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Investigation of a Bacteriocin Produced by a Probiotic Lactic Acid Bacterium (LAB) Isolated from Packaged *Lassi* Sample

Mainak Bhattacharjee[1]*, Monojit Banerjee[2], Jayashree Deb[3]

[1]* Department of Biotechnology, Heritage Institute of Technology, Chowbaga Road, Ananadapur, P.O-East Kolkata Township, Kolkata-700107, West Bengal.

Former Lecturer, Department of Microbiology, Duragapur College of Commerce and Science (DCCS), G.T.Road, Rajbandh, Durgapur-713212, West Bardhhaman Dist., West Bengal

[2] Department of Biochemistry, Triveni Devi Bhalotia (TDB) College, Raniganj, Dist-West Bardhhaman, West Bengal-713347

[3] Department of Biotechnology, Duragapur College of Commerce and Science (DCCS), G.T.Road, Rajbandh, Durgapur-713212, West Bardhhaman Dist., West Bengal

ABSTRACT

The present study involves isolation followed by morphological and physiological characterization of a bacteriocin-producing Lactic Acid Bacillus (LAB) species from a commercially available packaged *lassi* (curd or yoghurt based probiotic drink; widely consumed in Indian subcontinent) sample followed by the partial purification and characterization of the bacteriocin protein by ammonium sulphate precipitation and dialysis. Bacteriocins are proteinaceous compounds produced by a wide group of bacteria that have anti-bacterial activity against closely related species. The anti-bacterial activity of the crude bacteriocin produced by the *lassi* LAB isolate was tested against 3 indicator bacteria-*Bacillus subtilis, Staphylococcus aureus* and *E.coli* and it was observed that the bacteriocin was most effective at 37^0C temperature and at pH 3.0-4.0 as measured by the diameter of the inhibition zone against all the 3 indicator organisms. The protein retained considerable inhibitory effect up to 60^0C temperature indicating its partial thermostable nature. Maximum inhibitory effect was found against *Bacillus subtilis* while *E.coli* was the least sensitive. The proteinaceous nature of the crude bacteriocin was confirmed by trypsin treatment and as the protein was found to be degraded by increasing concentrations of trypsin treatment, it makes it safe for human consumption. UV-radiation was found to have no effect on the anti-bacterial action of the crude bacteriocin protein. Since the bacteriocin was considerably effective against *Staphylococcus aureus*, a common food-borne pathogen, it could be potentially used as a natural antibiotic against this bacterium and at the same time, it also has an industrial application as a bio-preservative for fermented milk products having acidic pH if more specialized methods for its purification and toxicity analysis are adopted.

KEYWORDS: Probiotic, Lactic Acid Bacteria (LAB), Bacteriocin, anti-bacterial activity, bio- preservative.

INTRODUCTION

Bacteriocins are peptides or proteinaceous substances that are produced by different types of Gram positive and Gram negative microorganisms-most importantly by Lactic Acid Bacteria (LAB) and exhibit bacteriostatic or bactericidal activities against closely related species[1]. The bacteriocins produced by diverse groups of bacteria vary in their biochemical natures, molecular weights and mechanism of action (Bhattacharyya and Bhattacharjee, 2007). The bacteriocins produced by the LAB are known to inhibit the growth of several pathogenic and food-spoilage causing bacteria (Caplice and Fitzgerald, 1999) and therefore, Lactobacilli bacteriocins are of much commercial importance. They have been assessed for preservation of milk, fermented milk products as well as packaged meat and vegetables due to their anti-bacterial properties and also because of their potential use as natural preservatives of such foods. The bacteriocins produced by LAB are classified into four distinct classes (Klaenhammer,1993) based on their primary structure, molecular mass, heat stability and molecular organization including: Class I, Lantibiotics; Class II, small (<10 kDa) relatively heat stable non-lanthionine containing peptides; Class III, large (>30 kDa) heat-liable proteins; and Class IV, complex bacteriocins containing lipid or carbohydrate moieties. Most of the genetically

*Corresponding author: Mainak Bhattacharjee | E-mail: microman.mainak@gmail.com

characterized LAB-bacteriocin-gene clusters are composed of three gene molecules: a) module that includes the structural genes, b) transport gene module and c) a module that include regulatory gene. On the other side, Probiotic foods are mainly fermented food products that contain live microbial flora that exerts positive effect on the intestinal health of an individual by various mechanisms including inhibition of colonization of pathogens and maintaining the balance of the normal intestinal flora. Probiotics are becoming increasingly popular now-a day because of their expanding roles in preventing diarrhea, constipation, flatulence, liver damage and even in improving mental health that has become an interesting arena of research. LAB are one of the most potential and wisely consumed probiotic bacterial flora mainly found to be present in various fermented milk products like curd, *lassi*, yoghurt and cheese all of which are popular milk products across India. The effectiveness of LAB as probiotic organisms is attributed to a large extent to the actions of bacteriocins produced by them. Although the bactericidal effects of LAB strains are not only due to the bacteriocin-like compounds but also due to other compounds having anti-bacterial activities like lactic and acetic acid that significantly reduces the pH of the food items, hydrogen peroxide, carbinol, carbon di-oxide and ethanol[2]**.** Several types of bacteriocins have been isolated and characterized from probiotic food-associated LAB strains[3-7.] The so called 'Lantibiotics' have huge potential as bio-preservatives of foods as well as replacement for currently used antibiotics used to treat gastro-intestinal infections. The current work therefore, focuses on LAB bacteriocins. The LAB isolates were obtained and characterized from commercially available (branded) packaged ready-to-drink "*lassi"* and were screened for their bacteriocin producing ability. *Lassi* is a cooling and refreshing probiotic drink prepared from fermented milk product; curd or yoghurt and is widely consumed in Indian subcontinent especially during summer season. For this reason, lassi contains many probiotic species of *Lactobacillus* and so, consumption of lassi has a positive influence on the intestinal health of the individuals*.* The bacteriocin obtained from the cell free extracts of one of the *lassi* LAB isolates were then subjected to partial purification and characterization in connection to temperature, pH, protease and UV-sensitivity tests and also determination of their anti-bacterial spectrum against both Gram positive and Gram negative indicator organisms.

Materials and Methods:

Sample collection:

Commercially available packaged Amul® *lassi* were purchased from a local super market store at Durgapur, West Bardhhaman, West Bengal and the sample was collected directly from the sealed packets into sterile screw-capped test tubes under aseptic conditions inside the Laminar Air Flow chamber.

The pure cultures for the 3 indicator bacteria viz. *Bacillus subtilis, Staphylococcus aureus and E.coli* were procured form The Department of Microbiology, The University of Burdwan, Golapbag Capus, Burdwan-713104, West Bengal and was maintained in the lab facility on Nutrient Agar slants (containing 2% agar; pH 7.0).

Screening of bacteriocins producing LAB isolates:

1ml.of the *lassi* sample was serially diluted (10^{-1} to 10^{-5} dilution factor) using sterile distilled water as the diluent. 0.1 ml aliquot of the *lassi* sample from the 10^{-5} dilution factor was then plated onto de Man Rogosa Sharpe (MRS) agar[8] medium by Pour Plate Method (in duplicates) and the plates were next incubated at 37^0C temperature for a period of 16-18 hrs. The colonies thus appeared on the plates were counted to determine the colony forming unit (CFU)/ml of the sample taken. A few colonies (10-12 as obtained from the MRS Agar plates) were chosen randomly and the selected bacterial isolates were then screened for bacteriocin production by well- diffusion method[9] against the indicator bacteria i.e. *E.coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Only 2 out of the 12 isolates exhibited anti-bacterial activity against the indicator organisms and the best one was identified and selected for the latter experiments on bacteriocin as well as for morphological and physiological characterization. The pure culture of the selected bacterium under study was maintained on MRS Agar slants (containing 2% agar, pH 7.0) and also in MRS broth medium for further experiments.

Charcterization and Identification of LAB Isolate:

The culture isolate from the packaged *lassi* sample that showed the best result for bacteriocin production in the earlier experiment was subjected to various morphological and physiological tests as well as studying of the colony features in order to characterize the organism as a species of *Lactobacillus*. The morphological tests include- Gram Staining, Endospore Staining and Capsule

Staining followed by microscopic observation, Motility Determination by Hanging Drop Method while the biochemical/physiological tests include- Methyl Red Test, Catalase Test, Oxidase Test, Arginine Hydrolysis Test, Coagulase Test, Carbohydrate Fermentation Tests utilizing Glucose, Sucrose, Lactose, Fructose, Mannitol and Maltose as sole carbon source in individual fermentation broth media, effect of varying pH (pH 3.0, 4.0, 5.0, 6.0, 7.0 and 9.0), effect of temperatures (10^0C, 25^0C, 37^0C, 45^0C, 60^0C and 80^0C), effect of NaCl concentrations (1.5%,2.5%, 5% and 7% of NaCl) and haemolysin production. For studying the effect of pH on growth of the isolated bacterium, the pH of the MRS broth media was adjusted accordingly by using appropriate volumes of 1M HCl and 0.5M NaOH [10]**.** To study the effect of temperature on the growth of the test isolate, the MRS broth media were seeded with 1ml.of the pure culture of the isolate and the inoculated media were treated at the defined temperatures for a period of 10 minutes prior to incubation and the tubes were next incubated at 37^0C for overnight in a shaker incubator. Likewise, to determine the effect of NaCl on the growth of the *lassi* isolate, appropriate amounts of pure NaCl were added to the MRS broth media (seeded with 1ml. of pure culture of the test isolate) and the individual tubes were incubated at 37^0C temperature for overnight in a shaker incubator. In each case, the optical densities (O.D.).of the MRS broth cultures were measured at 540 nm.wavelength spectrophotometrically by using a Spectrophotometer (purchased from Systronics, India; model no.-AU-2603) and the O.D.values were plotted against the respective parameters (Temperature, pH and NaCl concentration) to determine their effects on the growth of *lassi* LAB isolate. To study the production of haemolysin (i.e to study the.pathogenicity) of the isolate, the organism was grown on Blood Agar Medium plates (Nutrient Agar basal medium supplemented with 10% sheep blood) in duplicates and the Blood Agar plates were then incubated at 37^0C for 48 hrs. After the incubation period is over, the plates were observed for any clear zone that would appear on the Blood Agar surface around the colonies. Appearance of such clear haemolytic zone would indicate that the isolate is a haemolysin producer and hence, can be considered as a pathogen. All the chemicals/reagents required for the various biochemical tests mentioned as above were purchased from either Merck®, India or from SRL®, India and were of analytical grade.

Production of Bacteriocin:

1ml. of pure culture of the test LAB isolate was allowed to multiply for 16-18 hrs. at 37^0C temperature in 150 ml.of MRS broth medium (pH 7.0) in a 250 ml.Erlenmeyer flask in a shaker incubator (having a rotational speed of 150 rpm) to obtain a fully-grown liquid culture of the test bacterium that is supposed to contain the bacteriocin protein in the broth medium. The whole broth was then centrifuged at 10,000 rpm for 20 minutes at 4^0C and the cell-free supernatant thus obtained was used as the source of the crude bacteriocin.

Partial Purification of the Crude Bacteriocin:

The cell-free supernatant was gradually saturated with 60% solid ammonium sulphate and the mixture was stirred using a magnetic stirrer for 3 hrs.in order to precipitate out the protein[10]and the resulting mixture was next centrifuged at 10,000 rpm for 30 minutes at 4^0C temperature. The pellet obtained was dissolved in 30 ml.of 0.5M potassium phosphate buffer, pH7.0 (containing potassium di-hydrogen phosphate and di-potassium hydrogen phosphate, all of analytical grade; purchased from SRL®, India). The resultant protein solution was next dialyzed against the same buffer for overnight [11] in a dialysis bag at 4^0C temperature. The dialyzed protein was precipitated by adding 5% Tri-Chloro Acetic Acid (TCA) and the mixture was centrifuged at 10,000rpm for 15 minutes. The supernatant was discarded and the pellet (precipitated protein) was re-suspended in 0.5M potassium phosphate buffer, pH 7.0. This solution was treated as the partially purified bacteriocin for the latter experiments.

Bacteriocin Assay:

The antibacterial activity of the crude bacteriocin was estimated by well-diffusion method as described by Ivanova *et al* [12]. 30 µl. of the crude bacteriocin was taken within wells of 5 mm diameter. The wells were cut previously on the surface of individual MRS agar plates that were pre-inoculated with the pure cultures (in Nutrient Broth, pH7.0) of 3 of the indicator bacterial strains. The plates were then incubated at 37^0C temperature for overnight and after the incubation period, all the plates were observed for the zone of inhibition. The anti-bacterial activity was measured in terms of the diameter (in mm.) of the clear zone obtained on the plates around the wells loaded with the crude bacteriocin samples.

Effect of temperature on bacteriocin activity: The crude bacteriocin obtained from the *lassi* LAB isolate was treated at different temperatures i.e.10^{0}C, 25^{0}C, 37^{0}C, 45^{0}C, 60^{0}C and 80^{0}C for a period of 15 minutes in individual test tubes. After the heat treatment, aliquots (about 30µl.) of samples were drawn from each tube and they were assessed for their bacteriocin activity against the 3 indicator organisms on MRS agar plates by well-diffusion method as described above.

Effect of pH on bacteriocin activity: The crude bacteriocin sample obtained from the *lassi* LAB isolate was taken in separate test tubes (about 50 µl.aliquotes) and the pH values were adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 by using 1N HCl and 0.5M NaOH. The bacteriocin assay was performed for each of the different pH samples after incubating the tubes at normal room temperature for 2hrs. against the 3 indicator organisms on MRS agar plates by well-diffusion method as described.

Effect of proteinase (trypsin) treatment: In order to study the effect of the proteolytic enzyme trypsin on the crude bacteriocin, about 50 µl.aliquotes of the sample was taken in different test tubes and the tubes were then treated with varying concentrations of the commercially available pure trypsin enzyme (purchased from Merck®, India)- 200µg/ml, 400 µg/ml, 600 µg/ml. 800 µg/ml and 1000 µg/ml and all the tubes were incubated at room temperature for a period of 1hr. The bacteriocin samples were then assayed for their anti bacterial activities against the 3 indicator bacteria by the well-diffusion method using MRS agar medium. An appropriate control was used for comparison in which no trypsin was added to the bacteriocin sample.

Effect of UV-radiation: For studying the effect of UV-radiation on the bacteriocin activity, the crude bacteriocin sample was exposed to UV light from a 20W UV lamp at a distance of 100cm for 0, 5, 10 and 15 minutes. The samples were then tested for their anti-bacterial activity against the test bacterial strains by well-diffusion method on MRS agar plates.

Result and Discussion:

Result

Characterizations and Identification of LAB Isolate exhibiting bacteriocin activity:

Colony Features:

Colony Size	Small
Colony Colour	Creamy white
Colony Consistency	Sticky
Colony Margin	Entire
Colony Elevation	Flat

Morphological Features:

Gram Staining	Gram Positive elongated rods; isolated
Endospore staining	Non-spore Forming
Capsule Staining	Non-Capsulated
Motility Determination (by Hanging Drop Method)	Non-motile (non-flagellated)

Biochemical Features:

Methyl Red Test	Positive
Catalase Test	Positive
Oxidase Test	Negative
Arginine Hydrolysis Test	Positive
Growth on Blood Agar (Haemolysin Test)	No clear zone; Non-pathogenic
Coagulase Test	Negative

Carbohydrate Fermentation Profile:

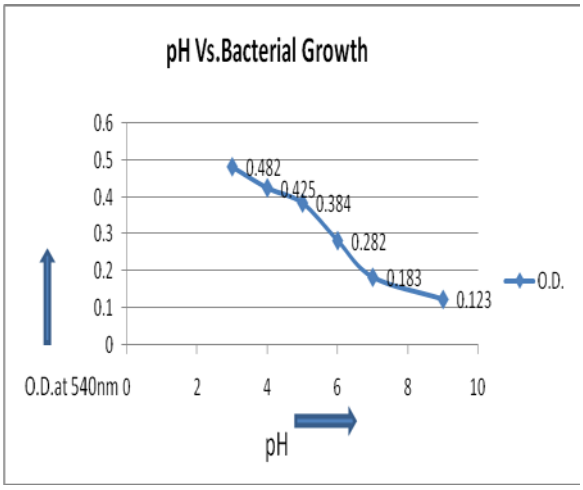
Sugar	Acid	Gas
Glucose	++	++
Sucrose	+	+
Lactose	++	++
Fructose	-	-
Mannitol	+	+
Maltose	++	++

+ indicates weak fermentation, ++ indicates strong fermentation, - indicates no acid or gas produced

Effect of pH on the growth of the test isolate:

The effect of different pH on the growth on the *lassi* isolate measured spectrophotometrically in

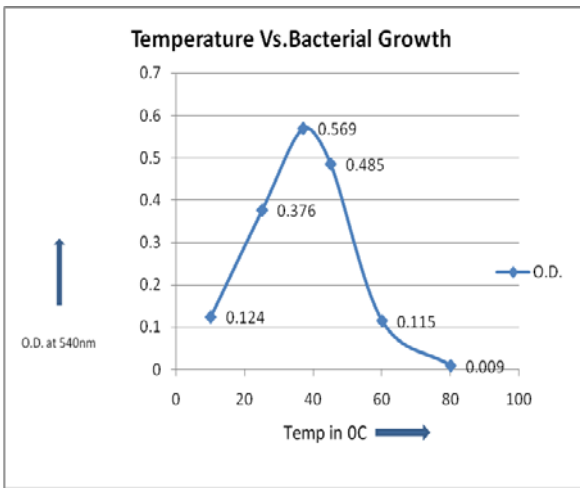
terms of O.D.at 540nm wavelength is represented in the following plot:



From the plot, it becomes evident that as the pH increases, the growth of the bacterium declines proportionately indicating that the isolate is an acidophile and its optimum pH for growth is around pH 3.0-4.0.

Effect of temperature on the growth of the test isolate:

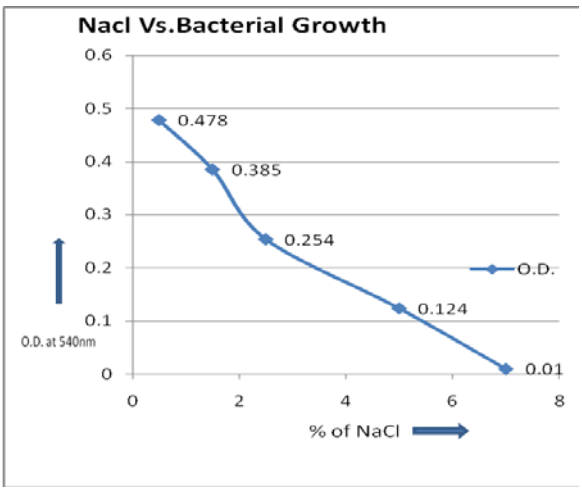
The following plot depicts the effect of temperature on the growth of the *lassi* isolate as measured spectrophotometrically at 540nm wavelength:



The plot indicates that the bacterium from *lassi* sample grows maximally at 40^0C temperature and growth is declined at temperatures higher than that. This data indicates that the isolated organism is a mesophile in nature.

Effect of NaCl on the growth of the test isolate:

The effect of different concentrations of sodium chloride on the growth of the test strain from *lassi* sample is represented as follows-



It could be concluded form the graph that the test organism grows optimally at 0.5% concentration of NaCl and growth reduces drastically at higher concentrations of NaCl indicating that the organism is a non-halophilic and non-osmophilic bacterium. Higher concentrations of NaCl (solute) inhibit its growth.

By compiling all the results of the colony features, staining and morphological features as well as biochemical/physiological characteristics, it could be said that the bacteriocin producing *lassi* isolate is a bacterium that belongs to the genus *Lactobacillus.*

Bacteriocin Assay:

The observation for the bacteriocin activity test (performed by well diffusion method) of the *lassi* LAB isolate against the 3 indicator bacteria is tabulated as below:

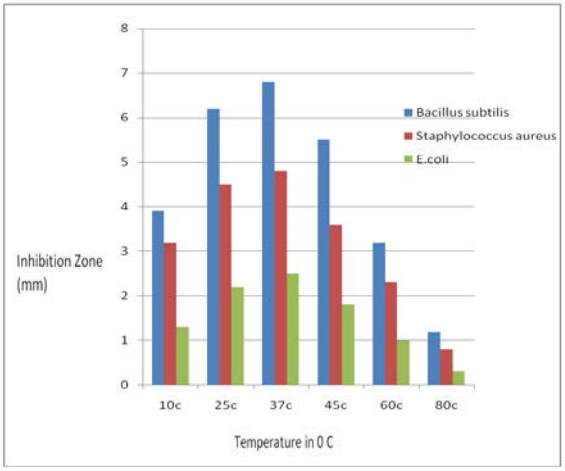
Source of Bacteriocin	Indicator Organism	Diameter of Zone of Inhibition (in mm.)
Lassi LAB isolate	*Bacillus subtilis*	8.2
	Staphylococcus aureus	5.8
	E.coli	3.4

According to the observation recorded as above, the bacteriocin activity is most effective against *Bacillus subtilis*. On the contrary, *E.coli* is least sensitive against the bacteriocin produced by the isolated LAB strain under study. This establishes the fact that bacteriocin produced by an organism is most effective against closely related species. Since the LAB and the *Bacillus subtilis* both are Gram positive rods, the latter is most sensitive against the bacteriocin produced by the LAB strain. *E.coli* is a

Gram negative bacterium that may the reason why the bacteriocin secreted by the Gram positive LAB isolate has the least activity against *E.coli*. *Staphylococcus* is also a Gram positive organism and hence, the LAB isolate bacteriocin exerted considerable anti-bacterial effect on the bacterium although it is lesser than that of on *Bacillus*.

Effect of temperature on crude bacteriocin activity:

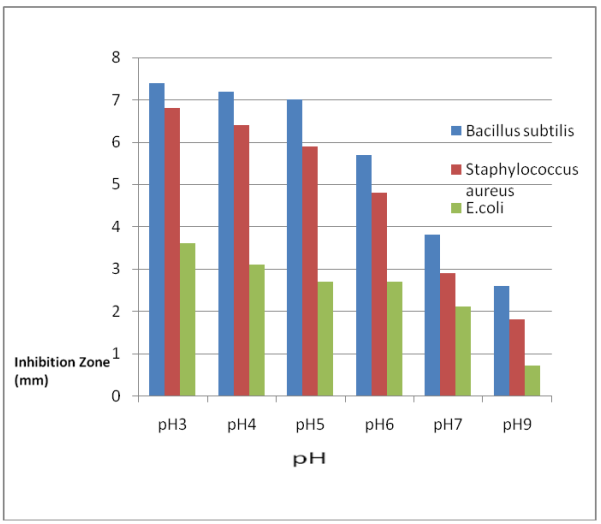
The influence of temperature treatment on the crude bacteriocin produced by the *lassi* LAB isolate on the 3 indicator organisms measured in terms of the diameter (in mm) of the inhibition zone is illustrated by the following plot:



The crude bacteriocin protein was found to exhibit its anti-bacterial activity up to 80^0C temperature which indicates its partially thermostable nature but its activity was declined with the rise in the temperature. This is in keeping with its proteinaceous nature and as high temperature denatures the protein structure, its activity on the indicator bacteria also reduces proportionately. The plot also revealed that 37^0C temperature is optimum for the activity of the bacteriocin since the maximum inhibitory effect against all the indicator organisms is observed at this particular temperature.

Effect of pH on bacteriocin activity:

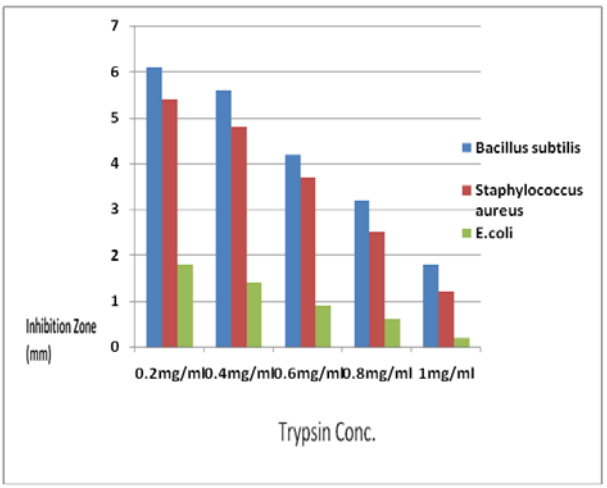
The plot below demonstrates the effect of pH on the anti-bacterial activity of the crude bacteriocin on the 3 indicator organisms as was observed by well-diffusion method and was measured in terms of the diameter (in mm) of the inhibition zone:



This graphical representation reveals that the best inhibitory effect of the bacteriocin on the 3 indicator bacteria is seen at pH 3.0-4.0 indicating that the production and activity of the crude bacteriocin of the LAB isolate collected from the *lassi* sample is maximum at this particular pH range. As the pH increases, the inhibitory activity is also reduced. The fact suggests that the bacteriocin protein is best active at acidic pH although it can partially retain its anti-bacterial activity up to pH 9.0.

Effect of trypsin treatment:

Treatment with different concentrations of the trypsin (a proteolytic enzyme) on the anti-bacterial activity of the bacteriocin on the 3 different indicator bacteria (measured in terms of the diameter of the corresponding zones of inhibition) yielded the following result:

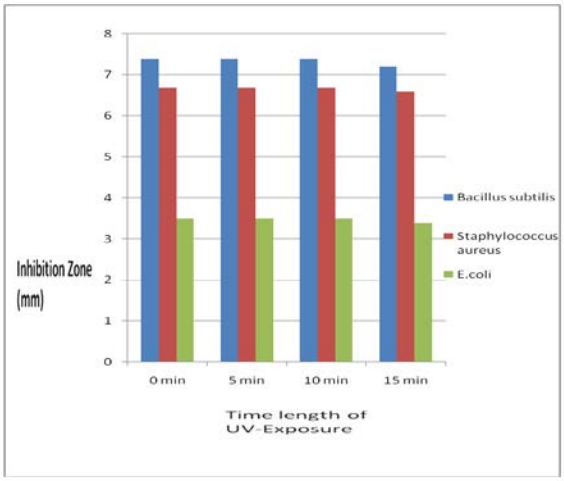


The plot above confirms the fact that the bacteriocin produced by the LAB isolate is protein in nature and as higher concentrations of the trypsin enzyme denatures the protein structure due

to proteolytic cleavage, its function is also lost as evident by the decreased anti-bacterial activity on all the 3 indicator bacteria. It may also be concluded that the bacteriocin protein partially retained its function up to 1mg/ml.of trypsin treatment.

Effect of UV-radiation:

Treatment with UV-radiation for varying periods of time on the crude bacteriocin protein revealed that UV exposure had almost no effect on the anti-bacterial activity of the said protein. It led to the fact that UV-radiation does not interfere with the structure (and hence, function) of the crude bacteriocin as depicted in the following plot:



Discussion

The bacterial strain isolated from the packaged *lassi* sample was characterized to be a species of *Lactobacillus* as it was found to be a Gram positive, non-sporing, Catalase positive rod that gave positive result for methyl red test and was also a strong fermenter for carbohydrates like glucose, lactose and maltose. The study revealed that the test isolate produces a bacteriocin protein that exhibited anti bacterial activity against the 3 indicator organisms i.e. *Bacillus subtilis, Staphylococcus aureus* and *E.coli* as evidenced by the clear zone of inhibition that appeared on the MRS agar plates following well-diffusion method. The optimum temperature and pH of the partially purified bacteriocin was found to be 37^0C and pH 3.0, respectively. This makes it useful to be used as a natural bio-preservative for packaged fermented dairy products that have an acidic pH. The partial thermostable nature of the bacteriocin is also an important aspect as far as its practical application is considered although its anti-bacterial function was partially lost at higher temperatures. The proteinaceous nature of the bacteriocin was confirmed by trypsin treatment since high concentrations of trypsin impaired its anti-bacterial function but at the same time it makes it safe for human consumption as the gastric juice contains trypsin that would lead to degradation of the protein within the alimentary canal. UV-exposure was not shown to have any effect on the function or activity of the bacteriocin. Since *lassi* is a probiotic drink prepared form fermented milk and is widely consumed (either commercially available or home-made) in India, this *Lactobacillus* present in the packaged *lassi* found to be a potent probiotic producer of bacteriocin. The fact that the bacteriocin is considerably effective against *Staphylococcus aureus*, a common food-borne pathogen makes it industrially exploitable and a novel target for strain improvement technologies to enhance its production and /or activity and its probable utilization as a replacement for antibiotics against Gram positive food-borne pathogens.

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