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Research Article

Genetic Localization and Screening of Bacteriocinogenic Positive Marker (Bac$^+$) in *Lactobacillus curvatus* KIBGE-IB44

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Abstract

Increased bacterial resistance towards effective drugs has become a debated issue all over the world in a last few decades. Numerous concerns have been reported against multidrug resistant organisms and are the matter of concerned. Newly emerging case of multidrug resistance in microorganisms responsible for the failure of drugs against these pathogens. This scenario has enforced the researchers to screen the natural antimicrobial compounds from microbial sources to resolve the issues of drug resistance in microbes. Current study is an effort for the screening of antimicrobial potential of bacteriocin like inhibitory substance (BLIS) produced by *Lactobacillus curvatus* KIBGE-IB44 [GenBank accession number: MG814033] isolated from yogurt. It exhibited broad antimicrobial potential against various bacterial and fungal pathogenic strains. Results revealed that BLIS was sensitive to proteolytic enzymes and resistant to heat treatment which confirms its bacteriocinogenic nature. Moreover, plasmid curing technique was also performed using acridine orange as a curing agent. After removal of plasmid, *L. curvatus* KIBGE-IB44 was lost its antimicrobial potential which indicated that bacteriocinogenic gene present on the plasmid of bacteria. The presence of bacteriocin gene on the plasmid of bacteria make it distinguish and competent enough to transfer its bacteriocinogenic property to other incompetent bacteria.

Keywords: Acridine Orange; Antimicrobial Potential; *Lactobacillus curvatus*; Plasmid Curing; Plasmid Borne Bacteriocin

Introduction

Microorganisms are capable of producing several extracellular metabolites including digestive enzymes, metabolic byproducts, toxic compounds, lytic agents, antibacterial and antifungal proteins. Toxic compounds usually act as a lytic agent though, lysozymes, lactic acid, antibiotics and bacteriocins are also act as a toxic compound for foreign competitors [1,2]. Bacteriocins are the antimicrobial proteins that only produced by bacteria and have an antimicrobial potential against both bacteria and fungi. Bacteriocins are found to be a target specific, an efficient antagonist and now become more eminent due to its broad antimicrobial potential against several species.

The genes responsible for the immunity and synthesis of bacteriocins are generally clustered and located on plasmids, chromosome and/or transposons with minimum genetic machinery. The genetic information of most of the organisms is encoded in DNA, but few viruses have ribonucleic acid (RNA) as their genetic material. Sometime in addition to DNA and RNA, some extra chromosomal elements are also present in the cytoplasm called plasmids. The plasmid constitutes 1.0 to 2.0% of the total cellular DNA and more than one plasmid may be present in one bacterium. Many non-lanthionine containing bacteriocins are also encoded by plasmid borne genes [3].

Bacteriocins have several applications in various industries. Bacteriocins play a dual role as an alternative therapeutic agent and probiotic in pharmaceutical industry. The research on bacteriocins is in full swing due to the emergence of multidrug resistant pathogens towards effective drugs. Especially in third world countries most of the effective drugs have now turn out to be virtually useless against most of the pathogenic organisms. The problem is

not only due to the microbes that developed different ways to resist effective antibiotics, but also due to the increase use and misuse of different antimicrobials in human medicines. This public health dilemma can be resolved by the discovery of a new antimicrobial compounds having broad spectrum of inhibition especially against multidrug resistant organisms present in our community.

Species from genus *Lactobacillus* are generally recognized as safe (GRAS) and have well known history for their nutritional and health benefits. Recently, *Lactobacillus* are the focus of interest for the production of target specific and broad spectrum antimicrobials. Therefore, the aim of the current study is to screen the antimicrobial potential and localization of gene responsible for the production of bacteriocin in *Lactobacillus curvatus*.

Materials and Methods

Microbial strains

In this study, *Lactobacillus curvatus* KIBGE-IB44 [GenBank accession number: MG814033] was used to produce bacteriocin like inhibitory substance (BLIS) isolated from yogurt [4]. The strain was maintained in 15% glycerol for long term storage. After identification, the strain was qualitatively screened for the antimicrobial potential against various pathogenic bacterial and fungal strains.

Production of bacteriocin like inhibitory substance (BLIS)

Initially, *L. curvatus* KIBGE-IB44 was grown in the MRS (De-Man Rogosa Sharpe, Oxoid) medium. Inoculum (10^8 CFU mL^{-1}) was transferred in to 100 mL flask and after 24 hours culture was again transferred into 900 mL of MRS medium and further incubated at 35ºC with an agitation of 135 rpm. Cell free supernatant (CFS) was collected through centrifugation under 4ºC at 14000 × g for 15 minutes and assayed for antimicrobial activity after filter sterilization (0.22 µm syringe filters, Millipore, USA) using agar well diffusion assay.

Confirmation of bacteriocinogenic nature of bacteriocin like inhibitory substance (BLIS)

For the confirmation of the bacteriocinogenic substance produced by *L. curvatus* KIBGE-IB44 several strategies were employed.

Neutralization of cell free supernatant (CFS)

To eliminate the effect of organic acids in the inhibition of indicator strains, pH of the CFS was adjusted to 6.0 through sterilized 5N NaOH and assayed for antimicrobial activity using agar well diffusion assay.

Elimination the effect of hydrogen peroxide

To eradicate the inhibitory effect of hydrogen peroxide (H_2O_2), neutralized CFS was treated with 1 mg mL^{-1} of catalase (Sigma-Aldrich Corporatio n, USA) at 25ºC for 30 minutes. Agar well diffusion assay was used to determine the antimicrobial activity of BLIS after treatment with catalase.

Treatment with Proteases

To confirm the proteinaceous nature of the BLIS produced by *L. curvatus* KIBGE-IB44 neutralized CFS was treated with different proteolytic enzymes. The proteolytic enzymes include: protease, pepsin, trypsin (Serva) and Proteinase-K (Invitrogen). All enzymes were dissolved in sodium acetate buffer (0.05 M, pH-6.0) except pepsin which was dissolved in 0.01 N HCl (pH-2.0). The CFS was treated with enzymes (1:1) at the final concentration of 1 mg mL^{-1} and incubated at 37ºC for 2 hours. After incubation, the samples were assayed for antimicrobial activity using agar well diffusion assay. Enzymes alone were used as a control.

Reverse slide technique

To rule out the possible involvement of lytic phages in the inhibition of indicator strains, reverse slide technique was used. *L. curvatus* KIBGE-IB44 was spread on nutrient agar plate and incubated at 37ºC overnight. Next day, agar was detached from the plate and flipped out on another sterilized petri plate. Indicator strain inoculated in nutrient soft agar was poured on the back surface of the nutrient agar in order to prevent to contact between the producer strain and indicator strain. Plate was incubated at 37ºC for 24 hours [5].

Heat treatment

To further confirm the proteinaceous nature of the BLIS, neutralized CFS was also heated at 100ºC for different time intervals. Afterwards, the samples were assayed for antimicrobial activity.

Salt precipitation

In order to confirm the presence of proteins (BLIS) in CFS, neutralized CFS was precipitated out using 80% ammonium sulphate saturation. After saturation, precipitates were dialyzed, re-dissolved in 0.05 M sodium acetate buffer pH 6.0 and assayed for antimicrobial activity. For long term storage partially purified precipitates were stored at -20ºC and used for further experimentation.

Quantitative screening of bacteriocin activity

L. curvatus KIBGE-IB44 was quantitatively screened for the activity of BLIS against various bacterial and fungal pathogenic strains using agar well diffusion assay.

Antibacterial assay

The antibacterial activity of BLIS was performed using agar well diffusion assay. Sterilized BLIS (100 µL) was added in wells on nutrient agar plates previously spread with 100 µL suspension of each indicator strain containing 10^6 CFU mL-1. The plates were incubated for 24 hours at 37ºC.

Antifungal assay

To examine the antifungal effect of BLIS, agar dilution assay was used. Briefly, potato dextrose agar (PDA) was prepared and spore suspension (10-5) was added into the medium. Afterwards, the medium was poured into the plate and allowed to solidify. Then, the well of 6 mm size were prepared in the center of the plate with sterile borer and 100 µL of bacteriocin was added into the well and incubated at 25ºC for 5 to 7 days. After incubation, the clear zone around the wells were measured and expressed in millimeters. Sodium acetate buffer was used as a negative control.

Localization of bacteriocinogenic positive gene (Bac+)

Plasmid curing

In order to localize the bacteriocinogenic genes, a plasmid curing assay was performed. For plasmid curing the logarithmic phase culture of *Lactobacillus curvatus* KIBGE-IB44 was transferred in MRS broth (2.0 mL) containing acridine orange as a curing agent in different concentrations from 0.025 to 1.0 mg mL-1. Tubes were incubated at 35ºC for 24 hours. Producer strain without any curing agent used as a control. After incubation, the tubes with the lowest concentration of the acridine orange with growth of the producer strain were selected and further processed for isolated colonies by making dilutions from 10^{-1} to 10^{-4}. The isolated colonies were appeared after 24 hours of incubation which were assayed for antibacterial potential by overlaying 5 mL nutrient soft agar containing 0.1 mL of standardized inoculum of the indicator strain (106 CFU mL^{-1}). Plates were re-incubated at 35ºC for further 24 hours to observe a clear zone of inhibition around the producer strain which were exposed to mutagen for plasmid curing [6].

Plasmid isolation and visualization

To confirm the curing of plasmid, plasmid was isolated from both cured and uncured producer cells using a method reported by Ansari., *et al*. [7] with slight modifications. Briefly, overnight culture was centrifuged at 35000 × g for 5 minutes. 100 µL of suspension buffer (gL^{-1}: Tris-HCl, 3.8; EDTA, 3.7; glucose, 9.0) and 150 µL of denaturation solution (gL-1: NaOH, 8.0; SDS, 10.0) were added into the pallet and kept at 30ºC for 5 minutes. Afterwards, 200 µL of neutralization solution (potassium acetate, 5.0 M; glacial acetic acid, 11.5 mL) was incorporated and again kept at 30ºC for 10 minutes. Then, the tube was centrifuged at 35000 × g for 5 minutes and the supernatant was collected in a new tube. In the next step, 2.5 volume of ethanol was added into the supernatant and centrifuged at 35000 × g for 10 minutes and the tube was kept at -20ºC for 20 minutes. Discard the supernatant and the pellet was washed with 200 µL of 70% ethanol. Then the tube was spin for 1 minutes and supernatant was discarded. While, the DNA in the pellet was vacuum dried and stored at -20ºC for further study.

After plasmid extraction, agarose gel electrophoresis was performed and the extracted plasmid was visualized in gel documentation system under UV transilluminator.

Results and Discussion

Lactobacillus curvatus KIBGE-IB44 [GenBank Accession number: MG814033] was isolated from yogurt and identified on the basis of morphological, biochemical and molecular analysis [5]. After identification, stab and overlay and cross and streak methods were used for the qualitative screening of *L. curvatus* for the production of bacteriocin like inhibitory substance (BLIS) against various pathogenic bacterial and fungal strains.

Production of BLIS

Production of bacteriocin like inhibitory substance (BLIS) from *L. curvatus* KIBGE-IB44 was carried out in MRS medium which is an enriched and selective medium for the cultivation of lactic acid bacteria [8]. After production, antimicrobial activity of BLIS was determined and zones of inhibitions were measured in term of millimeters.

Confirmation of BLIS

In order to confirm the antimicrobial activity of BLIS against various bacterial and fungal strains following parameters were con-

sidered. First of all to rule out the effect of organic acids, pH of the CFS was neutralized. pH neutralization is very necessary specially in case of BLIS produced by lactic acid bacteria. After the treatment with the catalase antibacterial potential of BLIS was also detected which showed that there is no role of hydrogen peroxide in the inhibition of indicator strains and the appearance of inhibitory zones were solely due to the presence of bacteriocinogenic agent. Catalase break hydrogen peroxide (H_2O_2) which usually produced during fermentation into water (H_2O) and oxygen (O_2) and rule out its inhibitory effect on the indicator strain. Ahmad., *et al*. [9] has also reported that H_2O_2 was not responsible for antagonistic activity against the indicator strains used.

In the preliminary characterization of inhibitory substance (BLIS), the effect of different proteolytic enzymes (protease, pepsin, trypsin and proteinase-k) were determined. Results demonstrating that the BLIS was sensitive to the proteolytic enzymes which confirms the bacteriocinogenic nature of inhibitory substance. Similarly, Benkerroum., *et al*. 2007 [10] also reported the sensitivity of BLIS to proteases. Moreover, antibacterial activity of BLIS was also observed after heat treatment at 100ºC. Appearance of zones of inhibition even after boiling indicating the presence of antimicrobial compound because most of the bacteriocins are heat resistant as compared to the bacteriophages [10-12]. The data for the confirmation of BLIS are presented in Table 1.

Treatments	Effect on BLIS
Neutralized CFS	+ve
Catalase	+ve
Proteinase-k	-ve
Pepsin	-ve
Protease	-ve
Trypsin	-ve
100 ºC	+ve

Table 1: Confirmation of bacteriocin like inhibitory substance (BLIS) produced by *Lactobacillus curvatus* KIBGE-IB44 after different
treatments.

+ve: Activity of BLIS, -ve: No activity of BLIS

Reverse slide technique also confirmed that there is no involvement of lytic bacteriophages in the inhibition. Results revealed that after 24 hours no growth of indicator strain was observed on the back surface of the agar. The inhibition of the indicator strain showed that BLIS was produced by *L. curvatus* KIBGE-IB44 and diffused into the agar and inhibited the bacterial growth because bacteriophages did not have an ability to diffuse and always need direct contact for the formation of plaques.

Besides all these, antibacterial activity was detected after precipitation, dialysis and re-dissolving of precipitates in the buffer which confirmed the presence of antibacterial proteins/peptides in the sample.

Antimicrobial potential of BLIS

Antimicrobial spectrum of bacteriocin like inhibitory substance produced by *L. curvatus* KIBGE-IB44 was determined against various bacterial and fungal pathogenic strains using agar well diffusion assay and the results are quantified in millimeters (Table 2). Results showed that BLIS exhibited a potential antimicrobial effect not only against most of the bacterial strains tested but also against the pathogenic strains of genus *Aspergillus*. Figure 1 showed the clear inhibition of *Aspergillus flavus* KIBGE-IB34 and *Escherichia coli* ATCC 8739 by BLIS. Genus *Aspergillus* comprises of both pathogenic and opportunistic pathogenic organisms which causes diseases especially in immune-compromised patients. Bacteriocins from *Lactobacillus curvatus* and other species of lactic acid bacteria are mostly recognized as a potent antimicrobials for the inhibition of pathogenic microorganisms [13-15].

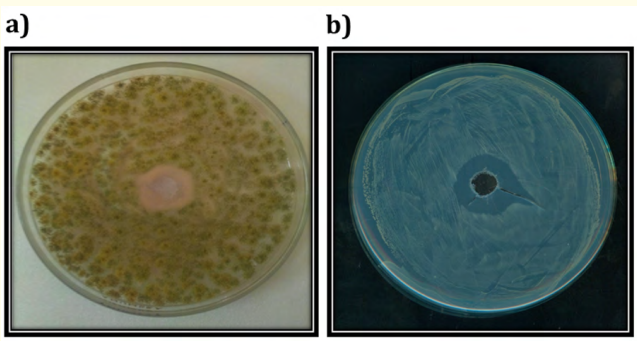

Figure 1: Pictorial illustration of antimicrobial activity of BLIS. a) Antifungal activity of BLIS against *Aspergillus flavus* KIBGE-IB34; b) Antibacterial effect of BLIS against *Escherichia coli* ATCC 8739.

Indicator Strains	Sources	Zones of Inhibition (mm)
Micrococcus luteus	KIBGE-IB20	16.0
Pseudomonas aeruginosa	Drinking water	14.0
Methicillin resistant staph aureus	KIBGE-IB23	13.0
Bacillus cereus	ATCC 11778	15.0
Escherichia coli	ATCC 8739	16.0
Enterococcus faecalis	ATCC 29212	14.0
Listeria monocytogenes	ATCC 7644	*-ve*
Salmonella typhimurium	ATCC 3632	-ve
Aspergillus flavus	KIBGE-IB34	13.0
Aspergillus niger	KIBGE-IB36	12.0
Aspergillus fumigatus	KIBGE-IB33	-ve
Aspergillus terreus	KIBGE-IB35	-ve

Table 2: Antimicrobial effect of bacteriocin like inhibitory substance (BLIS) on bacterial and fungal strains. -ve: Resistant

Localization of bacteriocinogenic positive gene (Bac+)

To localize the gene responsible for the production of bacteriocin like inhibitory substance, plasmid curing technique was used. Results revealed that the treatment with acridine orange at low concentration (0.25 mg mL^{-1}) leads to the loss of plasmid from the producer strain with approximately 62.0% curing rate whereas, rest of the concentrations were found to be lethal for producer strain. Agarose gel electrophoresis confirmed the removal of plasmid in cured colonies after the treatment with acridine orange however plasmid was present in uncured colonies (Figure 2). Moreover, after the curing of plasmid *L. curvatus* KIBGE-IB44 lost its antimicrobial activity. It clearly indicated that the gene responsible for the bacteriocinogenecity in *L. curvatus* KIBGE-IB44 was located on the plasmid because after removal of plasmid its ability to produce BLIS was lost. Therefore, BLIS produced by *L. curvatus* KIBGE-IB44 is plasmid borne and this property make the bacteria more distinguish and competent. Due to the presence of bacteriocinogenic gene on the plasmid, bacteria are capable of transferring its bacteriocinogenic property to other non-bacteriocinogenic bacteria to make it bacteriocinogenic in nature. Similarly, plasmid borne bacteriocins were also reported in *Lactobacillus curvatus* CWBI-B28 and *Bacillus subtilis* KIBGE-IB17 [7,16]. In contrast, few studies on *Lactobacillus* species reported the presence of bacteriocin positive genes on the chromosomes of the bacteria [4,17,18].

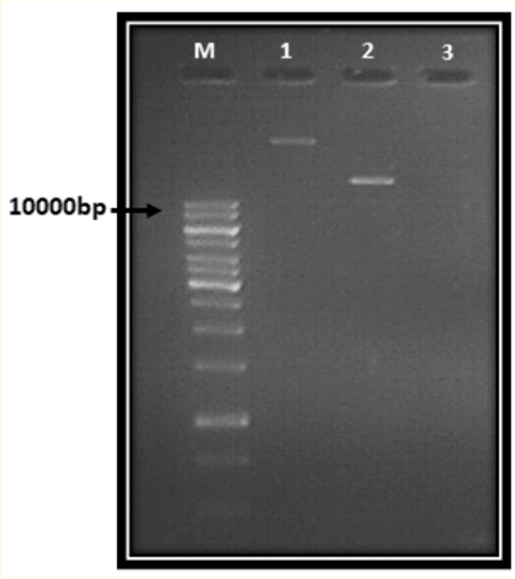

Figure 2: Agarose gel electrophoresis showing plasmid profile analysis. Lane M: DNA ladder (1.0 kb, Thermo fisher), Lane 1: Positive Control (Escherichia coli plasmid); Lane 2: Uncured cells of the *Lactobacillus curvatus* KIBGE-IB44, Lane 3: Cured cells of *Lactobacillus curvatus* KIBGE-IB44.

Conclusions

The emergence of multidrug resistant pathogenic microorganisms and the failure of commercially available drugs have enforced the screening of natural antimicrobial compounds. LAB bacteriocins have emerged as a great alternatives to the traditional antibiotics. In this study, bacteriocin like inhibitory substance (BLIS) produced by *Lactobacillus curvatus* KIBGE-IB44 was exhibited a broad antimicrobial potential against both bacterial and fungal pathogenic species. The inhibitory substance was found to be sensitive to various proteolytic enzymes and resistant to heat which confirms its bacteriocinogenic nature. Moreover, the bacteriocinogenic gene responsible for the production of BLIS from *Lactobacillus curvatus* KIBGE-IB44 was located on the plasmid. It was concluded from the current study that after complete characterization, this bacteriocin like inhibitory substance could be used as a potential candidate to resolve the unrestrained issues of pharmaceutical industries.

Conflicts of Interest

The authors declare that no conflicts of interest exist.

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