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Bacteriocin Production by a New Isolate of *Lactobacillus rhamnosus* GP1 under Different Culture Conditions

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Abstract: Bacteriocin producing *Lactobacillus rhamnosus* GP1 was isolated from Grape peel and characterized. The bacteriocin had wide spectrum of inhibitory activity against test strains of pathogenic and food spoilage micro organisms. The cell-free supernatant of *L. rhamnosus* inhibited the growth of *Bacillus brevis*, *B. pumilus*, *B. subtilis, Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio harveyi*, *Acinetobacter* sp. and *Arthrobacter* sp. The influence of culture conditions on the production of the bacteriocin by *L. rhamnosus* was evaluated. Maximum bacteriocin activity of 1200 AU/mL was obtained in MRS broth at 72 h at an initial pH of 6.0 in 30ºC. Increased bacteriocin production by *L. rhamnosus* GP1 was noted when MRS broth was supplemented with yeast extract (0.1%), tween 80 (0.1%), $MgSO_4$ (0.02% to 0.04%) and dextrose (2.0 to 3.0%). Addition of bacteriological peptone, beef extract, NaCl and SDS to the culture medium however, reduced the production of bacteriocin. Results of sensitivity studies revealed remarkable stability of the bacteriocin at different storage conditions, temperatures and pH tested.

Key words: Bacteriocin, characterization, culture conditions, *Lactobacillus rhamnosus* GP1, MRS broth

INTRODUCTION

The anti-microbial properties of Lactic Acid Bacteria (LAB) have enabled the extension of the shelf life of many foods through fermentation processes. The inhibition of food spoilage microbes could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins (Atrih *et al*., 1993). Many species of *Lactobacillus*, used in the manufacture of fermented dairy products, inhibit the growth of other bacteria including the intestinal pathogens and spoilage organisms by producing anti-bacterial compounds or bacteriocins. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity, against those bacteria which are closely related to the producer strain (Klaenhammer, 1988a; Tagg *et al*., 1976). The bacteriocins produced by Gram-positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative(Galvez *et al*., 2008) and therapeutic (Jack *et al*., 1995) potentials. Considering this quality, there has been an increased concern in recent years on usage of bacteriocins due to the wide spread over-prescribing of antibiotics and consequent increased development of antibiotic resistance. In order to use bacteriocin as food biopreservative and as a therapeutic agent, large-scale production is required with high level of activity. This paper presents the results of production of bacteriocin by *L. rhamnosus* GP1 by optimizing the culture conditions such as temperature, pH, incubation period and substrate concentration.

Though *Lactobacillus rhamnosus* is found in various habitats, only a few have been listed as producers of bacteriocins (Avonts *et al*., 2004; Todorov and Dicks, 2005a). In this study, we report on the bacteriocin produced by *L. rhamnosus* GP1 isolated from Grape peel. *L. rhamnosus*, being a clinically important LAB widely used as probiotic, its broad spectrum bacteriocin will be of much relevance in replacing the antibiotics. The studies on the same require the production of the protein in large amounts for which the conditions should be optimized for the maximum production of bacteriocin by the bacterium. The effects of various substrates on the production of bacteriocins and also the stability of the crude bacteriocin to different temperatures and pH are discussed.

MATERIALS AND METHODS

Bacterial strains and growth media: *Lactobacillus rhamnosus* GP1 was isolated using de Mann Rogosa Sharpe (MRS) medium from Grape peel. The *L. rhamnosus* GP1 thus isolated was characterized by cell morphology, carbohydrate fermentation and 16S rRNA sequencing (Mandal *et al*., 2008; Rouse *et al*., 2008). *L. rhamnosus* GP1 was grown in MRS medium (HiMedia -M369), stored in refrigerator in MRS agar slants and was

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used for all the experiments. MRS medium was used in all experiments except for optimization studies for which the medium was supplemented with different substrates. The antimicrobial activity was checked using pure cultures of *Acinetobacter*, *Arthrobacter*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *B. subtilis*, *B. brevis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Vibrio harveyi* and *Enterococcus faecalis* obtained from the laboratory stock. The experiments were carried out in biotechnology lab in CMFRI, Vizhinjam during January 2008.

Detection of antibacterial activity and assays: The bacteriocin produced by *L. rhamnosus* during the growth phase was detected by deferred method (Ray *et al*., 2001) and confirmed by well-diffusion assay (BSI, 1968). For the deferred method, MRS plate was spread with the diluted samples (10^{-3}) with 0.85% saline and incubated at 30ºC for 24 h. As soon as the colonies started appearing, 7.5 mL of melted soft agar (0.75% agar) seeded with 50 µL of the overnight grown indicator, *Vibrio harveyi* was poured onto MRS bottom agar. The presence or absence of inhibitory activity against the indicator organism was determined after incubating the agar plate for 24 h at 37ºC. For well-diffusion assay, 50 µL of the serial two-fold dilutions of the cell free culture filtrate in normal saline was transferred into the wells, while the uninoculated MRS medium served as the control. The plates were incubated at 37ºC for 24 h without inversion. At the end of incubation, diameter of zones of inhibition formed around the well was measured in mm. The quantity of bacteriocin production was calculated as arbitrary units. One Arbitrary Unit (AU) was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain (Ko and Ahn, 2000).

Determination of growth and bacteriocin production at different culture conditions: The 18 h-old culture of *L. rhamnosus* GP1 was inoculated (2%; v/v) into MRS broth (pH 6.5) and incubated at 30ºC, without agitation. Samples were taken every 24 h and examined for bacterial growth (OD_{600} nm), changes in culture pH, and antimicrobial activity (AU/mL) against *V. harveyi*. In a separate experiment, the effect of different temperatures and initial pH on the bacteriocin production was tested. MRS broth (100 mL) was inoculated with *L. rhamnosus* and incubated at different temperatures such as 20, 25, 30, 35 and 40ºC to study the effect of different temperatures on the bacteriocin production. The effect of initial medium pH on bacteriocin production was determined by adjusting the MRS broth to different pH levels of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. Each flask was inoculated with 2.0% v/v of an 18 h-old culture of *L. rhamnosus* and incubated at 30ºC for 96 h, without agitation. Changes in culture pH and bacteriocin production (AU/mL) were determined every 24 h interval, as described above.

Effect of medium composition on bacteriocin production: Cells of *L. rhamnosus,* grown in 10 mL of MRS broth for 18 h at 30ºC were harvested by centrifugation (10,000 *rpm* for 10 min) and the pellet was re suspended in 10 mL of sterile peptone water. A volume of 2 mL of this cell suspension was inoculated to 100 mL of MRS broth supplemented with varying concentrations of Yeast Extract (0.1, 1.0, 2.0, 3.0% w/v), Beef Extract (0.1, 1.0, 2.0, 3.0% w/v), Peptone (0.1, 1.0, 2.0, 3.0% w/v), NaCl (1.0, 2.0, 3.0% w/v), Dextrose (1.0, 2.0, 3.0% w/v), $MgSO_4$ (0.02, 0.03, 0.04, 1.0, 2.0, 3.0% w/v), Tween 80 (0.1, 1.0, 2.0, 3.0% v/v) and Yeast Extract (2.0, 2.5, 1.5 and 1.0% w/v) plus Tween 80 (1.0, 0.5, 1.5 and 2.0% v/v). The culture at every 24 h interval was withdrawn to determine growth (OD_{600} nm) and bacteriocin activity.

Stability of bacteriocin to pH and temperature: Samples of crude bacteriocin preparation were exposed to different temperatures from 30 to 100ºC for 5 min and autoclaved at 121ºC for 20 min, cooled and assayed for bacteriocin activity. For storage stability, crude bacteriocin samples were kept at 4, 0 and -20ºC and samples were withdrawn periodically and assayed for antimicrobial activity. The sensitivity to different pH was estimated by adjusting the pH of supernatant sample to pH 3, 4, 5, 6, 7, 8, 9 and 10 with NaOH and HCl and testing against the indicator strain after 30 minutes of incubation at room temperature (29±1ºC).

RESULTS AND DISCUSSION

Strain identification: The *Lactobacillus* isolated from Grape peel using MRS as the selective medium was Gram positive, catalase negative and non-spore forming long rods. In 16S rRNA sequencing and phylogenetic analysis, 99% similarity was noted with *Lactobacillus rhamnosus* St. LV108 and *L. rhamnosus* St. IDCC 3201 through the Neighbour Joining Method (Fig. 1). Hence, the isolated producer organism was characterized and designated as *L. rhamnosus* GP1. The bacteriocin produced by the strain was designated as bacteriocin GP1. The complete sequence (1536 bp) was deposited in GenBank with the accession number HM585368.

Antibacterial spectrum of the bacteriocin produced by *L. rhamnosus* GP1: The inhibitory activity pattern of bacteriocin GP1 against non-lactic acid bacteria is given in Table 1. The human pathogenic bacteria

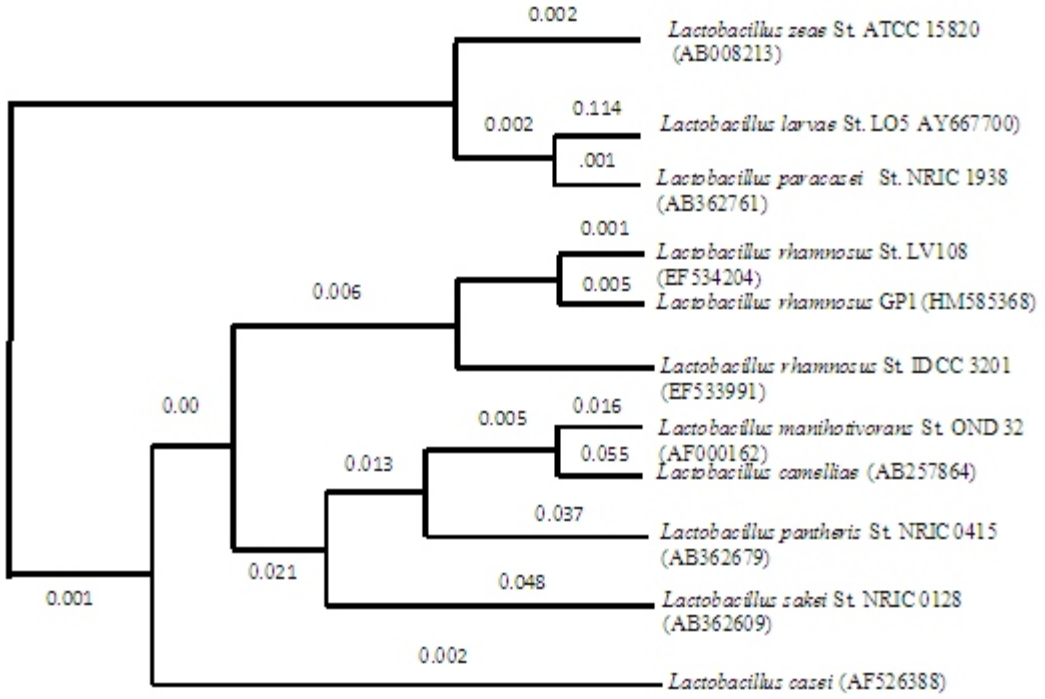

Fig. 1: Phylogenetic tree in MEGA 3.1 software using Neighbour Joining Method

Table 1: Antimicrobial spectrum of the bacteriocin produced by *L. rhamnosus* GP1 isolate

Bacterial strains	Deferred method	Well- diffusion assay* (AU/mL)
Acinetobacter sp.	+	200
Arthrobacter sp.	+	400
Bacillus brevis	+	600
Bacillus pumilus	+	400
Bacillus subtilis MTCC B121	+	600
Enterococcus faecalis	+	400
Escherichia coli	+	400
Klebsiella pneumoniae	-	-
Proteus vulgaris	-	-
Pseudomonas aeruginosa	++	800
Staphylococcus aureus	+	400
Vibrio harveyi	++	1000

++: Indicator strain strongly inhibited by bacteriocin
+: Indicator strain inhibited by bacteriocin
-: Indicator strain not inhibited by bacteriocin
*: Bacteriocin activity AU/mL

Staphylococcus aureus, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis* were strongly inhibited by *L. rhamnosus* and its bacteriocin; but it did not inhibit *Proteus vulgaris* and *Klebsiella pneumoniae* (Table 1). The bacteriocin effectively inhibited the fish pathogen *Vibrio harveyi* with maximum inhibitory activity, compared to other tested bacteria. Hence it was selected as the indicator strain for further experiments. In addition, the test strain exhibited stronger inhibition against the spoilage causing organisms viz., *Acinetobacter* sp., *Arthrobacter* sp., *Bacillus pumilus* and *Bacillus brevis*. The antibacterial activity of bacteriocins against food borne pathogenic, as well as spoilage bacteria has raised considerable interest for their application in food preservation (Galvez *et al*., 2008). Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved. Bacteriocins of Gram-positive bacteria generally exhibit antagonistic activity against Gram-positive bacteria and the activity against Gram-negative bacteria is an unusual phenomenon and has been reported for the bacteriocins produced by *Lactobacillus plantarum* (Chin *et al*., 2001), *Pediococcus* sp. (Mandal *et al*., 2008), and *Lactobacillus bulgaricus* BB18 (Simova *et al*., 2009). The present *L. rhamnosus* GP1 isolate showed inhibitory activity against six Gram positive and four Gram negative bacteria and hence it could be considered as possessing broad inhibitory spectrum (Table 1).

Pattern of bacteriocin production: The growth (OD600 nm) of the strain GP1 increased drastically between 24 to 60 h after inoculation entered the stationary stage at 60 h after inoculation and didn't increase at all after the 96th h of incubation. The bacteriocin production started 24 h after inoculation, showed the maximum quantity at 72 h, remained stable till the 96 h and decreased thereafter as could be noted from Fig. 1. Thus it can be inferred that the

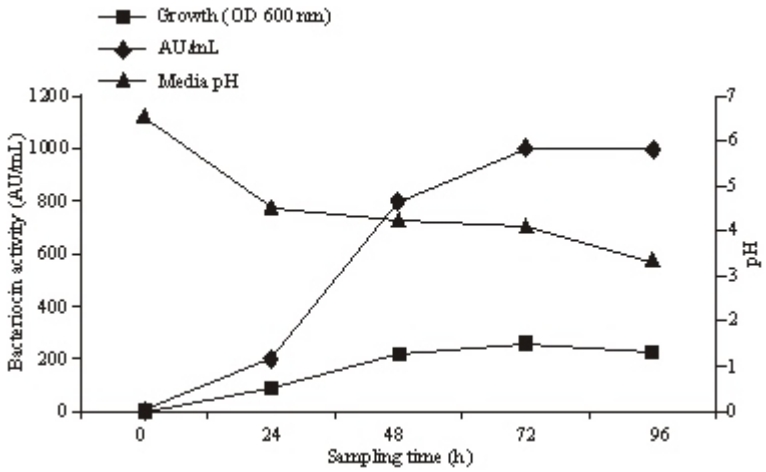

Fig. 1: Bacteriocin level and media pH changes in relation to the growth of *L. rhamnosus* GP1

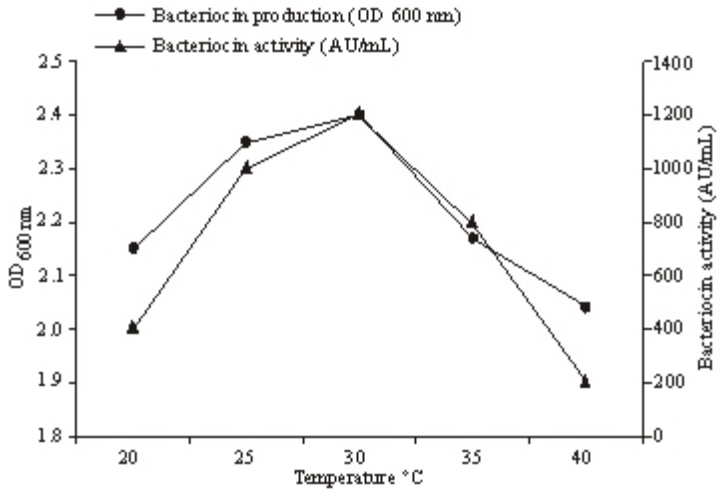

Fig. 2: Effect of temperature on the growth and bacteriocin production by *L. rhamnosus* GP1

L. rhamnosus GP1 isolate showed the growth stability and maximum bacteriocin production at the stationary phase. It has been reported that activity in broth cultures reached maximum only after the exponential growth had ceased (Jose *et al*., 1998). Such observations(Ko and Ahn, 2000; Chin *et al*., 2001) made earlier are in tune with the results of the present study. The growth was accompanied by production of acid as evidenced by the fall of pH from 6.55 to 4.53 after 24 h and 3.36 after 96 h. The bacteriocin activity by *L. rhamnosus* was detected at the early exponential growth phase and reached the maximum (1000AU/mL) at the stationary phase (Fig. 1).

Optimization of incubation period, temperature and initial medium pH: The pattern of antibacterial activity indicated that production of inhibitor started after 24 h and increased rapidly till maximum activity of 1000 AU/ml was reached after 72 h which remained constant up to 96 h. Thus it can be inferred that the *L. rhamnosus* GP1 isolate showed the growth stability and maximum bacteriocin production at the stationary phase. It has been reported that activity in broth cultures reached maximum only after the exponential growth had ceased (Jose *et al*., 1998). Such observations (Ko and Ahn, 2000; Chin *et al.* 2001) made earlier are in tune with the results of the present study. The results on production of bacteriocin right from the 24 h, which remained stable even at the 96 h indicated that the bacteriocin could be a primary metabolite of the *L. rhamnosus* GP1isolate. Similar results were reported for plantaricin Y (Chin *et al*., 2001) and bacteriocins produced by *Pediococcus acidilactici* (Lozano *et al*., 2002). As indicated earlier, the pH of the culture broth decreased from 6.55 to 3.36 after 96 h of fermentation. But, the activity of bacteriocin GP1 did not decrease further during 96 h of incubation at room temperature, suggesting that specific peptidases active against the bacteriocin were not produced as reported earlier (Todorov and Dicks, 2005a).

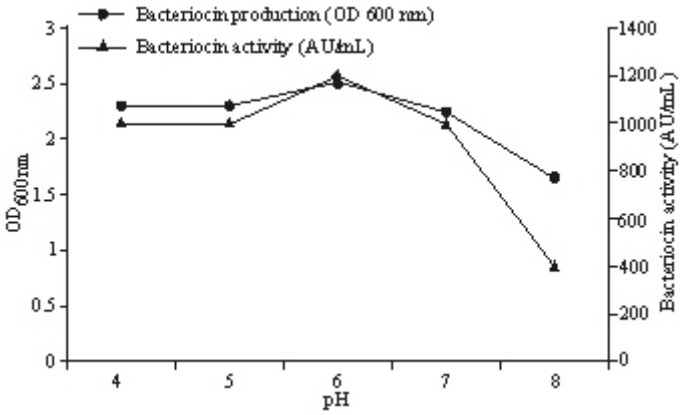

Fig. 3: Effect of pH on the growth and bacteriocin production by *L. rhamnosus* GP1

As for the effect of incubation temperature, the bacterial proliferation and the amount of bacteriocin production were most significant when cultured at 30ºC (Fig. 2). Temperature required for growth and bacteriocin production are often correlated, as observed for plantaricin (Li *et al*., 2008), enterocin 1146 (Parente and Ricciardi, 1994), *lactocin S*. (Abildgaard, 1995) and *nisin Z*. (Matsusaki *et al*., 1996). The bacteriocin activity recorded maximum in MRS media (HiMedia) at 30ºC (1200 AU/mL) compared to the other incubation temperatures (20, 25, 35 and 40ºC) suggesting that ambient growth temperature played an important role on the production of bacteriocin. The amount of the bacteriocin production also was affected by the initial medium pH. The bacteriocin activity was maximum (1200 AU/mL) at pH 6 and the same pattern was detected in the bacterial growth (Fig. 3). Increased production of bacteriocin was noticed even at low pH, i.e., 4 and 5 (1000 AU/mL at both pH) and at a pH of 7. However, further decrease or increase in pH led to low activity. Irrespective of the initial pH the end-pH value of the culture broth decreased to 3.36 and it could be inferred that the optimal production of the bacteriocin GP1 was favored by lowered pH as reported for the bacteriocins of *Lactobacillus plantarum* (Chin *et al*., 2001).

Effect of medium composition on bacteriocin production: The composition of medium influenced the production of bacteriocin by *Lactobacillus* strains. Results indicate that inclusion of yeast extract @ 0.1% w/v in MRS medium increased the bacteriocin production of *L. rhamnosus* GP1 isolate. Tween 80 in the growth medium @ 0.1% v/v in MRS medium also increased the bacteriocin production. Similar results were recorded for nisin (Simsek *et al*., 2009; Zhou *et al*., 2008) and pediocin AcH (Biswas *et al*., 1991). The reason for increased bacteriocin production is not clear and yet to be ascertained. Most of the bacteriocin producing organisms requires stabilizers or a unique medium composition for bacteriocin synthesis. It is probable that the yeast extract may in part serve to inactivate an inhibitor of bacteriocin synthesis (Fukushima *et al*., 1983). Being a surfactant Tween 80, might enable the discharging of the bacteriocin from the cell surface of the producer strain. This finding was supported by the increased bacteriocin production in the medium supplemented with different concentrations of yeast extract plus Tween-80. Previous studies showed that the addition of 2% yeast extract and 1% Tween-80 into MRS medium increased the bacteriocin production

Table 2: Bacteriocin activity of *L. rhamnosus* GP1 isolate in MRS medium with different substitutes in MRS broth

MRS substitutes	Conc. (%)	Final pH	Activity (AumL^{-1})
MRS broth	-	4.10	800
MRS + YE	0.1	4.07	1200
	1	4.09	800
	2	4.05	800
	3	4.05	800
MRS + Dextrose	0.1	4.05	800
	1	4.08	1000
	2	4.06	1200
	3	4.07	1200
MRS + $MgSO_4$	0.02	4.06	1000
	0.03	4.05	1000
	0.04	4.09	1000
	1	4.10	400
	2	4.06	400
	3	4.07	400
MRS + Beef Extract	0.1	4.09	400
	1	4.07	200
	2	4.10	200
	3	4.09	200
MRS + NaCl	1	4.07	400
	2	4.06	400
	3	4.07	400
MRS +	0.1	4.06	400
Bacteriological	1	4.07	400
peptone	2	4.06	200
	3	4.07	200
MRS + Tween 80	0.1	4.03	1200
	1	4.04	1000
	2	4.07	800
MRS + SDS	0.25	5.93	0
	0.5	6.01	0

Table 3: Effect of heat treatment, pH and storage conditions on inhibitory activity of *L. rhamnosus* GP1 cell-free supernatant

Treatment	Activity (AU/mL)
Untreated	1200
Heat (^{0}C)	
30	1200
40	1200
50	1200
60	1200
70	1200
80	1200
90	1200
100	1200
121	1000
pH	
2.5	800
3.5	1000
4.5	1000
5.5	1200
6.5	1200
7.5	1200
8.5	800
9.5	400
10.5	400
Storage temperature	
4	1200
0	1200
-20	1000

(Saharan *et al*., 1998). In the present study, the addition of 0.1% Tween-80 and 0.1% yeast extract increased the bacteriocin production (Table 2). An earlier study by the senior author (Sarika, 2003) revealed that in *L. plantaram* MTCC1746, maximum bacteriocin production could be achieved by providing 1.5% yeast extract and 1.5% Tween-80. The addition of $MgSO_4$ could make a slight impact on the production of bacteriocin. Activity of 1000 AU/mL was observed by the addition of this substrate at a lower concentration of 0.02 to 0.04%. The higher concentrations 1, 2 and 3%, however, bring about reduction in bacteriocin production. Also, the supplementation with NaCl, bacteriological peptone and beef extract have resulted in reduced activity. In contrast to the present observation, growth as well as bacteriocin production in the presence of bacteriological peptone or casamino acids and NaCl was reported to be higher by previous researchers (Todorov and Dicks, 2005b, Pingitore *et al*., 2009). Growth of *L. rhamnosus* MSUIS1 isolate in the presence of 1 g/L of dextrose yielded bacteriocin levels of 800 AU/mL (Table 2). In the presence of 20 and 30 g/L of dextrose, the activity levels increased to 1200 AU/mL, suggesting that higher quantities of dextrose stimulate the bacteriocin production. There was no effect of SDS up to 0.5% to MRS medium. Similar observation was made earlier in *L. acidophilus* (Saharan *et al*., 1998).

Stability of bacteriocin to pH and heat: The bacteriocin exhibited consistent stability over the wide range of pH (2.5 to 8.5) at room temperature with a reduction in activity at a high alkaline pH as reported earlier (Mandal *et al*., 2008; Ko and Ahn, 2000). The highest activity was recorded in the pH range of 5.5 to 7.5. The bacteriocin had a remarkable stability over heat treatment even at the autoclaving temperature for 20 min (Table 3). In respect to temperature sensitivity, the bacteriocin GP1 could be considered as similar to that of pediocin NV5 (Lozano *et al*., 2002). This molecular stability of the bacteriocin produced by *L. rhamnosus* suggests that it belongs to the Class II bacteriocin described by Klaenhammer (1988b). Storage of bacteriocin preparation at 4ºC and in the frozen state for two months did not influence its antibacterial activity.

CONCLUSION

The bacteriocin suspension of *Lactobacillus rhamnosus* GP1 grown in MRS broth had the best inhibitory effect against wide spectrum of bacteria. The present study demonstrated the production of the bacteriocin GP1 by *L. rhamnosus* GP1 isolate under different culture conditions. Its antimicrobial potency, pH stability, activity retention in low and high temperatures suggested its wide applicability in acidic pH conditions and in pre-processed food products.

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