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Screening, Production Purification and Potential Use of Bacteriocins From Lactic Acid Bacteria of Meat and Dairy Food Origin

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Abstract. The present paper focused on screening, production and purification strategies of bacteriocins from lactic acid bacteria isolated from meat and dairy products. A total of 19 isolates were screened for bacteriocin production by sandwich test. Isolates were subjected to inhibitory activity test using well diffusion method. The isolates from the meat and dairy samples were confirmed as *Lactobacillus sp., Lactococcus sp.* and *Pediococcus sp.* based on their morphological and biochemical characteristics. *Pseudomonas sp.* and *Proteus sp.* were found to be the least sensitive to inhibitory substances produced by the lactic culture as compared to other indicator strains. *S. aureus* exhibited the highest sensitivity to it. The optimal initial pH for higher production of the inhibitory factor was found to be 5.5 and incubation period of 24-48h for *Lactobacillus* and *Lactococcus.* Though, in case of *Pediococcus* pH of 6.5 and 48-72h of incubation time was observed to be the best. Bacteriocin recovery was achieved by partial purification step followed by repeated washing with water. The activity of bacteriocins was retained after purification process and activity was found to increase with increased purification.

Keywords: Lactic acid bacteria, Bacteriocins, Phytosanitary, Dairy products.

1. Lactic Acid Bacteria: An Introduction

The industrial importance of the lactic culture is further evidenced by their *'Generally Recognized as Safe'* (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surface. The lactic acid bacteria produce different antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins, which can inhibit pathogenic and spoilage microorganisms, extending the shelf life and enhancing the safety of food products (Puniya *et al.*,2012; Mehra *et al.,* 2012; Dhewa *et al.,* 2011; Dhewa & Singh *et al.,* 2011; Dhewa, 2011; Dhewa *et al.*,2010a;Dhewa *et al.,* 2010b; Dhewa & Goyal, 2009; Dhewa *et al.,* 2009; Puniya *et al.,* 2008).

1.1. Antimicrobial Potential of Lactic Acid Bacteria: Bacteriocins

Lactic cultures display a wide range of antimicrobial activities and renders protection of food from spoilage and pathogenic microorganisms. Lactic acid and acetic acid are the most important products produced. The other products produced are bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, exopolysaccharides, reuterin, reutericyclin and other enzymes of importance. Many strains produce bacteriocin and bacteriocin–like molecules that display antibacterial activity. Besides bacteriocin, some lactic culture are able to synthesize other antimicrobial peptides that may also contribute to food preservation and safety like, anti-fungal activity of organic acid and fatty acids, other low molecular mass metabolites and/or cyclic dipeptides (De Vuyst & Leroy, 2007).

1.2. Potential Applications of Bacteriocins

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Bacteriocins from lactic acid bacteria have demonstrated as food preservatives or as therapeutics for veterinary or medical uses or as phytosanitary for protection of plants. Bacteriocins have a remarkable potential in phytosanitary for plants protection. In the development of probiotics, bacteriocin appears to play their largest role. There is a great deal of effort directed towards the development of probiotic strains that are effective against a variety of human gastrointestinal tract pathogens such as *L. monocytogens* and *H. pylori* as well as against pathogens of oral cavity and vagina (Gillor *et al.,* 2008). It is possible that in future, probiotics will be used for different gastrointestinal diseases, vaginosis, or as delivery systems for vaccines, immunoglobulins, and other therapies (Ljungh & Wadstrom, 2009). Keeping in view the above aspects the present study is to design for **i**solation and biochemical characterization of bacteriocin producing lactic acid bacteria from Meat and dairy products, Production studies of bacteriocin in batch culture with the influence of initial pH and incubation period on its production and Purification and Characterization of the purified bacteriocin.

2. Methodology

2.1. Collection of Samples:

A total of 20 samples constituting of 8 meat samples and 12 dairy samples were collected in sterile containers from different butcher shop and dairies and stored at 4^{o}C for a maximum of 24h before analysis (Dhewa *et al.,* 2011).

2.2. Screening of Potential Bacteriocin Producers: A Sandwich Test

A 25g portion of each meat or dairy sample was aseptically transferred to a sterile flask containing 225 ml of buffered peptone water to obtain a 1:10 dilution. Plates were incubated at 35^{o}C for 48h. The overlay agar was seeded with 100µl of the indicator strain. The indicator strains used were *S. aureus* and *E. coli.* The plates were incubated at 35^{o}C for 24h. Colonies showing zones of inhibition were transferred to MRS broth and incubated at 35^{o}C for up to 72h. The cultures were then purified on MRS agar plates and incubated at 35^{o}C for 18h.

2.3. Maintenance and Propagation of Cultures

Isolates and the indicator strains were streaked and re-streaked on MRS agar medium and Nutrient agar medium (containing 0.6% yeast extract) respectively at frequent intervals of time. The stock cultures were preserved in a refrigerator at 4^{o}C (Dhewa *et al.* 2011, 2010).

2.4. 2.4 Purity and Confirmation of Cultures

The lactic acid bacteria isolates as well as those of the indicator bacterial strains were checked for their purity by performing Gram staining and microscopic examination at intervals of time (Dhewa *et al.,* 2011, 2010).

2.5. Detection of Antimicrobial Activity

The recovered isolates were subjected for antimicrobial activity. The isolates and the indicator strains (*S. aureus, E. coli, S. typhi, Klebseilla sp* and *Proteus sp)* were grown in MRS broth and soy casein digest broth (with 0.6% yeast extract) respectively. The MRS broth were inoculated with 1% (v/v) of the active culture of the recovered isolates and incubated at 35^{o}C for 48h. Cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4^{o}C and the supernatants were for antagonistic activity by well diffusion assay method (Anand *et al.,* 1984). The diameters of zone of inhibition were measured.

2.6. Morphological, Biochemical Characterization of Isolates

Morphological characters of the positive isolates like colony morphology (color, shape, margin, elevation and surface) and cell morphology (shape, arrangement, and Gram reaction) were studied Biochemical characterization on the basis of catalase, nitrate reduction and sugar fermentation test.

2.7. Production of Bacteriocins

For the preparation of the inoculums, 10 ml of MRS broth medium was inoculated with 0.1ml of freshly prepared culture of lactic acid bacteria isolate and was incubated for 12h at 35^{o}C. From this pre-culture, 1ml

was added to 100ml of MRS broth medium which after 12h of incubation at 35˚C, was used to inoculate the production media (Salha Hassan Al-Zahrani *et al.,* 2006).

2.7.1. Influence of Different Initial pH on the Production of Bacteriocin

To determine the effect of initial pH on the production of bacteriocin, the production media were adjusted to initial pH value of 4.5, 5.5, 6.5 and 7.5. Each medium was inoculated (2%v/v) with overnight culture of the bacteriocin producing lactic isolates and incubated for 48h at 35^{o}C. After incubation the samples were then checked for antibacterial effect. Sterile MRS broth medium adjusted to their corresponding pH were used as control.

2.7.2. Influence of Incubation Period on the Production of Bacteriocin

The effect of incubation time was determined by inoculating sterile MRS broth media with active overnight cultures (2%v/v) and incubated at 35^{o}C. The samples were checked for antibacterial activity at periods of 24, 48 and 72h.

2.8. Extraction and Purification of Bacteriocins

The extraction and purification performed in accordance by the methods given by Kacem Mourad *et al*. (2005); Gautam & Sharma (2009) with some modifications. The isolates propagated in MRS broth media for 48h at 35^{o}C were centrifuged at 8000 rpm for 30 minutes at 4^{o}C for the extraction of bacteriocin. The supernatant was then filtered through a filter paper. This solution was designated as the crude extract.

2.8.1. Partial Purification of Bacteriocin

Partial purification of bacteriocin was done by salt saturation method. The crude extract was saturated with 70% ammonium sulfate and stored at continuous stirring with the help of magnetic stirrer**,** for 24h to precipitate out the proteins. After 24h, centrifugation was done at 8000 rpm at 4^{o}C for 1h and pellet was re-suspended in 25ml of 0.1 M potassium phosphate buffer pH 7.0. To this solution 5% equivalent of TCA was added. The mixture was centrifuged at 8000 rpm for 20 minutes after which the supernatant was decanted. The resulting pellet was dissolved in potassium phosphate buffer.

2.8.2. Purification by WashingMethod

Partially purified bacteriocin was centrifuged at 8000 rpm for 10minutes at 4^{o}C. The supernatant was discarded and the pellet which contains bacteriocin was washed with water and centrifuged again at 8000rpm for 20 minutes at 4^{o}C.No inhibition zone indicates that the bacteriocin activity was retained only in the pellet. Bacteriocin activity was confirmed by well diffusion assay.

2.8.3. Characterization of the Purified Bacteriocin

Characterization was done according to the methods given by Gautam & Sharma (2009). The bacteriocins were characterized by heat resistance, sensitivity to pH and proteolytic enzymes.

2.8.3.1. Heat Resistance

The thermal stability of the purified bacteriocin was assessed by exposing it to different temperatures. To 4.5 ml of sterile Nutrient broth, 0.5ml of the purified bacteriocin was added in test tubes and heated to different temperatures (40, 50, 60, 70, 80, 90, and 100) for 15 minutes each. Preparation containing nutrient broth and bacteriocin (4.5ml+0.5ml) in test tubes, plugged with cotton and covered with aluminium foil was kept in autoclave at 121oC and 15lbs pressure for 15 minutes to check its stability at very high temperature. Then each of the test tubes was tested for antimicrobial activity by well diffusion method.

2.8.3.2. pH Sensitivity

The effect of pH on the purified bacteriocin activity was tested by adjusting tubes containing 4.5ml of Nutrient Broth to different pH ranges from 4-11 and then 0.5ml of the bacteriocins were added to each tube, incubated at room temperature for 1h and further tested for antimicrobial activity.

2.8.3.3. Sensitivity to Proteolytic Enzymes

The effects of proteolytic enzymes such as pepsin and trypsin on bacteriocin activity were studied. In a test tube 0.15ml of phosphate buffer (0.5M, pH 7.0), 0.15ml of the bacteriocin and 0.15ml of trypsin/pepsin

(0.25mg/ml) were added. This will be the enzyme reaction tube. In order to be sure that the inhibition is not caused by the phosphate buffer, enzyme control tube can be taken, containing 0.3ml of phosphate buffer devoid of bacteriocin and enzyme and another containing the 0.15ml phosphate buffer.

3. Observations and Results

From the meat and dairy samples a total of 19 isolates were recovered which showed possible bacteriocin production through Sandwich method. Zones of inhibition of the indicator pathogen were seen around colonies (Fig.3.1). These colonies were carefully picked up and inoculated in MRS broth and consequently in MRS agar media to obtain pure culture of the potential bacteriocins producer.

3.1. Antimicrobial Assay of the Recovered Isolates

Isolates recovered from the meat and dairy samples were tested for antimicrobial activity by well diffusion assay. Only 7 of them showed antimicrobial activity of bacteriocin by well diffusion assay (Fig. 3.2). The zone of inhibition was measured and recorded in mm after incubation of 48h. The sensitivity of various pathogenic bacteria to bacteriocins produced by Lactic acid bacteria were *S. aureus* (28.54%), *S. typhi* (20%), *E. coli* (18.23%), *Pseudomonas sp.* (14%), *Klebsiella sp* (14.40%) and *Proteus sp.*(18.23%).

3.2. Morphological and Biochemical Characterization of the Isolate

The 7 isolates showing antimicrobial activity of bacteriocin by well diffusion method were preceded for morphological examination and biochemical test. On the basis of these tests, the different patterns of sugar fermentation were compared using PIBWIN software and Bergey's manual of Determinative Bacteriology for the identification of the isolates (Table 3.1).

3.3. Production Studies

Out of 7 bacteriocin producing Lactic acid bacteria**,** 3 of them i.e., *Lactobacillus sp, Lactococcus sp* and *Pediococcus sp* which showed the best antagonistic activity were inoculated (2%v/v) in MRS broth medium of initial pH 5.5 and then incubated for 48-72 h at 35^{o}C.

3.3.1. Influence of Initial pH on the Production of Bacteriocins

Initial pH of the growth medium ranging from 4.5- 6.5 were found to favour bacteriocin production in *Lactobacillus* and *Lactococcus sp* with pH 5.5 as the best pH for production, but in case of *Pediococcus sp.* pH of 6.5 fostered the best production of bacteriocin (Fig.3.3; Table 3.2).

3.3.2. Influence of Incubation Period on Production of Bacteriocin

The effect of incubation period on the production of bacteriocin was also investigated. 24-48h incubation period was observed as the best production time for *Lactobacillus sp* and *Lactococcus sp* however, *Pediococcus sp* showed best bacteriocin production and activity at 42- 72h of incubation period (Fig.3.3; Table 3.2).

3.4. Extraction and Purification of Bacteriocins

The bacteriocins of two lactic isolates *Lactobacillus sp* and *Lactococcus sp* were proceeded for the extraction and purification process. Partial purification of the cell free supernatant was done by 70% ammonium sulfate precipitation and trichloroacetic acid (TC) precipitation. The precipitates so formed showed complete insolubility in water and Potassium phosphate buffer (pH 7.0, 1M). Partially purified bacteriocin of *Lactobacillus sp.* showed antagonistic activity against *S. aureus* and *E. coli* whereas, *Lactococcus sp.* showed activity against *E. coli* and *S. typhi* After that, the partially purified bacteriocin is purified further by repeated washing of the impurities by water, the purified bacteriocin was also checked for its activity. An increase in the activity of the bacteriocin against the pathogens as compared to partially pure bacteriocin was observed. Purified Bacteriocin of both lactic isolates produced large zones of inhibition against all three pathogens: *E. coli, S. aureus* and *S. typhi* (Fig. 3.4; Table 3.3).

3.5. Characterization of the Purified Bacteriocin

3.5.1. Heat resistance

The purified bacteriocins of both *Lactococcus* and Lactobacillus both are very sensitive to heat i.e. they are heat labile. With the continuous increase in temperature, loss of activity was noticed, with the complete loss of activity at around at around at around 60^{o}C (Table 3.4).

3.5.2. pH Sensitivity

Bacteriocin of *Lactococcus* shows activity in a wide range of pH with highest activity ay pH 6 and 7 and decreases as the pH increases. *Lactobacillus* bacteriocin retained their antimicrobial activity at pH ranging from 4-7. But complete loss of activity was observed at pH above 8.0 (Table 3.4).

3.5.3. Sensitivity to Proteolytic Enzymes

Bacteriocin of *Lactococcus* and *Lactobacillus* were fully or partially inactivated by the proteolytic enzymes such as trypsin and pepsin, which indicates their proteinaceous nature. Both of the bacteriocins activity was inactivated due to the action of trypsin. *Lactobacillus* bacteriocin was also inactivated by pepsin whereas the bacteriocins of *Lactococcus* retained its activity though it showed a significant decrease in its activity (Table 3.4).

4. Discussion

Bacteriocins producing lactic acid bacteria isolated from meat and dairy products could ensure the safety and extend the shelf life of these foods. A total of 19 isolates were recovered by sandwich test. Isolates were subjected to inhibitory activity test using well diffusion method. Only some of lactic cultures showed activity against the indicator organisms. The isolates from the meat and dairy samples were confirmed as *Lactobacillus, Lactococcus* and *Pediococcus* based on their morphological and biochemical characteristics. *Lactococcus sp* exhibited the broadest host range. The bacteriocins of all isolates inhibited the growth of at least 2 or 3 of the indicator strain. *Pseudomonas sp.* and *Proteus sp.* were found to be the least sensitive to inhibitory substances produced by the lactic culture as compared to other indicator strains. *S aureus* exhibited the highest sensitivity to it. The resistance pattern of most of the Gram negative bacteria is attributed to the particular nature of their cellular envelope. The optimal initial pH for higher production of the inhibitory factor was found to be 5.5 and incubation period of 24-48h for *Lactobacillus* and *Lactococcus.* However, in case of *Pediococcus* pH of 6.5 and 48-72h of incubation time was observed to be the best. Bacteriocin recovery was achieved by partial purification steps, ammonium sulfate precipitation and trichloroacetic acid precipitation followed by repeated washing with water. The activity of bacteriocin was retained after purification process and activity was found to increase with increased purification. Bacteriocins were insoluble in water and therefore, the impurities which were soluble in water were easily removed (Gautam & Sharma, 2009). The inhibitory activity of these substances were completely inactivated after heat treatment at 60^{o}C and above for 15 minutes indicating their heat-labile proteinaceous nature. The inhibitory activity of both bacteriocins was stable at pH 4-7 but is either decreased or inactivated after exposure to pH 8.0. The antagonistic effect produced by *Lactobacillus* and *Lactococcus* were partially or completely inactivated when preparations were treated with proteolytic enzymes suggesting that the substances are protein in nature or at least has a proteinaceous activator. With respect to the application of bacteriocins as biological preservative in foods and feeds, sensitivity to proteolytic enzymes suggests that their ingestion will not affect the microbial flora of the gastrointestinal tract. The produced bacteriocins showed a broad spectrum of activity to spoilage microorganisms and pathogens associated with food such as *S. aureus*, *S. typhi* and *Pseudomonas sp.* These results show the potential usefulness of these bacteriocins justifying a more in depth investigation for their identification and application as food bio preservatives. However, further work is needed to fully understand the molecular mechanisms, structure-function relationships and mechanisms of action of bacteriocins for exploration of applications.

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(a)

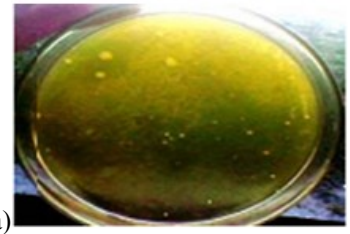

Fig. 3.1: Inhibition by potential bacteriocin producer of (a) *S.aurcus* in sandwich method

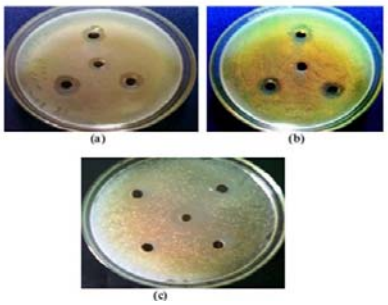

Fig. 3.2: Antimicrobial activity of Lactic acid bacteria against (a) *S.aurcus* (b) *S.typhi* (c) *E. coli* in well diffusion assays.

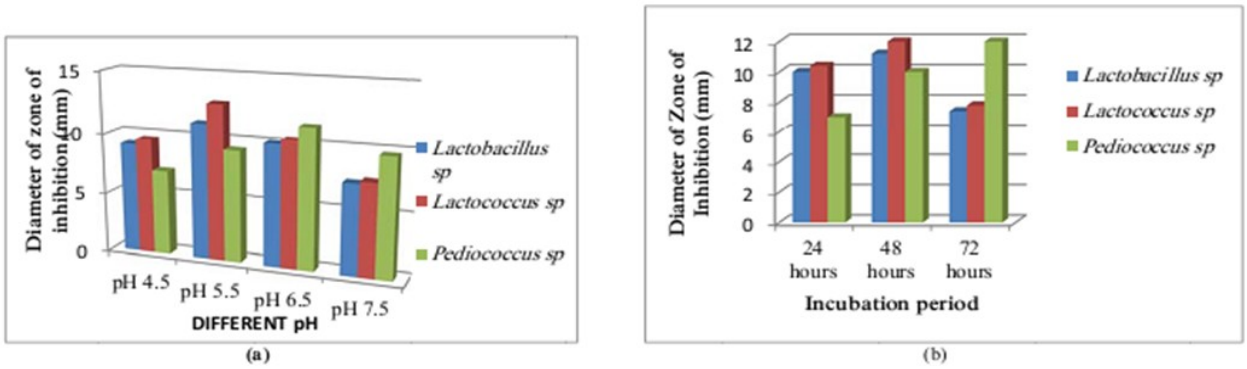

Fig. 3.3: Effect of (a) initial pH (b) incubation period, on the production of bacteriocin by Lactic acid bacteria

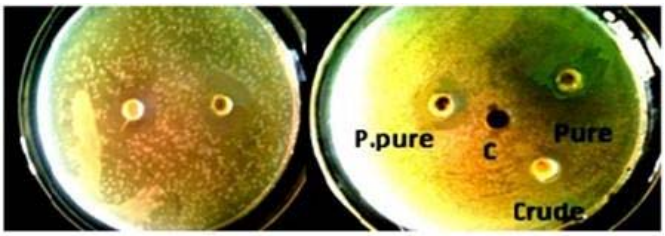

Fig. 3.4: Activity of Crude, partially pure and pure bacteriocion against (a) *E. coli* (b) *S. aureus*

Table 3.1: Sugar fermentation profile of the recovered isolates

Isolate	Sugars															Identified LAB
	Ar	De	Du	Fc	Ga	In	La	Ma	Mn	Mo	Mb	Rh	Sb	Su	Te	
L-2	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	*Lactobacillus sp*
L-6	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	*Lactobacillus sp*
L-9	+	+	+	+	+	+	+	-/+	+	+	+	+	+	+	+	*Lactococcus sp*
L-11	-	+	-/+	-/+	+	-	+	+	-	+	-	-	-	+	+	*Pediococcus sp*
L-15	-	-/+	-	+	+	-	+	+	-	+	-	-	-	-/+	+	*Pediococcus sp*
L-17	+	+	+	+	+	+	+	+	+	+	+	+	-/+	+	+	*Lactobacillus sp*
L-18	+	+	+	+	+	+	+	+	+	+	+	-/+	+	+	+	*Lactococcus sp*

Symbols: + = fermentation; - = no fermentation; -/+ = variable; Ar-arabinose, De-dextrose, Du-dulcitol, Fc-fructose, Ga-galactose, In- inisitol, La-lactose, Ma-maltose, Mn-mannitol, Mo- mannose, Mb-melibiose, Rh- rhamnose , Sb- sorbitol and Te-trehalose.

Table 3.2: Influence of initial pH and incubation period on bacteriocin production

Isolates	Influence of growth Conditions						
	Initial pH				Incubation Period		
	4.5	5.5	6.5	7.5	24h	48h	72h
Lactobacillus sp.	++	+++	++	+	++	+++	+
Lactococcus sp.	++	+++	++	+	++	+++	+
Pediococcus sp	+	++	+++	++	+	++	+++

Symbols: +=low, ++=moderate , +++ = High influence

Table 3.3: Activity of partially purified and purified bacteriocin upon pathogenic strains

LAB Isolate	Bacteriocins type	Diameter of zone of inhibition against indicator organism in mm (including 6mm well size)		
		S. aureus	*E. coli*	*S. typhi*
Lactobacillus sp	Partially pure	7	8	-
	Pure	14	9	8
Lactococcus sp	Partially pure	-	7.6	7
	Pure	7	10	9

Symbol: - : No activity.

Table 3.4: Effect of heat treatment, pH and proteolytic enzymes on purified bacteriocins

Bacteriocins Producer strain	Resistance to heating temp. in (15mins)								Sensitivity to different pH								Sensitivity to proteolytic enzymes	
	40	50	60	70	80	90	100	121	4	5	6	7	8	9	10	11	Pepsin	Trypsin
Lactobacillus sp	R	R	S	S	S	S	S	S	R	R	R	R	S	S	S	S	S	S
Lactococcus sp	R	R	R	S	S	S	S	S	R	R	R	R	R	R	S	S	R	S

Symbols: R = Resistant: Bacteriocin activity retained; S = Sensitive: Bacteriocin inactivated; Pepsin = Pepsin enzyme and Tryps = trypsin enzyme.