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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2022.796.802

Research Article
Partial Purification of Bacteriocin from *Lactobacillus pentosus* Strain 124-2 Isolated from "Dadih"

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Abstract

Background and Objective: Preservation using antimicrobials has been observed to inhibit the growth of pathogenic bacteria in food. Nowadays many people choose food preservatives that are safe for health and natural. Bacteriocins as food preservatives are safe because antimicrobials from the antimicrobial peptide group include GRAS (Generally Recognized As Safe). Bacteriocin-producing LAB can be found in various fermented foods, one of which is "Dadih". Bacteriocins are expected to inhibit the growth of pathogenic bacteria so that they can be developed as an alternative to food preservatives. **Materials and Methods:** In this study, all experiments were performed with two replicates and the results were expressed as Mean±Standard Deviation (SD). **Results:** Screening results showed that the DK8 isolate had the highest antimicrobial activity. The DK8 isolate was identified molecularly using 16s RNA sequencing, showing that the DK8 isolate had the highest similarity to *Lactobacillus pentosus* strain 124-2. Bacteriocins from DK8 isolate and partially purified using ammonium sulfate precipitation at concentrations of 50, 60 and 70%. The addition of ammonium sulfate with a concentration of 50% showed the highest antimicrobial activity against *Salmonella* sp. (12.63 mm) and *Escherichia coli* (11.33 mm) while the highest antimicrobial activity against *Staphylococcus aureus* was the addition of 60% ammonium sulfate (8.13 mm). **Conclusion:** Lactic acid bacteria isolate was identified to have the highest similarity with *Lactobacillus pentosus* strain 124-2 and precipitation using 50% ammonium sulfate showed the highest antimicrobial activity.

Key words: Bacteriocin, Dadih, *Lactobacillus pentosus* strain 124-2, antimicrobial activity, partial purification, lactic acid bacteria, natural preservative

Citation: Yuliana, T., F. Hayati, T. Rialita and Y. Cahyana, 2022. Partial purification of bacteriocin from *Lactobacillus pentosus* strain 124-2 isolated from "Dadih". Pak. J. Biol. Sci., 25: 796-802.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pathogenic bacteria are the cause of many foodborne illnesses. Several preservation methods have been developed to ensure food safety from microbial contamination[1]. In general, the food industry uses various approaches to maintain the shelf life of food products, including the use of food additives or preservatives, which are hazardous[2].

Alternative materials in food preservation are needed due to an increase in consumer awareness of the health hazards caused by food and increasing consumer demand for the availability of chemical-free food[3]. Therefore, the demand for more natural products as well as microbiological products that are safe for consumption is increasing[4] and the food industry is starting to use safer preservatives, one of which is bacteriocin produced by lactic acid bacteria[5].

Bacteriocin is one type of antimicrobial peptide (AMPs) that can inhibit the growth of prokaryotes and have the potential to be used against pathogens and bacterial strains that are resistant to antimicrobials[6] which usually exhibit a high degree of target specificity, which inhibits the growth of closely related bacteria and exhibits a wide range of antimicrobial activity[7]. Bacteriocins as food preservatives are safe to use because antimicrobials from the antimicrobial peptide group include GRAS (Generally Recognized As Safe)[8].

Lactic acid bacteria can be found in fermented foods, either directly or indirectly fermented. One of the traditional Indonesian fermented foods that involve lactic acid fermentation is Dadih. Dadih is typical fermented buffalo milk from West Sumatra, Indonesia[9].

Dadih is a fermented buffalo milk product, where bacteriocin can be extracted from lactic acid bacteria present in Dadih. Bacteriocins belong to a group of antimicrobials that are safe to use. Bacteriocins are expected to inhibit the growth of pathogenic bacteria so that they can be developed as an alternative to food preservatives.

MATERIALS AND METHODS

Study area: This research was conducted in April to September, 2021 at Food Microbiology Laboratory, Faculty of Agro-Industrial Technology, Universitas Padjadjaran, Bandung, Indonesia.

Material: The indicator bacteria used were *E. coli* and *Salmonella* sp. and *S. aureus* obtained from the food microbiology laboratory, Faculty of Agro-Industrial Technology, Universitas Padjadjaran, Bandung, Indonesia.

Screening of bacteriocin-producing lactic acid bacteria: Isolation and qualitative identification of lactic acid bacteria have been carried out in previous studies[10]. Isolation from "Dadih" obtained several LAB isolates, for this reason, it is necessary to select selected LAB for bacteriocin production[11]. Isolation selection was carried out by testing the antimicrobial activity of the neutralized LAB supernatant or cell-free supernatant (CFS). About 10% of lactic acid bacteria cells were inoculated into MRSB medium and then incubated for 18 hrs. Furthermore, the lactic acid bacterial cells were separated by centrifugation (6000 rpm for 10 min at 4°C). Then the supernatant was filtered using a 0.20 µm millipore and neutralized using NaOH to a pH of about 6.5[12]. Cell-free supernatants (CFS) were then tested for antimicrobial activity, isolates with the highest antimicrobial activity were selected and then identified molecularly.

Molecular identification of lactic acid bacteria: The results of the screening were selected isolates that have the highest antimicrobial activity, then selected as isolates producing bacteriocin which will be partially purified at a later stage. Selected isolates were then identified molecularly to see the structure of the DNA sequence using a molecular approach based on PCR analysis which can provide reliable identification results, where 16S rRNA sequencing is the most commonly used for bacteria[13]. For this study, the primers used were 785F and 907R.

The results of the sequencing were analyzed by contiguous coding DNA base sequences. The contiguous sequences were aligned with the GenBank database sequence data using the Basic Local Alignment Search Tool (BLAST) which was integrated with NCBI[14]. The results of the sequencing will also be analyzed to determine the relationship of the isolates which will be displayed in the form of a phylogenetic tree, using the MEGA application.

Extraction and partial purification of bacteriocin: Crude bacteriocins were obtained from the neutralized supernatant or CFS. The crude bacteriocin was then precipitated using ammonium sulfate. Precipitation was carried out using ammonium sulfate with a concentration of 50, 60 and 70%. The purified bacteriocins are stored at 4°C for 24 hrs then a precipitate will form[15].

Antimicrobial activity: The antimicrobial activity of bacteriocins was determined using the paper disk diffusion method based on the size of the inhibition zone formed against the indicator bacteria[16]. The indicator bacteria used in this study were *E. coli*, *Salmonella* sp. and *S. aureus*. About 100 µL of pathogenic bacteria were placed and spread on the

surface of solid MHA media. Sterile disc paper (6 mm) was dipped in the sample to be tested, then the disc paper was placed on the prepared MHA surface. Next, incubation was carried out for 24 hrs at 37°C and the clear zone formed was observed[15].

Statistical analysis: All of the experiments in this research were carried out with two replicates and the results were expressed as Mean±Standard Deviation (SD).

RESULTS

Screening of bacteriocin-producing lactic acid bacteria: In this study, the production of bacteriocins and the testing of their antimicrobial activity will be carried out. For this reason, isolates were selected to determine which isolates would be used as bacteriocin producers. The selection of isolates was carried out by testing the antimicrobial activity of liquid LAB isolates, as well as cell-free supernatants (CFS), which can also be referred to as crude bacteriocins. The results of the observations can be seen in Fig. 1.

The results showed that the isolate with the highest antimicrobial activity was shown by isolate DK8. For this reason, DK8 isolates will then be identified molecularly and selected as isolates for producing bacteriocins.

Molecular identification of lactic acid bacteria: Molecular identification was carried out by 16s rRNA gene sequencing. Based on sequencing analysis, lactic acid bacteria were identified as having the highest similarity to *Lactobacillus pentosus* strain 124-2. The BLAST results from DK8 isolates can be seen in Table 1.

The molecular identification results showed that the DK8 isolate had an e-value of 0 which indicated that the sequence of the DK8 isolate had a high level of homology with *L. pentosus* strain 124-2. The percentage of similarity between DK8 and *L. pentosus* strain 124-2 was high, which was 99.55%. To determine the evolutionary relationship of DK8 isolates, the phylogenetic tree was reconstructed by comparing DK8 isolates with several strains which can be seen in Table 2 and the phylogenetic tree can be seen in Fig. 2.

Based on the phylogenetic tree in Fig. 2, six species have bootstrap values above 70 which indicate a close relationship level, while isolates DK8 and *L. plantarum* (NR_042254.1) show bootstrap values below 70 which indicate that they are more distantly related to each other six other isolates. The location of the DK 8 isolate is in the out-group and has a large distance scale from other species. Based on observations, the DK8 isolate had the highest percentage of *Lactobacillus pentosus* strain 124-2 (99.55%) and had a close relationship with *L. plantarum*.

Extraction and partial purification of bacteriocin: Bacteriocin precipitation, mainly using ammonium sulfate, has been widely used for bacteriocin purification processes. In this study, precipitation was carried out in stages using ammonium sulfate with a concentration of 50, 60 and 70%. Graphs of the antimicrobial activity of crude bacteriocins and ammonium sulfate-precipitated bacteriocins can be seen in Fig. 3.

Table 1: Results of BLAST isolate DK8

Pengamatan	Isolate DK8
Description	*Lactobacillus pentosus* strain 124-2
Max score	1609
Total score	1605
Query coverage	95%
E-value	0
Percent identity	99.55%
Accession length	1519
Accession number	NR_029133.1

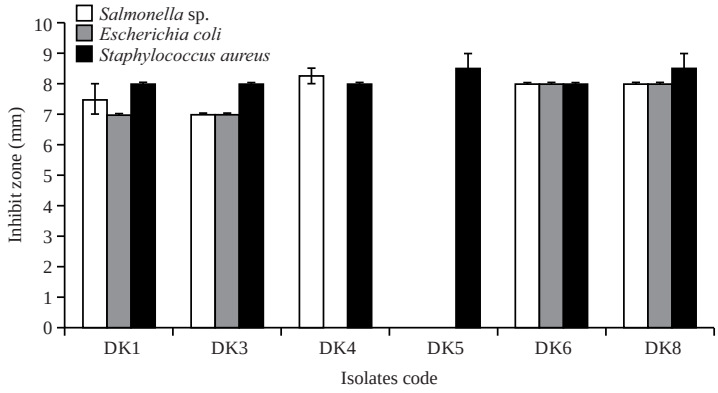

Fig. 1: Diagram of CFS antimicrobial activity against indicator bacteria

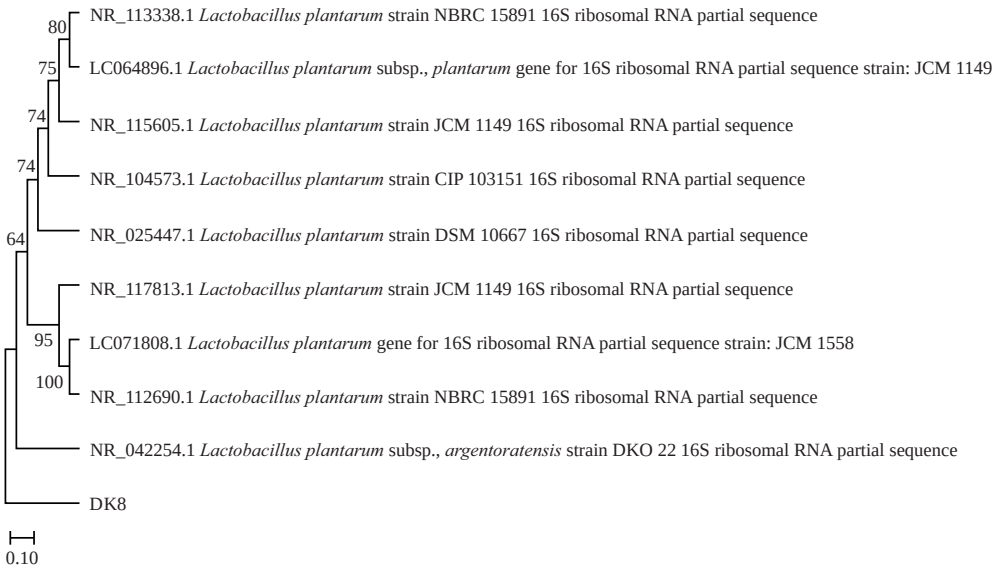

Fig. 2: Phylogenetic tree structure of isolate DK8

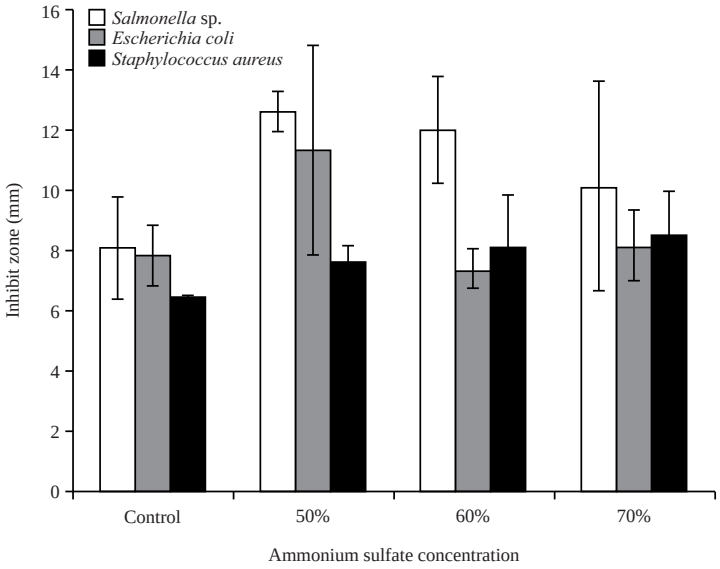

Fig. 3: Diagram of antimicrobial activity of crude bacteriocins and bacteriocins precipitated using ammonium sulphate

Table 2: Comparative sequences of DK8 isolate

Accession number	Description
NR_029133.1	*Lactobacillus pentosus* strain 124-2
LC064896.1	*Lactobacillus plantarum* subsp., *plantarum*, strain JCM 1149
NR_113338.1	*Lactobacillus plantarum* strain NBRC 15891
NR_115605.1	*Lactobacillus plantarum* strain JCM 1149
NR_104573.1	*Lactobacillus plantarum* strain CIP 103151
NR_025447.1	*Lactobacillus paraplantarum* strain DSM 10667
NR_042254.1	*Lactobacillus plantarum* subsp., *argentoratensis* strain DKO 22
NR_117813.1	*Lactobacillus plantarum* strain JCM 1149
NR_112690.1	*Lactobacillus plantarum* strain NBRC 15891
LC071808.1	*Lactobacillus pentosus* strain JCM 1558

The results showed that precipitation with the addition of ammonium sulfate had higher antimicrobial activity when compared to the control (CFS). Increased antimicrobial activity was seen especially in *Salmonella* sp. The addition of ammonium sulfate with a concentration of 50% showed the highest antimicrobial activity against *Salmonella* sp. (12.63 mm) and *E. coli* (11.33 mm) while the highest antimicrobial activity against *S. aureus* was the addition of 60% ammonium sulfate (8.13 mm).

DISCUSSION

The LAB is a Gram-positive bacterium, antimicrobial activity in LAB is due to the presence of various metabolites that act as antimicrobials. The antimicrobial activity of LAB against pathogens is species and strain-dependent. The antimicrobial activity of LAB is mainly due to the production of one or more active metabolites during its growth such as organic acids, hydrogen peroxide and bacteriocins[17]. One of the LAB metabolites is bacteriocin which is an antimicrobial peptide. Antimicrobial peptides produced by Gram-positive bacteria usually have a broad antimicrobial spectrum that can inhibit the growth of both Gram-positive and Gram-negative bacteria[18].

The results showed that the DK8 isolate had the highest antimicrobial activity so the DK8 isolate was selected as a bacteriocin-producing isolate and molecular identification was carried out. Based on observations, isolate DK8 had similarities with the highest percentage of *Lactobacillus pentosus* strain 124-2 (99.55%) and had a close relationship with *L. plantarum*. According to Zanon *et al.*[19], characteristics that distinguish it from *L. plantarum*, namely, *L. pentosus* can produce acid from D-xylose and glycerol.

Lactobacillus pentosus is a rod-shaped bacterium, Gram-positive, non-motile, growing at a temperature of 10-40°C but not at 45°C. Facultative anaerobic heterofermentative facultative producing D- and L-lactic acid. The acid produced from amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, galactose, gentiobiose, gluconate, D-glucose, glycerol, N-acetylglucosamine, lactose, D-mannose, mannitol, maltose, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Acid not produced from amidone, D-arabinose, L-arabitol, adonitol, erythritol, D-fucose, L-fucose, 2-ketogluconate, glycogen, 5-ketogluconate, inositol, inulin, a-methyl-D-mannoside, -methyl xyloside, D-lyxose, L-sorbose, D-tagatose, L-xylose and xylitol. Other properties are not producing catalase and arginine dihydrolase[19]. Certain strains of *L. pentosus* have been shown to have probiotic effects, in addition, the *L. pentosus* IG1 strain has shown bacteriocin activity against spoilage and pathogenic bacteria[20]. A phylogenetic tree was compiled using a neighbour-joining algorithm, bootstrapping was done with 1000 replications[21]. According to van de Peer and Salemi[22], a bootstrap value above 70 indicates relatively more stable data and the grouping is acceptable.

Based on the phylogenetic tree above, it showed that 7 species showed bootstrap values above 70 which indicated a close relationship level, while isolates DK8 and *Lactobacillus plantarum* (NR_042254.1) showed bootstrap values below 70 which indicated that they were more distantly related to the other 7 isolates. The location of DK8 isolates is in the out-group and has a large distance scale from other species. It can be concluded that the DK8 isolate is the ancestor of 9 other species.

Precipitation using ammonium sulfate is the most widely used bacteriocin purification step[23]. Ammonium sulfate is a type of salt that can be used in the salting-out process, which aims to separate easily aggregated proteins from soluble proteins, making it easy to use in the purification of small dissolved proteins[24]. Overall, the antimicrobial activity of bacteriocins in this study showed higher activity against *E. coli* and *Salmonella* sp., bacteria, compared to *S. aureus*.

Bacteriocins are cationic peptides that affect membrane permeability. In general, it is assumed that cationic antimicrobial peptides exert an effect on the electrostatic attraction of the negatively charged phospholipids of the membrane[25]. The effectiveness of bacteriocins can be influenced by the presence of resistant microorganisms, enzymes, the occurrence of reduction and oxidation reactions, as well as interactions with food components (fats, proteins, preservatives and pH)[26]. In addition, the growth phase also affects the total amount of bacteriocin produced. Because lactic acid bacteria start to produce bacteriocins during the initial phase of logarithmic growth and show maximum bacteriocin activity in the stationary phase, there is a positive correlation between the growth rate and the inhibition zone[27]. This is probably due to differences in the total bacteriocin produced at each growth phase.

According to Ahmad *et al.*[28], the cell-free supernatant (CFS) contained 2.721 mg mL^{-1} of bacteriocin (purification fold = 1), while the precipitation with 40% ammonium sulfate contained 1.836 mg mL^{-1} of bacteriocin (purification fold = 3.1). According to Therdtatha *et al.*[29], showed increased antimicrobial activity of crude bacteriocin (from *Lactobacillus salivarius* KL-D4) which was precipitated using 20% ammonium sulfate. Crude bacteriocins had a total protein concentration of 18,600 mg mL^{-1} (purification fold = 1), while those that were precipitated by ammonium sulfate had a total protein concentration of 2,150 mg mL^{-1} (purification fold 34, 57). Further purification of bacteriocins can be carried out to obtain higher purity bacteriocins, ion exchange chromatography is the most commonly used method[6].

CONCLUSION

The results of the selection of antimicrobial activity from liquid isolates and cell-free supernatant (CFS) found that DK8 isolate was the isolate with the highest antimicrobial activity. DK8 isolate was identified molecularly using 16S RNA

sequencing and confirmed to have the closest resemblance to *L. pentosus* strain 124-2. Partially purified bacteriocin using ammonium sulfate precipitation was able to inhibit the most against *Salmonella* sp., then against *E. coli* and the least against *S. aureus*. Precipitation of 50% ammonium sulfate showed the results of the antimicrobial test with the highest activity. The addition of ammonium sulfate with a concentration of 50% showed the highest antimicrobial activity against *Salmonella* sp. (12.63 mm) and *E. coli* (11.33 mm) while the highest antimicrobial activity against *S. aureus* was the addition of 60% ammonium sulfate (8.13 mm).

SIGNIFICANCE STATEMENT

This study discover indigenous lactic acid bacteria isolated from Dadih, identified as *L. pentosus* 124-2 which has potential as bacteriocin-producing bacteria. This study will help the researcher to uncover the novel bacteriocin from lactic acid bacteria indigenous isolated from "Dadih" through characterizing psychochemical properties and abilities as antimicrobials.

ACKNOWLEDGMENT

This work was financially supported by Universitas Padjadjaran Grant Research with grant number (1959/UN6.3.1/PT.00/2021) from the Ministry of Research, Technology and Higher Education, Indonesia.

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