On Study of application of bacteriocin produced by Lactic Acid Bacteria against isolated plant pathogenic bacteria responsible for bacterial wilt in Solanaceous plants

[1]Urvi K Bhanushali, [2]Dr.Reena G. Desai
[1]Student, [2]Guide (Head of department)
[1]Microbiology
[1]Dolat-Usha Institutes of applied sciences and Dhiru-sarla institute of management & commerce, Valsad, Gujarat, India

Abstract: Lactic acid bacteria are associated with habitats rich with nutrients, such as various dairy products, certain fermented food items as well as plant materials. They have also been found in human intestine where they are said to be inhibiting unwanted gut microorganisms by producing compounds such as organic acids, hydrogen peroxide, diacetyl and bactericidal proteins i.e. Bacteriocin. In the current study, Lactic acid bacteria were isolated from Dairy products using MRS medium. All the 16 isolates were screened for production of Bacteriocin against indicator strains available in college laboratory culture collection such as *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella paratyphi* A. Along with the known indicator strains, the bacteriocin activity was also checked for inhibition against isolated plant pathogen S2 and S3 from infected *Solanaceous* crops using Kelman's TZC medium (modified). Ten isolates found to be inhibiting the indicator strains were selected further for application of Bacteriocin against isolated plant pathogen. The bacteriocin from U1, U2 and U5 LAB isolates, giving the highest inhibition against the isolated plant pathogen S2 and S3, were selected for production of bacteriocin. The produced Bacteriocins from the selected strains were extracted from the cell free supernatant by Ammonium Sulfate precipitation followed by Dialysis. These partially purified bacteriocins can be used in the field to control the bacterial wilt disease without using any chemical control agents and hence the soil pollution by the chemical can be stopped.

Key words **-Bacteriocin, Lactic acid bacteria, Bacterial wilt, *Solanaceae.***

I. INTRODUCTION

Lactic acid bacteria (LAB) are Gram positive, non-sporulating anaerobic rods or cocci shaped bacteria, which possess negative catalase activity. They are comprised mainly of 20 genera in which *Lactobacillus*, Leuconostoc, *Streptococcus*, *Pediococcus* and a newly proposed genus *Lactococcus* form the core of the group (Salminen & Von, 2004). LABs have been found in nutrient rich environments such as plant materials, meat and dairy products as well as soil, sewage etc. Particular LABs have been found to be having inhabitation in human oral cavity, Intestinal tract as well as vaginal region and have a beneficial influence in these human ecosystems (Moghaddam et al, 2014). Lactic acid bacteria have a long history in food industries. They are best known for their use as starter cultures in manufacture of dairy products such as yogurt, cheese etc as well as they have also been associated to meat industry. LABs have been employed in these many food products because they significantly contribute to the flavor, texture, and in many cases, to the nutritional values of products (McKay & Baldwin, 1990). Metchnikoff (2004) proposed a role for lactobacilli in suppressing undesirable intestinal microflora, since then antimicrobial effects of LABs have been widely studied. The inhabitance in the human gut could be according to the production of inhibitory compounds such as organic acids like acetic acid and lactic acid, hydrogen peroxide, diacetyl and bacteriocin or antimicrobial protons during lactic fermentation (Holzpafel et al, 2001; Hirano et al, 2003). Organic acids and hydrogen peroxide impart broad-spectrum inhibition but antibacterial peptides mediate antagonistic activity in narrow spectra.

Bacteriocins are ribosomally synthesized peptides with antimicrobial activity, mostly applied in the food industries as biopreservatives and are GRAS in many countries. They are produced by almost all major groups of bacteria as well as archaea. The first bacteriocin was discovered by Gratia in 1925 from *Escherichia coli*, which was later named as "Colicin". A physiological function of bacteriocin in bacteria seems to inhibit the growth of competing microorganisms in a particular biological niche by killing those (Nes & Holo, 2000). The latest classification arranges bacteriocin into three classes based on their structural and physico-chemical properties namely class I, class II and class III bacteriocins (Zacharof & Lovitt, 2012). General process of biosynthesis of bacteriocin includes formation of prepeptide followed by modification reactions; then the leader peptide sequence undergoes proteolytic cleavage and then finally the modified prepeptide/ mature propeptide is translocated across the cytoplasmic membrane of the cell. Biosynthesis pathway of bacteriocin differs from class to up to some extent (And & Hoover, 2003). The bactericidal mechanism of bacteriocins are generally located in the receptor binding of bacterial surfaces, and then through the membrane which causes cytotoxicity (Yang et al, 2014).

Bacterial wilt is a commonly found disease in the Solanaceae family plants causing a great problem in the production of these crops. It is found to be caused by *Ralstonia solanacearum*, which is a Gram negative, rods shaped, strictly aerobic bacterium that

can be found in the xylem of the diseased plants or the soil surrounding the plant. A Kelman's selective nutrient triphenyl tetrazolium chloride(TZC) medium can differentiate the virulent and non-virulent colonies as the pathogenic bacteria grow in individual pink centered white or cream colored, irregularly shaped and highly fluidal colonies after 36 to 48 hours of incubation at 30±2°C (Kelman, 1954; Konappa et al, 2015). Management of the disease has been difficult and still threatens the commercial crop production. Certain chemicals have shown to be controlling the bacterial wilt disease, but have negative effect on the plant growth and crop yield. Hence, there has been a wide approach to use biocontrol agents for the remedy of the same.

The application of bacteriocins is generally found in the food industries. Nisin and Pediocin have been widely utilized as biopreservatives in European countries as a biopreservatives for dairy as well as meat products. Bacteriocins can also be utilized as a therapeutic agent against the multidrug resistant pathogens (Cotter et al, 2013). The bacteriocins have recently been found to be applicable as therapeutic agent in cancer therapy because the enhanced expression of negative charge on the cancer cells makes them prone to the cytotoxic activity of bacteriocin (Zhao et al, 2006). Apart from all these application, bacteriocin has been found to be suitable as biocontrol agent against plant pathogens. In this study, bacteriocins from Lactic acid bacteria have been studied their antagonistic activity against the plant pathogen of bacterial wilt isolated from the diseased plant sample.

II. Research Methodology

1. Enrichment and Isolation of lactic acid bacteria

Total of 10 samples were collected from the local region for isolation of lactic acid bacteria. This includes homemade samples, samples from local dairies as well as packaged curd and buttermilk; commercially available yogurt; goat milk and; cheese whey from local cheese manufacturer company. The 1 g or 1 ml of samples were inoculated into MRS (de Man Rogosa and Sharpe) broth and incubated for 24 hours at 37°C in anaerobic condition for enrichment of the resident lactic acid bacteria (Lade et al, 2007). The enriched samples were then serially diluted and plated on MRS agar medium at pH 6.7 indicated by blue color of medium due to Bromocresol purple dye, incubated at 37°C for 48 hours in an anaerobic jar (Lequitron, Labline stock centre). The colonies giving a yellow zone due to lactic acid production were picked and studied for Gram's reaction and motility. The Gram positive organisms were selected for further use and stored in MRS agar slants at 4°C.

2. Screening of bacteriocin production

Isolated LABs were inoculated into MRS broth, incubated at 37°C for 48 hours in anaerobic condition for production of bacteriocin in the broth. The broths were centrifuged 10,000 rpm for 15 min at 4°C for removal of cells. The cell free supernatant was collected and was adjusted to pH 6.5-7.0 with 1N NaOH to nullify the effect of organic acids.

For the screening of bacteriocin production various common organisms from the laboratory i.e. *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Salmonella paratyphi* A were used as indicator organisms. All the cultures were streaked across the Mueller-Hinton agar medium using cotton swabs. On the agar, 10 mm wells were prepared using sterile cup borer. The cell free supernatant was added into the agar well with 0.3 ml aliquot. The plates were kept at room temperature for 30 minutes to allow diffusion and then were incubated at 37°C for 24 hours. After the incubation time, antimicrobial activity was determined by measuring the inhibitory zone surrounding the wells (Indira et al, 2015).

3. Isolation of plant pathogenic bacteria

The suspected diseased plants of chilly and eggplant with symptoms of bacterial wilt like stunting and wilts with green leaves were collected from the nearby plants for the isolation of plant pathogen. The rhizospheric soil of the diseased plants and the plant tissues after surface sterilization were plated onto Kelman's TZC agar medium (modified) by serial dilution. The plates were incubated at room temperature i.e. 26±2°C for 24 to 48 hours. The suspected colonies were observed for colony characteristics, Gram's reaction and motility. They were further isolated and stored at 4°C (Konappa et al, 2015)

4. Application of bacteriocin against isolated plant pathogen

The isolates showing production of bacteriocin were screened for their ability for inhibition of isolated suspected plant pathogens using Kelman's medium (modified) with agar well diffusion assay in the same fashion as discussed above. The LAB incorporated with the cell free supernatant giving higher inhibitory zone against the isolated plant pathogens were selected for further use.

5. Bacteriocin production and partial purification

MRS broth without Tween 80 at pH 6.5 were inoculated with 48 hours grown culture of selected LAB and incubated anaerobically at 37°C for 48 hours. After the incubation period, the cells were removed by the centrifugation as described above and the pH of the cell free supernatant was adjusted to pH 6-7 using NaOH. This was determined to be crude antimicrobial peptide for the further studies.

The crude bacteriocin samples were partially purified using solid ammonium sulfate of 50, 55, 60 and 65& saturation. The powder of ammonium sulfate was added into the crude bacteriocin in small amounts while stirring it vigorously for 2.5 hours. Later it was centrifuged at 20,000 rpm for 30 minutes at 4°C. The precipitates were dissolved using 0.05 M potassium phosphate buffer of pH 7.0. This was followed by the dialysis in cellulosic Dialysis bag with pore size of 2.4 nm (HiMedia laboratories Pvt. Ltd. Mumbai, India) and molecular weight cut off 12-14 kDa in the same buffer for 24 hours. The bacteriocin activity of the dialyzed sample was done using agar well diffusion assay (Ogunbanwo et al, 2003).

The capacity of the partially purified bacteriocin to inhibit the isolated plant pathogen was checked using the same technique i.e. agar well diffusion assay.

III. Results and Discussion

From 10 samples, isolates were selected on the basis of their ability to produce organic acid, which could be observed as yellow colored zone surrounding the colonies on the MRS agar plates. Colonies of Gram positive organisms showing organic acid

production with different colony characteristics were selected for further screening. Total 16 isolates were obtained, out of which 10 were Gram positive cocci whereas the rest were Gram positive bacilli.

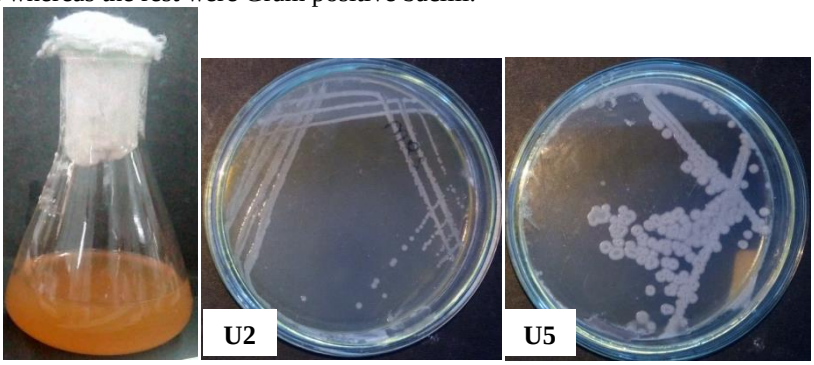

Figure 1: Results of isolation of lactic acid bacteria. Enriched curd sample is shown in the left most image.

In the screening procedure, cell free supernatant of 10 LAB isolates were found to be inhibiting *B. subtilis* ; 6 of them were observed to be inhibiting *S. epidermidis*; 3 of them inhibited the growth of *S. paratyphi* A. Whereas *E. coli* and *S. aureus* were not inhibited by any of the cell free supernatant of LAB. Six LAB isolates did not produced antimicrobial peptides and hence the cell free supernatants from those isolates did not inhibit any indicator strain or isolated plant pathogen. These results can be observed in the table 1 below.

Table 1: Inhibitory zone by cell free supernatant of LAB isolates against indicator organisms as well as isolated plant pathogen

Isolates	Inhibition zone (mm)		
	B. subtilis	*S. epidermidis*	*S. paratyphi* A
U1	18	14	11
U2	18	14	13
U3	17	12	-
U4	16	11	12
U5	17	12	-
U6	17	-	-
U7	16	-	-
U8	15	14	-
U10	12	-	-
U14	11	-	-

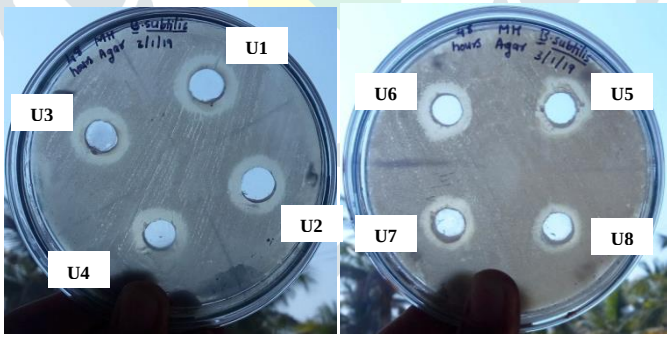

Figure 2: Result of antagonistic activity of bacteriocin produced by LAB against *Bacillus subtilis*

Total six suspected plant pathogenic bacteria were isolated from the plant tissues of Brinjal and chilly based on the colony characteristics like fluidal, white colored colonies with pink centre, which can be clearly seen in the Fig. 3 below.

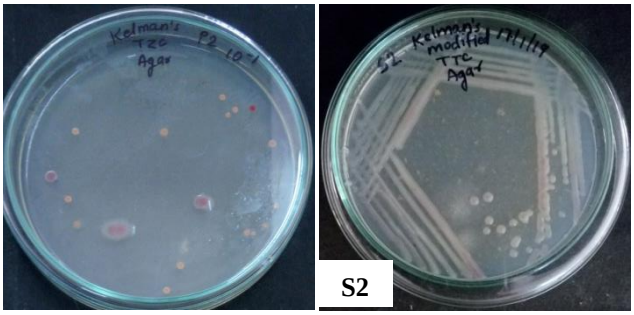

Figure 3: Results of isolation of plant pathogenic bacteria

Only S2 and S3 isolated plant pathogens were found to be inhibited by cell free supernatant of five LAB isolates whereas isolated plant pathogens S1, S4, S5 and S6 were not found to be inhibited by any of the cell free supernatant. Table 2 shows the zone of inhibition measured. This results show that cell free supernatant of LAB isolate U1, U2 and U5 give the highest zone of inhibition amongst all. Hence, these isolates were selected for further procedure of production of bacteriocin and partial purification of the bacteriocin.

Table 2: Result of antagonistic activity of crude bacteriocin against isolated plant pathogen.

Isolates	Inhibition zone(mm)	
	S2	S3
U1	16	17
U2	13	20
U3	14	14
U4	12	12
U5	14	16

After the production of bacteriocin from selected isolates U1, U2 and U5, partial purification of the crude bacteriocin was obtained. Results of the agar well diffusion assay of the partially purified and dialyzed bacteriocin, maximum antagonistic activity was found to be obtained using 55% saturation of ammonium sulfate. Figure 4 (right) inhibitory zones given by partially purified bacteriocin produced by U2 isolate against isolated plant pathogen S2. The result of partial purification of bacteriocin from LAB isolates U1, U2 and U5 can be seen in fig. 5 as chart of size of inhibition zone given by the partially purified bacteriocin from corresponding isolate against the isolated plant pathogen versus the ammonium sulfate concentration used for the partial purification of the bacteriocin. It can be said that the bacteriocin were well purified at 55% concentration of Ammonium sulfate which can be seen as highest zone of inhibition by the partially purified bacteriocin produced by isolate U2 at 55% ammonium sulfate concentration

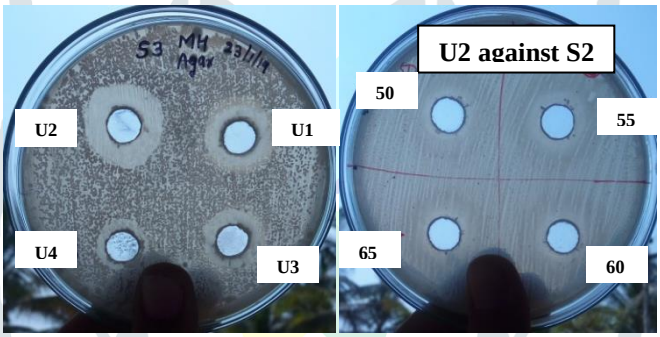

Figure 4: Results of agar well diffusion assay by isolated LAB against one of the isolated plant pathogen S3 (left) and the right image is the result of antagonistic activity of partially purified bacteriocin of isolated LAB U2 at 55% ammonium sulfate saturation against isolated plan pathogen S2.

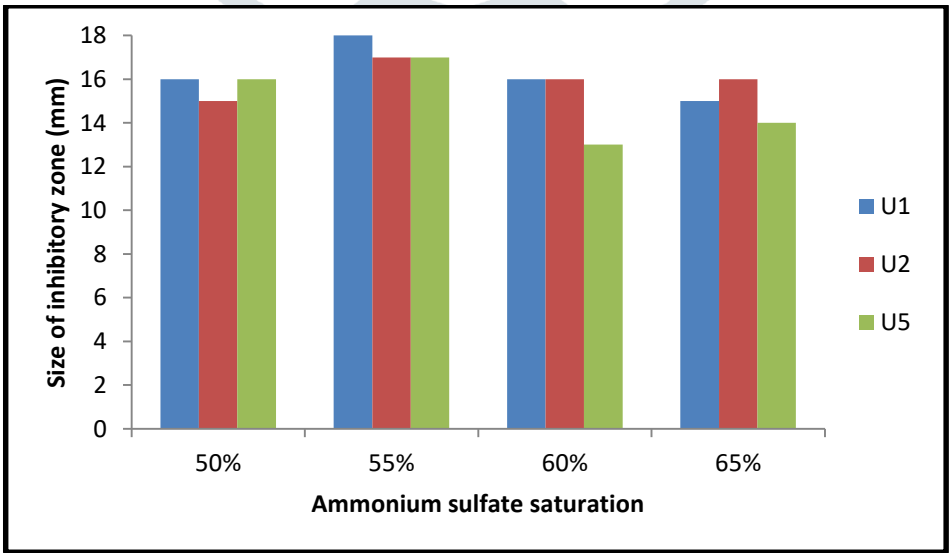

Figure 5: Results of Antagonistic activity of partially purified bacteriocin produced by isolate U1, U2 and U5 by ammonium sulfate followed by dialysis against isolated pathogen S2.

IV. CONCLUSION

The findings of the present study suggest that LABs isolated from the dairy products are bacteriocin producers. The bacteriocins produced by the isolated LABs have been found to be having narrow spectrum of inhibition. The results in the inhibitory effect of bacteriocins against plant pathogens suggest that these bacteriocins can be utilized as biocontrol agent of bacterial wilt in the Solanaceous crops. The use of chemical control agents in the agriculture fields as well as the environmental pollution caused by them would be avoided by the use of these bacteriocin producers against the bacterial wilt disease.

Future research should conduct the plant growth promoting activity of the isolated LAB towards the Solanaceous crops. Moreover, the bacteriocin should be characterized for commercial use as well as the mechanism of inhibition of the plant pathogen by the bacteriocin of LAB should also be studied

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