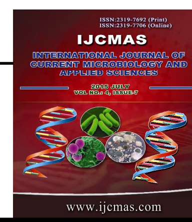

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Original Research Article

Isolation, Identification and Application of Bacteriocin-Like Inhibitory Substances Producing Bacterial Strains

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Keywords

Bacteriocin-like inhibitory substances (BLIS), bacterial isolates, food pathogenic microorganisms, food products

A B S T R A C T

Microbial activity is the first and most dangerous limitation of the food shelf life. Pathogenic and spoilage bacteria should be controlled to guarantee the microbial safety of foods. This study aims to isolate and characterize bacteriocin-like inhibitory substances (BLIS) producing bacterial strains. One hundred bacterial isolates were obtained from soil (32) and food (68) samples. Screening of isolates for the production of BLIS were studied using disc diffusion method against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Candida albicans* and *Aspergillus niger*. Twelve isolates showed measurable inhibition zone against all the 7 tested organisms. Cell-free supernatants of these twelve cultures were then added to luncheon and Edam cheese slices to extend its shelf life in a refrigerator. Mesophilic aerobic bacterial count generally decreased during storage period (15days) with 4 BLIS producing strains, which were identified with Biolog system as: *Micrococcus luteus, Cellulomonas hominis, Corynebacterium nitrilophilus* and *Rhodococcus rthodochrous.*

Introduction

Microbial activity is the first and the most dangerous limitation of a food's shelf life (Kesenkas *et al.,* 2006). In the production of food, it is crucial to take proper measures for ensuring its safety and stability during the shelf-like (Jeevaratnam *et al.,* 2005). Pathogen and spoilage bacteria should be controlled to guarantee the microbial safety of foods. A combination of factors can be an efficient way to warranty food safety keeping their organoleptic and functional properties (Antolinos *et al.,* 2011). Microorganisms are good sources for the production of biologically active substances.

The use of microorganisms and their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind (Galvez *et al.,* 2007). Protective cultures are food-grade bacterial, which may or may not be strains naturally present in the foods. They are selected for their ability to grow in a product and inhibit a pathogen or undesired microorganisms through the production of low molecular mass compounds such as organic acids, carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins (Kesenkas *et al.,* 2006). In biopreservation,

storage life is extended and /or safety of food products is enhanced by using natural or controlled microflora, mainly lactic acid bacterial (LAB) and/ or their antibacterial products (Rodgers *et al.,* 2002).

The term "bacteriocin" comprises a large and diverse group of ribosomally synthesized extracellular antimicrobial low molecular mass proteins or peptides which have a bactericidal or bacteriostatic effect on other closely related bacteria (Devlieghere *et al.,* 2004). To control food borne pathogens in food, role of baceriocin or bacteriocin like inhibitory substances (BLIS) might be the best alternative to chemical preservative because bacteriocin or BLIS provide safety against spoilage causing and food borne pathogens and it does not have any ill effect on health (Pingitora *et al.,* 2007). The term BLIS is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocins. They have been reported to inhibit a wide range of both Gram positive and gram negative bacteria. They are medically, industrially and agriculturally very important (Riley and Wertz, 2002).

The aim of this work is to study the following main points: Isolation and identification of bacterial strains having antimicrobial activity, screening of the isolated bacterial strains for production of bacteriocins and bacteriocin-like substances and application of bacteriocin-like inhibitory substances (BLIS) as biopreservation agents for luncheon and cheese.

Materials and Methods

Microbial strains

The following pathogenic bacterial strains were obtained the Research Park from the Faculty of Agriculture Cairo University: *Bacillus cereus, Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* and *Staphylococcus aureus* ATCC 29213 and were cultivated on trypticase soy agar (TSA) at 30 - 37°C. Also fungal strain of *Aspergillus niger* and *Candida albicans* were cultivated on potato dextrose agar at 30°C. The above pathogenic strains were used as indicator microorganisms for antimicrobial of bacteriocin and bacteriocin-like substances from isolated bacterial strains.

Collection and preparation of soil and food samples

In a systematic screening program for isolation of bacteria, 8 soil samples were collected from the school farms and gardens in Cairo from the upper layer around the roots where most of the microbial activity takes place and thus where dense bacterial population could be found. Soil samples (approximately 100g) were collected using some clean dry and sterile bags along with a sterile spatula, these were sandy, clay and silt soil samples of 5 food products were collected from local markets in Cairo governorates. These were raw, cream and rayeb milk, Kariesh cheese and pickling brine of cucumber. For solid samples such as Kariesh cheese, pickling brine of cucumber and soil samples, 10g of each were homogenized with 90ml of 0.85% w/v NaCl sterile saline to give suspensions. For liquid samples such as raw, cream and rayeb milk, 10 ml of the product were homogenized with 90ml of 0.85% w/v NaCl sterile saline to give food suspensions.

Isolation of bacteria

For isolation from soil samples, crowed plate technique was achieved using TSA medium to isolate bacterial strains which have antimicrobial activity against other microorganisms. One ml of soil suspensions

and its dilution was inoculated in TSA plates and incubated at 30°C for 48 hours. Colonies with clear inhibition zone were selected, tested and purified (Musliu and Salawudeen, 2012). Regarding to food samples, bacteriocin producing LAB were isolated using selective culture media and pour plate technique. One ml of the food suspensions and its dilutions were inoculated in MRS and M17 media by the pour plate method and incubated at 30°C for 5 days (Sharpe *et al.*,1966) and at 37°C for 2 days (IDF,1981) respectively. Colonies after 18–24 hours reached 1–2 mm diameter within 48 hours from M17 pates were selected (IDF, 1981). Colonies that grow on MRS plates were be selected and subjected to biochemical tests to identify the species of lactobacilli. All the colonies that were selected from each medium where gram stained in accordance with standard gram staining procedure by Todar *et al.* (2005) and tested for catalase enzyme. Bacterial isolates which were selected from each media were subcultured into each of its media's slants for preservation and to purify the isolates. They were kept in a refrigerator at 4°C for storage before, biochemical tests were applied for identification.

Screening of bacterial isolates for production of biosubstances

The antimicrobial activity of the bacterial isolates was tested by the disc diffusion assay (Bauer *et al.,* 1966). The disc diffusion or Kirby- Bauer disc method is a commonly used assay or microbial susceptibility. In this method, the antimicrobial substance-impregnated discs are placed on the agar surface inoculated with the test microorganism. The disc release the antimicrobial substance into the surrounding medium and an inhibition zone occurs if the test microorganism is susceptible to this substance.

The method described by Corsetti *et al.* (2004) was used. Before testing, the bacterial isolates were grown in each of their suitable broth medium at 35°C for 24h. Cells were removed from the culture by the centrifugation at 6400 rpm for 15 min. To eliminate the inhibitory effect of lactic acid and H_2O_2, the cell- free supernatant was tested for neutrality by the addition of BTB drop wise. If the color of the supernatant changed to light green means it is neutral, if not 1M NaOH was added drop wise till a color change to neutral and catalase enzyme was added to eliminate the effect of H_2O_2 followed by filtration through a cellulose acetate filter with pore size 0.22 µm (ADVANTEC MFS, Inc., Japan). The resulting supernatants were kept in a refrigerator for further tests. This supernatants were designated as crude bacteriocin-like substance (CBLS) (Xie *et al.,* 2009) To carry out the disc diffusion assay, agar base medium (1.5 % agar) was overlaid with 7ml of soft agar medium (0.7% agar) inoculated with 4% v/v of the indicator strain. The disc (Whatman no. 1; 5 mm in diameter was saturated with 50µl of the supernatant and placed on appropriate agar medium. The plates were incubated for 24h at the optimum temperature. Growth inhibition was measured using a ruler in the presence of a detectable clear zone diameter around the disc.

Effect of the bacteriocin-like inhibitory substances on shelf life of selected foods

Food samples treatment

Two millimeters of 12 different CBLS were sprayed on each surface of luncheon slices (10g). After assuring good contact of the inoculums with the slice surface, they were placed in sterile plastic bags and kept under refrigeration (4 ± 1°C) for 15 days. The samples were examined at intervals of 0, 3, 6, 9 12 and 15 days of storage for

microbiological changes. The experimental conditions were as follows: Samples not sprayed with CBLS as a control and samples sprayed with CBLS of isolates (10,12,13,17,19,20,32,36,49,51,54 and 61).

Microbiological sampling and analysis

On days 0, 3, 6, 9, 12 and 15, 10 grams of each sample were homogenized in 9 ml of sterile water in a sterile blender for 1 minute at normal speed at room temperature. Serial dilutions were prepared for analysis. The microbiological analysis included the determination of total viable bacterial count, appropriate dilutions, prepared in sterile water, were plated in duplicates on trypticase soy agar. The plates were incubated at 37°C for 3 days, and the counts per gram of the sample were calculated.

Culture media

Trypticase soy agar medium (APHA, 1978)

It is a highly nutritious general purpose medium. It can be used either as solid or broth for the total bacterial count and for the cultivation of pathogenic strains. It is composed of the following ingredients (g/l): Tryptone (17), Soy peptone (3), Sodium chloride (5), Dipotassium phosphate (2.5), Glucose (2.5), Yeast (6) and Agar (18). pH = 7.0 ± 0.2.

All components were added to one liter of distilled water then heated to boiling in order to dissolve the components. The melted medium was dispensed in flasks and autoclaved at 121℃ for 15 min.

Potato dextrose Agar (Biolife, 1991)

It's a medium used for the growth of microfungi. It is composed of the following ingredients (g/l): Potatoes, peeled and diced (200), D- Glucose (20) and Agar (20). Boil 200g of peeled, dried potatoes for 1h in 1 litre of distilled water. Filter, and make up the filtrate to 1 litre. Add the glucose and agar and dissolve by steaming. Distribute in 10- or 100-ml amounts in screw-capped

bottles and sterilize by autoclaving at 121℃

for 20mins.

MRS broth (De Man *et al.,* 1960)

It's a selective medium for the culture of lactobacilli. It is composed of the following ingredients (g/l): Peptone (10), Meat extract (10), Yeast extract (5), Glucose (20), Tween 80 (1), Dipotassium hydrogen phosphate (2), Sodium acetate (5), Triammonium citrate (2), Magnesium sulphate, hydrate ($MgSO_4 \cdot 7H_2O$) (0.2), Manganese sulphate, hydrate ($MnSO_4 \cdot 4H_2O$) (0.05). To get MRS agar use MRS broth, adding 13–15g/l of agar Bios LL. Dissolve the ingredients in one liter distilled water by steaming. Adjust the pH to 6.2–6.6. Distribute as required and sterilize at 121 °C for 15min. The final pH after sterilization should be 6.0–6.5.

M17 broth (Terzaghi and Sandine, 1975)

It is used for the isolation and enumeration of the lactic streptococci from yoghurt, cheese, starter cultures and other dairy products. It is composed of the following ingredients; Tryptic casein (2.5), Biomeat (2.5), Soy peptone (5), Yeast extract (2.5), Beef extract (5), Sodium Glycerophosphate (19), Magnesium sulphate (0.5), Ascorbic acid (0.5) and Lactose (5). To get M17 agar use M17 broth, adding 13–15g/l of agar Bios LL. Suspend 55.2g of M17 Agar or 42.2g of M17 Broth in 1000ml of cold distilled water.

Heat to boiling, distribute and sterilize at 121°C for 15 minutes, final pH 7.2± 0.1.

Identification of the bacterial isolates using Biolog system

Biolog™ micro- plates (Biolog, Inc., 3938 Trust way, Hayward, CA 94545, USA) test the ability of microorganisms to utilize a preselected panel of different carbon sources and amino acids. The test yields a characteristic pattern of purple wells, which constitutes a "Metabolic finger print" of the capacities of the inoculated organism (Jones *et al.,* 1993; Harris and Gudmestad, 1996). Tetrazolium violet is used as a redox dye to colorimetrically assay which indicated the utilization of the carbon sources**.**

Results and Discussion

Isolation and efficiency of bacterial strains for production of bacteriocin and bacteriocin-like substances

One hundred bacterial isolates in total were obtained from soil (32) and food (68) samples as follows: Clay soil (3), Sandy soil (7), Silt soil, (22), Raw milk (2), Cream milk (24), Rayeb milk (20), Kariesh cheese (18) and pickling brine of cucumber (4). Cultures were isolated from soil samples using crowded plate technique with trypticase soy agar. In food samples, cultures were isolated using dilution plate method with MRS and M17 media. All isolates were purified by streaking plate technique onto trypticase soy agar.

The 100 bacterial isolates in which 30 were isolated from soil samples and 70 from food samples were screened for bacteriocin and bacteriocin-like inhibitory substances using the disc diffusion assay. In this method, the culture supernatant is neutralized with NaOH and treated with catalase enzyme to eliminate the inhibitory effect of organic acids and H_2O_2, respectively (Corsetti *et al.,* 2004). The culture supernatant-impregnated disc is placed on the surface of the agar plate inoculated with the test microorganism (Bauer *et al.,* 1966).

A total of 100 bacterial isolates were screened for the production of bacteriocin and bacteriocin-like substances against 7 test strains which were *Bacillus cereus, Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853*, Salmonella typhimurium* and *Staphylococcus aureus* ATTCC 29213. Also fungal strain of *Aspergillus niger* and *Candida albicans* inhibition zone diameter were measured using disc diffusion method (Table 1). In this experiment, the possible inhibitory effect of organic acids or hydrogen peroxide was excluded. Twelve bacterial isolates (10,12,13,17,19,20,32,36,49,51,54 and 61) showed measurable inhibition zone against all 7 test strains. Similarly, results of Khanian *et al.* (2014) indicated that the neutralized supernatant fliud of the two isolates identified as *Lactobacillus brevis* LB32 and *Lactobacillus pentosus* LP05, were active against the growth of *Listeria monocytogenes*, *Salmonella enteritidis, Shigella dysenteriae, Staphylococcus aureus* and *Streptococcus pneumoniae*.

All inhibition diameter zones ranged from 6 to 23.5 mm. Twelve bacterial isolates (10,12,13,17,19,20,32,36,49,51,54 and 61) showed measurable inhibition zone against all 7 test strains. Isolate 90 and 91 showed no inhibition zone against the 7 test strains. This may be possibly due to lack of any antimicrobial substances in these bacterial isolates. The bacterial isolates strain 60 was the most active against *Staphylococcus aureus* with inhibition zone diameter being 23.5 mm compared to any other strain. This may be as a result of the fact most undesirable bacteria such as *Pseudomonas*

spp. and *Staphylococcus aureus* are many times more sensitive than the LAB (Olaoye and Ntuen, 2011) The least active bacterial isolate was strain 70 with inhibition zone diameter being 12 mm against *Pseudomonas aeruginosa*. The bacterial isolate strain 60 produced greatest effects against *Staphylococcus aureus* and *Bacillus cereus* with inhibition zone diameter being 23.5mm and 20.5mm, respectively compared to any other strain. In this respect, Rodgers (2001) reported that the target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the lipopolysaccharide and lack of phospholipids at the outer membrane of Gram-negative bacteria, they are generally effective against Gram-positive organisms. Similarly, Rehaim *et al.* (2009) found that cell- free supernatant of *Enterococcus faecium* could inhibit *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Listeria* species, but there was no inhibition of Gram- negative bacteria, *Hafnia*, *Serratia* and *E. coli.*

Application of crude bacteriocin-like substances (CBLS) on luncheon and cheese as a preservation factor

The use of microorganisms and their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind (Gálvez *et al.,* 2007). Biopreservation refers to the extension of the shelf-life and improvement of the safety of foods using microorganisms and/or their metabolites (Settanni and Corsetti, 2008). Bacteriocins may be considered natural preservatives or biopreservatives that fulfill these requirements, since it is assumed that they are degraded by the proteases in gastrointestinal tract (Cleveland *et al.,* 2001).

Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which, the important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins and plantaricins (Nettles and Barefoot, 1993).

In this study, the addition of crude bacteriocin or crude bacteriocin-like inhibitory substance was used as a biopreservation for refrigerated sliced luncheon and cheese were evaluated. The effect of each CBLS on the luncheon and cheese slices shelf life was studied. For each trial, two treatments were carried out, total viable bacterial counts were estimated in samples without CBLS as control and samples sprayed with CBLS (Tables 2, 3, 4 and 5). Since foods are complex ecosystems in which the different microbial populations interact (through cooperation, competition or antagonism, *etc*.) Treatment with antimicrobials such as bacteriocins affects both target bacteria and other members of the microbial community (Fangio and Fritz, 2014).

Identification of promising isolates

Four isolates (10, 12, 13 and 19) revealed the best antimicrobial activity during application of isolates extracts on luncheon and Edam cheese (Tables 2, 3, 4 and 5). These isolates were identified using Biolog system, the four isolates were; *Micrococcus luteus* (10), *Cellulomonas hominis* (12), *Corynebacterium nitrilophilus* (13) and *Rhodococcus rthodochrous* (19). All of the four strains were isolated from soil samples, which indicated that soil is a rich ecosystem with enormous biological diversity. Similarly, Lisboa *et al.,* (2006) isolated BLIS producing bacterial strain (*Bacillus amyloliquefaciens*) from Brazilian Atlantic forest soil as a unique ecosystem. Also, Ozdemir and Biyik (2012) in India isolated

BLIS producing thermophilic bacterial strain (*Geobacillus toebii*) from mud and soil samples.

Table.1 Inhibition zone diameter (mm) of the isolates extracts against sensitive strains

Isolate no.	Sensitive strains						
	Can.	*B.*	*E. coli*	*Ps.*	*Salm.*	*Staph.*	*A. niger*
10	10	9	9.5	9.5	7	6	11
12	10.5	11	8.5	11	8.5	11	13
13	10	13.5	11.5	8.5	9.5	10	9.5
17	10	9.5	11.5	8.5	10	9.5	9.5
19	13.5	10	10.5	15	10.5	10	10
20	12	10	10.5	8.5	10	8.5	10
32	11.5	13	9.5	8.5	17	13.5	11.5
36	10	10	10	9	11.5	12.5	12
49	10	10.5	9.5	10	8	10	8.5
51	7	7	8	10	15	10	8.5
54	13.5	12.5	12.5	8.5	14.5	11.5	10
61	19	20	9	8.5	7	19.5	11

B., Bacillus cereus
Can., Candida
Ps., Pseudomonas aeruginosa
Salm., Salmonella typhimurium
Staph., Staphylococcus aureus
A. niger., Aspergillus niger

Table.2 Effect of crude bacteriocin-like substances of different cultures (10, 12, 32, 51and 49) on total viable bacterial counts of luncheon slices during storage periods

Storage time(days)	Total viable bacteria count (cfu/g)					
	Control	**10**	**12**	**32**	**51**	**49**
0	1.58×10^{9}	1.07×10^{7}	8.20×10^{6}	3.10×10^{7}	1.37×10^{7}	1.00×10^{9}
3	1.35×10^{9}	9.50×10^{5}	9.00×10^{5}	9.80×10^{6}	1.15×10^{7}	1.15×10^{7}
6	7.50×10^{9}	9.70×10^{6}	1.85×10^{7}	1.18×10^{7}	1.05×10^{7}	1.05×10^{7}
9	1.25×10^{9}	2.25×10^{8}	2.15×10^{6}	2.20×10^{8}	2.60×10^{8}	2.30×10^{8}
12	2.65×10^{9}	1.55×10^{9}	2.45×10^{9}	2.48×10^{9}	2.45×10^{9}	1.35×10^{9}
15	8.00×10^{9}	3.00×10^{9}	2.80×10^{9}	2.55×10^{9}	1.20×10^{10}	2.50×10^{9}

Table.3 Effect of Crude bacteriocin-like substances of different cultures (13, 17, 19, 20, 54, 36 and 61) on total viable bacterial counts of luncheon slices during storage periods

Storage time (days)	Total viable bacteria count (cfu/g)							
	Control	**13**	**17**	**19**	**20**	**54**	**36**	**61**
0	$2.13x10^{7}$	$2.50x10^{6}$	$1.05x10^{6}$	$4.8x10^{7}$	$3.00x10^{7}$	$4.50x10^{5}$	$7.00x10^{5}$	$4.70x10^{5}$
3	$4.10x10^{6}$	$1.63x10^{6}$	$6.50x10^{7}$	$1.80x10^{7}$	$2.00x10^{6}$	$8.80x10^{6}$	$1.40x10^{7}$	$8.60x10^{6}$
6	7.50×10^{8}	3.00×10^{7}	9.50×10^{7}	3.00×10^{7}	8.70×10^{7}	1.60×10^{7}	2.45×10^{7}	1.52×10^{7}
9	9.00×10^{8}	9.10×10^{7}	9.60×10^{7}	9.10×10^{7}	9.00×10^{7}	9.30×10^{7}	9.00×10^{7}	2.90×10^{7}
12	$2.20x10^{9}$	$1.25x10^{8}$	$1.25x10^{8}$	$1.11x10^{8}$	$1.33x10^{8}$	$1.86x10^{9}$	$6.80x10^{8}$	$1.10x10^{8}$
15	$2.38x10^{9}$	$1.60x10^{8}$	$2.50x10^{8}$	$2.40x10^{8}$	$3.50x10^{9}$	$3.00x10^{9}$	$1.86x10^{9}$	$2.10x10^{8}$

Table.4 Effect of crude bacteriocin-like substances of different cultures (10, 12, 19, 20, 36 and 61) on total viable bacterial counts of cheese slices during storage periods

Storage time (days)	Total viable bacterial count(cfu/g)						
	Control	10	12	19	20	36	61
0	1.00×10^8	3.00×10^8	3.90×10^5	4.50×10^5	4.50×10^7	4.00×10^3	3.20×10^3
3	1.00×10^8	1.62×10^8	1.35×10^5	7.70×10^4	1.80×10^4	2.35×10^5	2.00×10^4
6	2.00×10^8	1.80×10^6	7.00×10^4	4.00×10^4	3.50×10^5	3.20×10^4	5.00×10^4
9	2.20×10^8	6.00×10^5	5.00×10^4	1.00×10^4	1.08×10^6	3.50×10^4	1.44×10^4
12	2.50×10^8	3.30×10^4	4.50×10^4	6.00×10^3	9.50×10^4	5.00×10^5	5.90×10^4

Table.5 Effect of crude bacteriocin-like substances of different cultures (13, 17, 32, 51, 49 and 54) on total viable bacterial counts of cheese slices during storage periods

Storage time (days)	Total viable bacterial count(cfu/g)						
	Control	13	17	32	51	49	54
0	4.20×10^5	3.80×10^7	5.00×10^7	4.10×10^6	7.30×10^7	5.60×10^6	5.20×10^7
3	1.16×10^6	3.00×10^5	1.11×10^6	6.30×10^6	1.31×10^5	2.90×10^5	8.00×10^5
6	4.80×10^4	2.80×10^5	5.00×10^5	4.10×10^5	9.30×10^4	1.46×10^4	1.33×10^5
9	2.65×10^4	2.00×10^5	1.27×10^5	4.10×10^5	1.08×10^6	6.50×10^4	5.90×10^5
12	1.20×10^6	5.70×10^4	1.31×10^5	7.40×10^5	1.44×10^5	8.70×10^5	3.30×10^5
15	9.10×10^7	1.75×10^4	1.52×10^7	3.00×10^7	3.00×10^7	2.12×10^7	3.00×10^7

However, BLIS producing strains could be isolated from other sources, BLIS producing strains of *Brevibacillus borstelensis* was isolated from Marcha, which is herbal cake used as traditional starter culture to ferment wine in North East India (Sharma *et al.,* 2014).

Therefore, it could be concluded that the use of bacteriocin-like inhibitory substance is most likely a promising applicable in the field of food biopreservation.

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