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Screening of Antimicrobial Spectrum of *Brevibacillus* sp. Isolated from Dairy Environment

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A B S T R A C T

Bacteriocins and Bacteriocin like Inhibitory Substance (BLIS) are ribosomal products exhibiting antimicrobial activity. The present study aimed to screen for production of antimicrobial agents from soil bacteria of dairy environment. Borer was used to dig soil and collected in sterile plastic bags. Isolation was done on tyrosine agar. To exclude non spore formers the soil suspension, was heated at 80ºC. The bacterial isolates were identified underpinning biochemical and carbohydrate utilization tests and screened for their antibacterial activity against methicillin resistant *S. aureus* and *E. coli* by agar well diffusion method. Among forty nine isolates three isolates were identified as *Brevibacillus brevis*. Screening of crude brevicin obtained from cell free broth inoculated with *B. brevis* exhibited a strong antagonistic activity against *S. aureus* but no zone of inhibition was recorded against *E. coli*. The spore forming bacterium, *B. brevis* produced narrow spectrum antibacterial agent. Strains of *Brevibacillus* are potent source of bacteriocins and these antimicrobial peptides can be used as an effective therapeutic agent in pharmaceuticals as well as can also be used as food preservatives in canned or packed food and dairy products, controlling pathogens with natural products instead of synthetic ones.

Introduction

Bacteria and their products have been proved to be beneficial as well as harmful in different sectors such as food and dairy, pharmaceutical, etc. Spoilage of food materials even after packaging is of great concern for industries. However, advancement in technology has ameliorated the losses. Fulfilling the consumer demands of ready to eat materials are on hike, placing a challenge for industries to provide quality food. Microbes are ubiquitous and very much adaptive and evolve new strategies to survive, resulting in altered food safety hazards, WHO food strategic planning meeting have noticeably have shown their concern on the issue (Food safety strategic planning meeting,

2001). Similarly, rapid spread of antibiotic resistant pathogens due to institutional abuse of antibiotics is an alarm to find alternative methods of combating infections. A biological tool of natural origin, which can be used in both the sectors, is needed. Bacteriocins are ribosomally synthesized peptides produced by bacteria itself, with ability to kill or inhibit microorganisms usually associated, but not always, to the producer strain (Klaenhammer, 1988). Recent studies have demonstrated the potential use of bacteriocins as natural preservatives and its therapeutic applications against bacteria with Multiple Drug Resistance (MDR) (Singh *et al.,* 2012; Galvez *et al.,* 2007; Bizani and Brandelli, 2002). Based on their molecular weight bacteriocins have been characterized into three classes; Nisin, one of the broadly studied bacteriocin from LAB fall into Lantibiotics or class I bacteriocin due to its small size and granted Generally Recognized as Safe (GRAS) by United States Food and Drug Administration (USFDA) (Galvez *et al.,* 2007; Klaenhammer, 1993; Maisnier-Patin, 1992). Among bacteriocin producing bacteria, the genus *Bacillus* is one of the diverse group reported to produce a variety of antimicrobial peptides or BLIS, such as, subtilin from *B. subtilis,* cerein from *B. cereus,* megacin from *B. megaterium*, lichenin from *B. licheniformis, B. stearothermophilus,* etc. (Pattnaik *et al.,* 2005; Hyung *et al.,* 2001).

Bacillus brevis, primarily as soil bacterium was reclassified into the genera *Brevibacillus* in 1996 (Shida *et al.,* 1996) and have been reported for production of peptide antibiotics Gramicidin and Tyrocidine. Different strains of *B. brevis* have shown antagonistic effect on various gram positive and gram negative organisms, the bacterium have also reported for extending the shelf life of curd (Anja *et al.,* 2014; Appaiah *et al.,* 2012; Gillor *et al.,* 2008). In consideration of the above background a strain of *Brevibacillus brevis* was isolated from soil samples collected from dairy environment with help of surface sterilized borer.

Materials and Methods

The research was conducted in Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad. Soil samples from SHUATS dairy environment for isolation of bacteriocin producing bacteria was collected and stored in sterilized zipper plastic bags. All experiments *i.e.* isolation, identification and screening of bacteriocin production was conducted in laboratory of Department of Industrial Microbiology, SHUATS.

Isolation and identification of bacterial strain

A total of one hundred soil samples were collected from a depth of 5-15 cm at different sites. One gram of each soil sample was transferred to 10 ml of sterilized Ringer's solution aseptically and serially diluted to 10^{-6}. Each of the dilution tube was heated at 80 ºC in a preheated water bath for 20 min to exclude non-spore formers. After cooling to room temperature, 1 ml of suspension from each tube was put onto the petriplates and sterilized molten tyrosine agar was poured over the suspension. As soon as the agar got solidified the plated were flipped upside down and incubated at 37 ºC. Observations were recorded after 48 to 72 hr; colonies with dark pigment around the colonies were quarantined on nutrient agar slants after purification with quadrant streaking. To identify the bacterial strain, isolates were carried through series of, cultural, morphological and physiological tests, including Grams reaction, spore staining, growth under various conditions, carbohydrate utilization, etc. according to "Bergey's Manual of Systematic Bacteriology" (Table 1).

Bacteriocin production

Muller Hinton Broth (MHB) (Hi-media) with pH at neutrality was used as production medium, 25 ml broth was prepared in 50 ml Erlenmeyer flasks and sterilized at 121 ºC for 15 min, selected isolates were inoculated as soon as the broth came to room temperature followed by incubation in shaking incubator at 35 ºC with 100 rpm for 24 and 48 hr.

Detection of antimicrobial activity

A cell free suspension (CFS) was prepared by centrifuging the MHB, at 4500 x g for 20 min and stored at 4 ºC. The antimicrobial activity determined by agar well diffusion method on Muller Hinton Agar (MHA) (Hi-media) against indicator organisms *i.e. E. coli* (MCCB 0017) and methicillin resistant *S. aureus* (MCCB 0139) provided by Microbial Collection Culture Bank, Department of Industrial Microbiology, SHUATS. A 12-18 hr old culture broth of indicator organisms (10^7 $CFUml^{-1}$) were swabbed on the MHA plates and 5 mm wells were cut out, an aliquot of 50 µl of CFS was filled in the wells followed by incubation at 37 ºC. The antagonistic activity in Arbitrary unit/ml ($AUml^{-1}$) was calculated (Bhaskar *et al.,* 2007) as a measure of bacteriocin production.

$$AUml^{-1} = \frac{\text{Diameter of zone of clearance (mm) X 1000}}{\text{Volume of CFS in the well (µl)}}$$

Results and Discussion

Isolation and Identification of bacterial strain

49 isolates were quarantined from hundred soil samples collected form diary environment, on the basis of the method mentioned by Edwards and Seddon (2000). Among these isolates three were *viz*. 18, 54 and 55 identified as *Brevibacillus brevis* (6%) and 20% as *B. licheniformis*, 18% as *B. megaterium*, 6% as *B. pumilus*, 4% as *B. coagulans* based on morphological and biochemical characteristics enlisted in Table 1.

The organisms were found motile, spore bearer, gram positive and strictly aerobic.

The carbohydrate profile (Table 2) of bacteria under investigation depicts that it could utilize glucose, fructose, glycerol, mannitol, sucrose, trehalose, maltose, ribose and melezitose with weak production of acid and little or no gas production; but could not utilize xylose, sorbitol, salicin, rhamnose, raffinose, melebiose, mannose, lactose, inositol and arabinose. Similar results have been reported by Ghadbane *et al.,* (2013).

Detection of antimicrobial activity

All three isolates were examined for their antibacterial activity against *E. coli* and methicillin resistant strain of *S. aureus.* Crude brevicin from isolate no. 18 displayed largest zone of inhibition among all the three isolates against *S. aureus*.

The crude brevicin obtained after 24 hr of incubation displayed higher activity than that of 48 hr incubation time. The results are summarized in the (Table 3). On calculating the effectiveness in terms of arbitrary unit per milliliter ($AUmL^{-1}$) (Bhaskar *et al.,* 2007; Bhuvaneswari *et al.,* 2015) the activity of crude brevicin was found with upper limit of 440 $AUmL^{-1}$ for isolate no. 18 against *S. aureus* followed by 360 $AUmL^{-1}$ and 210 $AUmL^{-1}$ for isolate no. 54 and 55 respectively (Fig. 1). In contrast, no activity was observed against *E. coli* (Fig. 2).

The genus *Brevibacillus* is mainly a soil organism, found readily in soil with high humus (Elo *et al.,* 2000).

Table.1 Morphological and biochemical characteristic of isolated no. 18

Characteristics studied	*Bre viba cill*	*B. meg ater*	*B. lich enif*
Gram reaction	+ ve	+ ve	+ve
Size (in µm)	3.0 - 4.3	2.0-4.6	1.6-2.8
Endospore	+ ve	+ ve	+ve
Growth at 45 °C	+ ve	+ ve	+ve
Growth at 65 °C	- ve	-ve	-ve
Growth at pH 5.7	+ ve	+ ve	+ve
Growth in 7% NaCl	+ ve	+ ve	+ve
Growth in lysozyme	+ ve	+ ve	+ve
Growth in anaerobic medium	- ve	-ve	+ve
Motility	+ ve	+ ve	+ve
Hemolysis	- ve	+ ve	+ve
Hydrolysis of esculin	+ ve	-ve	-ve
Hydrolysis of gelatin	+ ve	+ ve	±ve
Tyrosine degradation	- ve	+ ve	-ve
Hydrolysis of urea	+ ve	-ve	-ve
Catalase	+ ve	+ ve	+ve
Oxidase	+ ve	+ ve	+ve
Lysine decarboxylase (LDC)	- ve	-ve	-ve
Ornithine decarboxylase (ODC)	- ve	-ve	-ve
Indole production	- ve	-ve	-ve
Voges-Proskauer	-ve	-ve	-ve
Nitrate reduction	+ ve	+ ve	+ve
Egg yolk reaction	- ve	+ ve	+ve
Citrate utilization	+ ve	+ ve	-ve

Table.2 Fermentation profile of different carbohydrates by isolate no. 18

Carbohydrate	Br. brevis (Isolate no 18)	B. megaterium	B. licheniformis
	Acid /Gas	Acid /Gas	Acid /Gas
L-Arabinose	-/-	+/-	+/-
Cellobiose	+/-	+/-	+/-
Fructose	+/-	+/-	+/-
D-Glucose	+/-	+/-	+/-
Glycerol	+/-	+/-	+/-
M-Inositol	-/-	+/-	-/-
Lactose	-/-	+/-	-/-
Mannitol	+/-	+/-	+/-
D-Mannose	-/-	+/-	+/-
Maltose	+/-	+/-	+/-
Melebiose	-/-	-/-	-/-
Raffinose	-/-	-/-	-/-
Rhamnose	-/-	-/-	-/-
Ribose	+/-	-/-	+/-
Salicin	-/-	+/-	+/-
Sorbitol	-/-	-/-	+/-
Starch	+/-	+/-	+/-
Sucrose	+/-	+/-	+/-
Trehalose	+/-	±/-	+/-
D-Xylose	-/-	+/-	-/-

*Negative (-), Positive (+)

Table.3 Inhibitory effect of crude brevicin from different isolates against indicator organisms after 24 and 48 hrs respectively

Isolate no.	zone of inhibition		
	S. aureus MCCB 0139 (in mm)		*E. coli* MCCB 0017 (in mm)
	CFS after 24 hr	CFS after 48 hr	
18	22	20	-
54	18	16	-
55	10	9	-

*included well size of 5mm diameter; CFB: Cell Free Broth

Fig.1 Screening of antibacterial activity of brevicin against *S. aureus* MCCB 0139

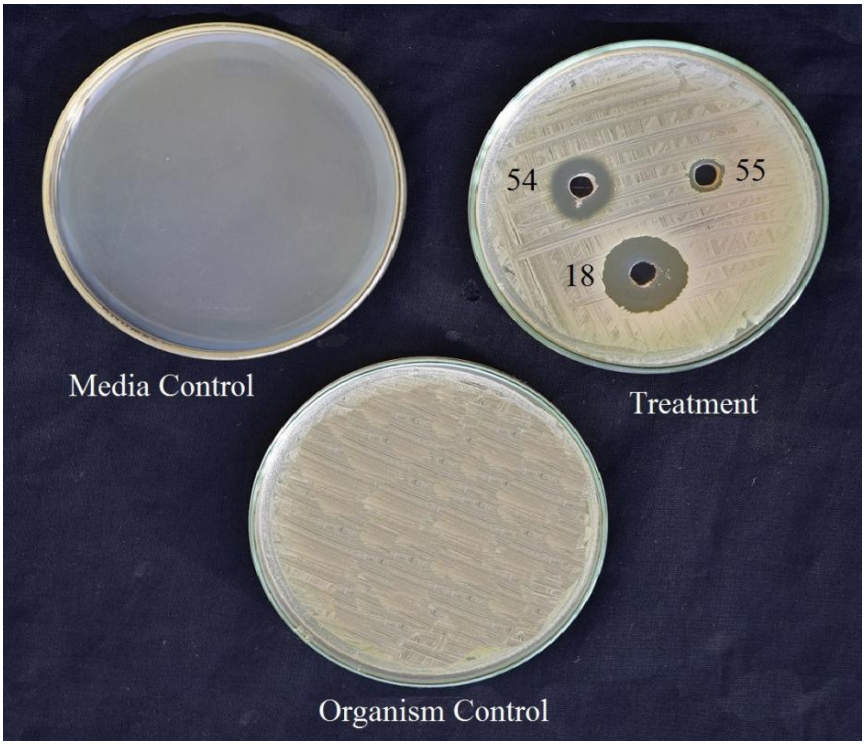
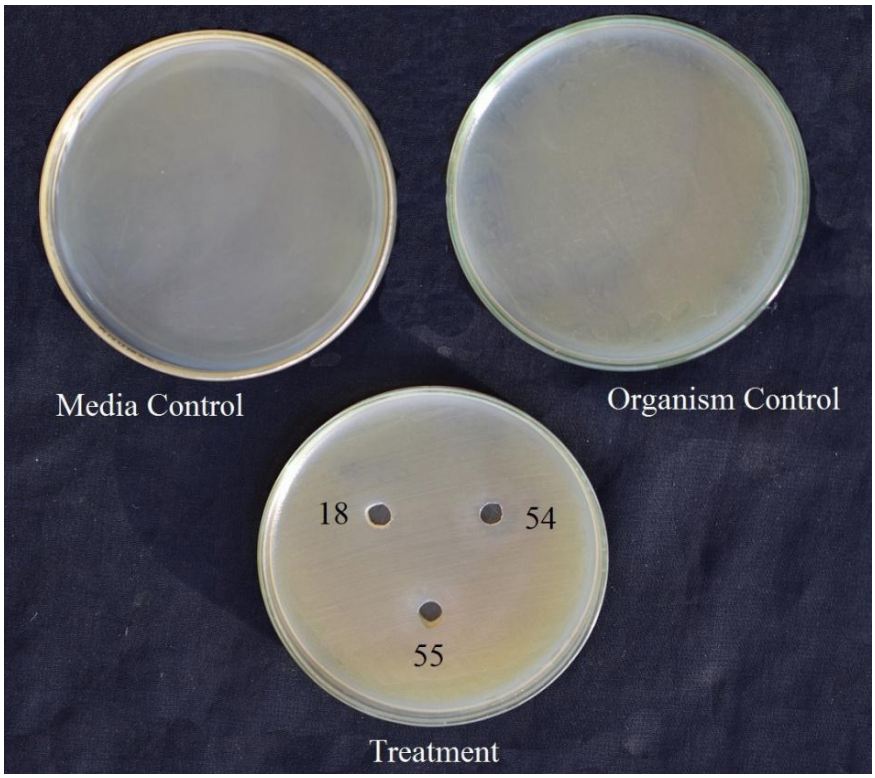


Fig.2 Screening of antibacterial activity of brevicin against *E. coli* MCCB 0017



But isolates of *Brevibacillus* sp. have also been reported from the airborne dust (Andersson *et al.,* 1999), food packaging products of paper and board (Pirttijarvi *et al.,* 2000), submerged rhizosphere of sea grass *Vallisneria americana* (wild celery) in an estuarine environment (Kurtz *et al.,* 2003). In the present study *B. brevis* was isolated from soil samples of dairy environment with 6.12 % occurrence level. The brown or black pigmentation produced on the agar plates was due to formation of melanin as a result of presence of tyrosine in the medium (Cubo *et al.,* 1988). Negative result for lecitinase production and hemolysis confers the organism as nonpathogenic entity (Nakamura, 1991).

Thus, from present study, it can be concluded that brevicin from *B. brevis* is having narrow spectrum of anitimicrobial activity, restricted to gram positive organisms. Similar results have been reported by (Saleem *et al.,* 2009), (Faheem *et al.,* 2007) and (Chalasani *et al.,* 2015) for brevicin. This can be explained on the basis of mode of action of anitimicrobial peptides, which primarily believed to act upon the cell membrane rich in teichoic and teichuronic acids by creating pores, these anionic structures in contrast to cationic antimicrobial peptide or bacteriocin activity, increases the negative surface charge, which is not possible with Gram negative organism. Since, it has a Lipopolysaccharide (LPS) outer membrane and a very thin layer of petidoglycan lacking teichoic and teichuronic acids (Wimley, 2010; Yeaman and Yount, 2003). However, (Hyung *et al.,* 2001) have reported bacteriocin with broad antimicrobial activity. Various species of *Bacillus* have also been reported for their Antimicrobial peptide (AMP) or BLIS having broad spectrum inhibitory activity (Singh *et al.,* 2012; Ghadbane *et al.,* 2013; Embaby *et al.,* 2014). In the present study the antibacterial activity of crude bacteriocin from *B. brevis* was detected to be restricted for gram positive bacteria. Maximum production was observed at 24 hr at 37 ºC with medium pH at neutrality. Strains of *Brevibacillus* are potent source of bacteriocins and these peptide antimicrobials can be used as a potent therapeutic agent in pharmaceuticals as well as can also be used as food preservatives in canned or packed food and dairy products.

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