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Optimization of Bacteriocin Production by *Pediococcus pentosaceus* 2397 in Inhibiting *Pectobacterium carotovorum* subsp. *carotovorum*

Usman Pato[1], Yusmarini Yusuf[1], Shanti Fitriani[1], Tartila[1], Fani Fadilah[1], Latifa Husnaini[1], Rahma Yeni[1], Indra Fuadi[2] and Rachmiwaty Yusuf[3*]

[1]*Department of Agricultural Technology, Faculty of Agriculture, Universitas Riau, Kampus BinaWidya KM 12.5 Simpang Baru, Pekanbaru Indonesia*
[2]*Food Crops and Horticultural Protection Agency of Riau Province, Indonesia*
[3]*Environmental Sciences Study Programme, Universitas Riau, Pekanbaru Indonesia*
[*]*Corresponding author:* rachmi_2608@yahoo.co.id

Abstract

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Soft rot disease by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) is one of the causes of post-harvest damage to economically important vegetables such as carrots, cabbage, cucumbers, and others. The study reported growth optimization to increase the antimicrobial activity of the bacteriocin from *Pediococcus pentosaceus* 2397 to inhibit Pcc. The antimicrobial activity of cell-free supernatant and crude bacteriocin was carried out using the well agar diffusion method. The results showed that the antimicrobial activity of the bacteriocin from strain 2397 could be increased by regulating the growth environment and adding nutrients to the growth medium. The optimum growth conditions for strain 2397 to produce bacteriocins that have high antimicrobial activity were 24 h incubation time, pH 6.3-7.0, and 7.5% starter concentration, the addition of 4% Carbon sources (sucrose, lactose, mannose, fructose, glucose), and Nitrogen sources (peptone, beef extract, yeast extract, ammonium sulfate) and 2% of tween (tween 20, tween 40 and tween 80). Thus, bacteriocin of *P. pentosaceus* 2397 strain isolated from dadih has the potential to be used as a safe bio-preservative in place of chemical preservatives to avoid the post-harvest damage to the fresh vegetables.

Keywords: Antimicrobial activity; *Pediococcus pentosaceus* 2397 *Pectobacterium carotovorum* subsp. *Carotovorum;* bacteriocin; bio-preservative

Introduction

Pathogens and pests cause global crop yield losses of between 20 – 30% (Savary et al., 2019). Flood damage is commonly found in the post-harvest stage, from harvesting, post-harvest handling, storage, processing, distribution to consumption. The Food and Agriculture Organization (FAO) in 2016 estimated that the total production of fresh vegetables reached 1.07 billion tons, and fresh fruits were 865 million tons worldwide. Unfortunately, the loss of vegetables and fruits can reach 50% before reaching the consumers. In addition, fruits and vegetables start to become infected with several types of fungi and bacteria through surface wounds during or after harvest due to poor handling (Elik et al., 2019).

Pectobacterium carotovorum subsp. carotovorum (Pcc), formerly known as *Erwinia carotovora* subsp. *carotovora* is a Gram-negative bacterium that is phytopathogenic, necrotrophic, and opportunistic (Davidsson et al., 2013). Pcc causes soft rot disease in various vegetables such as

carrots, cabbage, cucumber, eggplant, garlic, onions, pepper, potatoes, radishes, sweet potatoes, pumpkins, and tomatoes (Opara & Asuquo, 2016), where the disease can be detected in the field, ship, store, and market. For example, Pcc was reported as the cause of tomato damage during transportation or storage and soft rot and blackleg disease in potatoes (Blancard, 2012). Pcc damages the cell walls of the host plant, then colonizes the intercellular spaces and produces robust molecules. They are known as avirulence effectors (Avr) through the type III secretion system (Aizawa, 2014). *P. carotovora* subs. *atroseptica* is more likely to cause blackleg disease than Pcc, but Pcc is more widespread and grows faster on contaminated potatoes (Slack et al., 1996). Plant protection uses chemical compounds and growth-promoting agents such as pesticides, herbicides, and fertilizers to ensure sufficient and consistent yields. However, many of these chemical products can pose health hazards to humans and the environment, leading to a demand for safer agents (Anand and Sati, 2013; Nishimoto, 2019). A promising alternative agent is microbial-based products that can protect plants from diseases on and off farms. The application of bio-preservation and antimicrobial compounds has been a part of human life since the rise of human civilization. There is sufficient evidence to believe that humans have benefited from the bio-preservation of different fermented food products such as bacteriocins produced by lactic acid bacteria (LAB) (Cleveland et al., 2001; Bharti et al., 2015).

Bacteriocin is an antimicrobial peptide produced by microorganisms. Almost all bacterial species can produce bacteriocins as part of defense molecules with different antimicrobial spectra between genera, species, and even strains. LAB has been used in various foods as a preservative to extend the shelf life of products. The preservative properties of LAB's are based on the resulting antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl, reuterin, antifungal peptides, and bacteriocins (Heredia-Castro et al., 2015).

The bacteriocin produced by LAB is in great demand because of its potential use as a biological preservative in the food industry. The application of bacteriocins in food preservation increases due to their safety and stability in various food system conditions. A previous study showed that bacteriocin from *Pediococcus pentosaceus* 2397 had the highest microbial activity in inhibiting Pcc. However, the results of this study showed a relatively small inhibition zone of only 5.86 mm (Pato et al., 2020). Antimicrobial activity can be enhanced by setting growth conditions such as starter concentration, initial pH of the growth medium, addition of various sources of carbon and nitrogen in LAB growth (Danial et al., 2016; Sure et al., 2016; Fahim et al., 2017; Abbasiliasi et al., 2017). Therefore, the purpose of the present study was to determine the optimal growth conditions of strain 2397 to produce bacteriocin with higher antimicrobial activity to inhibit Pcc.

Materials and Method

Activation of *P. pentosaceus* 2397 and *P. carotovorum* subsp. *carotovorum*

The active culture was prepared by mixing 0.1 ml of *P. pentosaceus* 2397 culture in a test tube containing 5 ml of MRS broth (MRSB) medium, and the mixture was shaken then incubated at 37°C for 24 h. *P. carotovorum* subsp. *carotovorum* was activated by inoculating 0.1 ml of the Pcc sample into 5 ml of the nutrient broth (NB) medium, shaken, and incubated at 37°C for 24 h.

Optimization of production parameters

Effect of incubation time on the growth and antimicrobial activity

MRSB medium was prepared, inoculated with an active culture of strain 2397, and incubated at different incubation times, namely 12, 24, 36, 48, and 72 h at 37°C. After incubation, the growth at 540 nm and antimicrobial activity of strain 2397 cell-free supernatant against Pcc were measured.

Effect of different initial pH on the growth and antimicrobial activity

The MRSB medium was prepared in 250 ml Erlenmeyer flasks at different pH namely control, 4, 5, 6, 7, 8, 9, and 10 with 6 M HCl or 6 M NaOH, and then autoclaved at 121°C for 15 min. Each flask was inoculated with inoculums of strain 2397 and incubated at 37°C for 24 h. Then, the absorbance of the medium at 540 nm was measured, and the antimicrobial activity of strain 2397 cell-free supernatant against Pcc was tested.

Effect of different starter concentrations on the growth and antimicrobial activity

The MRSB medium was prepared and autoclaved at 121°C for 15 min, inoculated with a fresh culture of strain 2397 with different starter concentrations (2.5; 5.5; 7.5 and 10.0%), and incubated at 37°C for 24 h. Then, the absorbance of the medium at 540 nm was measured, and the antimicrobial activity of strain 2397 cell-free supernatant against Pcc was tested.

Effects of medium composition on the growth and antimicrobial activity

The MRSB medium was prepared in 250 ml Erlenmeyer flasks with different concentrations and sources of carbon,

nitrogen, and tween. The sources of C used were sucrose, lactose, mannitol, glucose, and fructose; sources of N were peptone, beef extract, yeast extract, and ammonium sulfate, and sources of fatty acids were tween 20, tween 60, and tween 80. The flasks were then autoclaved at 121°C for 15 min. Next, each flask was inoculated with inoculums of strain 2397 and incubated at 37°C for 24 h. Then, the absorbance of the medium at 540 nm was measured, and the antimicrobial activity of strain 2397 cell-free supernatant and crude bacteriocin against Pcc was tested.

Production of cell-free supernatant and crude bacteriocin

The crude bacteriocin was produced from strains 2397. LAB was propagation in MRS broth (1000 ml) seeded with a 7.5% inoculum of overnight culture before being incubated at 37°C for 24 h. After the incubation period, the whole broth was centrifuged at 10,000 rpm for 15 min, and the cell-free supernatant was collected. This cell-free supernatant was partly used to test its antimicrobial activity and partly used to produce crude bacteriocin. The cell-free supernatant was saturated with 70% ammonium sulfate and stored at 4°C for the precipitation of proteins. The ammonium sulphate-saturated cell-free supernatant was centrifuged at 10,000 rpm at 4°C for 30 min, and the crude bacteriocin was collected and tested for antimicrobial activity (Zhou et al., 2014; Fahim et al., 2017).

***In vitro* antimicrobial activity of cell-free supernatant and crude bacteriocin**

The antimicrobial activity of the cell-free supernatant and crude bacteriocin obtained from strain 2397 was determined using the well diffusion method described by Sure et al. (2016). First, strain 2397 was grown in MRSB medium under various conditions to obtain the optimum growth for this LAB which could inhibit the growth of Pcc. Next, Pcc's indicator bacterium was aerobically grown in NB medium at 37°C for 24 h. Following this, 100 µl of Pcc culture was placed and spread using glass hockey sticks on the surface of MRS agar. Each 50 µl cell-free supernatant or crude bacteriocin was inserted into a well (9 mm) perforated with a blue tip. The plates were incubated at 37°C for 24 h, and the diameter of the inhibition zone of growth was measured.

Results and Discussion

The incubation time is one of the physical growth factors determining the biomass and the primary and secondary metabolites produced. The growth of strain 2397 increased until the incubation period of 48 h marked by an increase in absorbance of the growth medium, after which the growth decreased. On the other hand, although the growth increased up to 48 h of incubation time, the highest antimicrobial activity of cell-free supernatant was obtained at 24 h of incubation time. After that, the antimicrobial activity decreased from 36 – 72 h of incubation time (Figure 1). Strain 2397 grew very slowly from 12 – 24 h of incubation and achieved optimal growth at 48 h of incubation. After that, the growth of strain 2397 decreased drastically at 72 h of incubation. This phenomenon is because nutrient content in the medium has begun to decrease, and there has also been an accumulation of primary metabolite compounds such as lactic acid, which inhibits the growth of strain 2397. This statement follows Rakhmanova et al. (2018) statement that during growth, LAB hydrolyzes simple sugars into lactic acid as the main metabolite product, which causes a decrease in the pH of the medium, which can inhibit the growth of LAB itself. The optimum growth of *Lactobacillus plantarum* was found at 48 h incubation (Sure et al., 2016) and *Leuconostoc mesentroides* M8 at 24 h of incubation (Danial et al., 2016). These data showed that the optimal growth of each LAB varies depending on the genus, species, and strain of LAB.

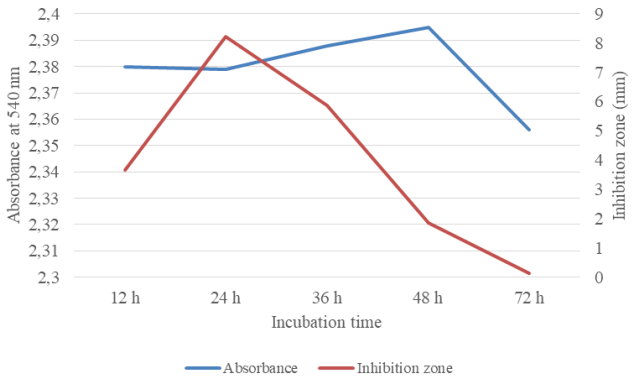

Fig. 1. Effect of incubation times on the growth of *P. pentosaceus* 2397 and antimicrobial activity of cell-free supernatant against *P. carotovorum* subsp. *carotovorum*

The highest antimicrobial activity was obtained at 24 h of incubation and then decreased from 36 to 72 h of the incubation period. This fact may be due to the incubation period between 12 and 24, and LAB growth has been in a static phase where optimal secondary metabolites are formed in the form of bacteriocins that inhibit Pcc's growth. This statement follows previous studies that showed that the bacteriocin from *Leuconostoc mesentroides* was able to inhibit the growth of *K. pneumoniae* ATCC700603 and *P. aeruginosa* ATCC27583 after 24 h incubation period. However, some

researchers reported the highest zone of inhibition was obtained after 24 h of incubation, namely 30 h (Fahim et al., 2017), 48 h (Sure et al., 2016), 72 h (Sharma et al., 2011; Amortegui et al., 2014). Thus, the difference in incubation time concerning antimicrobial activity is most likely due to differences in antimicrobials, especially the bacteriocins produced by different LAB.

P. pentosaceus 2397 could grow well in a pH range between 5 and 10, including control with a pH of 6.3, but the growth was not so good under acidic conditions at pH 4 to 5. Although the growth of this strain was good at pH 5 – 10, the highest antimicrobial activity of cell-free supernatant was obtained at a pH range of 6 – 7 (Figure 2).

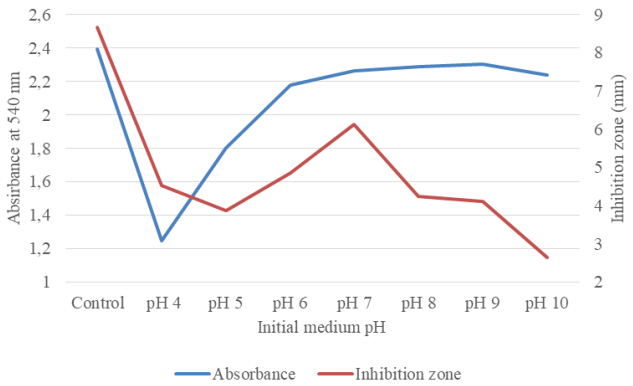

Fig. 2. Effect of various pH on the growth of *P. pentosaceus* 2397 and antimicrobial activity of cell-free supernatant against *P. carotovorum* subsp. *carotovorum*

Strain 2397 can grow in a long-range from 5 to 10. Similar findings were reported by Kaur and Tiwari (2017) that *Pediococcus pentosaceus* LB44 could grow at pH 5.0 to 8.0, with an optimum pH of growth ranging from 6.0 – 6.5. Although *P. pentosaceus* 2397 grew well in a pH range between 6 and 10, the highest antimicrobial was obtained at pH 6.3 (control) with an inhibition zone of 8.67 mm followed by pH 7. Beyond this pH, the zone of inhibition is less than 4.52 mm. Similar results were reported by Danial et al. (2016), Sure et al. (2016), Kumar et al. (2012) and Fahim et al. (2017) using *Leuconostoc mesentroides, Lactobacillus viridescence* NICM 2167, *Lactobacillus casei*, and *Lactococcus lactis* subsp. *lactis* as well as *Enterococcus avium* HF86, respectively. It appears that despite the differences in bacteriocin-producing LAB, they have the same pH range, which shows the highest range of antimicrobial activity around neutral pH. Dodamani & Kaliwal (2014) reported that pH 6 is the ideal pH of *Lactococcus garvieae* to produce higher bacteriocin activity. The previous finding showed variation in the optimal pH for the growth of *P. pentosaceus*. *P. pentosaceus* LB44 could grow and showed antimicrobial activity at acidic and near-neutral pH. In contrast, *P. pentosaceus* LM85 showed growth and activity at acidic, near neutral, and very alkaline pH (Kaur and Tiwari, 2017). *P. pentosaceus* MTCC 5151 showed optimum antimicrobial activity at pH 5.5 (Agrawal and Dharmesh, 2012), and *P. pentosaceus* NRC AM1 and *P. pentosaceus* NRC AM4 pH 4.0 to 8.0 (Mabrouk et al., 2014). Yoghurt with an acidity of 0.70 – 0.80% has a pH of approximately 4.7. *L. acidophilus* in acidophilus yogurt has been proven to suppress the growth of *S. aureus* during fermentation at this pH (Othman et al., 2017).

The optimal growth of strain 2397 was obtained at the addition of 5.0% starter and decreased slightly from 7.5 to 10.0%. Optimal growth was achieved using a 5.0% starter, but the highest antimicrobial activity of cell-free supernatant was obtained when using a starter of 7.5% with an inhibition zone of 13.48 mm (Figure 3).

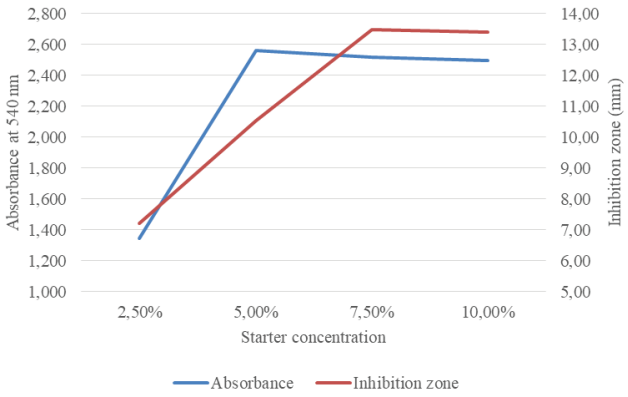

Fig. 3. Effect of various starter concentrations on the growth of *P. pentosaceus* 2397 and antimicrobial activity of cell-free supernatant against *P. carotovorum* subsp. *carotovorum*

The highest growth of strain 2397 increased from starter concentration of 2.5 to 5%, and the growth of strain 2397 had a slight decrease in growth from the starter concentration of 7.5 to 10.0%. This fact may be because if the starter concentration is less than 5.0%, the growth was slow, whereas if it is more than 5.0%, the greater the amount of LAB at the beginning of fermentation, the more LAB is fighting for nutrients in the medium so that the growth decreased. Thus, the present study results were in contraction with Danial et al. (2016), who reported that the higher the amount of inoculum used, the higher the LAB growth from 2.5 to 10.0% of starter number.

Although the optimum LAB growth was found in the use of a 5.0% starter, antimicrobial activity of cell-free supernatant was found in 7.5% of starter. This condition is presumably because, at this concentration, the LAB growth has entered a stationary phase where secondary metabolite compounds such as bacteriocins were formed, which can inhibit the growth of Pcc. The results were similar to the highest antimicrobial activity shown in the stationary phase of *L. bulgaricus* (Kim et al., 2004; Cheigh et al., 2002).

The addition of various carbon, nitrogen, and tween sources generally did not affect the growth of strain 2397 than controls (Figure 4). However, there was a slight increase in growth in the addition of bovine extract (Figure 4A), while the addition of sources, especially tween, slightly decreased the growth of strain 2397 (Figure 4B). Likewise, the use source concentrations of C, N, and tween from 2 to 4% generally did not affect the increase in strain growth 2397.

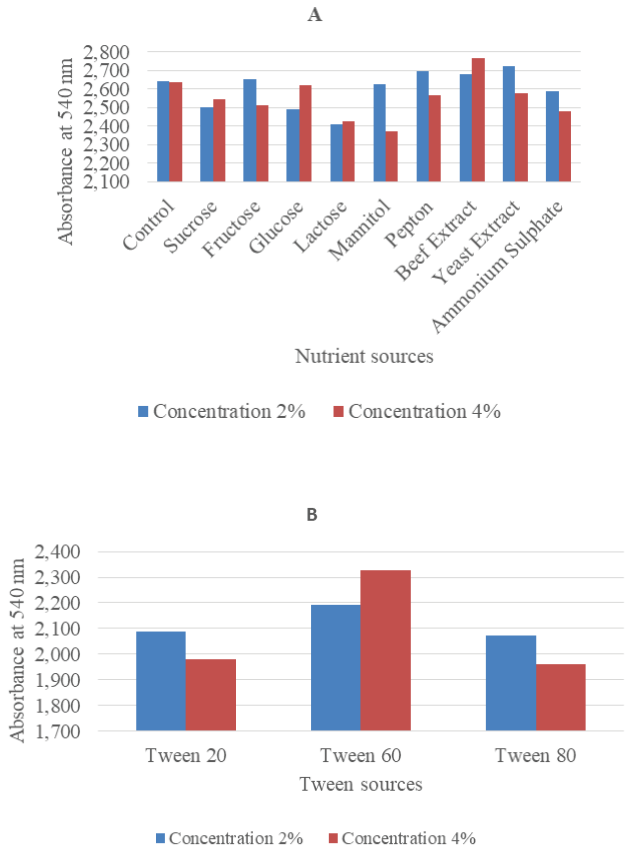

Fig. 4. Effect of various nutrient sources on the growth of *P. pentosaceus* 2397

Several factors can affect the growth of LAB and the formation of metabolite compounds, including the growing environment like pH of the medium, temperature, inoculum, and media composition such as carbon, nitrogen, and tween sources. The highest growth of strain 2397 in control was due to the MRSB medium containing sufficient nutrients to support the growth of LAB in general. MRS Broth is an improved medium for lactobacilli and other LAB to support good growth and is particularly useful for some fastidious strains which grow poorly on other general media. The nutritional content in MRSB includes an enzymatic digest of casein, meat extract, yeast extract, D (+) glucose, di-potassium hydrogen, phosphate, tween 80, di-ammonium citrate, tri-ammonium citrate, sodium acetate, magnesium sulfate heptahydrate, magnesium sulfate monohydrate, magnesium sulfate tetrahydrate (Merck, 2021). Based on this composition, it can be seen that MRS broth already contains sources of C, N, and tween, minerals, a growth factor which are suitable to support the growth of strain 2397. Thus, adding more C, N, and tween sources to this medium generally does not affect the growth of this LAB. The results of this study contradict the research of Kaur and Tiwari (2017), who found an increase in optimal growth of *P. pentosaceus* strains LB44 and LM85 with the addition of 10% glucose and 30% lactose. The difference in the results of these studies may be due to differences in concentration of C source and the *P. pentosaceus* strains used.

The addition of nutrients in the form of various C, N, and tween sources increased the antimicrobial activity of cell-free supernatant and crude bacteriocins from strain 2397 at concentrations of 2 and 4%, respectively, compared to control (without additional nutrients). In general, the addition of various sources of C, N, and tween increased the clear zone in cell-free supernatant and crude bacteriocin at 2 and 4% concentrations. However, source C showed lower activity than the addition of other nutrient sources, except for source C at a concentration of 4%. Thus, the antimicrobial activity with the addition of nutrients was 4% greater than 2% and crude bacteriocin greater than cell-free supernatant (Figure 5). Although the addition of C, N, and tween sources did not increase the growth of strain 2397, it could increase the antimicrobial activity of cell-free supernatant and crude bacteriocin against Pcc. This phenomenon is because monosaccharides and disaccharides added to the growth medium are not used for growth but are most likely used as energy sources to synthesize antimicrobials, especially bacteriocins. This finding is supported by a report that the amount of glucose greatly influenced the bacteriocin synthesis during the growth cycle of *L. mesenteroides* L124 and *L. curvatus* L442 (Mataragas et al., 2004) and *P. pentosaceus* LB44 (Kaur &

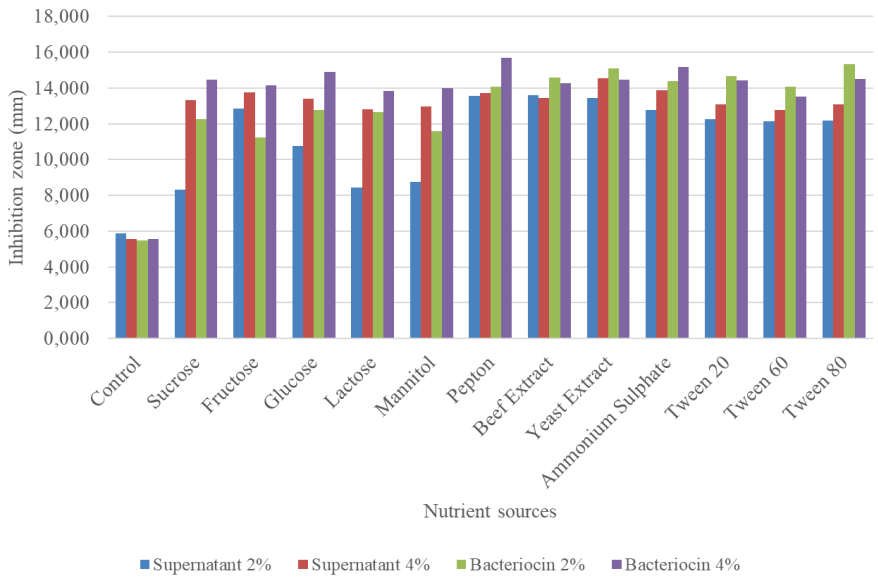

Fig. 5. Effect of various nutrient sources on the antimicrobial activity of cell-free supernatant and crude bacteriocin against *P. carotovorum* subsp. *carotovorum*

Tiwari, 2017), and lactose for *P. pentosaceus* LB44 (Kaur & Tiwari, 2017).

Increased antimicrobial activity of cell-free supernatant and crude bacteriocins were also found in the medium with various N sources. This fact is probably because organic and inorganic N was used to synthesize bacteriocins. The precise regulatory mechanisms for bacteriocin biosynthesis are not fully understood. Due to their protein properties, all bacteriocins are synthesized by ribosomes in producing microorganisms. The necessary information is supplied from the genetic code, which can sometimes be assigned to the plasmid, the parent chromosomes, or cellular elements such as transposons (Cintas et al., 2001; Cleveland et al., 2001; Riley & Wertz, 2002). Because it involves ribosomes, it is suspected that the added N source in the medium was used for bacteriocin synthesis. The addition of N sources such as peptone, beef extract, yeast extract, and ammonium sulfate produced bacteriocins that have relatively the same antimicrobial activity against Pcc. However, Fahim et al. (2017) reported that peptone produces bacteriocins with higher antimicrobial activity than beef extract, yeast extract, and ammonium sulfate in inhibiting *Lactobacillus sakei* LMGT 2313. Mataragas et al. (2004) stated that glucose and several N sources play a role in bacteriocin synthesis.

The addition of various tween types, namely tween, 20, 40, and 80, can increase the antimicrobial properties of cell-free supernatant and crude bacteriocin from strain 2397 against Pcc, although the zone of inhibition is slightly lower than that of the C and N sources. These results are consistent with Malheiros et al. (2015), who reported an increase in the inhibition zone of *Lactobacillus sakei* subsp. *sakei* 2a against *Listeria monocytogenes* with the addition of Tween 20 to the growth medium. The addition of tween 80 was able to increase the antimicrobial properties of bacteriocins from *Lactococcus lactis* subsp. *cremoris* J46 (Huot et al., 1996). These results contradict Dodamani & Kaliwal (2014) findings, who found that the addition of tween 20 or tween 80 decreased the antimicrobial activity of bacteriocins produced by *Lactococcus garvieae* against several types of pathogenic bacteria. Suresh et al. (2014) also reported that bacteriocin activity was not affected by treatment with Tween 40 and Tween 80.

The results showed that the antimicrobial activity of cell-free supernatant was lower than the crude bacteriocin from strain 2397. This fact was because crude bacteriocins were mainly composed of bacteriocins, while cell-free supernatant contained bacteriocins and fluids from the growth medium. C and N sources with a concentration of 4% had higher antimicrobial activity than 2%. The addition of each 2% lactose and mannitol and 2.2% peptone and peptone protease, respectively, increased the antimicrobial activity of the bacteriocin from *E. avium* HF86 (Fahim et al., 2017). MRS broth supplemented with 5.5 g / L glucose showed antimicrobial activity to bacteriocins from *Lactobacillus sakei*

subsp. *sakei* 2a (Malheiros et al., 2015). Similar results were also found for the antimicrobial activity of cell-free supernatant on the medium from various types of tween sources. However, the antimicrobial activity of bacteriocin from a medium with the addition of various sources of tween with the concentration of 2% was higher than the concentration of 4%. Previous studies reported that the use of 1% respectively of tween 40 and tween 80 resulted in high bacteriocin activity of *S.haemolyticus* MSM (Suresh et al., 2014). MRS broth supplemented with 1.05% Tween 20 produced bacteriocins from *Lactobacillus sakei* subsp. *sakei* 2a, which has high antimicrobial activity (Malheiros et al., 2015). In contrast, the use of 1% tween 80 decreased the bacteriocin activity of *Lactococcus lactis* subsp. *cremoris* J46 (Huot et al., 1996). This study showed the differences in bacteriocin activity at various concentrations and types of C, N, and tween sources added to the growth medium. This result may be due to differences in the type of bacteria and the growth medium used.

Conclusion

The results showed that the increase in the antimicrobial activity of the bacteriocin from strain 2397 could be done by regulating the growth environment and adding nutrients to the growth medium. The optimum growth conditions for strain 2397 to produce bacteriocins that have high antimicrobial activity were 24 h incubation time, pH 6.3-7.0 and 7.5% starter concentration, the addition of 4% C sources (sucrose, lactose, mannose, fructose, glucose), and N sources (peptone, beef extract, yeast extract, ammonium sulfate) and 2% source of the tween (tween 20, tween 40 and tween 80).

Acknowledgments

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