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Improvement of antimicrobial activity of *Pediococcus pentosaceus* strain 2397 in suppressing *Escherichia coli*

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Abstract. *Riftyan E, Yusmarini, Rossi E, Pato U. 2024. Improvement of antimicrobial activity of* Pediococcus pentosaceus *strain 2397 in suppressing* Escherichia coli. *Biodiversitas 25: 2500-2506.* Lactic acid bacteria exhibit antibacterial activity that suppresses spoilage and pathogenic bacteria. Due to their antagonistic action against foodborne pathogens such as *Escherichia coli*, LAB isolated from dairy products have attracted considerable attention as a potential food preservative. Furthermore, bioactive compounds generated by forming LAB biofilms may exhibit efficacy against pathogenic microorganisms and their toxin. Combining LAB biofilms and other LAB products, such as bacteriocins, could offer a significant method against pathogenic microorganisms and their biofilms. The aim of this study was to evaluate the effects of optimization of media composition and incubation conditions on the antimicrobial activities of cell-free supernatant (CFS) from *Pediococcus pentosaceus* strain 2397 to inhibit *E. coli* FNCC-19. The antimicrobial activity of CFS was determined using agar well diffusion method. The results revealed that antimicrobial activity of *P. pentosaceus* strain 2397 was enhanced by optimizing the media composition and incubation conditions. *Pediococcus pentosaceus* strain 2397 exhibited excellent antimicrobial activity against *E. coli*. In MRS, 5% (v/v) of *P. pentosaceus* strain 2397+2.0% (w/v) yeast extract with pH 7.0 showed an inhibition zone of 10.44 mm. Therefore, strain 2397 may be effective in producing bacteriocin as a preservative for food products.

Keywords: Antimicrobial activity, cell-free supernatant, *Escherichia coli*, lactic acid bacteria, *Pediococcus pentosaceus*

INTRODUCTION

Escherichia coli is a rod-shaped, Gram-negative, and facultative anaerobic coliform bacterium. Warm-blooded endotherms typically have *E. coli* in their lower digestive tracts (Mohamed 2019). While the largest group of *E. coli* strains are not harmful, a few serotypes have, in rare instances, been linked to food recalls and can give their hosts severe food poisoning (Torso et al. 2015). *Escherichia coli* may continue to proliferate in fecal matter for up to 48 hours after it has been expelled, and *E. coli* can also survive for extended periods in specific conditions after it has been released into the environment (Barrera et al. 2019). Moreover, *E. coli* promotes the growth and spread of antimicrobial-resistant organisms by producing extended-spectrum beta-lactamase (ESBL), a significant contributor to antimicrobial-resistant infections. The ESBL serves as a model indicator of the distribution of antimicrobial-resistant organisms transmitted via the fecal-oral route due to their frequent detection in fecal waste streams. Treatment of infections induced by ESBL-producing *E. coli* is challenging (Berendes et al. 2020). Because of the recurring outbreaks of microbial diseases, consumers and food safety regulatory authorities worldwide pay close attention to foodborne infections, which have turned into an alarming social challenge. With a case-fatality rate of 3-5%, up to 10% of those with Shiga toxin-producing *E. coli* (STEC) infection can develop hemolytic uremic syndrome (HUS). In general, HUS is the most common cause of acute renal failure in children under five. It can induce neurological issues (such as seizures, strokes, and coma) in up to 25% of HUS patients, as well as persistent renal sequelae in up to 50% of survivors (World Health Organization 2018).

Lactic acid bacteria (LAB) can be utilized as a natural preservative in food processing because they have antimicrobial activity that inhibits spoilage and pathogenic microbes and are usually safe for human health. There are two ways to apply LAB as a bio-preservative: adding LAB culture as an inoculum in food products and using antimicrobial metabolites as natural preservatives for food. Metabolites produced by LAB include H_2O_2, diacetyl, CO_2, acetaldehyde, and bacteriocins (Bharti et al. 2015). Bacteriocins are proteins excreted by bacteria that inhibit spoilage and pathogenic microorganisms (Saeed et al. 2014). While certain bacteriocins exhibit antimicrobial action across a wide variety of genera, others show antimicrobial activity that is restricted to strains related to the producer (Hegarty et al. 2016). Numerous elements control the generation of bacteriocin, i.e., pH, temperature, and growth media (Fernandez et al. 2013; Guinane et al. 2015; Turgis et al. 2016). LAB bacteriocins are typically categorized into three distinct categories (Class I, II, and III). Alvarez-Sieiro et al. (2016) examined a comprehensive collection of 238 LAB genomes made available in public databases. The results indicated the presence of 137 putative bacteriocin-encoding gene clusters in class I, 514 in class II, and 97 in class III. Class II bacteriocins are the most prevalent antimicrobials in LAB. Class IIb bacteriocins, contains the two-peptide

bacteriocins. Approximately equivalent concentrations of each peptide are necessary to exhibit maximum antibacterial activity. The effectiveness of lactococcin G in combating foodborne pathogens, including *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., and *L. monocytogenes*, has significantly expanded the class IIb variety (Yi et al. 2022).

The LAB have been shown to reduce spoiling bacteria and pathogens (Bharal and Sohpal 2013; Lim 2016; Syukur et al. 2014; Zhang et al. 2018). In the previous analysis, *P. pentosaceus* strain 2397 exhibited optimal growth in MRS broth incubated at 37°C for 24 hours with a starting inoculum size of 2.5% (v/v). However, it had relatively low antimicrobial activity against *E. coli*. The findings were remarkably identical to those of prior studies, revealing that the diameter of the clear zone produced by cell-free supernatant (CFS) and crude bacteriocin was only 3.63 and 5.30 mm in diameter, respectively (Pato et al. 2021). Even though *P. pentosaceus* strain 2397 was able to grow well in MRS broth, it was necessary to increase its antimicrobial activity by adjusting the growth conditions of LAB and adding appropriate nutritional sources. Several studies have succeeded in increasing antimicrobial activity and bacteriocin production by modifying LAB growth conditions and adding various nutritional sources such as carbon, nitrogen, and tween sources (Danial et al. 2016; Fahim et al. 2017; Pato et al. 2020; Malheiros et al. 2015; Saraniya and Jeevaratnam 2014). The aim of this study was to evaluate the effects of optimization of media composition and incubation conditions on the antimicrobial activities of cell-free supernatant (CFS) from *P. pentosaceus* strain 2397 to inhibit *E. coli* FNCC-19.

MATERIALS AND METHODS

Culture collection and maintenance

The culture of *P. pentosaceus* strain 2397, was isolated from dadih in Bukittinggi, Indonesia. *Escherichia coli* FNCC-19 was collected from the Gajah Mada University Food and Nutrition Culture Collection (FNCC) in Yogyakarta, Indonesia. Strains 2397 and *E. coli* were activated and cultured in MRS Broth (Merck, Indonesia) and Nutrient Broth (Merck, Indonesia), respectively. MRSB was utilized to activate strain 2397, whereas NB was employed to activate *E. coli* FNCC-19. To produce an active culture, strain 2397 and *E. coli* were placed in 5 mL of suitable medium along with 0.1 mL of culture stock and cultured for 24 hours at 37°C.

Preparation of cell-free supernatant

The active culture of strain 2397 was administered into sterile MRSB and then incubated at 37°C for 12, 24, 36, 48, and 72 hours of incubation times. The production of strain 2397 was then measured at 540 nm using spectrophotometer. Active culture strain 2397 was cultured in 1 L MRSB with a 10% overnight culture inoculum and incubated at 37°C for 24 hours. After incubation, the medium was centrifuged for 15 minutes at 10,000 rpm at 4°C to evaluate the antibacterial activity against *E. coli* (Pato et al. 2020).

Influence of incubation time on the growth and antibacterial activity of strain 2397

Active culture strain of 2397 was added to sterile MRSB and incubated for 12, 24, 36, 48 and 72 hours, at 37°C. After incubation, the growth of strain 2397 CFS against *E. coli* at 540 nm and its antibacterial activity were evaluated.

Influence of different pH on bacterial growth and antibacterial activity of strain 2397

The MRSB solution was prepared in 250 mL Erlenmeyer flasks at various pH levels ranging from 4 to 10 using either HCl or NaOH. Subsequently, the solution was sterilized by autoclaving at a temperature of 121°C for approximately 15 minutes. MRSB was inoculated with *P. pentosaceus* strain 2397 and incubated for 24 hours at 37°C temperature. The growth of strain 2397 was monitored by measuring the absorbance at 540 nm. The antibacterial activity against *E. coli* FNCC-19 was assessed using agar well-diffusion experiment.

Influence of different inoculum concentrations on bacterial growth and antimicrobial activity of strain 2397

Strain 2397 was inoculated into sterile MRSB at different initial concentrations (2.5, 5.5, 7.5, and 10%) and incubated at 37°C for 24 hours. The cell concentration was determined by measuring the absorbance at 540 nm, while the antimicrobial activity of the culture filtrate was examined against *E. coli*.

Influence of medium composition on bacterial growth and antimicrobial activity of strain 2397

MRSB was supplemented with varied concentrations (2 and 4%) of carbon, nitrogen, and tween sources to provide a medium for developing LAB and producing antimicrobial compounds (bacteriocins). Carbon sources included sucrose, lactose, mannitol, glucose, and fructose; nitrogen sources included ammonium sulfate, yeast extract, beef extract, and peptone; and fatty acid sources included tween-20, tween-60, and tween-80. For 15 minutes, the tubes were autoclaved at 121°C. Strain 2397 was used to propagate each vial, which was then subcultured at 37°C for 24 hours. The absorbance of the medium at 540 nm and antibacterial activity of strain 2397 CFS against *E. coli* were subsequently evaluated (Pato et al. 2020).

Antimicrobial activity test of CFS

The evaluation of antimicrobial activity of CFS isolated from strain 2397 was assessed using agar well diffusion method (Sure et al. 2016; Rossi et al. 2021). In order to determine which growth parameters effectively inhibited the growth of *E. coli*, strain 2397 was initially cultivated in MRS-B under different conditions. *E. coli* was subsequently cultured aerobically in NB for 24 hours at 37°C. Following this, 100 L of *E. coli* culture was inoculated into nutrient agar and distributed with a glass spreader. A 50 L of CFS was added to a well with a 9 mm diameter and a sterile blue tip. The inhibitory zones were

measured in diameter after 12, 24, 36, 48, and 72 hours of incubation at 37°C.

Data analysis

The mean and standard deviation of antimicrobial activity data were obtained from three replicates and described descriptively.

RESULTS AND DISCUSSION

Result showed that incubation time affected strain growth and generation of primary and secondary metabolites (Figure 1). The growth of strain 2397 decreased after 48 hours of incubation. In contrast, while growth continued to increase for up to 48 hours after the incubation period, the antimicrobial activity of CFS peaked at 36 hours. After that, antibacterial activity declined drastically until 72 hours of incubation. Between 12 and 24 hours of incubation, *P. pentosaceus* strain 2397 grew extremely slowly and achieved its maximal growth rate after 48 hours. After 72 hours of incubation, the development of strain 2397 significantly slowed down.

This effect results from the inhibition of growth of strain 2397 by the reduced food supply of the medium and primary metabolite chemicals such as accumulated lactic acid. This is in line with a previous study that LAB hydrolyze monosaccharide into lactic acid as the primary product metabolite during development, thus lowering the pH of the medium and inhibiting LAB growth (Rakhmanova et al. 2018). *Lactiplantibacillus plantarum* subsp. *plantarum* grows optimally after 48 hours of incubation (Sure et al. 2016), whereas *L. mesentroides* M8 after 24 hours of incubation (Danial et al. 2016). These results indicate that the ideal growth conditions for each LAB vary according to the genus, species, and strain.

The highest antimicrobial activity was observed after 36 hours of culturing, and it then declined from 48 to 72 hours. During the incubation period between 12 and 24 hours, strain 2397 grew in a static phase during which secondary metabolites such as bacteriocins were generated that were excellent for preventing the growth of *E. coli*. This observation follows a prior study demonstrating that *L. viridescence* NCIM 2167 produced optimum bacteriocins after 48 hours of incubation (Sure et al. 2016). *Lactobacillus brevis* UN isolated from *dulliachar* produced the most bacteriocin during the early stationary phase, inhibiting spoilage microbes and foodborne pathogens such as *L. mesenteroides*, *L. plantarum* subsp. *plantarum*, *C. perfringens*, *L. monocytogenes*, *B. cereus*, and *S. aureus* (Gautam et al. 2014). However, other studies indicate the maximum antimicrobial activity before 48 hours of incubation, precisely 24 hours (Danial et al. 2016) and 30 hours (Fahim et al. 2017).

The difference in optimal incubation time for bacteriocin production may be attributed to the optimal growth conditions of the species and the type of bacteriocin being produced. Strain 2397 thrived well at a range of pH 5-10, including the control pH of 6.3, but in an acidic environment at pH 4-5. Although strain 2397 showed favorable growth at pH values of 5-10, the maximum antimicrobial activity of CFS was observed when strain 2397 was inoculated in MRS with an initial pH of 6-7 (Figure 2).

When *P. pentosaceus* strain 2397 was cultured in MRS broth, it grew at various initial pH values ranging from 5 to 10. Similar results were indicated by Kaur and Tiwari (2017), who found that *P. pentosaceus* LB44 could grow in a pH range of 5.0 to 8.0, with 6.0 to 6.5 being the ideal range. Although strain 2397 did well at pH values between 6 to 10, its antibacterial activity peaked at pH 7.0, with an inhibition zone of 8.66 mm, and its antimicrobial activity was lowest at pH 4. Similar results were reported for *L. mesenteroides*, *L. viridescence* NICM 2167, *E. avium* HF86, *L. casei*, and *L. lactis* subsp. *lactis* by a few studies (Danial et al. 2016; Fahim et al. 2017; Sure et al. 2016; Kumar et al. 2018). The different species of LAB showed comparable optimum pH ranges of 6 to 7 for maximum bacteriocin production, suggesting the most incredible antibacterial action near-neutral pH. According to Dodamani et al. (2013), the optimal pH for *L. garvieae* to generate more activity of bacteriocin is 6. The earlier finding indicated that the ideal pH for the growth of *P. pentosaceus* varied. At acidic and near-neutral pH, *P. pentosaceus* LB44 thrived and exhibited antibacterial activity. In comparison, *P. pentosaceus* LM85 grew and functioned at extremely alkaline, neutral, and acidic pH values (Kaur and Kumar 2017). *Pediococcus pentosaceus* NRC AM1 and AM4 exhibited optimal antimicrobial activity between pH 4.0 and 8.0 (Mabrouk et al. 2015).

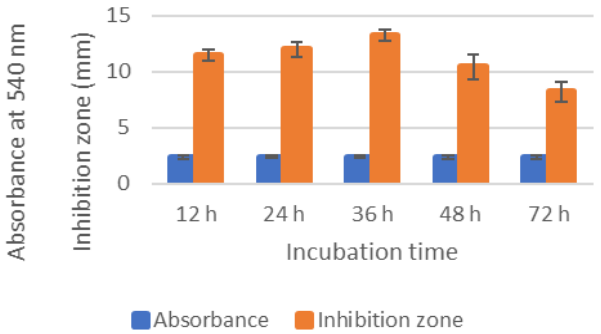

Figure 1. Influence of different time of incubation on *P. pentosaceus* strain 2397 growth and CFS antimicrobial activity against *E. coli*

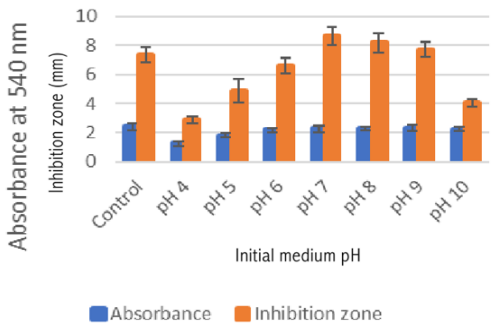

Figure 2. Influence of different pH on *P. pentosaceus* strain 2397 growth and CFS antimicrobial activity against *E. coli*

The ideal proliferation rate of strain 2397 was reached with a starting concentration of 5.0%, slightly lowered from 7.5 to 10.0%. Even though the inoculation of 5.0% culture produced the highest cell concentration, the initial inoculum concentration of 7.5% yielded the highest antimicrobial activity (Figure 3). Strain 2397 proliferated from 2.5 to 5% inoculum concentration, but grew slowly from 7.5 to 10%. This finding may be linked to the slow growth when the inoculum concentration was less than 5.0%. When the inoculum was over 5.0%, the more LAB was present at the start of incubation, the more it competed for nutrients in the medium, resulting in lower proliferation.

Thus, the findings of current study corroborated the results of Danial et al. (2016), who demonstrated that LAB growth increased from 2.5 to 10.0% of the initial inoculum size when the inoculum concentration was raised. Although 5.0% inoculum was optimal for LAB development, a 7.5% inoculum was ideal for the production of antimicrobial activity present in CFS. This observation is consistent with the growth of strain 2397 at 7.5% starting inoculum size, in which it reached the stationary phase earlier than the other conditions, allowing the culture to produce more secondary metabolites, such as bacteriocins. The outcomes align with the maximum antimicrobial activity observed in the static phase of *P. pentosaceus* strain 2397 (Pato et al. 2021).

In general, the addition of carbon, nitrogen, and fatty acid sources had no detectable effect on the development of strain 2397 compared to controls. Nonetheless, its growth was slightly enhanced by the addition of 2 and 4% beef extract, but was slightly inhibited by the addition of 20 to 80% beef extract. Similarly, increasing carbon, nitrogen, and tween sources from 2 to 4% in the source media did not affect the growth of strain 2397 (Figure 4).

MRS broth is an optimal medium for *Lactobacilli* sp., and other LAB promote the development and is especially beneficial for some fastidious strains that do not thrive on other media. MRS broth contains several compounds, such as 1) phosphate, 2) Tween 80, 3) di-/tri-ammonium citrate, 4) sodium acetate, 5) magnesium sulfate tetrahydrate, and others (Merck 2023). MRS broth provides carbon, nitrogen, tween, minerals, and growth factors that aid in the growth of strain 2397. Therefore, adding more carbon, nitrogen, and tween sources to this medium did not produce a noticeable rise in the growth rate of strain 2397. These findings contrast those of Kaur and Kumar (2017), revealing that the addition of 10% glucose and 30% lactose enhanced the optimum growth of *P. pentosaceus* strains LB44 and LM85. Variations in the concentration of the carbon source and the *P. pentosaceus* strains utilized may contribute to the different results of these tests.

Adding nutrients in the form of various carbon, nitrogen, and tween sources enhanced the antimicrobial activity of CFS from strain 2397 by 2 and 4%, respectively, when compared to the control. Carbon sources exhibited less activity than other nutrition sources (Figures 5 and 6).

Although adding carbon, nitrogen, and tween sources could significantly promote the growth of strain 2397, CFS isolated from strain 2397 demonstrated increased antibacterial activity against *E. coli*. It is most likely that monosaccharides and disaccharides supplied to the growth media are used as energy sources to generate bacteriocins, an antibiotic compound. During the growth of *L. lactis* Gh1, fructose significantly affected the production of bacteriocins (Jawan et al. 2020) and *P. pentosaceus* LB44. However, lactose does not impact the production of bacteriocins by *P. pentosaceus* LB44 (Kaur and Kumar 2017).

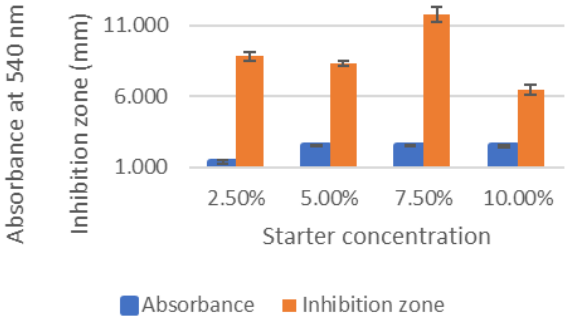

Figure 3. Influence of various inoculum concentrations on *Pediococcus pentosaceus* strain 2397 growth and CFS antimicrobial activity against *E. coli*

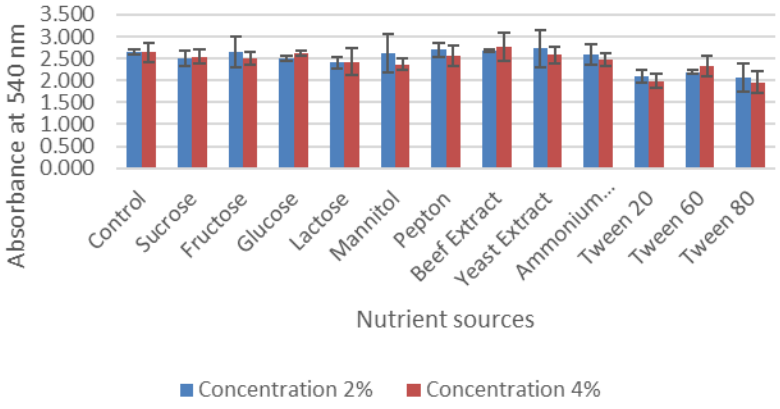

Figure 4. Influence of various nutrient sources on *Pediococcus pentosaceus* strain 2397 growth

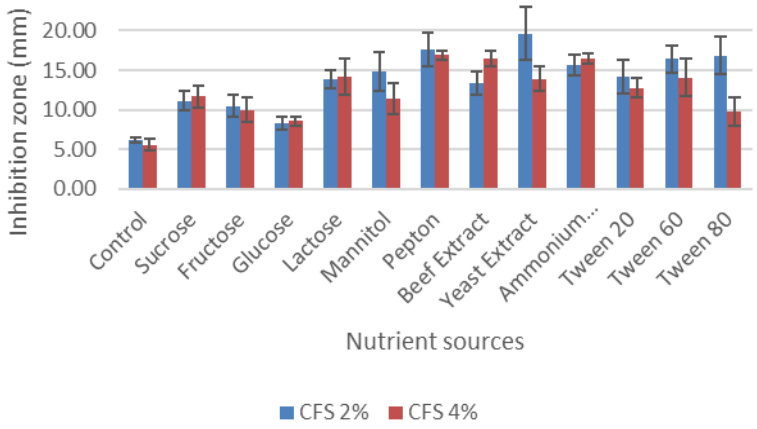

Figure 5. Influence of varied nutrient amounts and sources on the CFS antimicrobial activity of *Pediococcus pentosaceus* strain 2397 against *E. coli*

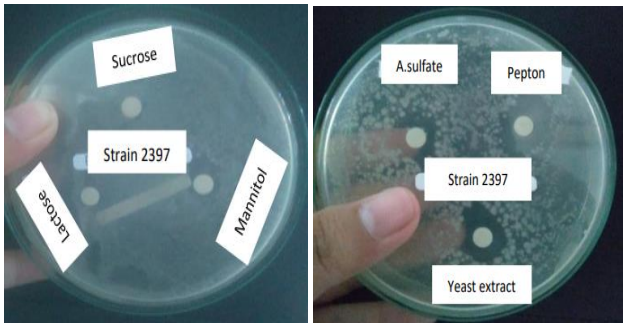

Figure 6. Inhibition zone of CFS (strain 2397) against *E. coli*

In addition, the antibacterial activity of CFS was found to be more effective in a medium containing various nitrogen sources. Bacteriocins were likely to be formed because both organic and inorganic nitrogen sources were present. Nevertheless, the precise regulatory mechanisms that control the biotransformation of bacteriocins remain uncertain. On the other hand, ribosomes of the producing bacteria synthesize all bacteriocins because of their proteinaceous constitution. The required information is extracted from the genetic code often found on plasmids, parental chromosomes, and cellular components, including transposons (Zhang et al. 2022; Polikanov et al. 2018). According to Guinane et al. (2015), Fernandez et al. (2013), and Turgis et al. (2016), synthesis of bacteriocins is controlled by several different parameters, including pH, temperature, and growth conditions. Since ribosomes take part in the process, it is hypothesized that the nitrogen sources added to the medium are used to synthesize bacteriocins. Compared to carbon and tween sources, nitrogen sources such as peptone, beef extract, yeast extract, and ammonium sulfate contributed to relatively significant antimicrobial activity against *E. coli*. This finding suggests that nitrogen derived from different sources is utilized as raw material for producing antimicrobials, particularly bacteriocins. According to Jawan et al. (2020), fructose and various other nitrogen sources are involved in the production of bacteriocins. The addition of 2% yeast extract to the growth medium led to the maximum antimicrobial activity. Fahim et al. (2017) observed that bacteriocins produced from the supplementation of peptone were more effective at inhibiting *L. sakei* LMGT 2313 than those produced from beef extract, yeast extract, or ammonium sulfate.

With the addition of a new source, the inhibition zone became slightly shorter than that of nitrogen, but much broader than that of carbon. Compared to the control (without the addition of nutritional sources), adding multiple types of tweens, namely Tweens 20, 40, and 80, may increase the antibacterial activity of CFS from *P. pentosaceus* strain 2397 against *E. coli*. These findings support Pato et al. (2021) who discovered that the addition of Tween 20 to the growth medium increased the inhibition zone of *L. sakei* subsp. sakei 2a against *L. monocytogenes*. Meanwhile, Tween 80 enhanced the antimicrobial activity of bacteriocins isolated from *L.* subsp. *plantarum* B21 (Parlindungan et al. 2021). However, these findings conflict with Dodamani et al. (2013) who reported that adding Tweens 20 or 80 reduced the antimicrobial activity of *L. garvieae* bacteriocins against various pathogenic bacteria. Moreover, Suresh et al. (2014) observed that Tweens 40 and 80 did not affect bacteriocin activity.

In general, 2% nutritional supplementation produced higher antibacterial activity than the 4%. This may be due to the additional 2% food sources which fulfilled the nutritional needs of strain 2397 for the production of antimicrobial chemicals, namely bacteriocins. The antibacterial activity of bacteriocins from *E. avium* HF86 was enhanced by adding 2% lactose, 1% mannitol, and 2.2% peptone (Fahim et al. 2017). Furthermore, MRS broth with 5.5 g/L glucose is proven effective against *L. sakei* subsp. *sakei* 2a bacteriocins (Malheiros et al. 2015). Similarly, bactericidal activity of CFS was observed in a tween-derived medium. According to previous studies,

adding 1% Tween 40 or Tween 80 significantly increased the bacteriocin activity of *S. haemolyticus* MSM (Suresh et al. 2014). The growth of *L. sakei* subsp. *sakei* 2a in MRS broth containing 1.05% Tween 20 produced bacteriocins with a broad spectrum of action (Malheiros et al. 2015). Conversely, the addition of 1% Tween 80 considerably reduced the bacteriocin activity of *L.* subsp. *plantarum* B21 (Parlindungan et al. 2021). This study showed variations in bacteriocin activity with the addition of different carbon, nitrogen, and tween sources to the growth medium. Carbon, nitrogen, and fatty acid sources added to the growth medium have varying impacts on the accumulation of bacteriocins depending on their concentration.

In conclusion, the antimicrobial activity of *P. pentosaceus* strain 2397 could be increased by optimizing the environmental and nutritional regulation of the growth medium. The highest antimicrobial activity of *P. pentosaceus* strain 2397 against *E. coli* was observed after inoculating 7.5% (v/v) inoculum in MRSB (pH 7.0) supplemented with 2% yeast extract.

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