LWT - Food Science and Technology 78 (2017) 303–310

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Antimicrobial, cytotoxic effect and purification of bacteriocin from vancomycin susceptible *Enterococcus faecalis* and its safety evaluation for probiotization

Venkatesh Perumal, Arul Venkatesan*

Department of Biotechnology, School of Life Sciences, Pondicherry University, Puducherry 605014, India

ARTICLE INFO

Article history:
Received 20 April 2016
Received in revised form
18 December 2016
Accepted 26 December 2016
Available online 27 December 2016

Keywords:
Enterococcus faecalis
Bacteriocin
Chicken intestine
MTT assay
Virulence genes

ABSTRACT

A vancomycin susceptible *Enterococus faecalis* CV7 produced bacteriocin was purified by size exclusion chromatography followed by RP-HPLC C-18 column. Tricine-SDS PAGE unrevealed molecular mass was further confirmed by MALDI-TOF MS as 4.829 kDa. This bacteriocin showed broad spectrum of antibacterial activity against important food borne pathogens mainly against *Salmonella typhi* and this activity also found to be withstood after treatment with different proteolytic enzymes, temperature, pH, solvents and detergents. Cytotoxicity of bacteriocin CV7 by MTT assay showed that lower survival inhibition on HeLa than HT-29 cell line at lower concentrations conclude its potentiality in food biopreservation. Results obtained from PCR amplifications revealed that strain *E. faecalis* CV7 does not harbor virulence genes *esp, ace, cylB, cylA* and *efaAfs* but contains *asa1*. With this, surviving in the presence of low pH and bile oxgall promising its safety as probiont to control *Salmonella* infections.

1. Introduction

After the first time discovery of bacteriocins in 1925, they became a part of thrust area of research and then now being as a potential tool in modern biotechnology. Bacteriocins of lactic acid bacteria (LAB) are antimicrobial proteins or peptides which are synthesized and secreted by bacteria. These bacteriocins are secondary metabolites which can able to inhibit closely related and competitive bacterial strains (Hwanhlem, Biscola, & Jaffre, 2013; Kjos, Snipen, Salehian, Nes, & Diep, 2010). The majority of bacteriocins reported small peptides, net positive to slightly acidic pH (amphiphilic) in nature and diverge in spectrum such as molecular masses, thermo stabilities, active pH and genetic determinants (Javed, Masud, Ul Ain, Imran, & Maqsood, 2011; Zacharof & Lovitt, 2012).

Due to consumers' awareness, nowadays the demand on natural preservatives has increased drastically for healthy, secured and fresh products (Foulquié Moreno, Callewaert, Devreese, Van Beeumen, & De Vuyst, 2003). In last two decades enterococci and enterocci producing bacteriocins attained wide scientific view for their safe use. For instance, in the agenda of the FAIR Programme (project CT97-3078), a number of enterococci related to food fermentations were selected in association with their functional aspect and safety. The project entitled Tradisausage has given award of selection grade as probiotics to some more enterococci isolates of fermented sausages (Talon, 2005; Laukov et al., 2011).

However, Enterococci often been reported that causing agent of nosocomial infections and may cause human diseases such as bacteremia, endocarditis or urinary infections. Pathogenic traits of *E. faecalis* strains often contain many antibiotic resistances, and virulence factors such as cytolysin and haemolysin. When this enterococci considering as probiotic, their security and harmful activities are always under debate. However, there is a necessity to consider each strain in the aspects whether it contains virulence genes (hemolysin, gelatinase, Esp protein, etc.) as well as valuable probiotic properties such as proteolytic, lipolytic, and esterolytic activities. Most likely the clinical isolated enterococci harboured most of the virulence factors than the food and other sources (Franz et al., 2001). Though, Genus *Enterococcus* comprised in LAB group and preferably used in many commercial probiotic feed additives to poultry, cattles, etc., due to Generally Recognized As Safe (GRAS) status (Fernandez, Martinez-Bueno, Martin, Valdivia, Maqueda, 2007) and the bacteriocins of this strains has not yet been reported any risk while using in food preservation. Hence, extensive studies have been carried out on isolation and purification of bacteriocins

* Corresponding author.
E-mail address: varul18@yahoo.com (A. Venkatesan).

http://dx.doi.org/10.1016/j.lwt.2016.12.048
0023-6438/

of *E. faecalis* by many researchers.

Enterococci are Gram-positive, facultative anaerobic, catalase-negative, cocci-shaped bacterium and are widely distributed and isolated from different sources such as animals, in some human outbreaks, soil, surface waters, plants and a few vegetables (Mareková, Lauková, Skaugen, & Nes, 2007). Commonly, *Enterococci* producing bacteriocins known as enterocins, *Enterococcus* strains of different origins can synthesize different enterocins i.e. different in molecular masses and amino acid sequences. Frequent demonstration on isolation of bacteriocins of *Enterococcus faecalis* from different sources such as human wound exudates, surface of a traditional cheese, feces of minipigs, grass silage, rice bran, corn silage, cheese, beef product, Spanish sheep's cheese have been reported (Galvez, Gimenez-Gallego, Maqueda, & Valdivia, 1989; Maisnier-Patin, Forni, & Richard, 1996; Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000; Nilsen, Nes, & Holo, 2003; Yamamoto, Togawa, Shimosaka, & Okazaki, 2003; Sparo et al., 2006; Izquierdo, Wagner, Marchioni, Aoude-Werner, & Ennahar, 2009; Liu et al., 2011; Cebrián et al., 2012). However, purification and partial characterization of bacteriocin producing strain *E. faecalis* isolated from country chicken intestine has not yet reported or scarce.

This study reports the purification and partial characterization of bacteriocin as well as probiotic attributes and safety evaluation of strain *E. faecalis* CV7 isolated from indigenous avian chicken intestine, which could give an idea to use of this bacteriocin as a biopreservative in food industries as well as to use strain *E. faecalis* CV7 as biocontrol agent to control some important pathogens in place of antibiotics particularly *Salmonella* sp., in poultry flocks.

2. Materials and methods

2.1. Sampling, isolation and identification

Intestinal homogenate of indigenous domestic bird chicken was collected aseptically, then serially diluted and spread over MRS (deMan, Rogosa and Sharpe) agar plates, incubated at 32 °C for 48–72 h. Around 60 milky white single cell colonies picked and inoculated in 2 ml MRS broth and screened based on its anti-bacterial activity against bacterial pathogens (data not showed). Among these, one isolate, which showed good anti-bacterial activity against *Salmonella typhi* 743 was selected, and identified as *Enterococcus faecalis* CV7 by gene sequencing (Microsynth, Switzerland) using 16 S rRNA universal primers. The sequence was submitted to GenBank with accession number KF724942. The strain was maintained in MRS agar medium and glycerol stock (50% v/v, −20 °C). Each time, the strain was rejuvenated in MRS medium from stock prior to use. The bacterial pathogenic strains were purchased from MTCC Chandigarh, Pune, India. All reagents used were analytical grade.

2.2. Growth kinetics of bacteriocin production

One percentage of inoculums (8.0 log CFU/mL) was inoculated in 1 L of MRS medium and incubated at 32 °C. This one in every 4 h, 1 mL of sample was taken and the viable cell count was determined in CFU/mL and anti-bacterial activity (Agar well diffusion assay) was observed with another 1 mL of CFS (cell free supernatant) (pH 6.5) against indicator strain *Salmonella typhi* 743 for up to 48 h.

2.3. Crude preparation and partial purification of bacteriocin

The broth culture in MRS medium (32 °C, 24 h) was centrifuged at 8500 × *g* for 10 min at 4 °C. The CFS was collected and neutralized to pH 6.5 ± 0.1 with 1N NaOH, prior to study the anti-bacterial activity against indicator strain *S. typhi* 743 by agar well diffusion assay. Then the CFS was ultrafiltered (GE Healthcare, Uppsala, Sweden) by employing a 10 kDa cutoff membrane catridge filter, since the desirable bacteriocin was suspected to be below this molecular weight. The procured filtrate was gradually supplemented with ammonium sulfate to reach upto 90% saturation at 4 °C and kept for overnight. Then the precipitated proteins collected by centrifugation (10 000 × *g*, 20 min, 4 °C) and dissolved in a minimal quantity of 10 mM ammonium acetate buffer (pH 6.0) and dialyzed for 16 h against the same buffer at 4 °C with 1 kDa pore size dialysis membrane (Spectrum labs, USA).

2.4. Purification of bacteriocin by HPLC

The dialyzed bacteriocin was subjected to partial purification by Akta Prime plus protein purification system (GE Healthcare, Sweden) which was connected with a size exclusion column (C10/20) and Sephadex G-25 as matrix. Two ml of dialyzed bacteriocin was injected through the column which was equilibrated with 10 mM ammonium acetate buffer (pH 6.0) and containing 0.01 M sodium chloride at a flow rate of 0.5 mL/min. The eluent was collected as 2 mL size of fractions. Each fraction was tested for anti-bacterial activity and protein profiling by Tricine SDS-PAGE. The antimicrobial activity showed fraction was further purified by reverse phase liquid chromatography (RP-HPLC) (Shimadzu, Japan). Briefly, 25 μL of the concentrated bacteriocin which was collected from Akta prime plus column purification and able to inhibit pathogens was injected into an analytical C18 reverse-phase column (Luna 5 μm, 4.6 × 250 mm; Phenomenex, CA, USA.). The elution was performed at a flow rate of 1 mL/min using a linear gradient from 90% solvent A [0.1% (w/v) trifluoro-acetic acid (TFA) in 5% (v/v) acetonitrile in water] and 10% solvent B (0.1% TFA in 100% acetonitrile) to 42% and 58% of solvents A and B, respectively, within 46 min. The peptide fractions were detected spectrophotometrically by measuring the absorbance at 220 nm and collected manually. The fractions were then lyophilized and dissolved in an ammonium acetate buffer (10 mM, pH 6.0) and used in the bacteriocin activity, and molecular mass determination analysis.

2.5. Molecular mass determination of purified bacteriocin

The molecular size of purified bacteriocin CV7 was primarily determined by Tricine SDS-PAGE (10%) (Schagger and Von Jagow, 1987), along with a low molecular mass protein marker ranging from 1 to 7 kDa (Merck, Bioscience). The gel was stained by coomassie brilliant blue staining method. Further, the molecular mass of bacteriocin CV7 was confirmed by mass assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) at SAIF, IIT Madras, India.

2.6. Anti-bacterial activity of bacteriocin

One hundred microliter of partially purified bacteriocin was loaded into the wells (6 mm in diameter) of MRS agar plates (2% agar w/v) which seeded with the bacterial pathogenic strains (6.0 log CFU/mL) including *Salmonella* sp., *S. enterica* 3219, *S. typhi* 734, *Escherichia coli* DH5α, *Vibrio fischeri* 1738, *Listeria monocytogenes* 1143, and *Staphylococcus aureus.* After incubation (32 °C, 18 h) the plates were scrutinized for the presence of zone of inhibition.

The activity of purified bacteriocin in polyacrylamide gel was performed by running the tricine SDS-PAGE under nonreducing conditions (Perumal, Repally, Dasari, & Venkatesan, 2016).