IJBPAS, July, 2014, 3(7): 1044-1053

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS* SP SH.ZH2 ISOLATED FROM SOIL CONTAMINATED WASTEWATER, LOCAL YOGURT

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ABSTRACT

Bacteriocins are ribosomally synthesized antimicrobial peptides widely distributed in nature. This peptide biodiversity is supported by several differences in their structures. Bacteriocin production seems to be aimed to compete against other bacteria which are present in the same ecological niche .Several researches have been focused on the production of bacteriocins from members of the LAB group, mainly *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Enterococcus* sp., and *Pediococcus* sp. *Lactobacillus* bacteriocins are found within each of the four major classes. L. sp sh.zh2 was isolated from Soil Contaminated Wastewater, Local Yogurt. L. sp sh.zh2 was grown in MRS broth at 37°C in 48 h. after incubation, the broth was centrifuged at 9000 rpm for 30 min and the cells were separated out. Supernatant was used as a crude bacteriocin. Different concentrations of ammonium sulphate were added to the supernatant. Precipitates formed were collected by centrifugation at 9000 rpm for 40 min and redissolved in phosphate buffer with pH=7.0.So, salting out dialysis. Assay of the antimicrobial activity was determined and estimate the molecular mass of bioactive peptides, SDS-PAGE was carried out on 15% acrylamide gels. *Lactobacillus* sp sh.zh2 was grown on MRS broths. Bacteriocin activity in supernatant was determined at several growth temperatures. Bacteriocin activity was observed in supernatants of cell-free culture incubated at 37 C. Analysis of the isolated after dialysis by SDS-PAGE, and direct detection of antimicrobial activity on the

electrophoresis gel indicated that molecular mass of the two bacteriocins is approximately between 25kDa and 28 kDa. More ever, soil microorganisms are highly capable of producing metabolic products in the environments such as soil, wide spread genetic exchanges take place in microorganisms and extend the ability of producing metabolic products among themselves. Some metabolic products include bacteriocin, enzyme and antibiotic. Due to the genetic exchange among soil micro organisms and LAB bacteria in soil environments.

Keywords: Bacteriocins, SDS-PAGE, Dialysis, Soil Contaminated Wastewater

INTRODUCTION

Worldwide, the increased awareness of the consumers regarding the effect of food on health has caused an increasing trendfor consumption of functional foods. In the years 2000, 2005 and 2010, theworld-widemarket share for functional foods were US$33 billion, US$ 73.5 billion, and US$ 167 billion, respectively **[1]**. Probiotic food products are regarded as a significant part of functional foods market, so that they comprise between 60%and 70% of the total functional food market **[2]**. "Probiotics" are live microorganisms (not molds) with the potential of settling mainly in host (humans/animals) intestine and comprising certain health advantage(s) for it. *Lactobacillus* and *Bifido bacterium* are the most currently used probiotic species in fermented milks **[3]**. The use of probiotics in aquaculture has not only resulted the reduction of use of harmful antimicrobial compounds, particularly the antibiotics, but also improved the appetite and/or biogrowth performance of the farmed species in an eco-friendly and sustainable manner **[3-6]**.

Lactobacilli, as well as some species of the genus *Bifidobacteria* and *Streptococci*, are micro-organisms which are classified in the GRAS (Generally Regarded as Safe) group. They have also been proposed as probiotics for both the gastrointestinal and urogenital tracts **[7]**.

Bacteriocins are proteinaceous, bactericidal substances synthesized by bacteria. They usually have a narrow spectrum of activity, meaning that they only inhibit strains of the same or closely-related species **[8]**.The lactic acid bacteria which produce bacteriocin are widely used in probiotic products for human and animal consumption to prevent pathogen growth in the gastrointestinal tract **[9,10,11]**. The bacteriocins of LAB were classified by (12) into four classes on the basis of common, mainly structural, characteristics. Most of the bacteriocins isolated so far belong to classes I or II. Class I bacteriocins named lantibiotics,

are small (<5 kDa) membrane-active peptides, which contain post-translationally modified amino acid residues like lanthionine. Nisins are the best-studied and known lantibiotics **[13]**. These classes of the bacteriocin are shown in the **Table 1**. Classification of bacteriocin from Gram Positive bacteria **[12]**.

Table 1: Classes of Bacteriocins Based on Their Molecular Weight

Class	Molecular Mass	Characteristics and Subclass
I	**< 5 KDa**	**Ribosomally produced peptides that undergo extensive post – translational modification, small peptides containinglanthionin and beta methyl lanthionine**
II	**< 10 KDa**	**Low molecular weight, heat stable peptides. Formedexclusively by unmodified amino acids, ribosomallysynthesized as inactive prepeptides that get activated by posttranslational cleavage of the N terminal leader peptide.II a: anti – listerial single peptides that contain YGNGV aminoacids motif near their N termini.II b: two peptide bactreriocins.IIc: thiol – activated peptides**
III	**> 30KDa**	**High molecular weight, heat labile proteins**
IV		**Complex bacteriocins carrying lipid or carbohydrate moieties**

MATERIALS AND METHODS

Bacterial Strains and Growth Media

The bacterial strains used as indicator micro-organisms for the screening of bacteriocin production and evaluation for antimicrobial activities are shown in Table 2.All LAB were propagated in MRS broth (Merck,Germany) at 37_C.*Escherichia coli* were grown in Luria-Bertani (LB) broth medium (Merck,Germany) at 37_C. *Staphylococcus aureus* and *Pseudomonas aeroginosa* was cultivated in Brain-Heart (BH) broth medium (Merck,Germany) at 37_C.

The Stock Cultures Were Maintained at -70_C in MRS Broth Containing DMSO

Isolation of the bacteriocin-producing strains: The lactic acid bacteria were isolated from Soil Contaminated Wastewater, Local Yogurt, by appropriate dilutions with NaCl physiological. Dilutions (10^{-1} - 10^{-6}) were prepared and plated on MRS agar medium (Merck, Germany) to isolate the *Lactobacillus* spp and incubated at 37°C for 48 - 72 h at condition anerobic jar **[14]**. The strains were subcultured onto MRS agar incu-bated at 30°C for 24 h and preserved in DMSO at −80°C. One of the isolates was selected for further studies. It exhibited strong inhibitory activity against indicator strains. It was identified on the basis of growth, cell morphology, gram staining and catalase activity. Further, identification was performed according to carbohydrate fermentation patterns and growth at 15°C and 45°C in the MRS broth based on the characteristics of the lactobacilli as described in Bergey's Manual of Determinative Bacteriology **[15]**.

Antimicrobial Activity Assay

The antimicrobial activity of cell-free supernatant and partially purified bacteriocin was determined by well diffusion method **[16]**. To investigate the antibacterial activity spectra of LAB strains by well diffusion assay, 100 µl culture of one of the test bacteria **(Table 2)**, grown to the early stationary growth phase in nutrient medium, was added to 20 ml of soft nutrient agar (0.8%, w⁄ v). Wells were made in the lawn of hardened soft agars in petri dishes. Aliquots (100µl) of supernatant of overnight cultures (16–18 h) were poured in the wells. The plates were left for1 h at room temperature in sterile conditions before incubating them to the adequate temperature of growth of the test micro-organism. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

Identification of LAB Strain

Bacteriocin-producing strain sh.zh2 was characterized and identified on the basis of its morphological, biochemical and genetic properties. All reagents for catalase reaction, oxidase test and gram staining **(Table 3)**.

Chromosomal DNA used for polymerase chain reaction (PCR) was prepared by using phenol-chloroform method **[17]**. The DNA fragments containing 16S rDNA were amplified from chromosomal DNA with primers pairs 27F (5- AGAGTTTGATCMTGGCTCAG -3) and 1492R (5- GGTTACCTTGTTACGACTT -3) (Turner et al. 1999). PCR reactions were performed in a DNA thermal cycler (Biorad.USA) in a total volume of 50 µl containing Master mix (Takara, Japan). Amplification consisted of a 1 min denaturation step at 94_C, a minute annealing step at 59_C and a minute extension step at 72_C. The first cycle was preceded by incubation for 5 min at 94_C. After 35 cycles, there was a final 10-min extension at 72_C. Negative controls containing no DNA template were included in parallel. PCR products were separated in a 1.5% (w/v) agarose gel and were subsequently visualized by ultraviolet (UV) illumination after ethidium bromide staining.

Purification of Bacteriocin

A 18-h-old culture of the bacteriocinogenic LAB strain was centrifuged (9000×g, 20 min, 4ºC) and the peptidic fraction precipitated from the cell-free supernatant with 70% saturated ammonium sulphate **[18]**. Some bacteriocins can precipitate at lower ammonium sulphate concentrations, or even in a small range of saturation, then is important to assay which is the concentration of salt that precipitates the peptide of interest. The suspension was incubated overnight at

4°C and agitated with a magnetic stirrer. Salted-out proteins were precipitated by centrifugation (10000×g for 20 min) and dissolved in a small volume of 10 mM phosphate buffer (pH 7.0) or distilled water. The suspension can be desalted by dialysis with phosphate buffer at 4°C during 12h by using benzoylated membranes (molecular weight cut off 1200; Sigma-Aldrich) or with dialysis cassettes with cut-off of 2000 to 3500 (Pierce Biotechnology, Inc). Since most bacteriocins have a size smaller than 10000 Da, the use of regular dialysis bags with cut-off of 10000 -12000 Da is inappropriate for this procedure **[19]**.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

To estimate the molecular mass of bioactive peptides, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% acrylamide gels. A low molecular mass protein marker with size ranging from 15 to 200 kDa (Sigma) was used as standard. To determine the apparent molecular mass of the bacteriocin, the gel was cut into two vertical parts after SDS-PAGE. The part of the gel containing the molecular mass marker and the samples was stained with Coomassie blue R-250 for 24h, while the remaining part, containing only samples, was extensively washed with regularly replaced sterile MilliQ water for Repeat 3 times and Decolorizing for 3h.

Table 2: Antimicrobial Activity Spectrum of the Cell-Free Culture Supernatant and Partially Purified Bacteriocin of *Lactobacillus* sp sh.zh2

Indicator strain	Antimicrobial activity*
	Supernatant Purified bacteriocin
Staphyloccocus aereus	++
E.coli	++
Pseudomonas aeroginosa	+++

-, no inhibition; +, inhibition zone <5 mm; ++, inhibition zone 10–15 mm; +++, inhibition zone >15 mm.
***Antimicrobial activity was determined by the well diffusion assays described in Materials and methods**

Table 3: Physiological and Biochemical Characteristics of *Lactobacillus* sp sh.zh2

Morphology	Gram Reaction	Catalase	motility	pH range for growth	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
Bacilli	+	-	-	5-7.5	+	-	-	-	-	-	-	-	-	-

RESULTS AND DISCUSSION

Isolation of Bacteriocin-Producing Strain

Ten LAB isolated from Soil Contaminated Wastewater, Local Yogurt were tested for their inhibitory activities against several food-borne pathogenic bacteria by well diffusion method as described in Materials and methods section. Five isolates showed a significant growth inhibition against food-borne pathogens such as *E. coli*, *Staph. aureus* , *Pseudononas aeroginosa*, (data not shown). For further analyses only one isolate (sh.zh2) was selected as it showed the highest antimicrobial activity.

Antimicrobial Activity Assay

The proteins from an *Lactobacillus* sp sh.zh2 cell-free culture supernatant (CFCS). The antimicrobial activity of CFCS and purified protein (PP) from CFCS were tested against food-borne pathogenic bacteria by well diffusion assay. The results are shown in **Table 1** and **Figure 1**. The CFCS and PP exhibited an antibacterial effect on a broad range of bacterial species. CFCS and PP from *Lactobacillus* sp sh.zh2 culture inhibited closely related gram-positive bacteria like pathogenic *Staphylococcus aereus*, also the gram negative bacteria *E. coli* and *Pseudomonas aeroginosa* .

Purification of sh.zh2 Bacteriocin

Lactobacillus sp sh.zh2 was grown on MRS broths. Bacteriocin activity was observed in supernatants of cell-free culture incubated at 37 C. Bacteriocin from CFCS was recovered by Ammonium sulfate precipitation. In this step, some bacteriocins can precipitate at different ammonium sulphate concentrations, or even in a small range of saturation. Salted-out proteins were precipitated by dialysis. Analysis of the fractions isolated after dialysis by SDS-PAGE, and direct detection of antimicrobial activity on the electrophoresis gel indicated that molecular mass of the two bacteriocins is approximately between 25 and 28 kDa **(Figure 2)**.

The purified antimicrobial substance was analyzed by 15% SDSPAGE: Gel stained with Coomassie blue R-250: line M, peptide ladder of with molecular mass ranging from 15 to 200 kDa, and line sample , the purified protein.

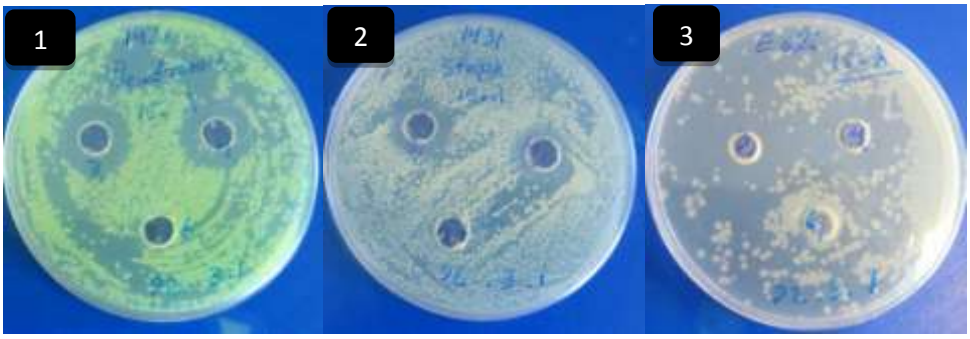

Figure 1: Antimicrobial Activity of CFCS of *Lactobacillus* sp sh.zh2 against 1: *Pseudomonas aeroginosa* 2: *S.aereus* 3: *E.coli*

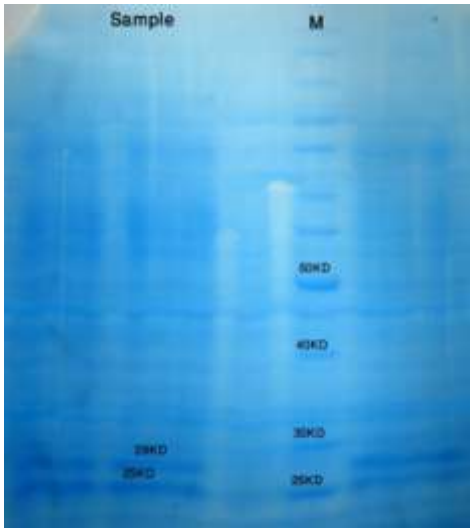

Figure 2: Analysis and Identification of the Purified Antimicrobial Substance from *Lactobacillus sp* sh.zh2.

DISCUSSION

Over the past few years, studies concerning bacteriocins produced by LAB have received an increasing interest because of the potential use of bacteriocins as food preservatives **[20, 21]**.

Bacteriocin-producing isolate sh.zh2 was isolated from by Soil Contaminated Wastewater, Local Yogurt direct plating method. The isolate sh.zh2 showed the broadest antimicrobial spectrum against several food-borne pathogens including *E. coli*, *Staph*. aureus and *Pseudomonas*. The strain was identified as *Lactobacillus plantarum* on the basis of its morphological and biochemical characteristics, carbohydrate fermentation profile and 16S rDNA sequence. However, the spectrum of inhibitory activity of these bacteria suggests a potentially useful means for controlling the growth of food-bornethogens bacteria. Development of a step purification procedure allowed the separation of two bacteriocins. These lactocins named Ha1 and Zh 1 are peptides with molecular

masses of 25000 and 28000 Da. All these characteristics allow us to assert that bacteriocins Ha1 and Zh1 from *Lactobacillus sp* sh.zh2 would belong to the class III of bacteriocins **[12]**. These two polypeptides/large proteins are individually/separately active. This is the first report on the isolation and characterization of bacteriocins produced by LAB from Soil Contaminated Wastewater, Local Yogurt .We assume that bacteriocins Ha1 and Zh1 could be used in the food preservation.

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