Characterization of bacteriocin and chitinase producing bacterial isolates with broad-spectrum antimicrobial activities

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Abstract

There is a need for more efficient and eco-friendly approaches to overcome increasing microbial infections. Bacteriocins and chitinases from *Bacillus* spp. can be powerful alternatives to conventional antibiotics and antifungal drugs, respectively. The purpose of this study was to assess the inhibitory potential of bacteriocins and chitinase enzymes against multiple resistant bacterial and fungal pathogens. Bacterial isolates were selected by growth on minimal salts medium and after that were morphologically and biochemically characterized. The physiochemical characterization of bacteriocins was carried out. The inhibitory potential of bacteriocins towards six pathogenic bacteria was determined by the well diffusion assay while chitinase activity towards three fungal strains was determined by the dual plate culture assay. Two bacterial strains (WW₂P₁ and WRE₄P₂), out of nine showed inhibition of *K. pneumoniae*, *P. aeruginosa*, *E. coli* and MRSA while WW₄P₂ was positive against *S. typhimurium* and *E. coli* and WRE₁₀P₂ against *P. aeruginosa*, *S. pneumoniae*. Two bacterial isolates (WW₃P₁ and WRE₁₀P₂) were chosen for further study on the basis of their antifungal activities. Of these, WW₃P₁ isolate was more effective against *A. fumigatus* as well as *A. niger*. The proteinaceous nature of the bacteriocins was confirmed by treatment of the crude extract with proteinase K. It was found that the inhibitory activity of strain WW₃P₁ against *E. coli* was highest at 20 °C, and against *S. pneumoniae* it was at 20 °C and pH 10 after treatment with EDTA. Inhibition by strain the WRE₁₀P₂ against *P. aeruginosa* was highest at 20 °C and pH 14. It was found that EDTA increased the inhibitory activity of strain WW₂P₁ against *P. aeruginosa*, *K. pneumoniae* and *E. coli* by 2 ± 0.235 , 3.5 ± 0.288 , 2.5 ± 1.040 times, respectively, of strain WRE₄P₂ against *P. aeruginosa* and *E. coli* by 2.5 ± 0.763 , 2.7 ± 0.5 times, respectively, and of strain WRE₁₀P₂ against *S. pneumoniae* by 3 ± 0.6236 times. The isolates have promising inhibitory activity, which should be further analyzed for the commercial production of antimicrobials.

Article highlights

1. The current study aimed to isolate the microbiome from wheat plant (*Triticum aestivum* L.), to screen for bacteriocin production and to assess its antimicrobial activity against human pathogens.
2. Forty-one phenotypically different bacterial colonies were subjected to bacteriocin purification from which 25 colonies showed positive reactions.
3. These 25 bacterial isolates were screened against six different human bacterial pathogens using the well diffusion method to check the antimicrobial activity. Out of nine bacterial isolates, WW3P1 and WRE10P2 were able to degrade the chitin and utilize it as their sole energy source. Strain WRE4P2 exhibited partial inactivation in its activity against MRSA after treatment with proteinase K.

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1 Introduction

The genus *Bacillus* represents a large group of gram-positive bacteria that belongs to the phylum *Firmicutes*. They are rod-shaped with aerobic or facultative anaerobic metabolic characteristics [1]. They inhabit a large variety of ecological niches: soil, water, air, the surfaces and rhizosphere of plants, the gastrointestinal tract of animals and many extreme environments. A key feature of the *Bacillus* species is its diverse secondary metabolism and its ability to produce a wide variety of structurally different antagonistic substances. Strains of *Bacillus subtilis* have approximately 4–5% of their whole genomes dedicated to synthesis of secondary metabolites, with the capability of producing more than two dozen structurally diverse antimicrobial compounds [2].

The antimicrobial compounds produced by bacteria are of two main categories; the antibiotics and the bacteriocins. Antibiotics are metabolic end products while bacteriocins are proteinaceous substances synthesized by ribosomes [3]. The main difference between antibiotics and bacteriocins is that antibiotics have broad spectrum activity and can inhibit the growth of bacteria across genera even with restricted activity which does not affect the closely related bacterial strains, while bacteriocins commonly show activity against closely related species or the strains of same species [4].

The microbiome residing in plant rhizosphere and biotopes helps them in the struggle for nutrients and the fight against pathogens. One of the methods of fighting pathogens is by the production of bacteriocins. Bacteriocins are synthesized by ribosomes as precursors and undergo post-translational modification for extracellular secretion [5, 6]. Bacteriocins have been reported from both, gram-positive and gram-negative bacteria and are further classified into sub-classes on the basis of heterogeneity, variation in molecular weight, biochemical characteristics and, mode of production and action [7]. They act either as bacteriostatic or bactericidal antimicrobial peptides (AMPs) and possess narrow-spectrum or broad-spectrum activities against different pathogens. Bacteriocins from gram-positive bacteria, e.g. *Bacillus*, can be categorized into four major classes, namely, Class I, II, III, and IV. AMPs have a significant role in the pharmaceutical and food industries [8].

As chitin is the second most abundant biopolymer in nature, it gets recycled this way [7]. There are three glycosyl hydrolase (GH) families of chitinolytic enzymes numbered 18, 19, and 20. Only families 18 and 19 glycosidases are considered as chitinases because they

catalyze chitin polymers and have been demonstrated to suppress several fungal phytopathogens, such as certain pathogenic fungi that are responsible for infections which damage body tissues in immunocompromised individuals [9]. For example, *Aspergillus* causes a serious pulmonary infection in cancer patients called aspergillosis [10, 11]. The *Bacillus* spp. has the ability to divide rapidly and display resistance to high temperature and other unfavorable environmental conditions such as salinity. Consequently, their bacteriocins and chitinases have been widely explored for their role as the agents of disease management [12].

In this study, *Bacillus* spp. from wheat (*Triticum aestivum* L.) rhizosphere were isolated and screened for their ability to produce bacteriocins and chitinases. Antimicrobial activities of the bacterial isolates were determined against pathogenic bacteria and fungi. Following antimicrobial assays, the isolates were subjected to physicochemical characterization for their potential use as antimicrobial agents.

2 Materials and methods

2.1 Sample collection

Three wheat plant (*Triticum aestivum*) samples were taken from a farm field in the Lahore district (Mohlanwal; 31.3695°N, 74.1768°E) following aseptic techniques and were carefully transferred to the Microbiology Research Laboratory. The samples were divided into two parts, i.e., phyllosphere and rhizosphere, and were kept in sterile polyethylene packets at 4 °C.

2.2 Isolation of bacteria from plant samples

For the isolation of phyllosphere bacterial isolates, 3 g of phyllosphere leaves were thoroughly washed and immersed in sterile distilled water for 10 min. The leaves were then crushed in a sterile pestle and mortar using 10 mL of autoclaved 0.85% normal saline solution (NSS). Endophytic bacteria were also isolated from the roots of the wheat plant. Three grams of root were thoroughly rinsed in sterile distilled water and re-suspended in 10 mL of NSS. The sample was completely crushed to a refined slurry in a sterile pestle and mortar, and stored aseptically. Rhizosphere bacterial isolations were performed from 1 g of root-adhered soil suspended in 10 mL of NSS. The

soil sample was incubated at 37 °C for 24 h in a shaking incubator.

Serial dilutions from phyllosphere, rhizosphere, and plant roots were prepared up to 10^{-5} [13]. Following serial dilutions, 100 µL of each dilution were taken and individually spread on Lauria Bertani (LB) and nutrient agar (NA) plates. The plates were sealed and incubated at 37 °C for 24 h. Chitinolytic bacteria were isolated by inoculating 1 mL of previously enriched culture into mineral salt medium (MSM) and keeping it in the shaking incubator for 7 days. For the preparation of colloidal chitin, the protocol of Rodriguez et al. [14] was followed with slight modifications. According to this method 20 g of chitin from crab shell was dissolved in cold concentrated HCl and placed in refrigerator for 24 h. The mixture was filtered through glass wool into a flask containing 2L of chilled ethanol with continuous stirring. The chitin suspension was then centrifuged at 10,000 rpm for 20 min. The chitin pellet was washed several times with distilled water until the pH was neutral.

2.2.1 Code designation to bacterial isolates from plant samples

The bacterial strains were given codes according to the following criteria:

P = plant, P1 = Plant # 1, P2 = Plant # 2, P3 = Plant # 3, W = Wheat, W1 = after 1st wash, W2 = after 2nd wash, W3 = after 3rd wash, W4 = after 4th wash, R = rhizosphere.

2.3 Assessment of the antimicrobial activity of isolates

2.3.1 Antibacterial activity

Isolates were screened for their antibacterial activity against six human pathogenic bacteria (*K. pneumoniae*, *P. aeruginosa*, MRSA (Methicillin resistant *Staphylococcus aureus*), *S. typhimurium*, *E. coli*, and *S. pneumoniae*) through agar well diffusion assay [15]. Identified bacterial pathogens were obtained from microbial culture collection of University of Central Punjab. All bacterial isolates and pathogens were cultivated/grown in 10 mL LB broth and kept overnight in shaking incubator at 37 °C. Following incubation, 100 µL of each pathogen bacterial culture was evenly spread on separate LB agar plates using a sterile spreader. Three 6 mm wells were dug on the LB agar plates using sterile blue tips and 25 µL of each test bacterial culture (1×10^7 cells/mL) was filled in each well. The plates were sealed with parafilm and placed in a static incubator at 37 °C for 24 h. They were observed for clear zones of growth inhibition around the agar wells.

2.3.2 Detection of Antifungal activity

Antifungal activity of bacterial isolates was tested using the dual-culture method. The medium used as a source of nutrition for bacteria and fungi was a combination of 50% potato dextrose agar (PDA) and 50% NA as described by Yanti et al. [5]. Three *Aspergillus* strains, namely, *A. niger*, *A. fumigatus* and, *A. nidulans* were obtained from the Chughtai Lahore laboratory. Pure fungal cultures previously incubated for 5–7 days at 30 °C were used to inoculate test plates. A fungal plug of 5 mm² was cut from the stock plate and placed at one end of the prepared test plates. A single bacterial colony of the test isolate was picked from freshly grown bacterial culture and streaked as a straight line on the other end of agar plate (2 cm apart). The plates were wrapped with parafilm tape and placed in an incubator at 32 °C for 5 days to observe fungal inhibition.

2.4 Identification of bacterial isolates

Those isolates which showed considerable antimicrobial activity were selected for biochemical identification using the QTS-24 bacterial identification kits (DESTO Laboratories, Karachi, Pakistan) and proceeded for standard biochemical assays to characterized Gram negative and Gram positive bacteria [16–18].

2.5 Cytochrome oxidase test

Individual colonies of bacterial isolates were rubbed on CO strips using the QTS-24 kit. Change in the color of the CO strip from white to blue was considered as positive.

2.6 Partial purification of bacteriocins

Bacterial isolates showing antimicrobial activity were subjected to bacteriocin purification using ammonium sulphate precipitation, as described by Pingitore et al. [19]. For bacteriocin production, bacterial isolates were inoculated separately in 100 mL of LB broth at standard culture conditions, for 24 h. After incubation, cells were removed by centrifugation at 10,000 rpm for 10 min and the cell-free supernatant (CFS) was taken, followed by filtration through a syringe filter, with a pore size of 0.45 µm. The filtered supernatant was saturated by adding ammonium sulfate (40–50%) and allowed to settle overnight at 4 °C. The protein precipitates were recovered the following day by centrifugation at 10,000 rpm for 12 min and redissolved in 500 µL of 5X phosphate buffer saline (PBS). The

protein pellet with PBS was considered partially purified bacteriocin and assayed for activity.

2.7 Effect of proteinase K on bacteriocin activity

The proteinase K enzyme was used to confirm the proteinaceous nature of partially purified bacteriocins. Isolated bacteriocins were treated with 1 mg/mL of proteinase K for 2 h at 37 °C and subjected to antibacterial assay against bacterial pathogens [20].

2.8 Physicochemical characteristics of bacteriocins

In order to examine how temperature affects the activity of bacteriocins, they were previously incubated at five different temperatures i.e., 20, 40, 60, 80 and 100 °C. Temperature-treated bacteriocins were used for antibacterial assay and the inhibition zones were measured.

The effect of pH on the activity of bacteriocins was tested according to the protocol of Mandal et al. [21]. Bacteriocins isolated from 100 mL of each bacterial culture were adjusted to different pH (2, 4, 6, 8, 10, 12 and 14) with 1 M HCl and 1 M NaOH respectively, and incubated for 2 h at room temperature. The antibacterial activity of each bacteriocin aliquot was then recorded.

EDTA and SDS were used to analyze the effect of surfactants on the activity of bacteriocins. Partially purified bacteriocins were treated with 1% (v/v) of the surfactants and incubated at room temperature for 2 h, following the determination of antibacterial activity against pathogens [3]. All tests were performed in triplicate and the mean was calculated. Untreated, partially purified bacteriocins of each bacterial isolate were used as controls.

3 Results

3.1 Isolation and selection of bacteria

Forty-one phenotypically different bacterial colonies, which were isolated from rhizosphere, roots, and phyllosphere samples, were subjected to purification, purified bacteriocins showed positive activity for 25 bacterial isolates. Of these 25 isolates, 9 showed inhibition of pathogens, and were selected for detailed characterization. Partially purified bacteriocins from the isolates were analyzed for physicochemical analysis by treating them with proteinase K, surfactants and incubation at different pH and temperatures. All of these 25 isolates were screened against 6 human bacterial pathogens by agar well-diffusion assay.

Out of 9 bacterial isolates, 2, namely WW₃P₁ and WRE₁₀P₂, were able to degrade chitin and utilized chitin as the sole energy source.

3.2 Morphological and biochemical characteristics of bacterial isolates

3.2.1 Morphology and biochemical tests

Figures 1 and 2 illustrates morphologically distinct purified bacterial isolates. Biochemical characterization of bacteria was based on the QTS-24 identification kits as shown in Fig. 2. Table 1 gives an outline for results of all biochemical tests and preliminary bacterial identification.

Fig. 1 Growth of bacterial isolates on MSM Agar plates

Fig. 2 Biochemical identification of bacterial strain WW₂P₁ by using QTS-24 identification kit

3.2.2 Cytochrome oxidase test

All bacterial isolates shown negative results for cytochrome oxidase production when rubbed on CO test strips provided with QTS-24 bacterial identification kits.

3.3 Partial purification of bacteriocins and evaluation of antibacterial activity

The antibacterial activity of partially purified bacteriocins from ammonium sulfate precipitation of the 25 isolates was assessed against six different human bacterial pathogens. Nine isolates showed positive results while the remaining 16 did not show any antibacterial activity towards bacterial pathogens. Of the 9 isolates, WW₂P₁ and WRE₄P₂, inhibited three pathogens: WW₂P₁ suppressed *K. pneumoniae*, *P. aeruginosa* and *E. coli* while WRE₄P₂ was showed considerable inhibition of *P. aeruginosa*, MRSA and *E. coli*. Isolates that were active against 2 pathogens were WW₄P₂ which was active against *S. typhimurium* and *E. coli*, and WRE₁₀P₂ showing inhibition of *P. aeruginosa* and *S. pneumoniae*. The remaining 5 isolates showed inhibition of only one pathogen. All of the tests were performed in triplicates and standard errors were calculated. Table 2 summarizes the zones of inhibition and antibacterial activities of rhizosphere bacterial isolates.

3.3.1 Evaluation of antifungal activity

When tested against fungal pathogens, isolate WW₃P₁ inhibited the growth of *A. fumigatus* as well as *A. niger*, whereas, isolate WRE₁₀P₂ was most effective against *A. fumigatus*. Both isolates showed inhibition against *A. nidulans*. The inhibition zones were visible and different interaction patterns were observed. Control plates showed considerable increased growth of the fungal cultures, however, rhizosphere bacterial isolates in test culture plates showed considerable inhibition of fungal mycelia (Fig. 3).

3.4 Physicochemical analysis of bacteriocins

3.4.1 Effect of proteinase K on bacteriocin activity

Proteinase K-treated bacteriocins demonstrated a considerable reduction in antibacterial activities as compared to the results of controls in which bacteriocins without proteinase K treatment were added. Strain WRE₄P₂ exhibited partial inactivation against MRSA (1.2 ± 0.5 mm) after treatment with proteinase K (Control value: 3 ± 0.29 mm). Furthermore, WW₂P₁, WW₃P₁, WW₄P₁, WW₁₁P₁, WW₄P₂, WRE₅P₂, and WRE₁₀P₂ strains manifested complete inactivation in their antibacterial potential against various pathogenic strains, which confirms their proteinaceous characteristics (Table 3, Fig. 4).

3.4.2 Effect of surfactants

The effect of the surfactants SDS and EDTA on the activity of bacteriocins was determined against different pathogens and compared with controls by noting the zone of inhibition around the wells. The results in Table 4 clearly indicate that EDTA increased the antimicrobial activity of WW₂P₁ against *P. aeruginosa*, *K. pneumonia* and *E. coli*. WRE₄P₂ showed remarkable activity against *P. aeruginosa* and *E. coli*. WRE₁₀P₂ was active against *S. pneumoniae*. SDS showed a slight increase in the activity of bacteriocins of only two isolates, WW₂P₁ against *K. pneumonia* and WRE₄P₂, against *E. coli* (Table 4).

3.4.3 Effect of temperature

The present study found substantial stability of bacteriocins against bacterial pathogens at the temperature range of 20–80 °C. Against *P. aeruginosa*, maximum antimicrobial activity was shown by WW₂P₁ and WRE₄P₂ at 80 °C, and by WW₄P₁ and WRE₅P₂ at 20 °C. Bacteriocins from WW₂P₁, WW₄P₂, WRE₁₀P₂, WW₁₁P₁ and WRE₆P₂, indicated highest antimicrobial activity at 20 °C against *K. pneumonia*,

Table 1 Biochemical identification of bacterial strains based on QTS-24 bacterial identification kits

Sr. No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Identification
Bacterial Isolates	ONPG	CIT	MALO	LDC	ADH	ODC	H ₂ S	UREA	TDA	IND	VP	GEL	GLU	NO ₃ /N ₂	MALT	SUC	MANN	ARAB	RHAM	SORB	INOS	ADO	MEL	RAF	CO	
WW ₂ P ₁	+	-	+	-	-	-	+	+	+	-	-	+	+	-/+	+	+	+	+	+	+	+	+	+	+	-	<i>Proteus vulgaris</i>
WW ₃ P ₁	+	+	+	-	-	+	+	+	+	-	+	+	+	-/+	+	+	+	+	+	+	+	+	+	+	-	<i>Bacillus sp.</i>
WW ₄ P ₂	+	-	+	-	+	-	-	+	-	-	+	+	+	-/+	+	+	+	+	+	-	-	-	+	-	-	<i>Burkholderia cepacia</i>
WRE ₁₀ P ₂	+	-	-	-	-	-	-	-	-	-	-	+	+	-/+	+	+	+	+	+	+	+	+	+	-	-	<i>Bacillus sp.</i>

(+): production, (-): no production

Symbols: ortho-nitrophenyl-β-galactosidase (ONPG), sodium citrate (CIT), malonate (MALO), lysine decarboxylase (LDC), arginine dihydrolase (ADH), ornithine decarboxylase (ODC), hydrogen sulfide (H₂S), urease (UREA), tryptophan deaminase (TDA), indole (IND), voges-proskauer (VP), gelatin hydrolysis (GEL), nitrate reduction (NO₃), nitrogen gas production (N₂), glucose (GLU), maltose (MALT), sucrose (SUC), mannitol (MANN), arabinose (ARAB), rhamnose (RHAM), sorbitol (SORB), inositol (INOS), adonitol (ADO), melibiose (MEL), raffinose (RAF) and cytochrome oxidase (CO)

S. typhimurium, *S. pneumoniae*, *P. aeruginosa* and MRSA, respectively. In case of *E. coli*, the highest antimicrobial activity was shown by WW₂P₁ at 60 and 80 °C, by WW₃P₁ and WW₄P₂ at 20 °C and by WRE₄P₂ at 60 °C (Table 5).

3.4.4 Effect of pH

The influence of pH ranging from 2 to 14 on the activity of purified bacteriocins against various pathogens was observed by calculating their zone of inhibition. Bacterial strains WW₂P₁, WW₄P₁, WRE₄P₂ and WRE₅P₂ showed maximum stability against *P. aeruginosa* at pH 10, 14, and 2 respectively. Against *E. coli*, maximum stability was observed by bacterial strains WW₂P₁ and WW₄P₂ at pH 14, by WW₃P₁ at pH 4 and by WRE₄P₂ at pH 2. In case of MRSA, WW₁₁P₁, WRE₄P₂ and WRE₆P₂ showed maximum stability at pH 4, 12 and 2 respectively. Bacteriocins from WW₂P₁ had high stability against *K. pneumonia* at both pH 10 and 14. The maximum stability of WW₄P₂ against *S. typhimurium* and WRE₁₀P₂ against *S. pneumoniae* and *P. aeruginosa* was at pH 2, 10 and 14, respectively (Table 6, Fig. 5).

4 Discussion

Appropriate alternatives to conventional antibiotics which are safer, cheaper, biodegradable and easily administered is the focus of much current research [22]. This study was aimed at isolating the bacterial strains which could synthesize both chitinase and bacteriocins, that could hinder fungal and bacterial growth, respectively. The antibacterial and antifungal potential of isolates was determined through agar well diffusion and dual plate culture assay, respectively. The isolates were screened for their antimicrobial activities against six validated bacterial and three fungal pathogens affecting humans.

Of the 41 phenotypically different bacterial isolates of wheat, 25 showed the production of bacteriocins. These 25 isolates were screened against six human bacterial pathogens by agar-well diffusion method. In total, 9 bacterial isolates of wheat showed considerable suppression of bacterial pathogens and further evaluated under treatment of different physicochemical parameters such as treatment with proteinase K, temperature, pH and surfactants. Upon treatment with proteinase K, significant reduction in antibacterial activities of wheat rhizobacteria was observed confirming the proteinaceous nature of bacteriocins. This reduction has been demonstrated by several bacteriocin producing bacteria previously where treatment with proteinase K has decreased their antibacterial potential [23, 24].

Parameters like the effect of temperature, pH and surfactants on the activity of bacteriocins against pathogens

Table 2 Screening of purified bacteriocins for antibacterial activity

Bacterial strains	Pathogens					
	<i>E. coli</i>	<i>K. pneumoniae</i>	MRSA	<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>S. pneumoniae</i>
WW ₂ P ₁	2.33 ± 0.29	1.67 ± 0.29	—	3.17 ± 0.29	—	—
WW ₃ P ₁	2 ± 0.5	—	—	—	—	—
WW ₄ P ₁	—	—	—	3.5	—	—
WW ₁₁ P ₁	—	—	1.17 ± 0.29	—	—	—
WW ₄ P ₂	1.67 ± 0.29	—	—	—	1.67 ± 0.29	—
WRE ₄ P ₂	2.83 ± 0.29	—	2 ± 0.5	3.17 ± 0.29	—	—
WRE ₅ P ₂	—	—	—	2.5	—	—
WRE ₆ P ₂	—	—	3.5	—	—	—
WRE ₁₀ P ₂	—	—	—	2.17 ± 0.29	—	1.67 ± 0.29

*Numbers in the table indicate zones in mm. MRSA: Methicillin-resistant *Staphylococcus aureus*, (—): no activity

Fig. 3 Inhibitory action of isolates towards *Aspergillus* strains

were also demonstrated. Bacteriocin activity was greatly affected by changes in temperature and pH, and the presence of surfactants [8, 25, 26]. These results are in accordance with previously reported literature where bacteriocins were shown to be influenced by these parameters. For example, bacteriocin ST712BZ had maximum production when incubated at 30 °C instead of 37 °C [3]. For the assessment of heat stability of purified bacteriocins, bacteriocins were subjected to different temperatures followed by the assay of their antibacterial potential. Bacteriocins from WW₃P₁, WW₄P₁, WW₁₁P₁, WW₄P₂, WRE₅P₂, WRE₆P₂ and WRE₁₀P₂ were active against different pathogens at 20 °C. Whereas, bacteriocins from isolates WW₂P₁ and WRE₄P₂ were most stable at 60 and 80 °C against different

pathogens. Bacteriocin activity was greatly reduced above 80 °C.

Knowledge of the stability of bacteriocins resulting from pH alteration has been used for the preservation of various fermented foods [3]. Sharma et al. (2018) reported that bacteriocins named LR₆ and LD₃ were stable at pH 2 to 6 [27]. WW₂P₁, WW₄P₁, WRE₄P₂ and WRE₅P₂ showed maximum stability at pH 10, 14, and 2, respectively, and showed significant inhibition of *P. aeruginosa*. Against *E. coli*, maximum stability was observed by bacteriocins isolated from bacterial strains WW₂P₁ and WW₄P₂ at pH 14, by WW₃P₁ at pH 4 and by WRE₄P₂ at pH 2. In case of MRSA, WW₁₁P₁, WRE₄P₂ and WRE₆P₂ bacteriocins showed maximum stability at pH 4, 12 and 2, respectively. Bacteriocins from WW₂P₁

Table 3 Effect of proteinase K on bacteriocins activity

Bacterial strains	Pathogens	Control	Enzyme (Proteinase K)
	mm		
WW ₂ P ₁	<i>P. aeruginosa</i>	2.33 ± 0.29	—
	<i>K. pneumoniae</i>	1.67 ± 0.29	—
	<i>E. coli</i>	3.17 ± 0.29	—
WW ₃ P ₁	<i>E. coli</i>	2 ± 0.5	—
WW ₄ P ₁	<i>P. aeruginosa</i>	3.5	—
WW ₁₁ P ₁	MRSA	1.17 ± 0.29	—
WW ₄ P ₂	<i>S. typhimurium</i>	1.67 ± 0.29	—
	<i>E. coli</i>	1.67 ± 0.29	—
WRE ₄ P ₂	<i>P. aeruginosa</i>	3.17 ± 0.29	—
	MRSA	3 ± 0.29	1.2 ± 0.5
	<i>E. coli</i>	2.83 ± 0.29	—
WRE ₅ P ₂	<i>P. aeruginosa</i>	2.5 ± 0.29	—
WRE ₆ P ₂	MRSA	3.5 ± 0.5	—
WRE ₁₀ P ₂	<i>S. pneumoniae</i>	1.67 ± 0.29	—
	<i>P. aeruginosa</i>	2.17 ± 0.29	—

(-): no activity

had high stability against *K. pneumoniae* at both pH 10 and 14. The maximum stability of WW₄P₂ against *S. typhimurium* and WRE₁₀P₂ against *S. pneumoniae* and *P. aeruginosa* was at pH 2, 10 and 14 respectively.

Deraz et al. [23] have reported that treatment of bacteriocins with reagents like EDTA or SDS enhanced their inhibitory activity on account of proteins getting unfolded, which then directly influences their antimicrobial activity [23]. In this study, two of the most common detergents i.e., SDS and EDTA were used at certain specified concentrations to determine their effect against different pathogens. Results demonstrated that SDS caused decreased bacteriocin activity. However, EDTA treatment increased the bacteriocin activity of WW₂P₁ against *P. aeruginosa*, *K. pneumoniae* and *E. coli*, of WRE₄P₂ against *P. aeruginosa* and *E. coli* and of WRE₁₀P₂ against *S. pneumoniae*.

Studies have shown that use of bacteriocins in food preservation is safe and possible [20]. Being cheaper and safer, they can also be used as an alternative to antibiotics and have the advantage of overcoming the problem of bacterial resistance to antibiotics, which is a growing problem [8]. More research is needed on ways to manipulate bacteriocins through genetic and metabolic engineering to enhance their potency.

Fig. 4 Effect of proteinase K on bacteriocins activity

Table 4 Effect of surfactants on bacteriocins activity

Bacterial strains	Pathogens	Control	Detergents	
			SDS	EDTA
			mm	
WW ₂ P ₁	<i>P. aeruginosa</i>	1.87 ± 0.5	1 ± 0.5	2 ± 0.235
	<i>K. pneumoniae</i>	1.5 ± 0.763	2 ± 0.408	3.5 ± 0.288
	<i>E. coli</i>	2 ± 0.408	1.23 ± 0.5	2.5 ± 1.040
WW ₃ P ₁	<i>E. coli</i>	2 ± 0.5	–	1.87 ± 0.408
WW ₄ P ₁	<i>P. aeruginosa</i>	3.5 ± 0.408	–	3 ± 0.5
WW ₁₁ P ₁	MRSA	2 ± 0.408	1 ± 0.5	1.5
WW ₄ P ₂	<i>S. typhimurium</i>	2.7 ± 0.408	2.1 ± 0.5	2.57 ± 0.235
	<i>E. coli</i>	1.57 ± 0.408	–	1.57 ± 0.5
	<i>P. aeruginosa</i>	2.4 ± 0.408	1.5 ± 0.5	2.5 ± 0.763
WRE ₄ P ₂	MRSA	2.5 ± 0.408	1.87 ± 0.763	2.5
	<i>E. coli</i>	1.5 ± 0.763	1.67 ± 0.5	2.7 ± 0.5
	<i>P. aeruginosa</i>	2 ± 0.6236	–	1.5 ± 0.5
WRE ₅ P ₂	MRSA	2.5 ± 0.408	–	1.2 ± 0.5
WRE ₁₀ P ₂	<i>S. pneumoniae</i>	1.89 ± 0.408	1.5 ± 0.5	3 ± 0.6236
	<i>P. aeruginosa</i>	2 ± 0.288	1 ± 0.763	1.5 ± 0.5

(-): no activity

Chitinases produced by bacteria degrade the chitin which is an important ingredient of fungal cell wall and lead to fungal growth inhibition. Out of forty-one wheat bacterial isolates two *Bacillus* isolates showed significant fungal inhibition of *Aspergillus* species. Similar results were reported by Rao and coworkers [28].

WW₃P₁ and WRE₁₀P₂ bacterial isolates showed different inhibitory patterns towards selected *Aspergillus* spp. strains. According to this study, WW₃P₁ showed maximum activity against *Aspergillus fumigatus* followed by *Aspergillus niger*, whereas, WW₃P₁ showed good activity against *Aspergillus fumigatus*. *Bacillus* spp. were also tested against *Aspergillus nodulans*, where both exhibited a moderate inhibition of fungal growth. Similarly, a study reported that *Bacillus solani* inhibited the growth of different fungal plant pathogens, giving a promising outlook for the use of the genus *Bacillus* as bio-control agents against fungal plant pathogens [29].

A more convenient and natural alternative to antibiotics and antifungal drugs is possible. The promising results from our research are a step forward to the future application of bacteriocin and chitinase antimicrobials in medical and agricultural industry.

Table 5 Effect of temperature on bacteriocins activity

Bacterial strains	Pathogens	Temperature				
		20 °C	40 °C	60 °C	80 °C	100 °C
		mm				
WW ₂ P ₁	<i>P. aeruginosa</i>	2 ± 0.235	1.5 ± 0.5	1.87 ± 0.408	5 ± 0.288	1 ± 0.623
	<i>K. pneumoniae</i>	2.5 ± 0.408	1.92 ± 0.763	1.5	1.5 ± 0.5	–
	<i>E. coli</i>	2 ± 0.235	2 ± 0.5	2.5 ± 0.623	2.5 ± 0.408	–
WW ₃ P ₁	<i>E. coli</i>	2 ± 0.5	1.5 ± 0.763	1.5 ± 0.408	–	–
WW ₄ P ₁	<i>P. aeruginosa</i>	3.5 ± 0.288	2.5 ± 0.235	2 ± 0.408	1.23 ± 0.763	–
WW ₁₁ P ₁	MRSA	3.2 ± 0.5	2 ± 0.408	1.5 ± 0.288	1.34 ± 0.5	–
WW ₄ P ₂	<i>S. typhimurium</i>	3 ± 0.288	2.34 ± 0.763	2.5	1.75 ± 0.5	–
	<i>E. coli</i>	2.13 ± 0.288	2 ± 0.408	1.87 ± 0.5	1.87 ± 0.5	–
WRE ₄ P ₂	<i>P. aeruginosa</i>	2 ± 0.235	1.5 ± 0.408	3 ± 0.5	4 ± 0.408	1 ± 0.5
	MRSA	1.5 ± 0.235	2 ± 0.623	3.5 ± 0.408	3 ± 0.288	–
	<i>E. coli</i>	2 ± 0.408	1.97 ± 0.5	4 ± 0.235	2.5 ± 0.40	–
WRE ₅ P ₂	<i>P. aeruginosa</i>	2.5 ± 0.5	1.5 ± 0.235	1.13 ± 0.235	–	–
WRE ₆ P ₂	MRSA	2.5 ± 0.288	2 ± 0.408	1 ± 0.5	–	–
WRE ₁₀ P ₂	<i>S. pneumoniae</i>	2.87 ± 0.288	2 ± 0.763	1.87 ± 0.408	1.5 ± 0.5	–
	<i>P. aeruginosa</i>	3	2.5 ± 0.763	2 ± 0.408	1.5 ± 0.40	–

(-): no activity

Table 6 Effect of pH on bacteriocins activity

Bacterial strains	Pathogens	pH						
		2	4	6	8	10	12	14
		mm						
WW ₂ P ₁	<i>P. aeruginosa</i>	2.166±0.288	1.5±0.5	2±0.5	1.833±0.288	2.5±0.5	2.166±0.763	2±0.5
	<i>K. pneumonia</i>	2	1.66±0.288	2±0.5	1.833±0.763	2.5±0.408	1.833±0.6236	2.5±0.408
	<i>E. coli</i>	Nd	1.5±0.408	1.833±0.235	2±0.408	2.5±0.408	3±0.408	3.33±0.6236
WW ₃ P ₁	<i>E. coli</i>	3.66±0.7637	3.833±0.763	2.833±0.763	2±0.5	1.5±0.5	1.833±1.040	3.5±0.5
WW ₄ P ₁	<i>P. aeruginosa</i>	2±0.5	3.5±0.5	2.5±0.763	6±0.5	7±0.5	7.166±0.7637	7.5±0.5
WW ₁₁ P ₁	MRSA	2.166±0.763	8±0.55	2±0.763	2.5±0.5	3±0.408	2.5±0.5	4±0.763
WW ₄ P ₂	<i>S. typhimurium</i>	6±0.5	2±0.763	3.833±0.763	1.833±0.763	4.5±0.5	3.88±0.763	2±0.5
	<i>E. coli</i>	5±0.5	2.166±0.288	2.5±0.5	2.5±0.763	3.5±0.5	4.5±0.5	6.5±0.5
WRE ₄ P ₂	<i>P. aeruginosa</i>	2.5±0.5	1.833±0.267	1.5±0.5	1.5±0.5	2.5±0.408	1.833±0.40	4±0.1.833
	MRSA	1.833±0.6236	2±0.5	2.5±0.288	1.5±0.763	2.5±0.408	3	2.5±0.5
	<i>E. coli</i>	4±0.5	2.5±0.763	1.5±0.5	1.5±0.5	2.5	2.5±0.5	3.5±0.822
WRE ₅ P ₂	<i>P. aeruginosa</i>	11±0.5	7.5±0.5	5.25±0.763	7±0.5	7.5±0.5	8±0.763	9±0.823
WRE ₆ P ₂	MRSA	6.5±0.763	4±0.5	3.5	3.5±0.5	3.5±0.833	4±0.5	6±0.763
WRE ₁₀ P ₂	<i>S. pneumoniae</i>	1.5±0.5	1.5±0.267	4±0.763	5±0.408	6±0.5	2±0.267	3
	<i>P. aeruginosa</i>	1±0.5	2±0.288	2.5±0.267	2.5	3±0.5	3	4.67±0.5

Fig. 5 Effect of different pH on bacteriocin activity against specific pathogens

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Data availability We are willing to share our data upon request. Please contact Dr. Basit Zeshan (email: dr.basitzeshan@ucp.edu.pk) for this purpose.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval An approval from institution review board was obtained before starting the research work.

Consent to participate All authors have participated equally in the current research work.

Consent for publication All authors have read the guidelines related to the publication and are agree to publish this research work in SN Applied Sciences.

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