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Original article

Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512

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Abstract

The aim of the present study was to investigate the characteristic diversity and stability of antimicrobial compounds produced by two probiotic strains of *Bacillus licheniformis* (MCC2514 and MCC2512). Antimicrobial compounds from the two strains notably varied, related to stability and potency. The inhibitory spectrum of *B. licheniformis* MCC2512 was higher than MCC2514, but, related to the effect on *Micrococcus luteus* ATCC9341, MCC2514 (LD_{50} = 450 AU ml^{-1}) was more potent than MCC2512 (LD_{50} = 750 AU ml^{-1}). The compounds were thermo-resistant and stable at a wide range of pH and exhibited considerable resistance to digestive enzymes and bile salts (anionic biological detergents), contributing to their appropriate application in various food systems. The isolate *B. licheniformis* MCC2512 gave a positive response to *Bacillus subtilis*-based biosensors BSF2470 and BS168.BS2, confirming the mode of action on the cell wall and subtilin-type, respectively. For *B. licheniformis* MCC2514, the mode of action was characterized by constructing *B. subtilis* reporters that interfered in five major biosynthetic pathways, i.e., biosynthesis of DNA, RNA, protein, the cell wall and fatty acids. *B. licheniformis* MCC2514 responded to the *yvg*S reporter, indicating it as an RNA synthesis inhibitor. Overall, the investigation reveals variability of the antimicrobial compounds from *B. licheniformis* of different origins and for their possible application as biopreservative agents.

Keywords: *Bacillus* sp.; Probiotic; Biosensor; Antibacterial activity; Inhibitory spectrum

1. Introduction

The increasingly fast-paced life style of consumers has increased the need for processed foods and has encouraged their industrialization. Environmental contamination of these processed foods is a serious problem faced by the food industry as it causes enormous economic losses. Chemical preservatives and antibiotics have been extensively used to safeguard processed foods; however, they have a drastic effect upon nutritional properties. Hence researchers have focused on a range of compounds produced by microbial sources that can maintain the nutritional value of products and increase their shelf-life. In addition, the emergence of multidrug-resistant pathogens and imposed restrictions on the use of antibiotics both in clinical settings and food additives have increased the quest for natural antimicrobial compounds from microbial sources. In this regard, probiotic strains with defined health benefits have been extensively studied for their antimicrobial activity. Among them, lactic acid bacteria (LAB) have created a growing interest among food industries because of their biopreservative activities against food-borne pathogens through simple fermentation processes [1,2].

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of bacteria that are often effective against closely related species [3,4]. Nisin produced by *Lactococcus lactis* subsp. *lactis* is the only bacteriocin approved by the US-FDA for commercial application in food products, but its use is limited because of weak activity at neutral or alkaline pH. Many non-LAB have also been reported to produce antimicrobial peptides. The genus *Bacillus*

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includes a large number of species known to produce bacteriocins or bacteriocin like substances such as subtilin, sublancin, bacillocin and subtilosin from *Bacillus subtilis* [5–7], coagulin from *Bacillus coagulans* [8], bacitracin and lichenin from *Bacillus licheniformis* [9–12] and megacin from *Bacillus megaterium* [13]. Unlike LAB, the *Bacillus* spp. exhibits broad spectrum inhibitory activity [4,6,11]. Numerous studies have been carried out on *Bacillus* bacteriocins in food products to address food safety aspects and their application in clinical studies [4,10,11,14].

In recent years, whole cell bacterial biosensors containing reporters which can be specifically induced via selected promoters are widely used in identifying specific mode of action (MOA) of antimicrobial compounds [12,15,16]. This is a rapid screening method that is robust, sensitive and very specific. In this context, bacteria have been genetically engineered to respond to the presence of specific chemicals or stress by synthesizing reporter proteins. These bacterial biosensors have been used as a tool in the present study to analyze the MOA of antimicrobial compounds.

Given the wide diversity of *Bacillus* spp., the present work focused on characterizing the antimicrobial compound produced by two strains of *B. licheniformis* (MCC2514 and MCC2512) originating from raw milk from sheep and rhizobial soil of *Hedychium coronarium*, respectively. These two cultures have been previously characterized for their potential probiotic properties [17]. Sheep milk is highly nutritious and represents an ideal growth medium for microorganisms [18]. Similarly, *H. coronarium* or the white ginger lily has high medicinal value. The essential oil from fresh and dried rhizomes is known to have antifungal, antibacterial and cytotoxic activity [19,20], as well as analgestic and anti-inflammatory properties [21].

In the present investigation, we report the differential activity of antimicrobial compounds produced by two strains of *B. licheniformis* in terms of their stability, inhibitory spectrum and mode of action.

2. Materials and methods

2.1. Media chemicals and reagents

All microbial media chemicals, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside), ONPG (O-Nitrophenyl-β-galactoside) and standard reference antibiotics were purchased from Hi Media Pvt Ltd, Mumbai, India. Sodium chloride, organic solvents such as butanol, toluene, acetone, chloroform and ethanol were purchased from Sisco Research Laboratory, Bangalore, India. Enzymes (trypsin, pepsin, proteinase K, α-amylase), bile salts (sodium glycocholic acid and sodium taurocholic acid) and DEAE cellulose were procured from Sigma–Aldrich Inc, USA. All chemicals used were of analytical grade reagent unless otherwise mentioned.

2.2. Bacterial cultures and growth conditions

Native strains of *B. licheniformis* MCC2514 and MCC2512 previously characterized in the laboratory for their probiotic properties [17] were grown in Luria Bertani (LB) medium at 37 °C under constant shaking (120 rpm). For antimicrobial activity, the indicator organisms such as *Micrococcus luteus* ATCC9341, *Yersinia enterocolitica* MTCC859, *Aeromonas hydrophila* NRRL B445, *Staphylococcus aureus* FRI722, *Salmonella typhimurium* MTCC1251, *Escherichia coli* CFR02 and *Klebsiella* sp. were procured from the American Type Culture Collection (ATCC), USA or the Microbial Type Culture Collection Center (MTCC) Chandigarh, India. *Listeria monocytogenes* ScottA was kindly provided by Dr. A.K. Bhunia, USA. All indicator cultures were grown in brain heart Infusion (BHI) media at 37 °C under constant shaking (120 rpm). Reference standard culture *B. subtilis* 168 and whole cell biosensor *B. subtilis* 168.BS2 (W168 *amy*E::-P_{SpaS}:*lac*Z, P_{SpaRK}-SpaRK), a subtilin-specific reporter, were kindly provided by Prof. K.D. Entian, Germany. The reporter strain *B. subtilis* BSF2470 (CU1065 lial::pMUTIN) [22] was used for confirming cell-wall stress-producing antimicrobial substances. *B. subtilis* ATCC6633 and *Bacillus flexus* MCC2011 were used as positive and negative controls, respectively, for cell-wall stress-inducing antimicrobial compounds.

2.3. Antimicrobial activity of Bacillus isolates

2.3.1. Preparation of the antimicrobial compound (AMC)

Bacillus cultures individually grown in LB broth were centrifuged at 10,000 rpm for 15 min at 4 °C. The cell-free supernatant or crude AMC was filter-sterilized (0.2 μm membrane) and stored at 4 °C until use.

2.3.2. Inhibitory activity of the AMC

Antimicrobial activity of *Bacillus* isolates against various indicator organisms was tested by the agar well diffusion assay as described by Xie et al. [23]. The antibacterial activity was further quantified by the twofold serial dilution method and results were expressed as AU ml^{-1}. A unit is defined as the reciprocal value of the highest dilution at which the zone of inhibition was observed.

2.4. Effect of enzymes on antimicrobial activity

The effect of enzymes including pepsin, trypsin, proteinase K and α-amylase was tested on cell-free supernatant of *Bacillus* spp. An aliquot of cell-free supernatant or crude AMC was treated with the respective enzymes at a final concentration of 2 mg ml^{-1} at 37 °C for 30 min. After incubation, the activity was quantified and expressed as AU ml^{-1} as described elsewhere. An untreated cell-free supernatant and the enzymes alone in the buffer (pH 7.0) served as controls.

2.5. Purification of the AMC

2.5.1. Optimization of the extraction procedure

AMC in the cell-free supernatant (CFS) of test cultures was extracted by various methods to determine a suitable protocol for maximum recovery. Method 1: CFS was precipitated with

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