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SCREENING OF BACTERIOCIN PRODUCING BACTERIA FROM SOIL SAMPLE AGAINST *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

In industrialized countries 30 percentage population suffer from food borne disease yearly. Food processing runs the risk of significant economic loss annually due to food spoilage resulting from microbial contamination. Although chemical preservatives may provide a solution, the use of such preservatives is generally avoided, since they have negative consequences for human health. Moreover, the problems associated with food safety especially of microbial contamination appear to be alarmingly high. To address the increasing bacterial resistance to conventional antibiotics, bacteriocins are now considered as alternative antimicrobials for the treatment of human and possibly animal infections. Further, processed foods without chemical preservatives are in demand by consumers. Research in natural antimicrobial agents such as bacteriocins becomes inevitable. Members of the Bacillus group are good producers of antimicrobial substances such as peptide and lipopeptide antibiotics, as well as bacteriocins. Interestingly, Bacillus represents an alternative genus for the identification of bacteriocins because it includes many industrial species and has a history of safe use in the food industry. In the present study screening of bacterial isolates for bacteriocin production were screened from soil samples, from them thirty bacterial isolates obtained were tested for the bacteriocin activity against indicator bacteria *Staphylococcus aureus*. Among them MA5 designated strain found to be Gram's positive rod bacteria. Based on the phenotypical characterization identified as *Bacillus sp.* and exhibited antagonistic activity against S. aureus. The antagonistic bacterial strain MA5 found to inhibit the growth of *S. aureus*. Antibacterial activity was identified by agar well diffusion assay. The sensitivity of the indicator strains were estimated based on the inhibition zones. To estimate the molecular weight of the bacteriocin, SDS-PAGE was performed and the protein in the active fraction was resolved and the molecular weight of the single protein band that exhibited antimicrobial activity was estimated to be 10 kDa.

Keywords: Bacteriocin, Methicillin Resistant, SDS-PAGE, *Staphylococcus aureus.*

INTRODUCTION

The emergence and dissemination of antibiotic resistance pathogenic bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) becomes an increasing fundamental problem in the public health worldwide (Schmitz *et al.,* 1998). Moreover, MRSA strains tend to accumulate additional new antibiotic resistance such as mupirocin. New strategies for controlling MRSA and multiresistant staphylococci are urgently needed. Bacteriocins are ribosomal synthesized antibacterial peptides. These compounds are produced by a broad variety of different bacteria belonging mainly to the genus Bifidobacterium, to which health promoting properties have frequently been attributed.

However, the identification of Bifidobacterium-associated bacteriocins was first reported in 1980 and that they exhibit antimicrobial activity against pathogenic microorganisms such as Listeria monocytogenes, Clostridium perfringens, and *Escherichia coli*, relatively little information is still available about the antimicrobial compounds produced by strains of this genus (Martinez *et al*., 2013).

Bacteriocins are proteinaceous antibacterial compounds, which are ribosomal synthesized antimicrobial peptides have novel applications other than food preservation. Various microorganisms were adapted to antibiotics by several mechanisms and they become as multidrug resistant bacteria (Kayalvizhi and Gunasekaran, 2008). The mode of action of bacteriocins is differing from conventional antibiotics so they are considered as novel source for the control of these microbial pathogens. Bacteriocins are produced by both gram positive and gram negative bacteria these microbial pathogens. Bacteriocins are produced by both gram positive and gram negative bacteria (Tagg and Given, 1971).

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Bacteriocin from Lactic acid bacteria exhibit a broad spectrum of activity includes protozoa, fungi, and yeast (Reddy *et al.,* 2004). Some of the bacteriocins are cytotoxic, with activity against sperm and tumor cells. This feature makes them attractive for formulation in feminine health care and contraceptive products (Reddy *et al,* 2004; Aranha *et al.,* 2004). The bacteriocins produced by lactic acid bacteria are widely used in medical and personal care applications. In addition to all these applications the antioxidative activity of lactic acid bacteria was also reported (Collins *et al.,* 1999). Extensive use of antibiotics in human health for various diseases results in the emergence of antibiotic resistance bacteria as well as multi drug resistant pathogens. The products produced by lactic acid bacteria exhibiting antimicrobial activity especially bacteriocins, hydrogen peroxide, enzymes and lactic acid helps in prevention and therapeutic purposes in controlling the pathogens (Collins *et al.,* 1999).

Bacteriocins from lactic acid bacteria have a wide potential application such as, for example food bio preservatives and health care (Cotter *et al.,* 2005; Suskovic *et al.,* 2010). Antimicrobial activity is often proposed as an important functional characteristic of probiotic strains, because bacteriocin biosynthesis by probiotics can contribute to eliminate undesirable bacteria during host colonization, thereby helping to prevent host from pathogen proliferation. Nisin and pediocin PA-1 produced by lactic acid bacteria are the only bacteriocins used in food preservation (Altuntas, 2013). Each bacteriocin has unique properties and a particular efficiency in targeting microbial pathogens. Therefore isolation and purification of new bacteriocins will be always beneficial (Elegado *et al.,* 1997; Merzoug *et al.,* 2015).

Bacteriocins received interest due to their potential application in the food industry as natural preservatives, given that they have the advantage of being reliable, nontoxic to eukaryotic cells and rapidly digested by proteases within the gastrointestinal tract (Parada *et al.,* 2007). For the industrial-scale production of bacteriocins required for therapeutic use, an efficient, inexpensive, and scalable purification scheme with high recovery is needed.

Members of the Bacillus group are good producers of antimicrobial substances such as peptide and lipopeptide antibiotics, as well as bacteriocins (Stein, 2005). Most bacteriocins are extremely potent, exhibiting antimicrobial activity at nanomolar concentrations, as opposed to the peptide antimicrobials produced by eukaryotic cells, which normally have lower activities (Jennsen *et al.,* 2006). Interestingly, the producer cells are immune to their own bacteriocins (Cotter *et al.,* 2005). The classification of bacteriocins has been revised from time to time. The latest classification arranges bacteriocins into three major classes based on their structural and physio-chemical properties (Zacharof and Lovitt, 2012).

Bacteriocins are antimicrobial peptides secreted by every bacterium to inhibit the growth of similar or closely related bacterial strains in a bacterial pool which has competition for obtaining Nutrients (Tagg *et al.,* 1990). They consist of molecules which act on the defense system of other bacteria, fungi, parasites and viruses. These compounds have gained importance in the fields of health care and food preservation as microbes are showing increasing resistance towards commonly used antibiotics and preservatives. However, the low quantity of peptides obtained from direct purification is, to date, still a remarkable bottleneck for scientific and industrial research development. These compounds can protect against broad array of infectious microbes. Antimicrobial peptides have a very good future in pharmaceutical and food industries. One traditionally sidestepped but ever-present issue is that of defining what constitutes a bacteriocin.

MATERIALS AND METHODS

Screening of bacteriocin producing bacteria from biomedical waste were carried by microbiological methods and identification of bacteriocin producing bacteria against *Staphylococcus aureus* done by following the methods given below.

Isolation of bacterial strains

Bacterial strains were isolated from soil samples using the standard protocol. Soil samples were suspended and serially diluted in sterile saline solution (0.89% w/v^{-1} NaCl). Tubes containing 0.1 ml of appropriately diluted solution were plated on nutrient agar (Himedia, Mumbai, India) plates and were incubated at 30°C for 24 hrs.

Morphologically distinct single colonies were subculture on nutrient agar plates and screened for antibacterial activity (Figure 1 and Table 1).

Table 1. Identification of microbial colonies by morphological and biochemical tests.

Morphological Tests	
Media used for inoculums	LB Medium
Colony character	Irregular , White
Colony on LB media	White
Grams staining	Positive Rods
Biochemical Tests	
Indole tests	Negative
Methyl red tests	Negative
VP tests	Negative
Citrate utilization tests	positive
Oxidase tests	positive
Nitrate tests	positive
Catalase tests	positive
Gelatin	positive
Casein hydrolysis	positive
Urease	positive
Lipase	positive
Starch hydrolysis	Negative

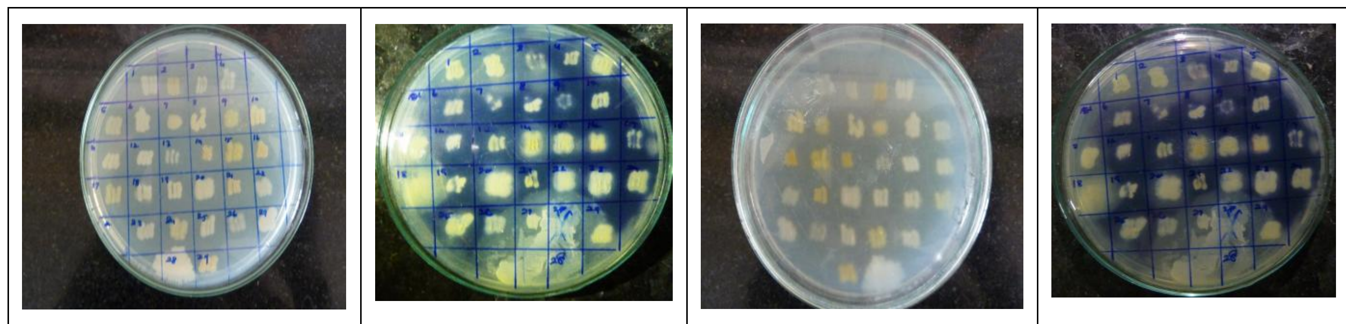

Figure 1. Screening of bacteriocin producing bacteria from biomedical waste.

Detection of antibacterial strains

The antibacterial activity of the isolates was determined by a deferred-antagonism plating assay (Tagg *et al.,* 1976). Nutrient agar plates were streaked with test organisms and incubated at 30°C for 24 hr and then the plates were overlaid with soft agar (0.75% agar) containing 10^6 CFU ml^{-1} of the stationary-phase culture of indicator strains. The plates were incubated at 30°C for 24-48 hrs and examined for the zone of inhibition.

Agar well diffusion assay

The strain which was selected as potential bacteriocin producers were grown in luria broth at 37ºC for 24 hrs. Cells were separated by centrifugation at 10,000 rpm for 10 min at room temperature. Around 6mm diameter wells were made on preinoculated agar media and each well was 100 µl of culture supernatant added. Inhibitory activity was performed against *Staphylococcus aureus*. Inhibition zones around the wells were measured and recorded.

Gram staining

Gram staining is used to detect the fundamental difference in the cell wall composition of bacteria. A clean, grease free glass slide was taken and wiped with alcohol. A thin smear of the organisms was made on the slide and it was fixed by air drying. The smear was flooded with crystal violet (primary stain) for 60 second and then washed with distilled water. Few drops of Gram's Iodine were added on the smear and kept for 60 seconds followed by washing with distilled water. Decolorization of the smear was done by using alcohol. Then the smear was stained by counter stain safranin and kept for 20 seconds followed by washing with distilled water. The slides were finally washed with distilled water, air dried and viewed under the oil immersion objective of the light microscope to obtain colony morphology.

Biochemical characterization

The microbial colonies were identified by using the following biochemical test (Table 1). 1.3 g of Luria broth taken and 100 ml of H_2O added, then culture inoculated with the help of sterile loop in the Laminar flow to prevent contamination. Culture was grown at 37°C for 24 hrs. After culture grown, culture can be added to Biochemical kit. Biochemical test done by three steps they were as follows.

Indole Test

1-2 drops of Kovac's reagent (R008) were added. Development of reddish pink color within 10 seconds indicated the positive reaction. Reagent remained pale colored if the test was negative.

Methyl Red Test

1-2 drops of Methyl Red reagent (1007) were added. Reagent remained red in color if the test was positive. Reagent decolorized and become yellow if the test was negative.

Voges Proskaeur's Test

1-2 drops of Baritt reagent A (Ro29) and 1-2 drops of Baritt reagent B (R030) were added. Pinkish red color development within 5-10 minutes indicated a positive test. No change in color or a slight copper color (due to reaction of Baritt reagent B) denoted a negative reaction.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular weight of the bacteriocin-like protein was confirmed on SDS-PAGE. The electrophoresis was carried out using a 16.5% w/v polyacrylamide gel using Tris-Tricine buffer system. The gel was quick-stained with Coomassie brilliant blue (CBB) G-250 and then destained. Protein molecular weight markers (Bangalore Genei, India) in the range of 14.3 to 97.4 kDa were used as the standard. To localize the *in situ* bacteriocin activity, the gel was cut and separated before staining and the gel was fixed and washed. The washed gel were placed on a glass plate and overlaid with 0.7% agar containing 10^6 ml^{-1} of *S. aureus*. The overlaid gel was incubated overnight at 37°C for 24 hours and examined for zone of clearance.

RESULTS

Screening of bacterial isolates for bacteriocin production

Thirty bacterial isolates were obtained from soil samples and they were tested for the bacteriocin activity against indicator bacteria *Staphylococcus aureus* as described in materials and method section. Among them, one bacterial isolate exhibited the antagonistic activity against *S. aureus.* The antagonistic bacterial strain designated as MA5 found to inhibit the growth of *S. aureus*.

Antibacterial activity

The agar well diffusion assay was used to study the antibacterial activity of the MA5 strain of bacteriocin bacteria isolated from soil sample were shown to produce inhibition zones against *Staphylococcus aureus*. The sensitivity of the indicator strains were estimated based on the diameter (mm) of the inhibition zones. The bacterial strain MA5 produced higher level of bacteriocin (100 AU ml^{-1}).

Gram staining

The microbial isolates were identified by Grams staining using the method of Bergey's Manual of Determinative Bacteriology and were viewed under the oil immersion objective of the light microscope. All the microbial isolates investigation in this study were found to be Gram positive as the cells retain the Crystal violet and remain purple to dark blue colour. The microscopic characteristics of the isolates were observed and recorded. Bacteriocin producing bacteria were Grams positive rod bacteria.

Biochemical test

The isolated strain MA5 was identified based on its morphological, biochemical characterization. The microscopic analysis showed rod in shape, motile, spore former and can grow at 37°C. Various biochemical and physiological tests showed that MA5 is catalase, urease, casein hydrolysis, nitrate broth, citrate utilization, gelatin liquefaction, oxidase and lipase positive; however, starch hydrolysis, methyl red, voges-proskauer and indole negative. Based on the characterization MA5 stain was phenotypically identified as *Bacillus* sp.

SDS-PAGE and Zymogram analysis

The molecular weight of the bacteriocin was estimated and the protein in the active fraction was resolved by 15% tricine SDS-PAGE. Based on the electrophoretic mobility, the molecular weight of the single protein band that exhibited antimicrobial activity was found to be 10 kDa (Figure 2).

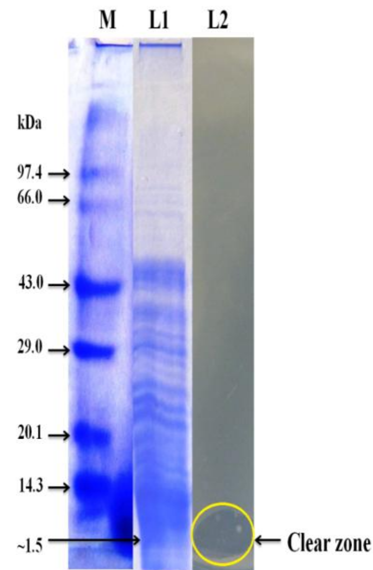

Figure 2. The fraction of protein in antimicrobial activity.

DISCUSSION

Bacteriocins are isolated from microorganisms which ribosomally synthesize antimicrobial protein or peptide,common among many gram-positive bacteria especially Bacillus as reported by Cherif et al., 2003. Recently, as reported by Mahrous et al., 2013,Lactobacillus species produce antimicrobial substances which assimilate bacteriocins.Vijayabaskar and Somasundaram (2008) isolated Bacillus sp. and used them as a probiotic in fresh water fish Tilapia (Oreochromis mossambicus) against the most common fish pathogen Aeromonas hydrophila. Higher antagonistic activity showed similar to the present study. Present results showed similarities with Kayalvizhi and Gunasekaran (2008) results that showed the SDS–PAGE analysis of the proteins in the culture filtrate which revealed a protein with an approximate molecular mass of 1.5 kDa that exhibited antibacterial activity against K. gibsonni GCS6 as showed in this study.

CONCLUSION

Food processors run the risk of significant economic losses annually due to food spoilage resulting from microbial contamination. Although chemical preservatives may provide a solution, the use of such preservatives is generally frowned upon, as many, such as nitrite, can have negative consequences for human health. Moreover, the extent of problems associated with food safety as a result of microbial contamination appears to be alarmingly high. To address increasing bacterial resistance to conventional antibiotics, bacteriocins are now considered as alternative antimicrobials for the treatment of human and possibly animal infections. Furthermore, since minimally processed foods with no chemical preservatives are in demand by consumers, research into natural antimicrobial agents such

as bacteriocins. Members of the *Bacillus* group are considered to be good producers of antimicrobial substances such as peptide and lipopeptide antibiotics, as well as bacteriocins. Interestingly, identified *Bacillus* represents an alternative genus for the identification of bacteriocins because it includes many industrial species and has a history of safe use in the food industry.

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