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ISOLATION AND CHARACTERIZATION OF BACTERIOCINOGENIC LACTIC ACID BACTERIA FROM INDIGINOUS DAIRY SOURCE AND ITS ANTIMICROBIAL POTENTIAL

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The aim of this research was to isolate lactic acid bacterial (LAB) strains, characterize them and classify the bacteriocin producing LAB strain from indigenous dairy sources. Antimicrobial potential of LAB strain was tested against *L. monocytogens*. The antimicrobial compound was also tested for maintaining the decreased colony forming unit (CFU) for minimally processed vegetables like carrots, cabbage and lettuce in second phase of the research. The initial identification was based on conventional morphological and biochemical analysis while the final confirmation was done by utilizing advanced molecular tools. Prior to all these manipulations the growth conditions were carefully optimized for the respective strains. The study finally led us to conclude that *Lactococcus lactis* subsp. *lactis* was the most abundant type of lactic acid bacteria found in indigenous dairy products (sour cream and cheese) samples studied. A bacteriocin (Lacticin SC07) produced during the growth of *Lactococcus lactis subsp. lactis* was purified partially by using biochemical technique ammonium sulfate precipitation in different percentages (60% and 80%). These precipitations lead to a 437-fold increase in total lacticin SC07 activity. SDS-PAGE electrophoretic pattern of lacticin showed that it is a single peptide band of 1.7 kDa. But, 3.7 kDa dimers also showing lacticin SC07 activity. The findings of my research revealed that the isolated LAB strain has good potential for bacteriocin production and antimicrobial potential that exerts in the usage of this bacteriocin (lacticinSC07) as a natural preservative in minimally processed vegetable industry.

Keywords: Bacteriocin, antimicrobial potential, indigenous dairy sources, purification, electrophoretic pattern, natural preservative.

INTRODUCTION

Food-borne infections are globally midst the utmost critical and inflated public health issues, as a main cause of morbidity. The reported numbers of food-borne diseases and intoxications still increased over the past decade because all the food safety techniques and food handling processes cannot overcome the risks relevant to food spoilage and food pathogens. *Campylobacter, Salmonella, Listeria* and viruses caused the most common food-borne infections in the European Union and affect more than 380,000 citizens per year (EFSA, 2009). Fresh-cut lettuce from more than a decade has been one of the major concerned raw material which accounts for 80% of the total production of fresh cut. The salad bars now became popular and demand of freshly cut lettuce also increased, hence available in convenience stores, supermarkets, and introduced new environment that favors the growth of food spoilage and food-borne pathogenic bacteria like *Listeria monocytogenes* (Gombas *et al*., 2003).

Global food market now introduces novel foods and ready to serve, minimal processed or fresh cut foods. Hence these foods may need a longer and more complicate food chain which increase the chances of microbial contamination. So, there is always a gap for continuous innovative food preservation techniques that fulfill the demand from "farm to fork". Recently special courtesy has been given to bioprotection/biopreservation of food products to enhance the hygienic quality and shelf-life of food products. Biopreservation also improves the sensory and nutritional characteristics of fresh cut vegetable products. Bioprotection/biopreservation with use of naturally existing microorganisms and their metabolic products has an excellent history of benign use with high antimicrobial potential.

Bacteriocins are produced proteins or peptides having greater antibacterial potential towards closely related strains. Many of the bacteriocins have relatively high molecular weight (>80 kDa) which destroy food spoilage and food pathogenic bacteria (Cascales *et al*., 2007). Recently, small, heat-stable cationic peptides produced from Gram positive bacteria are defined as bacteriocin, which have a broad antimicrobial inhibition spectrum (Cotter *et al*., 2005).

Many LAB bacteriocins have excellent antibacterial spectrum for food spoilage and food-borne Grampositive bacteria. External membrane protects the Gram-negative bacteria intrinsically. Nevertheless, recent techniques applied in fresh-cut vegetable processing manufacturers have enhanced shelf

life and maximum quality of the products but food safety is still a matter of worry (Devlieghere *et al*., 2004; Cleveland *et al*., 2001).

A fascinating way to enhance microbiological food safety of fresh-cut vegetables is the application of natural microorganisms and their metabolic antibacterial peptides as a food biopreservative approach. The application of microorganisms such as LAB and their antimicrobial metabolites, has been recommended to improve the quality of fresh-cut vegetables (Leverentz *et al*., 2006). LAB forms several antibacterial products, among which bacteriocins are of special attention. Bacteriocins are regarded as natural and safe biopreservatives, because they are tarnished by enzyme proteases for the gastrointestinal tract and can be helpful for controlling food spoilage and food-borne pathogens as a primary hurdle (Cleveland *et al*., 2001). Nisin is a commercially available and widely studied bacteriocin. But, other bacteriocins such as lacticin, plantaricin and pediocins have greater applications in food environments (Deegan *et al*., 2006). Previous studies have shown that use of bacteriocins in combination may be more effective approach towards natural food biopreservation (Limonet *et al*., 2004; Bari *et al*., 2005).

The present study is designed to produce bacteriocin by bacteriocinogenic LAB strain isolated from indigenous dairy sources. The ability of bacteriocin to inhibit food-borne pathogens will be assessed. Moreover, the antimicrobial potential of bacteriocins as biopreservativesin fresh-cut vegetables will be evaluated. Keeping in view all the benefits of bacteriocins to be used as biopreservative, the present study has been designed forisolation and characterization of bacteriocinogenic LAB strains from indigenous dairy sources as well as production and characterization of bacteriocin from indigenous LAB strains.

MATERIALS AND METHODS

***Isolation and identification of bacteria*:** Indigenous dairy (sour cream and cheese) samples were collected from local market of Faisalabad. The samples were kept under refrigerator temperature at 4°C in sterile sample holders and used to isolate the *Lactococcus* spp.10^{-1} – 10^{-6}dilutions of 1 gram of sample (sour cream) were made. Sample dilutions were plated on medium MRS agar to isolate the *Lactococci* strains. The strains were subcultured onto MRS agar medium and incubated for 24 hours at 30C then preserved in solution of 20% glycerol. One of the isolate (*L. lactis*) was taken for future studies which showed excellent antimicrobial potential against target organisms. The LAB isolate was identified and characterized on the basis of cell morphology,growth, gram staining and catalase activity, sugar fermentation profile and other basic physiological and biochemical tests.

***Production of crude bacteriocin*:** After biochemical tests were confirm bacterial colony, then that colony culture was inoculated into MRS broth and incubate it for 48 hrs. Centrifugation was performed at 10,000 rpm for 15 min. at 4C and then cells were separated from the growth medium. The cell-free supernatant was used as crude bacteriocin and maintained at pH 6.0 using 1N NaOH.

***Purification of bacteriocin*:** It was performed with 60 and80% of the ammonium sulphate precipitation, stirred at room temp. The precipitate was resuspended with 20mM buffer as potassium phosphate at pH 6.0 and temperature 5°°C. Membrane dialysis and ion exchange and gel filtered chromatography method was used for complete purification of isolate. The protein was precipitated and these precipitations contain our desired.20kDa dialysis membrane was used for the purpose of dialysis. The enzyme specific activity units were measured by Gelatin Digestion Unit (GDU) Assay. 1 GDU is defined asthe amount of enzyme, liberated one milligram of amino nitrogen from a standard gelatin solution at pH 5.5 or pH 4.5 at 45°C after 20 min of digestion.

***SDS-PAGE for the measurement of molecular weight of bacteriocin*:**15% sodium dodecyl sulfate polyacrylamide gel electrophoresis was used for the determination of molecular weight of bacteriocin from LAB isolate.

***Antibacterial effect of bacteriocin*:** Agar well diffusion method was used for the antimicrobial potential of the bacteriocin from *Lactococcus lactis*. The supernatant culture 48 hours after incubation of *Lactocccus lactis* was filtered through 045 mm membrane filter, the sterile supernatant was put in 4 mm diameter wells that had been cut in Mueller-Hinton agar with the target organism. After 12 to 18 hours of incubation, it showed the diameters of the zones of growth inhibition.

RESULTS AND DISCUSSION

***Isolation and identification of bacterial strain*:** The bacteriocin producing LAB strain was isolated from indigenous dairy source and the selected strain was identified as *L. lactis* subsp. *lactis* based on its biochemical and physiological properties as cell shapes comprised cocci, rods and tetrad-forming cocci. All isolates were Gram-positive, catalase–negative, non-spore formers capable of growth under anaerobic conditions. These are characteristics of lactic acid bacteria (Axelsson, 2004).(Table 1, Fig. 1). Figure 4 shows the biomass and bacteriocin production. LAB strain *L. lactis* produced a bacteriocin in MRS broth as shown in Figure 4. The stationary phase was the best in production of bacteriocin, whereas biomass production was maximum at 20 hours. Moreno *et al.* (1999) have isolated the different strains of LAB from different food products like milk and cheese. The antimicrobial potential was measured through agar well diffusion method on agar plates (Toro, 2005). To avoid antagonism hydrogen peroxide also used for inhibiting antagonism with catalase by adding to the culture medium;

phosphate was added to the solid medium as buffer to avoid inhibition with the use oforganic acids. LAB are used in the production of foods prepared by lactic fermentation such as dairy products, fermented vegetables, fermented meats, and sourdough bread (Moulay *et al*., 2013). LAB have a wide range of antimicrobial activities, among these activities, the production of lactic acid and acetic acid is obviously the most important. On the other hand, certain strains of LAB are known to produce bioactive molecules like ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity (Aween *et al*., 2012).

Table 1. Biochemical characteristics of *L. lactis.*

Biochemical test	***Lactococcu lactis***
Growth in	Uniform
Gram staining	Gram positve
Catalase test	Negative
Indole	Negative
Methyl red	+
Voges	–
Carbohydrate fermentation	+

Table 2. Sugar fermentation profile.

Sr.	**Isolates**	**Xylose**	**Ribose**	**Galactose**	**Raffinose**	**Maltose**	**Sucrose**	**Sorbitol**	**Lactose**	**Mannose**	**Glucose**
1	LC_1	-	+	+	-	+	-	+	+	+	+
2	LC_2	-	+	+	-	+	-	-	+	+	+
3	LC_3	-	+	+	-	+	+	-	+	+	+
4	LC_4	-	+	+	-	+	-	-	+	+	+
5	LC_5	-	+	+	-	+	-	-	+	+	+

LC= *Lactococcus lactis* subsp. *lactis*

In general, LAB used in fermentation of milk products are proteolytic due to the instability of milk proteins. The proteolytic system of LAB is important for growth of microorganisms and in native free amino acid of milk. Proteolytic system is involved in casein utilization within LAB cell contributing to development of organoleptic properties of fermented milk products (Yamina *et al*., 2013). The identification of lactic acid bacteria was established many years ago because of the need to determine the strains that can be used in the industry to characterize the properties and the marketing value of the strain, and above of that is to confirm the safety of the strain to be used in the food application or even in pharmaceutical application. Historically, the identification of LAB was based on phenotypic and chemical methods. These methods depend on the activity of the LAB,carbohydrates fermentation, gas production, motility and spore production (Ashmaig *et al*., 2009).

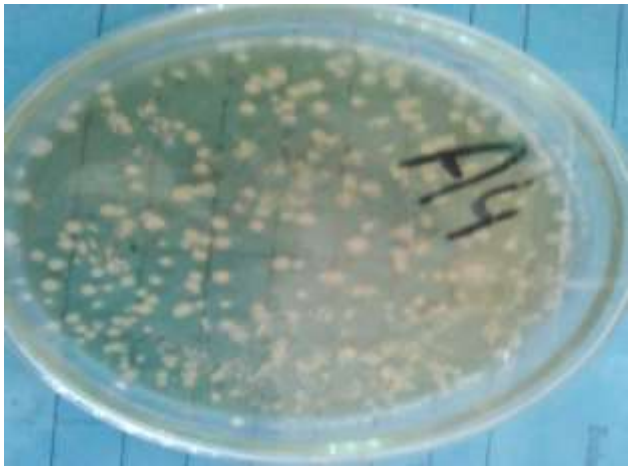

Figure 1. Lactic acid bacterial colonies (LAB)/ Growth of Lab.

Figure 2. Streak plates of LAB isolates for purification.

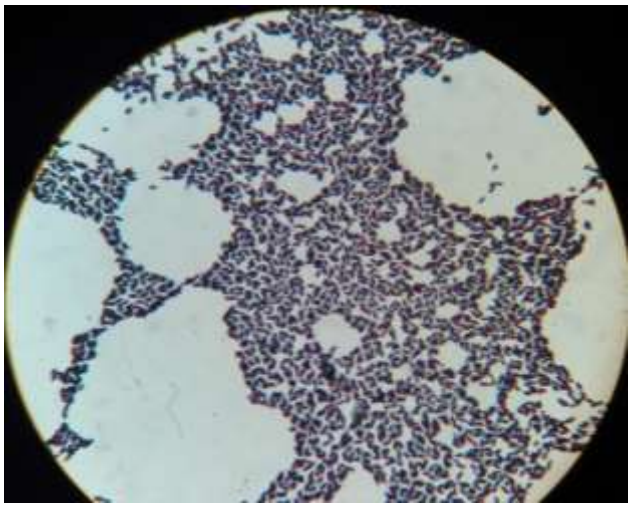

Figure 3. Gram's stained slide of isolated LAB strain (*L. lactis).*

Table 3. Purification of bacteriocin (lacticin SC07).

Sample	Total activity (u)	Total protein (mg/ml)	Specific activity U/mg	Recovery fold	Fold purification based on activity
Crude	6×10^3	109	55×10^1	1	1
60 % Saturation	10.2×10^2	17.56	58×10^3	1.7	106
80 % Saturation	26×10^5	12	218×10^4	437	3972
Dialysis	28×10^5	8	345×10^5	460	62740
IEC Extract	34×10^5	6	573×10^5	573	104176
Gel filtered Extract	58×10^5	1.8	320×10^6	9630	5836232

***Bacteriocin production and harvesting of biomass*:** Harvesting of biomass and production of bacteriocin is shown in Fig. 4. Figure 4 showed the biomass and bacteriocin production. LAB strain *L. lactis* produced a bacteriocin in MRS broth. The LAB strain *L. lactis* showed excellent bacteriocin potential at NaCl 1.5%, pH 6.0 and at 30°C as 2294 AU/ml. The stationary phase was the best in production of bacteriocin, whereas biomass production was maximum at 20 h.

A study was conducted and reported the optimal bacteriocin production during late log phase and early stationary phase of the culture. This might be because of partial degradation and/or adsorption on producer cells. Highest bacteriocin titers were always been obtained after eight hours of incubation at 35° C. But current findings suggest highest titer was obtained after 20 hours of incubation during the early stationary phase. It has been well cited in literature that bacteriocin is produced during late exponential and early stationary growth phase. Various authors have reported the decrease in bacteriocin activity after 48h of incubation. The researchers found that production of crispacin A in MRS broth was dependent on phase of bacterial growth. Callewaert *et al.*, (2013) described that bacteriocin production starts early in growth cycle and continues till the beginning of the stationary phase. During the stationary phase bacteriocin producing cells are killed. The inhibition of adsorption of the bacteriocin molecules to the cell surface by ethanol can prevent subsequent cell death due to a limited immunity of bacteriocin producer cells. According to Parante and Ricciardi (2005), bacteriocin production rate improves in continuous fermentations where high growth rates can be maintained. Cherif *et al.*, (2001) recovered inhibitory activity of culture supernatant at mid logarithmic phase and during stationary phase. Maximum bacteriocin production in *L. acidophilus* DSM20079 was obtained at the end of exponential growth phase (Deraz *et al.*, 2007). Bacteriocin showed maximum production during late log phase and early stationary phase after 20 hours of incubation as 5800 AU/ ml and 6000 AU/ml after 24 hours of incubation as shown in figure 6. Similar trends have been reported in other bacteriocin production studies (Ghrairi *et al.*, 2008; Huang *et al.*, 2009; Todorov and Dicks, 2009).

Figure 4. Lyophilized isolated bacterial mass after centrifugation. Figure clearly showed the bacterial biomass and crude bacteriocin production.

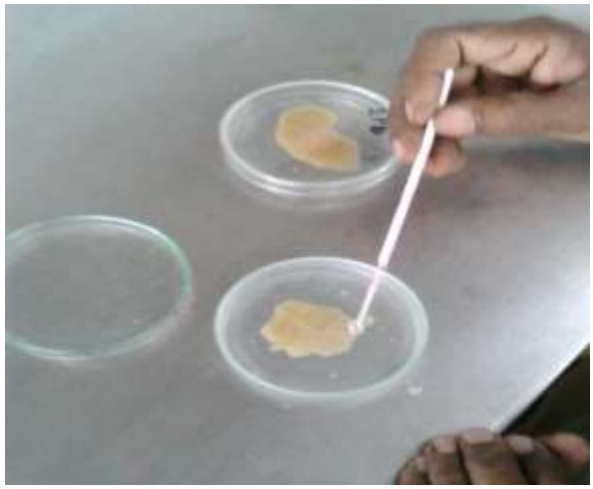

Figure 5. Freeze dried isolated bacterial biomass which was aseptically packed in polythene bags for further studies.

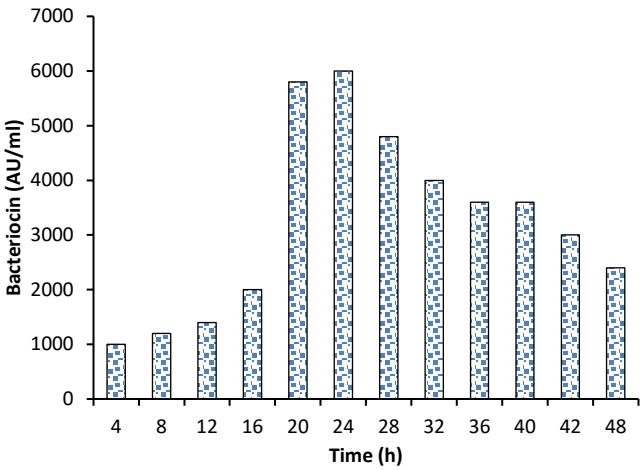

Figure 6. Bacteriocin production at 600 nm OD in different time intervals.

***Purification of bacteriocin*:** The recoveries and purification steps of bacteriocin production are shown in Table 2. At 60%V ammonium sulfate saturation, a 1.7-fold increase in total activity of bacteriocin was recovered in pellet. Upon subsequent treatment with ammonium sulfate at 80% saturation activity in the resulting pellet measured 437-fold higher than that in the initial active supernatant. Desalting of the active extract gave the indistinguishable bacteriocin titer. Thus, a significant increase in specific activity occurred during this step of purification. Taking into account the increase in total activity, the specific activity rose by a factor 3972. However, on the basis of proteins levels it appeared that bacteriocin (lacticinSC07) had been purified 102-fold. Centrifugation was used for the filtrate culture purification, and 80% ammonium sulphate precipitation and dialysis methods used for the protein concentration. Cold environment was maintained in a room for all the procedures. Bacteriocin was purified from culture supernatant extracellularly >12. The specific-activity was found markedly increased after ammonium sulphate precipitation (Alam *et al*., 2011). Other researchers also used 80% Ammonium sulphate saturation to precipitate bacteriocins from *B. thuringiensis* subsp. *entomocidus*, *B. cereus* and *B. subtilis* LFB112 (Cherif *et al*., 2008; Risoen *et al*., 2004; Xie *et al*., 2009) respectively. The antimicrobial substance produced by *B. amyloliquefaciens*MBL27 was precipitated readily by adding 40% saturation with about 98% recovery of the antimicrobial protein. Ammonium sulphate fractionation was used as the first step in purification protocol for *B. thuringiensis* (Ahern *et al*., 2003). The maximum yield and antimicrobial activities are summarized in Table 2.

***Molecular weight determination in SDS-PAGE*:** SDS-PAGE gel electrophoresis used for determination of molecular weight of bacteriocin as shown in Figure3. This bacteriocin was stained with Comassie blue and it showed single protein band which is the clear evidence of protein purity. The calculated molecular weight of the bacteriocin which was purified was about 3.7 kDa.To confirm the identity of 1.7-kDa band fractions derived from the Bac^{-} Bac^{+}*L. lactis* subsp. *lactis* which were analogous to lacticin SC07. Eluting fractions were recovered and analyzed by SDS-PAGE. No 1.7-kDa band or activity was detected (Fig. 2, lane 4) confirming that the 1.7-kDa purified band from *Lactococcus lactis* subsp. *lactis* was lacticin SC07. So far, various bacteriocins isolated from LAB in meat and dairy products have been reported. These bacteriocins are as follows: acidocin D20079 (6.6 kDa) produced by *L. acidophilus* DSM 20079 (Deraz *et al*., 2005), bacteriocin KCA2386 (8.1 kDa) produced by *Lactococcus lactis* (Ko and Ahn, 2000), plantaricin 35d (4.5 kDa) produced by *L. plantarum* 35d (Messi *et al*., 2001), bacteriocin ST44AM (6.5 kDa) from *Pediococcus pentosaceus* ST44AM (Todorovand Dicks 2009), bacteriocin AMA-K (2.9 kDa) from *L. plantarum* AMA-K, bacteriocin ST414BZ (3.7 kDa) from *L. plantarum* ST414BZ (Todorov and Dicks, 2010), sakacin C2 (5.5 kDa) from *L. sakei* C2 (Gao *et al*. 2010). The molecular weights generally range from 3 kDa to 10 kDa. Bacteriocins with the molecular weights higher than 10 kDa are not common. In this paper, the molecular weight of bacteriocin lacticin SC07 was 3.7 kDa.

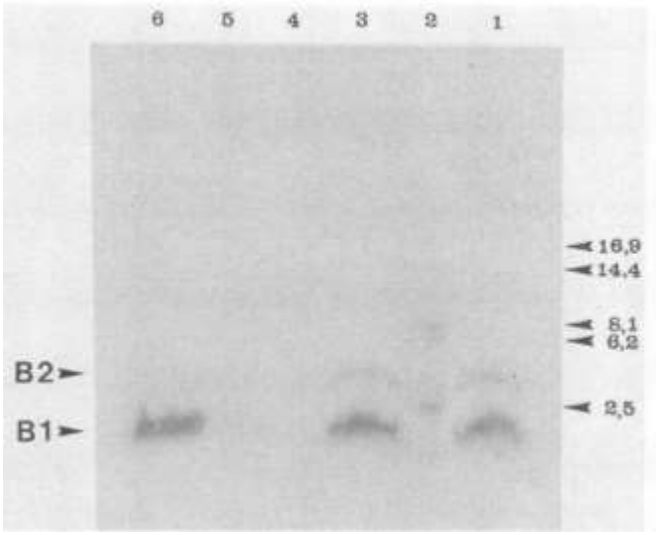

Figure 6. Coomassie blue-stained bacteriocin containing protein bands from a Sodium Dodecyl Sulfate-PAGE for the determination of molecular weight of bacteriocin. Lanes: 1 and 3. Ammonium sulfate saturated and IEC aliquots respectively: 4. The gel filtered extract from *L. lactis* subsp. *lactis*: 5 dialysis aliquot: 6 molecular weight standards. Two protein bands are visible on the membrane: B1 is a 1.7-kDa peptide and B2 is a 3.7-kDa peptide.

***Antibacterial potential of bacteriocin*:** *L. monocytogens* susceptibilities with culture supernatant to growth of *L. lactis* are presented in Figure **7**. LAB is well known to produce different low molecular weight antimicrobial substances which may prevent other bacterial growth. The antimicrobial potential might be because of the organic acids especially lactic acid and acetic acids, bacteriocins, hydrogen peroxide, ethanol, carbon dioxide, and diacetyl which were produced during fermentation processes (Elyass, 2010). The agar well diffusion method was found better option to examine the antibacterial potential of LAB bacteriocins which were studied against 6 communal plant/food spoilage organisms and bacterial pathogens at 5 and 20°C (Sharpe, 2009; Yang, 2011).

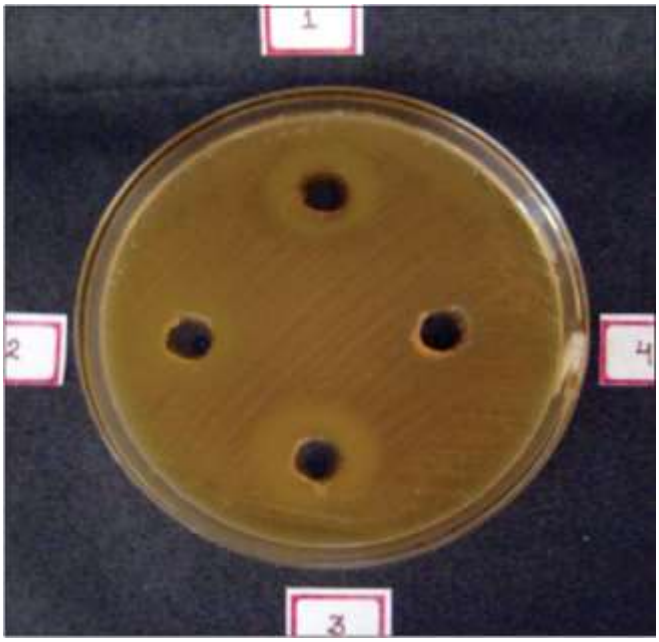

Figure 7. Antimicrobial potential of crude bacteriocin solution against *L. monocytogen.*

***Conclusion*:** This research effort has demonstrated the isolation and characterization of bacteriocinogenic lactic acid bacteria and production of bacteriocins. LAB bacteriocin (lacticin) showed excellent antimicrobial potential during the present research work. One of the prime application of this property is the utilization of bacteriocinogenic strains of LAB as biopreservative in minimally processing industries of vegetables. This will help to improve the microbiological quality of the product through controlling the growth of other, unwanted, microorganisms. In the end, the acceptance and use of purified bacteriocins in food preservation as safe and natural additives is expected to expand rapidly. It is, therefore, recommended that further probing and exploration of bacteriocionogenic strains of bacteria, particularly LAB, should be undertaken. It is hoped that the presently flourishing local food industry should back such efforts, as well as seek ways of making practical use of research findings in this field. This will certainly be more than welcome in view of the increasing demand by consumers for more "natural" food preservation methods.

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