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Characterization of Bacteriocin Produced by Lactobacillus SP and Optimization of Cultural Conditions

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Abstract- Lactic acid bacteria (LAB) play an important role in food fermentation and preservation either as natural microflora or as starter cultures. LAB displays numerous antimicrobial activities. This is mainly due to the production of organic acids, but also of other compounds, such as bacteriocins and antifungal peptides. Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum), or across genera (broad spectrum). In recent years, bacteriocin producing LAB have attracted significant attention because of their potential use as safe additives for food preservation that could, at least partially, replace chemical preservatives. LAB were isolated using MRS media. The agar diffusion bioassay was used to screen for bacteriocin or bacteriocin-like substances (BLS). 28 lactobacilli were isolated from 20 different samples. Among the isolate 4 strains of *Lactobacilli* were observed to have activity against 3 diferent strains and also against *S.aureus, MRSA, and Pseudomonas aeruginosa.* Thus only 14.2% of the isolated strains were found to be producing bacteriocin like substance and all of them were isolated from the milk samples. Bacteriocin sensitivity to physical conditions and chemical substances were also evaluated. The test bacteriocin was found to be a sensitive to choloroform and resistant to catalase treatment.

Index Terms- Lactobacillus, Bactriocin like substance, Lactic acid bacteria, Nisin

I. INTRODUCTION

Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory toward sensitive strains and are produced by both gram positive and gram negative bacteria (1). Bacteriocins are produced by bacteria and possess antibiotic properties, but bacteriocins are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can potentially illicit allergic reactions in humans (2).They are ribosomally synthesized polypeptide possessing bacteriocidal activity that are rapidly digested by proteases in the human digestive tract and relatively hydrophobic and heat stable (3, 4). The Bacteriocins (as colicins) were originally defined as bacteriocidal proteins characterized by lethal biosynthesis, a very narrow range of activity and adsorption to specific cell envelop receptors (4).

Lactic acid bacteria are used extensively in food processing such as diary, beverage and meat products (5) .The most important role of the lactic acid bacteria (LAB) is to inhibit growth of spoilage and pathogenic bacteria in food (6). Lactic acid bacteria produce a variety of antibacterial agents including organic acids, diacetyl, H_2O_2 and bacteriocins (7, 8, and 9).The bacteriocins from LAB are cationic, hydrophobic or amphiphilic molecules composed of 20 to 60 amino acids residues. The anionic lipids of cytoplasmic membrane are the primary receptor for bacteriocins of LAB for initiation of pore formation (10, 11)

Bacteriocins can be classified into 4 groups on the basis of their molecular mass, thermo stability, enzymatic, sensitivity, and presence of posttranslational modified amino acids and mode of action (12). The genes encoding bacteriocin production and immunity are usually organized in operon clusters (13) Bacteriocins gene clusters can be located on the chromosome as in case of subtilin (14) plasmid as in case of divergicin A (15) or transponsons as in the case of nisin (16). Bacteriocin molecules itself apparently act as an external signal to auto regulate its own biosynthesis via signal transduction (17). Induction usually occurs under stressful condition such as nutrient depletion or over crowding (18). In a mixed fermentation environment production of bacteriocins may prove advantageous for a producer's organism to dominate the microbial population (19). The bacteriocins are produced through out the experimented growth phase and not solely during late logarithmic or early stationary phase (20).

Bacteriocin shows maximum activity on organism belonging to the same species consequently *Lactobacillus* bacteriocins shows less activity towards gram negative species (21). The resistant gram negative cells of *E.coli. Erwinia carotovora*, *Pseudomonas aeruginosa* and *Serratia marcescens* can be made sensitive o the bacteriocin of strain *Lactobacilli plantarum* by transforming the gram negative cells to spheroplast (21) or by the use of chelating agents (EDTA) that function to diminish the barrier properties provided by the outer LPS membrane of gram negative (22). Bacteriocins are produced by only a few strains within a species which give them distinct survival advantages over others. For example only 7% of the strains were found to be producing bacteriocin in a study conducted with 150 strains of *L.lactis* subspecies *cremoris (*23).

The antagonistic effect of culture supernatants of bacteriocin producing strain can be detected using agar well diffusion assay (24). Another method of checking bacteriocin activity is agar spot method. It is reported that only a few strain tested positive using the spot on the lawn method gave positive result in the well diffusion assay. This may be because of aggregation, non diffusible bacteriocins, and protease inactivation and concentration effects (25). There are reports that the supernatant of bacteriocin producing strains are resistant to autoclaving conditions and to heat treatments (26). It has also been reported that some bacteriocins produced by lactorbacillus strains were inactivated by 10-15 minute heat treatments of 60º -100 º C (26)

Bacteriocins differ greatly with respect to sensitivity to pH. Most of the lactobacilli bacteriocins are considerably more tolerant of acid than alkaline pH values. (27) A studies of effect of various pH values from 1-12 shows that maximum activity occurs at pH4 and 5(28). Generally bacteriocins are destroyed by proteinase K, pronase E and trypsin treatments (29). In growth media, the key factors affecting bacteriocin production are optimal pH and supplementation of medium with specific nutrients. It is also reported that key factors differ with different strains and species. Conditions that favor high cell density result in a comparable increase in bacteriocin yield, which is indicative of primary metabolite. Maximum bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors such as sugars, vitamins and nitrogen source by regulating pH or by choosing the best adapted culture medium (30). It has been reported that highest bacteriocin activity was obtained when glucose and peptone were varied to 0.25% and 0.5% in the constituted MRS broth (31). Modification of nutrients of cultivation media should be considered for maximum production of bacteriocin that has potential use as a food biopreservative (32). A study of effect of Nacl on bacteriocin production showed no changes at 1% Nacl, but production was inhibited with increasing amount of Nacl (33). It has also been reported that large amount of bacteriocin were synthesized when the medium was supplemented with glucose (1%), Tween 80 (0.5%) yeast extract (2-3%) and Nacl (1-2%). (34) There are reports that growth beyond the stationary phase result in decrease bacteriocin activity. This decrease could be due to the activity of extra cellular endogenous proteinases induced during this growth phase (35).

Other factors affecting bacteriocin production are incubation time and temperature. Optimum temperature and incubation time vary from different strain and species (36). The effect of media pH on bacteriocin production is also extensively studied. Bacteriocin production tends to differ greatly based on pH of medium. Acidic pH is tending to be favorable for bacteriocin production by Lactobacilli, still the optimal pH of each starin varies (36). It has been reported that use of combinations of various bacteriocins enhance antibacterial activtivity (37).

II. MATERIALS AND METHODS

i. Screening of different sample for bacteriocin producing *Lactobacilli*

Sample collection and processing

Samples like curd, milk and fruit juices were collected from different sources. The samples were serially diluted and 0.1ml of diluted sample was spread over MRS plate using a spreader and incubated at 30˚C for 48 hours. 20 samples were collected and 28 isolates were obtained.

Identification of isolates

Morphological characteristics such as size, shape and colony characteristics were observed. Microscopic characteristics were based on Gram staining and spore staining. Biochemical characterization was based on motility, catalase test, Gelatin liquefaction test, sugar fermentation test, Nitrate reduction test.

ii. Detection of bacteriocin activity

Each lactobacilli isolate was tested for activity against all other isolates and other indicator strain such as *S. aureus MRSA, E. coli, Klebsiella pneumoniae, P. aeruginosa, Enterococcus faecalis.*

a) Detection of inhibitory activity

The inhibitory activity was screened by agar well diffusion assay. Overnight culture was inoculated in MRS broth and incubated at 24 hours at 37 ºC. Cells were removed by centrifugation at 10,000 rpms for 30 min at 4 ºC. The supernatant fluid was adjusted to pH-7 with 1N NaOH. The wells of 5 mm in diameter were cut into the agar plate by using a cork and culture supernatant was placed into each well. The plates were incubated over night at 37 ºC and observed for zone of inhibition.

b) Bacteriocin assay

The critical dilution assay was used to quantify the inhibitory activity exhibited by the bacteriocin against the respective sensitive indicator stain. A serial two fold dilution of supernatant fluid adjusted to pH 7 was made in sterile distilled H2O. The activity of each dilution was determined by agar well diffusion method. The titer was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units (AU) per ml. Bacteriocin assay was done only for the isolates with maximum zone size. Assay was also carried out on the standard strain (*Lactobacillus acidophilus* NCIM 2042)

iii. Selection of test producer & indicator strain

Lactobacillus isolates which showed the highest activity was selected as the test producer strain and the isolate which showed the maximum sensitivity was taken as the test indicator strain.

iv. Characterization of bacteriocin

Bacteriocin was checked for its temperature tolerance, pH stability and sensitivity to catalase enzyme and chloroform.

a) Sensitivity to heat:

Culture supernatant was heated for 10 min at 40 ºC, 50 ºC, 60 ºC, 70 ºC, 80 ºC, 90 ºC, 100 ºC and agar well diffusion assay was performed to detect residual activity.

b) Sensitivity to different pH values

The pH of the culture supernatant was adjusted to 3 4.5, 7.0 and 9.0 and then kept at room temperature for 4 h. Residual activity was determined by agar – well diffusion method.

c) Sensitivity to catalase and chloroform

The catalase enzyme was added to the supernatant at a final concentration of 0.5 mg/ml. The mixture was poured onto the culture plate wells one well having no catalase enzyme was used as the control. The culture plates were examined after 18-24 h of incubation. The presence of inhibition zone around well both with and without catalase were determined to be the effect of bacteriocin.

The culture supernatant was mixed with an equal volume of chloroform and kept at room temperature for 4 h. And the activity was tested using well diffusion method.

v. Optimization of culture condition to improve bacteriocin production

A) Optimization of medium composition

The influence of medium components on bacteriocin was evaluated by supplementing the media with glucose (1%), Tryptone (1%), NaCl (1%), Yeast extract (1%). The inoculated cultures were incubated for 24 hrs. and the activity was assayed using agar well diffusion method o serially diluted supernatant.

B) Optimization of growth conditions

The effect of incubation period was studied by inoculating the producer organism into individual MRS medium and incubating at 37 ºC for period of 24, 30, 48 and 60 hours. Supernatants were serially diluted and the activity was determined by agar well diffusion method.

i) Effect of incubation period

The effect of incubation period was studied by inoculating the producer organism in to individual MRS medium,and incubating at 37 o c for a period of 24,30,48,60 hours. Supernatants were serially diluted and the activity was determined by agar well diffusion method

ii) Effect on initial pH

The effect of initial pH on production of bacteriocin was determined by adjusting the initial pH of the MRS media to 4.5, 5.5, 7, 8, 9 using .1 N HC1 and 1 N NaOH. Each medium was inoculated with an overnight culture of bacteriocin producing organism and incubated at 30 ºC for 24 h. The supernatant was serially diluted. The activity was assayed by well diffusion method.

iii) Effect on incubation temperature

The effect of incubation temperature on production of bacteriocin was determined by inoculating the producer organism into sterile MRS broth and incubating at 25, 30, 37, and 45 for 24 hrs. Activity was checked by agar well diffusion method for the serially diluted supernatants.

III. RESULT AND DISCUSSION

Lactobacilli are important organisms which are recognized for their fermentative ability as well as their health and nutritional benefits. Antimicrobial properties of Lactobacilli are of special interest in food industry. The aim of the study was to isolate bacteriocin producing lactobacilli from various sources and optimize the cultural conditions for bacteriocin production.

28 lactobacilli were isolated from 20 different samples. The culture supernatants of all the 28 lactobacilli isolates were tested for activity against the same group of lactobacilli and also against *E.coli. Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus, Methicillin resistant S. aureus, Enterococcus faecalis*.(Table2) Among the isolate 4 strains of *Lactobacilli* were observed to have activity against 3 diferent strains and also against *S.aureus, MRSA, and Pseudomonas aeruginosa*. However none of the 4 bacteriocin inhibitory effect on *E.coli, Enterococcus faecalis Klebsiella pneumoniae.*

Thus only 14.2% of the isolated strain was found to be producing bacteriocin and all the bacteriocin producing strains were isolated from milk samples. No lactobacilli could be isolated from fruit juices. Though 10 isolates were obtained from curd, none of them were bacteriocin producers.

Table 1: Isolated *Lactobacilli* and screening for bacteriocin production

Sample	No. of sample	No. of lactobacilli isolate	No.of bacteriocin producing isolate
CURD	6	10	0
MILK	14	18	4
Fruit juice	1	0	0

Table 2 Producer Strains vs. Indicator strains

Producer Strains

Indicator	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	Standard
C1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+
M6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+		+	-	-	-	-	-	-	-	-	-	-	-	+
M7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+		-	-	-	-	-	-	-	-	-	-	-	+
M8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-		+	-	-	-	-	-	-	-	-	-
E.coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MRSA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Psudomonas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Enterococcus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*C1-C10 – isolates from curd
*M1-M18- isolates from milk

Table3: Activity spectrum of the producer isolates

Producer strain	Sensitive strain	No of sensitive strain
M5	M6, M7, *MRSA*, *S.aureus*	4
M6	M5,M7,*Pseudomonas aeuriginosa*, *S.aureus*, *MRSA*	5
M7	M5, M6, *S.aureus*, *Pseudomonas aeuriginosa*	4
M10	*S.aureus*	1

Isolate number M6, was selected as the test producer strain owing to its highest bacteriocin activity against M5 which was selected as the test indicator strain

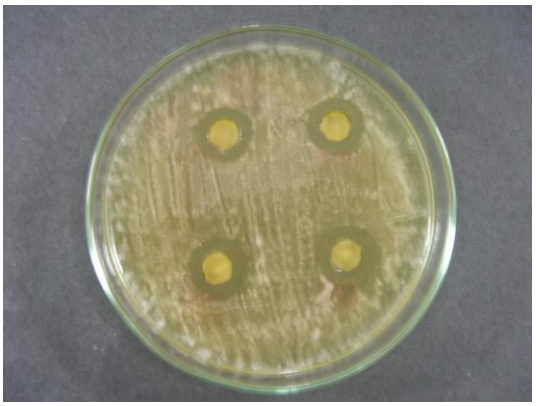

Figure 1: Bacteriocin from producerM6 on indicator M5

Isolate number M6 was found to be having highest bacteriocin production (32 AU/ml) compared to the other strain and was selcted as the test strain for further investigation. Sensitivity of bacteriocin to physical conditions and chemical substances were also evaluated. The test bacteriocin was found to be a lipid containing because of its sensitivity to chloroform. It was also resistant to catalase treatment which indicate that the activity is not due to hydrogen peroxide(table4). It was also found to be sensitive to temperature above 60ºC. (Table 5). It was stable between pH3 and 7 but sensitive to pH 9 (Table 6). This coincident with the result of a previous study (1).

Table 4: Effect of chloroform & Catalase enzyme

Reagent	Sensitivity
Chloroform	Sensitive
Catalase	Resistant

Table 5: Effect of temperature

Temperature	Sensitivity
40ºC	R
50ºC	R
60ºC	S
70ºC	S
80ºC	S
90ºC	S
100ºC	S

R = Resistant, S = Sensitive

Table 6: Effect of pH

pH	Sensitivity
3	R
4.5	R
7	R
9	S

The effect of media composition on production of bacteriocin wa also evaluated. There was no considerable increase in bacteriocin production when 1% Tryptone, glucose or yeast extract were introduced into the media. Where as 1% Nacl was found to have an inhibitory effect on bacteriocin production (Table 7). There are reports that increasing amount of Nacl actually inhibit bacteriocin production and there are contradictory reports as well (33,34). The effect of Nacl may vary depending upon the strain (34). Incubation temperature was also found to have a considerable effect on bacteriocin production with 24ºC showing the optimum production and 40ºCthe least(Table8). Media pH below 5 and above 8 were found to inhibit bactericin production while pH ranges of

5-8 showed highest bacteriocin production (Table 9). It was seen that an incubation period of 24-30 hours give maximum bacteriocin production(Table10). Bacteriocin yield was low in cultures incubated for more than 30 hours similar observations have been made previously (35). This decrease could be due to the effect of extra cellular endogenous proteinase evolved during prolonged incubation (35).

Table 7: Optimization of media composition

Media components	Activity (AU/ml)
Tryptone (1%)	32
Glucose (1%)	32
Nacl (1%)	16
Yeast extract (1-2%)	32

Table 8: Effect of incubation temperature

Temperature	Activity (AU/ml)
24ºC	32
37ºC	16
40ºC	8
44ºC	8

Table 9: Effect of Media pH

pH	Activity ()
4.5	16
5	32
7	32
8	32
9	16

Table 10: Effect of Incubation Time

Incubation Time	Activity (AU/ml)
24 h	32
30 h	32
48 h	16
60 h	16

IV. CONCLUSION

Over all bacteriocin M6 showed good activity against both gram negative and gram positive food spoilage and pathogenic bacteria. This makes it a good antagonistic agent for exploitation in the field of biotechnology.

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