The growth of strain 2397 increased with incubation time from 12 to 48 hours, which was indicated by an increase in the medium's absorption. This is because the medium's nutrient content was still widely available for energy sources and cell division of strain 2397. The increase in the number of cells in the medium causes an increase in the medium's absorption value. However, the growth of strain 2397 decreased when incubated for 72 hours. This is probably because the amount of nutrients in the medium has decreased, and primary metabolites have also been formed, such as lactic acid, which has accumulated in the medium and inhibited the growth of strain 2397. *L. casei subsp. casei* R-68, LAB, which was also isolated from dadih, grew well on skim milk medium with optimal growth achieved at 15 hours after incubation time, and at 18 - 21 hours showed decreased LAB viability [23]. In kefir manufacturing, the best incubation time was obtained at 16 hours [24]. This difference in optimal growth is due to different growing mediums. During growth, the LAB in a medium will produce lactic acid as the main metabolite product, which causes a decrease in the medium's pH and can inhibit the growth of LAB itself [25].

The antimicrobial activity of the cell-free supernatant of strain 2397 against *Listeria monocytogenes* is presented in Figure 2.

Figure 2. Antimicrobial activity of cell-free supernatant from *Pediococcus pentosaceus* strain 2397 at various incubation times against *Listeria monocytogenes*
The antimicrobial activity at 12 hours of incubation time showed a relatively high inhibition zone of 12.56 mm but decreased at 24 and 36 hours of incubation. Then starting at 48 hours, the antimicrobial activity increased again and reached the highest inhibition zone of 16.11 mm at 72 hours of incubation. This is because, during the incubation period between 48 - 72, the growth of LAB has entered a stationary phase where optimal secondary metabolite is formed in the form of bacteriocins, which inhibit the growth of *L. monocytogenes*. This statement is following previous research, which showed that bacteriocin from strain 2397 was able to inhibit the growth of *L. monocytogenes* [17]. Several researchers have also reported the ability of LAB to inhibit the growth of *L. monocytogenes* [26][6][26].

Figure 3 shows the growth of *P. pentosaceus* strain 2397 at various initial pH of the medium. This LAB was able to grow well in the pH range between 6 to 9, including control, which had pH 6.3, but the growth was not so good at acidic conditions of pH 3 to 5 and alkaline conditions of pH 10. *P. pentosaceus* has a lower optimum temperature for growth (28–32 °C) than *P. acidilactici* (40°C), but the latter grows at 50 °C. The optimum pH for growth is 6.0–6.5. Half of the species grow at pH 4.2, and most of them (except *P. damnosus*) grow at pH 7.0. *Pediococcus pentosaceus* LB44 could demonstrate similar growth at pH 5.0 to 8.0 [28].

**Figure 3.** Growth of *Pediococcus pentosaceus* strain 2397 in various initial pH of MRS broth (Control has a pH of 6.3)

**Figure 4.** Antimicrobial activity of cell-free supernatant from *Pediococcus pentosaceus* strain 2397 at various initial medium pH against *Listeria monocytogenes* (Control has a pH of 6.3)
Although *P. pentosaceus* strain 2397 can grow well in a pH range between 6 to 9, the highest antimicrobials were obtained in the range, especially in controls with a pH of 6.3 with an inhibition zone of 13.67 mm followed by a pH of 6 of 7.8 mm. Beyond this pH, the cell-free supernatant strain 2497 had very little antimicrobial activity against the growth of *L. monocytogenes* (Figure 4). This study's results are somewhat contradictory to the results of research by Kaur et al. [28]. They reported that *P. pentosaceus* LB44 could grow and produce bacteriocin under acidic and alkaline conditions at 37°C.

Figure 5 shows the effect of starter concentration on the growth of *P. pentosaceus* strain 2397 on MRS Broth medium.

![Figure 5. Growth of *Pediococcus pentosaceus* strain 2397 in various starter concentrations](image)

The higher the starter concentration used, the lower the growth of strain 2397. The highest growth of strain 2397 was obtained using a starter concentration of 2.5% and the lowest at 10.0%. This is because the higher the concentration used, the greater the amount of LAB at the beginning of fermentation that competes for nutrients in the medium so that the LAB growth decreases, which is indicated by a decrease in absorbance. In kefir manufacturing, a starter concentration of 10.0% produces the best quality kefir in terms of pH and lactic acid production. The difference in the best concentration depends on the medium used [24]. In this study, the MRS Broth medium was used, while in the manufacture of kefir, milk was used as the growth medium so that there was a difference in the best starter concentration for microbial growth.

An increase in the starter concentration not only decreased the growth of *P. pentosaceus* strain 2397 but also decreased the LAB's antimicrobial activity against *L. monocytogenes* (Figure 6). The highest antimicrobial activity indicated by an inhibition zone of 12.3 mm was obtained when the starter concentration was 2.5%, and the lowest inhibition zone was 4.2 mm at a starter concentration of 10.0%. The decrease in antimicrobial activity against *L. monocytogenes* was due to the growth of strain 2397, which was not optimal, along with the increase in the number of starters used. The optimal growth of strain 2397 in a medium will produce metabolic compounds, especially secondary metabolites such as bacteriocin, which can inhibit the growth of *L. monocytogenes*. Several previous researchers have reported the ability of LAB grown optimally in a medium to inhibit the growth of *L. monocytogenes* [29][30][31][32].
4. Conclusion
The growth of *P. pentosaceus* strain 2397 was influenced by the incubation time, initial pH of the medium, and the concentration of the starter used. These growth factors further influenced the antimicrobial activity of *P. pentosaceus* strain 2397 against *L. monocytogenes*. The supernatants from strain R-55 were able to inhibit the growth of LM with various inhibition zones. The optimal growth conditions for *P. pentosaceus* strain 2397 to perform its antimicrobial activity against *L. monocytogenes* were at 72 h of incubation time, pH 6.3, and 2.5% of starter concentration with an inhibition zone of 12.3 mm.

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