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Optimizing The Bacteriocin Production In Strain Of *Lactobacillus pentosus* Isolated From Cheese

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Abstract

MRS agar was used for the isolation of *Lactobacillus* strain. This strain was recognized through biochemical tests and 16S rRNA ribotyping, as *Lactobacillus pentosus*. Its antibacterial effects were detected by utilizing cell free supernatant (CFS). *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were used as test strains. CFS showed antimicrobial activity against the test strains. CFS was treated with proteinases for the confirmation of loss of antimicrobial activity. Loss of antimicrobial activity on exposure to proteinases indicated the presence of bacteriocin in CFS. CFS was also studied for its antimicrobial effect at different temperatures and pH. Optimum antimicrobial effect was recorded at pH 7 and at temperature 45°C. The current study indicates the antimicrobial activity of strain of *L. pentosus* against *E. coli*, *B. subtilis* and *S. aureus*.

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Introduction

Recently probiotics are defined as those microorganisms which have ability to affect certain functions of living organisms through activation of different metabolic pathways (Bomba *et al.,* 2012). Lactic acid bacteria (LAB) are acknowledged as probiotic for their beneficial applications. *Lactobacillus* strains are renowned as GRAS (generally recognized as safe). Their advantages include the development of healthy microbiota. Their role is also reported as antidiabetic, immunoregulatory, anticancer and protective for cardiovascular system by controlling the cholesterol level (Saarela *et al.,* 2000; Asahara *et al.,* 2011). In urinogenital system and intestine, *Lactobacillus* is the most prevailing type of bacterial genus (Tuohy *et al.,* 2007). These bacteria are more effective to control disease causing microorganisms (Pereira and Gibson, 2002; Hirano *et al.,* 2003; Sabia *et al.,* 2014). These have broad spectrum of applications from food preservation to medicines (Rossi *et al.,* 2008). This value is because of synthesis of different metabolites including hydrogen peroxide, diacetyl, lactic acid, acetic acid, reuterin and bacteriocins (Hirano *et al.,* 2003; Todorov, 2009; Sabia *et al.,* 2014).

Bacteriocins are the defensive tools for various bacteria against competitor bacteria either within same species or species of different genera (Drider *et al.,* 2016). These are intrinsically made ribosomal proteins which have anti-bacterial effects against correlated species (De Vuyst and Leroy, 2007; Drider and Rebuffat, 2011). Particularly bacteriocins synthesized by LAB are generally harmless for human body. They have inhibitory effects against the foodborne disease causing microbes like *Listeria monocytogenes*, *E. coli*, and *S. aureus* (Grosu-Tudor *et al.,* 2014; Zhao *et al.,* 2016). So their use as safe food bio preservatives is increasing (Drider *et al.,* 2016). Further bacteriocin proteins are active at low pH, heat resistant and easily degraded in GIT by proteases enzymes. These properties make them an alternative natural bio therapeutic agent to conventional antibiotics which have inhibitory effects on gut pathogenic strains and maintain natural gut biota balance (Cotter *et al.,* 2013).

The objective of the current work was to isolate and identify bacteriocin producing *Lactobacillus*. The antimicrobial activity of bacteriocin was detected using well diffusion assay. Bacterial test strains were *E.coli*, *B. subtilis* and *S. aureus*. The antimicrobial activity was detected with CFS of *L. pentosus*. Bacteriocin stability on various temperatures and pH was also analyzed in terms of its potential as an antimicrobial agent.

Materials and methods

Isolation and identification of lactobacillus strain

From local market, cheddar cheese was purchased to isolate the *Lactobacillus* strain. For the isolation, the sample was serially diluted in distilled water and its one drop was spread over MRS agar plates with pH equivalent to 6.5 (Ivanova *et al.,* 2000). For bacterial growth, an incubation period of 48 hours was provided at 37°C with anaerobic conditions. Bacterial colonies were isolated through streaking.

Strain of *Lactobacillus* was recognized through morphological and biochemical methods. Molecular characterization was also done. Parameters considered for the colony morphology were colony size, shape, texture, margins, elevation, color and opacity. Biochemical identification was based on the bacterial growth on selective media, growth at different temperature and pH, Gram staining, catalase test, cytochrome oxidase test and sugar fermenting ability tests by using Bergey's Manual of Determinative Bacteriology (Ewing, 1986).

The sugars used for the fermentation tests were glucose, mannose, sorbitol, rhamnose, sucrose, mellibiose, amygdalin and arabinose. Other biochemical attributes were defined in terms of Tryptophan Deaminase (TDA), Hydrogen sulphide (H_2S), Ornithine Decarboxylase (ODC), Lysine Decarboxylase (LDC), Arginine Dihydrolase (ADH), *o*-Nitrophenyl-D-galactoside (ONPG), Gelatinase, Urease, Oxidase, Citrate, Voges Proskauer (VP) and Indole test. Commercial service was availed by First

BASE Laboratories Sdn Bhd (604944-X) Malaysia through Advance biosciences Lahore for molecular identification of strain. Molecular characterization was based on 16 S ribotyping. This molecular method (16S rRNA ribotyping) was based on PCR and 3730xl genetic analyzer. The obtained sequences were analyzed using BLAST at NCBI. The phylogenetic tree for the novel strain was developed using the same tool.

Growth rate of lactobacillus strain

Optical density (O.D) of MRS broth inoculated with *L. pentosus* was measured after 24, 48, 72, 96 and 120 hours at 600 nm wavelength with the help of UV 1100, UV/VIS spectrophotometer by ROBUS TECHNOLOGIES to study the growth rate of the strain. Growth rate was studied to check the growth trend of *L. pentosus* by varying the incubation period.

Isolation and identification of test strains

Three test strains, belonging to *B. subtilis*, *S. aureus* and *E. coli,* were utilized. Soil sample was utilized to isolate the strain of *B. subtilis*. Strain of *E. coli* was isolated from the sewage water. *S. aureus* was isolated from meat sample. All the test strains were cultured on nutrient agar. Gram-staining, catalase, oxidase, sugar fermentation (glucose, arabinose, sorbitol, inositol and sucrose), H2S, methyl red, citrate, indole and urease tests were performed for the identification.

Isolation and detection of bacteriocin

Isolated strain of *Lactobacillus* was inoculated to MRS broth for growth. Broth was incubated at 37°C for 48 hours. Then it was centrifuged at 6000 rpm till 15 minutes. The pH of supernatant was maintained at 7 by mean of 1M NaOH. It resulted in the control of inhibitory action of organic acids (Bogovic-Matijasic *et al.,* 1998). Similarly catalase enzyme was added to CFS to remove the possible inhibitory activity of H_2O_2. Treated CFS was used as crude bacteriocin.

Two tests (Ninhydrin test and Biuret test) were executed for detection of protein contents in the CFS (Meyer, 1957; Boyer, 2000). Furthermore, proteinase test was conducted to find bacteriocin activity in CFS (Ivanova *et al.,* 1998).

Optimization of bacteriocin production

pH of broth was modified to assess impact of pH on bacteriocin synthesis from *Lactobacillus*. pH was modified to 3, 5, 7, 9, and 11. *Lactobacillus* inoculum was given in the 5ml of treated broth.

It was incubated on 37°C for 48 hrs. Centrifugation of test broths was done on 6000 rpm up to 15 minutes. CFS was treated with1M NaOH to neutralize it and was filtered by using Millex, 0.22µm Millipore filter. Finally it was analyzed for antimicrobial activity against test strains including *B. subtilis*, *E. coli* and *S. aureus*.

Effect of temperature was examined by incubating the *Lactobacillus* in the MRS broth at various temperatures 30°C, 35°C, 40°C, 45°C and 50°C for 48 hours with pH 7. After centrifugation CFS was analyzed for antagonistic action against test strains through agar well diffusion method. The zones for restricted growth were observed.

Characterization of bacteriocin

Bacteriocin stability was observed regarding pH stability and heat stability. Aliquots of CFS were heated up at 40°C, 50°C, 60°C, 70°C and 100°C for 10 minutes in water bath. Bacteriocin stability was checked through agar well diffusion assay. Similarly pH of cell free supernatant was set at 3, 5, 7, 9 and 11. Bacteriocin stability was checked through agar well diffusion assay at different pH.

Antimicrobial assay

To find the bacteriocin production, antimicrobial effects of bacteriocin were observed through agar well diffusion assay (Iqbal, 1998). Wells were made on nutrient agar plates already seeded with test strains.

The aliquots of 100 µl CFS (cell free supernatant) were poured in them. Zones of restricted growth of test strains nearby the wells were recorded (Bhaskar *et al.,* 2007).

Statistical analysis

One way ANOVA was applied for comparing the means of zone of inhibition formed at different temperature and pH. For multiple comparisons post hoc test (duncan's test) was applied.

Results

Isolation and Identification of the Lactobacillus strain

Creamy, smooth, elevated and round colonies with entire margins were observed. Gram staining showed positive result. The positive results of sugar fermentation tests for glucose, manitol, sorbitol, arabinose, mellibiose and amygdalin and the negative results for fermentation of rhamanose, sucrose and inositol indicated the presence of *Lactobacillus* strain. Voges Proskauer (VP) test showed positive results while Tryptophan Deaminase (TDA) Test, Hydrogen sulphide (H2S) Test, Ornithine Decarboxylase (ODC) Test, Lysine Decarboxylase (LDC) test, Arginine Dihydrolase (ADH) Test, *o*-Nitrophenyl-D-galactoside test (ONPG), Gelatinase test, Urease production test, Catalase production test, Oxidase Test, Citrate test, and Indole production test showed the negative results. Fig. 1. shows the results of 16 S ribotyping. Molecular characterization based on 16 S ribotyping confirmed that the isolated strain is *Lactobacillus pentosus* (similarity index=99%, score 2756 bits (1492), Expect= (0).

Table 1. Colony morphology for test strains.

Colony Parameters	Strain I	Strain II	Strain III
Size	2-3um/0.7-0.8um	0.5 µm – 1.5 µm	0.25–1.0 µm
Shape	Circular	Circular	Circular
Margin	Undulate(Wavy)	Entire	Entire
Color	Milky white	yellow	Colorless
Elevation	Flat	convex	Raised
Texture	Dry or rough	Smooth & Shiny	Smooth & Shiny
Opacity	Opaque	Opaque	Translucent

Growth rate of lactobacillus pentosus

Growth curve for *L. pentosus* with respect to incubation period is shown in Fig. 2.

Isolation and identification of test strains

Results for parameters of colony morphology including colony size, shape, texture, margins, elevation, color and opacity for all the three test strains are given in the table 1. Results of gram staining show that strain I and strain II were gram +ive and strain III was gram -ive. Results for sugar fermentation tests for test strains are given in Table 2. The other tests performed for the characterization of the strains are shown in Table 3. These results indicate that strain I was belonging to *Bacillus subtilis*. Strain II was *Staphylococcus aureus*. Strain III was the strain of *Escherichia coli*.

Isolation and detection of bacteriocin

Positive result was observed for both the Ninhydrin test and Biuret test. These result confirmed the presence of protein contents in CFS. Furthermore when CFS was treated with proteinase enzyme no zone of inhibition appeared during the well diffusion assay. Bacteriocin loses its antimicrobial activity due to proteinase activity.

Table 2. Sugar fermentation test results for test strains.

No.	Sugar fermentation	Strain I	Strain II	Strain III
1	Glucose	+	+	+
2	Manitol	+	+	+
3	Sorbitol	+	-	+
4	Sucrose	+	+	-
5	Arabinose	-	-	+

Optimization of bacteriocin production

At different pH after 48 hours of incubation period of inoculated MRS broth, different O.D values were observed which shows variable growth rate of *L. pentosus* (Fig. 3). Further zones of inhibition against all the three test strains show bacteriocin production at different pH (Table 4). At different temperature after 48 hours of incubation period of inoculated MRS broth different O.D values were observed which shows variable growth rate of *L. pentosus* (Fig. 4).

Further zones of inhibition against all the three test strains show bacteriocin production at different temperatures (Table 5).

Table 3. Confirmatory biochemical tests for test strains.

No.	Chemical test	Strain I	Strain II	Strain III
1	Catalase test	+	+	+
2	Oxidase test	-	-	-
3	H2S test	-	-	-
4	Methyl red test	-	+	+
5	Citrate test	+	+	-
6	Indole test	-	-	+
7	Urease test	-	+	-

Antimicrobial assay

Cell free supernatant isolated from treated MRS broth formed the clear zones of inhibition against test strains except the broth established at pH 11(Table 4). Bacteriocin produced at pH 9 formed the biggest restricted growth zone against *B. subtilis* (Table 4). While bacteriocin produced at pH 7 formed the largest restricted growth zone against *S. aureus* and *E. coli* (Table 4).

Table 4. Effect of variation of pH on antimicrobial activity of bacteriocin against test strains.

No.	pH	Zone of inhibition		
		Bacillus subtilis Mean± S.E.M	*Staphylococcus aureus* Mean± S.E.M	*Escherichia coli* Mean± S.E.M
1.	3	4.21 ± 0.117^{a}	3.4 ± 0.218^{c}	3.33 ± 0.188^{c}
2.	5	2.15 ± 0.087^{c}	1.53 ± 0.273^{d}	2.18 ± 0.093^{d}
3.	7	3.03 ± 0.117^{b}	5.25 ± 0.126^{a}	6.33 ± 0.188^{a}
4.	9	4.25 ± 0.161^{a}	4.38 ± 0.196^{b}	4.38 ± 0.205^{b}
5.	11	0.00^{d}	0.00^{e}	0.00^{e}

p≤0.0001. Means with same alphabets are not statistically significantly different.

At various temperatures cell free supernatant of inoculated broth has showed antagonistic effects against all the three test strains including *B. subtilis*, *S. aureus* and *E. coli*. Clear zones of inhibition were formed around wells. Bacteriocin produced at 40°C formed the largest zone of inhibition against *B. subtilis* (Table 5). Largest zone of inhibition against *S. aureus* formed by bacteriocin produced at 45°C (Table 5). Similarly bacteriocin produced at 35°C formed the largest zone of inhibition against *E. coli* (Table 5). No bacteriocin production occurred at 50°C as no zone of inhibition was formed (Table 5). So it is concluded that the values of pH and temperature affect the synthesis of bacteriocin. Furthermore they affect its efficacy against different test strains.

Table 5. Antimicrobial activity of bacteriocin against test stains at different temperatures.

No.	Zone of inhibition			
	Temperature	*Bacillus subtilis* Mean± S.E.M	*Staphylococcus aureus* Mean± S.E.M	*Escharichia coli* Mean± S.E.M
1.	30	5.45 ± 0.029^{b}	5.35 ± 0.058^{b}	4.75 ± 0.058^{c}
2.	35	5.43 ± 0.145^{b}	4.43 ± 0.073^{c}	5.6 ± 0.029^{a}
3.	40	7.6 ± 0.087^{a}	5.31 ± 0.164^{b}	5.58 ± 0.093^{a}
4.	45	5.71 ± 0.164^{b}	5.81 ± 0.060^{a}	5.36 ± 0.073^{b}
5.	50	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}

p≤0.0001. Means with same alphabets are not statistically significantly different.

Characterization of bacteriocin

Bacteriocin was found stable when heated at 40°C, 50°C, 60°C, 70°C and 100°C for 10 minutes. Clear zones with restricted growth were made when well diffusion assay was performed. Likewise bacteriocin was found active at 3, 5 and 7 pH as clear zone appeared. At pH 9 and 11, the bacteriocin loses its activity as no zone was appeared.

Fig. 1. Results for 16 S ribotyping,

Discussion

Present research was conducted to isolate and to identify the strain of bacteriocin producing *Lactobacillus*. Optimum conditions for the bacterial growth and bacteriocin synthesis were also analyzed. Antagonistic activity of *Lactobacillus* strain was checked against some pathogenic strains to check the efficacy of its bacteriocin. Molecular characterization based on 16S ribotyping identified the strain as *L. pentosus* due to its 99% homology. Lash *et al.* (2005) conducted a similar research and observed antagonistic activity of *Lactobacillus plantarum* CFS at different pH and temperature. They concluded that CFS depicts its activity at 4 to 5 pH range. Present study is in agreement with different studies where it was proved that bacteriocin synthesis relies on temperature, pH and incubation period (Todorov and Dicks, 2005; Biscola *et al.,* 2013). *L. pentosus* produced maximum bacteriocin at 45°C and pH 7. Similarly, for *Lactococcus lactis subsp. lactis* optimum conditions for bacteriocin production have been described which were 18 hours of incubation period, pH 5 and temperature 37°C (Aslam *et al.,* 2012). In the same way Sharma *et al.,* (2010) described that *Lactococcus lactis CCSULAC1* synthesized maximum bacteriocin at 7.5 pH and 35°C. Other researchers described optimal temperature 30°C and 37°C separately for *Lactococcus lactis* (Ivanova *et al.,* 2000; Ahmed *et al.,* 2006). Aslam *et al.* (2012) also described optimum bacteriocin synthesis at pH 5 and after pH 7 its production decreased. In contrast to Aslam *et al.* (2012) in present study, bacteriocin production from *L. pentosus* was maximum at pH 7 and range was 6.5 to 9 pH. At pH 11 no growth and bacteriocin synthesis was observed. Similarly Sharma *et al.* (2010) and Ivanova *et al.* (2000) testified optimum bacteriocin synthesis at pH 7.5 and 5.5 respectively.

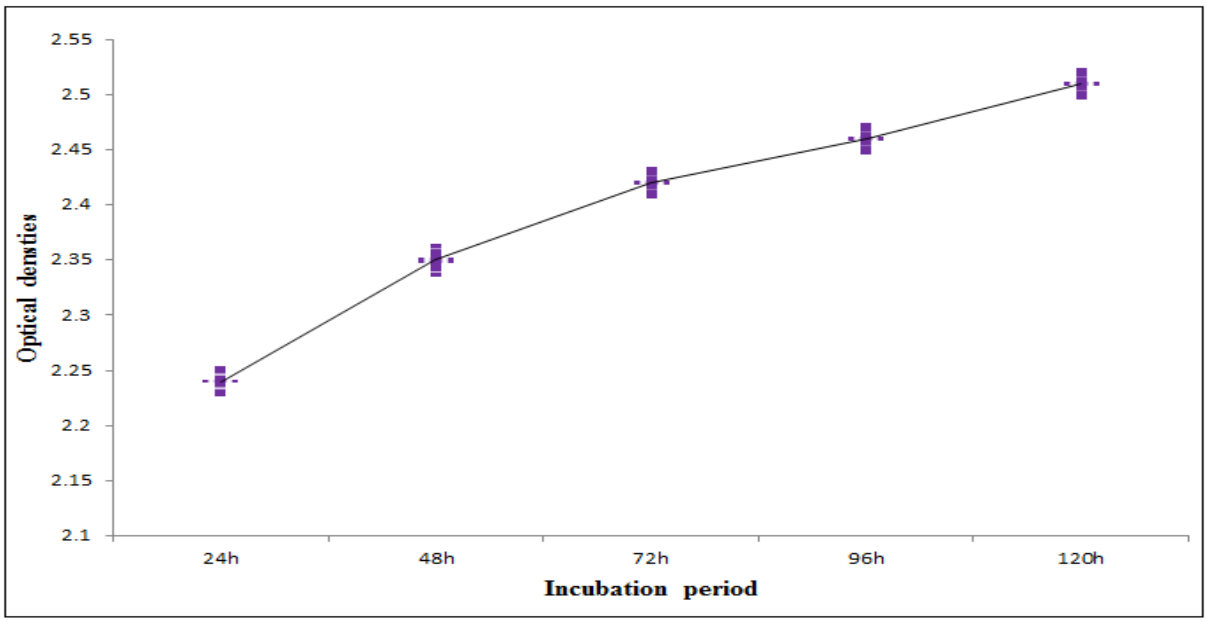

Fig. 2. Growth curve for *Lactobacillus pentosus*.

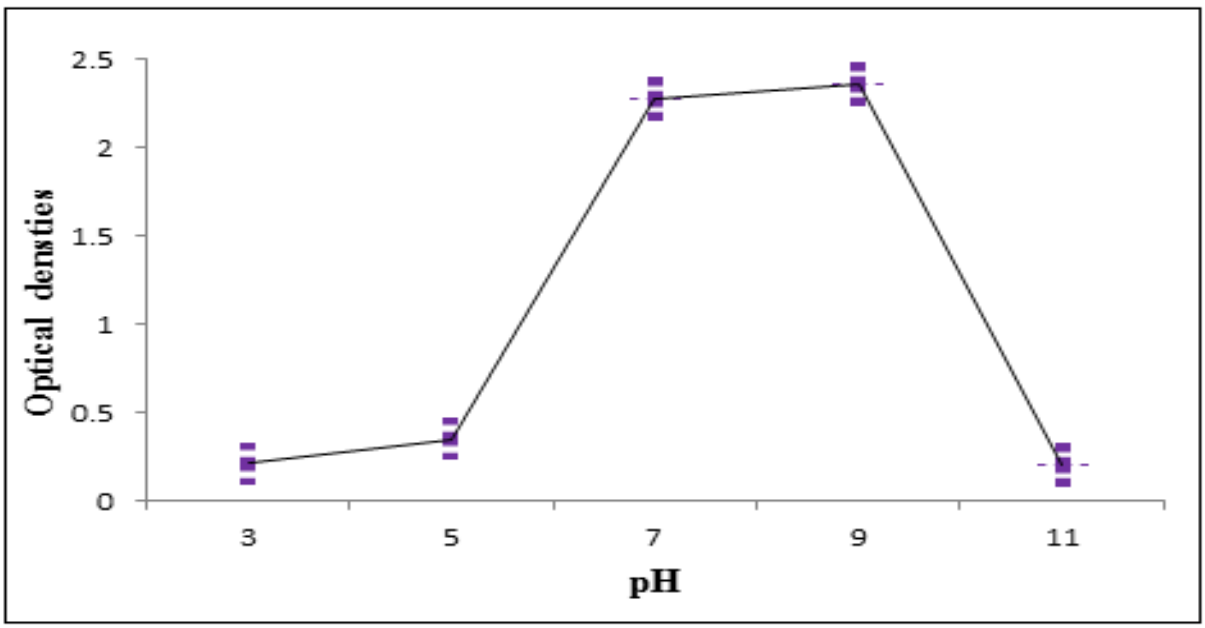

Fig. 3. Impact of pH on *Lactobacillus pentosus* growth.

Three strains of *Lactococcus lactis* showed optimum pH 4.88, 4.89 and 4.82 for bacteriocin synthesis (Ahmed *et al.,* 2006). In other researches, it was reported that *Lactobacillus acidophilus* shows antagonistic effects counter to *B. subtilus; S. aureus, E.coli, Klebsiella spp.* and *S. typimurium* while *L. rhamnosus* shows antagonistic effects counter to *S. aureus, P. aeruginosa, E. coli, B. subtilis,* and *E. faecalis* (Karthikeyan and Santosh, 2009; Sarika *et al.,* 2010). Our study also showed that *L. pentosus* has inhibitory effects counter to *E. coli*, *B. subtilis* and *S. aureus*. Further this research outcome was in compliance with Chavan *et al.* (2016). They noticed no change in inhibitory effects of cell free supernatant after heating at 60°C, 70°C, 80°C, 90°C and 100°C. Same case was with present work where heating CFS at 40°C, 50°C, 60°C, 70°C and 100°C did not affect antimicrobial activity which showed that proteinaceous compound in CFS is stable at these temperatures. In present study *L. pentosus* was isolated from fermented milk product i.e cheese. Precedents exist about isolation of *Lactobacillus*

rhamnosus GP1, L. plantarum, Lactococcus lactis from fermented foods like grape peel and fermented vegetables respectively (Joshi *et al.,* 2006; Sarika *et al.,* 2010).

Antibacterial effect of CFS was lost from pH 6 to 11 which was due to destabilization of protein structure or its proteolytic degradation. This result is in accordance to the finding of Aasen *et al.* (2000). Results about bacteriocin stability at different pH are contrary to *Joshi et al.,* (2006) as *Lactococcus lactis* D53 and D23 bacteriocin afficacy was lost at alkaline pH 8 whereas in present study bacteriocin activity lost at acidic pH 6.

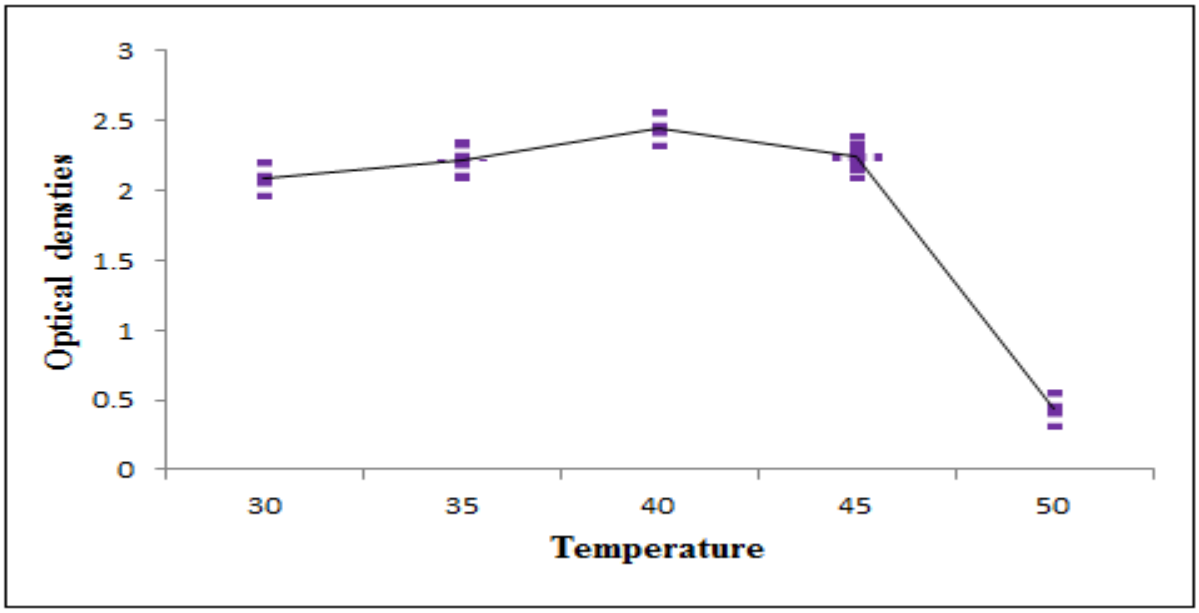

Fig. 4. Impact of temperature on *Lactobacillus pentosus* growth.

Fig. 5. Antimicrobial activity of bacteriocin against test strains.

In a study more bacteriocin activity was reported between pH 3 to pH 5 produced by *Lactococcus lactis* OC1 similar effects have been observed for *L. pentosus* in present study which shows maximum activity at acidic pH from 2 to 5 (Akcelic *et al.,* 2006). In different researches LAB revealed their antimicrobial effects counter to gram positive and negative microbes (Pascaul *et al.,* 2008; Barman *et al.,* 2017). Present study is in agreement with these studies as *L. pentosus* depicts its antimicrobial effects

counter to gram positive (*S. aureus* and *B. subtilis*) and negative (*E. coli*) microbes. Furthermore in previous studies maximum bacteriocin production for *Lactobacilli pentosus* 31-1 and *Lactococcus lactis ssp. lactis* CN1. 10a was observed in exponential growth phase. Bacteriocin synthesis reduced when bacteria entered the stationary phase (Liu *et al.,* 2008; Indriati *et al.,* 2014). Present study also showed similar results for bacteriocin production.

Conclusion

L. pentosus was isolated from cheese. It produces bacteriocin which has bactericidal effects against *B. subtilis, S. aureus* and *E. coli*. This study also determined the growth spectrum of pH and temperature for *L. pentosus*. Further heat and pH stability of bacteriocin suggested its potential use as bio preservative in food industry. It can be used against foodborne pathogens and to cure the diseases caused by pathogenic microbes. For test strains, methods of identification were based only on biochemical tests. The study can be expanded by using tools of identification on the basis of ribotyping. The basic tests were performed for the identification and confirmation of protein. Advance methods were not used. For further analysis, advance methods can be performed for the analysis of proteins under observation. This research will invite the attention of pharmaceutics for Large-scale synthesis of new, more stable and biologically active antimicrobial molecules with an extensive range of inhibitory action. Furthermore it will provide a new line of work for them to make analogues for these substances as antibiotics.

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