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Lactobacillus plantarum subsp *plantarum*: Influence of growth parameter on bacteriocin production and characterization

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Abstract: This study examine the influence of growth parameters on bacteriocin production and also characterized the crude bacteriocin obtained using the agar well diffusion method. Results were interpreted as the zone of inhibition measured in millimeter. Analysis variance observed a significance at $p \leq 0.05$. At initial pH levels below 7 bacteriocin production was observed to be growth associated while no influence of NaCl concentration and temperature on bacteriocin was observed. The crude bacteriocin produced was characterized as thermostable, aciduric and efficient at - 20^{o}C temperature of storage. The crude bacteriocin showed inhibitory activity against *Bacillus cereus* CGMCC 1.260, *Enterococcus faecalis* CGMCC 1.2629, *Lactobacillus plantarum* CGMCC 1.2707 and *Listeria monocytogenes* CGMCC 1.10753 used as indicator strains indicating its potentials as a biopreservative.
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Keywords: *Lactobacillus plantarum;* subsp *plantarum;* growth parameter' bacteriocin' production; characterization

1. Introduction

Biopreservation, the control of one organism by another has received much attention in the last decade (Magnusson *et al*, 2003). And among these natural biological antagonist, lactic acid bacteria (LAB) have several potential applications and are widely used for the production of fermented foods and also are part of the intestinal microflora (Dalie *et al*, 2010). Due to their nutritional requirements, LAB are generally cultured in enriched media and are found in dairy products, meat, meat-derived products and cereal products (Carr *et al*,2002). Among the LAB is the genera Lactobacillus. The genera lactobacillus consist of a genetically and physiologically diverse group of rod-shaped gram positive, non-spore forming, non-pigmented (Hasan and Frank 2001), catalase negative and microaerophilic to strictly anaerobic organisms (Vernoux *et al*, 2003). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bactericidal products during lactic fermentation (Lindgren and Dobrogosz 1990). Magnusson *et al* (2003) has attributed their antimicrobial efficiency to three mechanisms: the yield of organic acid, competition for nutrients and production of antagonistic compounds. This antagonistic compounds are termed bacteriocins. Bacteriocins of lactobacillus origin from different environments have been described (Klaenhammer 1988, Olasupo 1996). These bacteriocinogenic strains of lactobacillus plantarum are naturally present in good products and contribute not only to the organoleptic characteristics of the products, but play an essential role in natural biopreservation of these products (Todorov 2009).

The objective of this study was to evaluate the influence of growth parameters on bacteriocin production, characterize and application of the crude bacteriocin of *Lactobacillus plantarum* subsp *plantarum* isolated from Ogi a cereal fermented food product.

2. Materials and Methods

2.1. Strain cultivation

Lactobacillus plantarum subsp *plantarum* previously isolated from the fermentation of Ogi was grown in Mann Rogosa Sharpe broth (Oxoid) at 37^{o}C for 24h and stocked at -20^{o}C in 50% glycerol supplemented with MRS broth. Bacillus cereus CGMCC 1.260, Enterococcus faecalis CGMCC 1.2629, Lactobacillus plantarum CGMCC 1.2707 and Listeria monocytogenes CGMCC 1.10753 were used as indicator organisms. The strains were obtained from the China General Microbiological Culture Collection Centre and grown in beef extract agar at 30^{o}C, yeast extract agar and Mann Rogosa Sharpe agar at 37^{o}C for 24 h before use respectively.

2.2. Optimization of growth parameters for bacteriocin production

2.2.1. pH

The influence of pH on the growth and bacteriocin activity was studied using the method of Chanprasert and Gasaluck (2011) by preparing MRS broth adjusted to initial pH 2, 4, 6,8,10 using sterile 1M NaOH or HCL. An 18h L plantarum (1%) was inoculated into each initial pH of MRS broth; sampling was at 12h interval for 48h. Growth was monitored by checking the OD at 600_{nm} and bacteriocin activity was assayed using the well diffusion method of Xiraphi *et al* (2005) Cell-free supernant (CFS) was obtained by centrifuging an 18h L plantarum MRS broth culture at 10,000×g for 15min at 4ºC. pH was adjusted to 6.5 using sterile 1M NaOH or 1M HCL and treated with catalase at a final concentration of 2mg/ml for 30min at room temperature. The CFS was filtered through a 0.22µm Millipore express PEG membrane filter (Darmstadt, Germany). Nutrient agar plates seeded with 100µl overnight broth culture of indicator organisms. Two millimetre diameter hole was bore inside the nutrient agar plates and 50µl of CFS was applied in the holes allowed to diffuse and incubated at 37ºC for 24h. The bacteriocin activity was measured as the diameter of zone of inhibition (mm) surrounding the wells.

2.2.2. NaCl

The influence of salt on the growth and bacteriocin activity was studied using the method of Chanprasert and Gasaluck (2011) by preparing MRS broth containing different concentrations of NaCl 1.5%,3.5%,5.5%,7.5%,9.5%. An 18h L plantarum (1%) was inoculated into each initial MRS broth; sampling was at 12h interval for 48h. Growth was monitored by checking the OD at 600_{nm} and bacteriocin activity was assayed using the well diffusion method Xiraphi *et al* (2005).

2.2.3. Temperature

The influence of temperature on the growth and bacteriocin activity was studied using the method of Chanprasert and Gasaluck (2011) by inoculating 1% 18h culture of L plantarum into MRS broth and incubating at 15ºC, 37ºC and 45ºC; sampling was at 12h interval for 48h. Growth was monitored by checking the OD at 600_{nm} and bacteriocin activity was assayed using the well diffusion method Xiraphi *et al* (2005).

2.3 Characterization of crude bacteriocin

2.3.1. Heat stability: 2ml aliquots of crude bacteriocin was taken in sterile eppendorfs and overlaid with drops of paraffin oil to prevent evaporation. Samples were exposed to heat treatment at 60ºC, 70ºC, 80ºC, 90ºC, 100ºC and at 121 ºC for 20mins at 15psi (Kaur and Garg, 2013). The heat treated crude bacteriocin samples were assayed for bacteriocin activity using the well diffusion method of Xiraphi *et al.* (2005).

2.3.2. Effect of pH: Bacteriocin activity at different adjusted pH 2 to 10 with 1M NaOH or HCl were assayed using the well diffusion method.

2.3.3. Effect of enzymes: The sensitivity of crude bacteriocin to various enzymes was tested. Samples were treated with Catalase, Lysozyme, Pepsin and Trypsin. Enzyme stocks were prepared in 50mM PBS (pH 7.0) and enzymes were added at a final concentration of 3mg/ml. The mixture was incubated at 37ºC for 3h. After incubation the sample was subjected to heat treatment in boiling water bath for 5mins in order to inactive enzymes. The heat treated crude bacteriocin samples were assayed for bacteriocin activity using the well diffusion method of Xiraphi *et al* (2005).

2.3.4. Effect of organic solvents: Crude bacteriocin was treated with 50% v/v of different organic solvents including ethanol, acetone, chloroform, butanol, methanol and propanol-2. The mixture was incubated at 25ºC for 1h, evaporated and assayed for bacteriocin activity using the well diffusion method.

2.3.5. Effect of surfactants: Crude bacteriocin was treated with 1% w/v of different surfactants including Triton-100, Tween -20, Tween-80, Urea, EDTA and sodium dodecyl sulphate (SDS). Samples were incubated at 37ºC for 5h (Kaur and Garg, 2013) and assayed for bacteriocin activity using the well diffusion method.

2.3.6. Stability of bacteriocin: Crude bacteriocin was stored at -20ºC, 4ºC and 37ºC for 2months. Bacteriocin activity was assayed every 15 days interval using the well diffusion method.

3. Results

3.1. Optimization of Growth Parameters for Bacteriocin Production

3.1.1. Effect of pH

Effect of pH on the growth of *Lactobacillus plantarum* subsp *plantarum* is shown in Fig.1. At the different pH ranges an effect was observed on the growth rate *Lactobacillus plantarum* subsp *plantarum*. At pH ranges between 2 and 6 *Lactobacillus plantarum* subsp *plantarum* grew optimally. While at pH range 8 and 10 a retarded growth was observed.

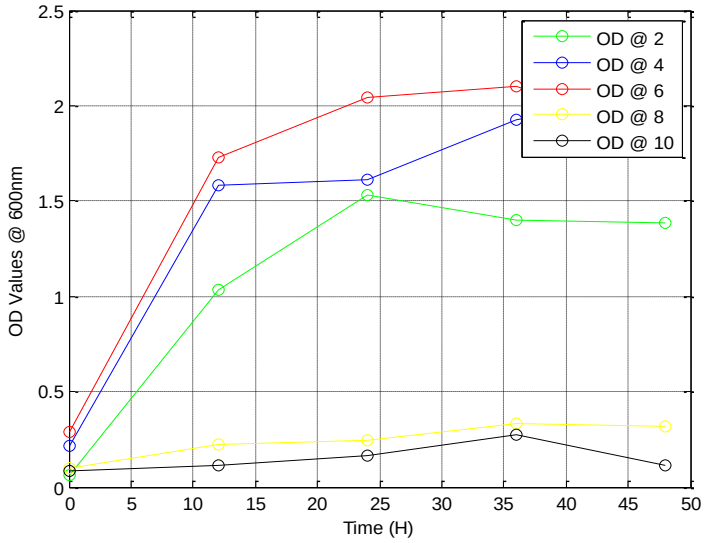

Fig.1 Influence of Initial pH on Growth of *Lactobacillus plantarum* subsp *plantarum*.

The effect of pH on the inhibitory activity of *Lactobacillus plantarum* subsp *plantarum* is shown in Fig.2. pH of incubation of *Lactobacillus plantarum* subsp *plantarum* was observed to have effect on the inhibitory activity of the indicator strains. After 12 and 48h of incubation respectively no inhibitory activity was detected against indicator strains for pH 8 and 10. At pH ranges 2-6 *Lactobacillus plantarum* subsp *plantarum* retained its inhibitory activity.

3.1.2. Effect of NaCl

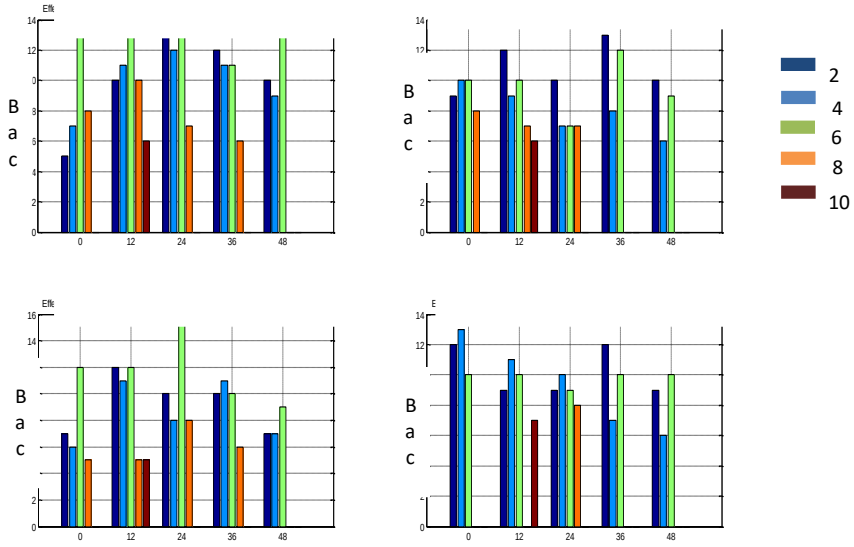

Fig.2 Influence of Initial pH on Bacteriocin Activity of *Lactobacillus plantarum* subsp *plantarum*.

Effect of NaCl concentration on growth of *Lactobacillus plantarum* subsp *plantarum* is shown in Fig.3. The different NaCl adjustment was observed to affect the growth rate and pattern of *Lactobacillus plantarum* subsp *plantarum* monitored over a period of 48h. Optimal growth was observed at NaCl concentration of 1.5%. There was no effect of NaCl concentration on bacteriocin activity of *Lactobacillus plantarum* subsp *plantarum* was observed Fig.4.

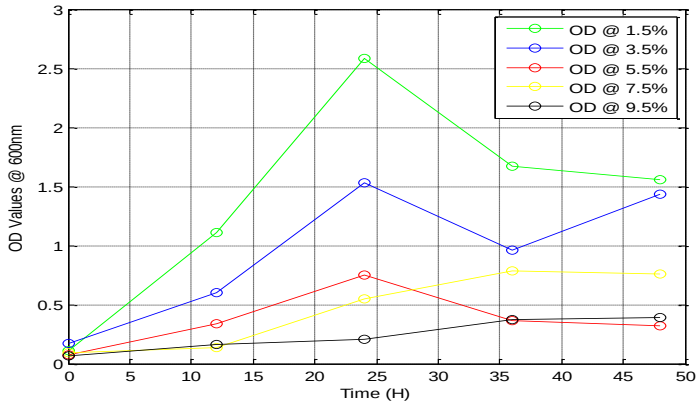

Fig.3: Influence of NaCl on Growth of *Lactobacillus plantarum* subsp. *plantarum*.

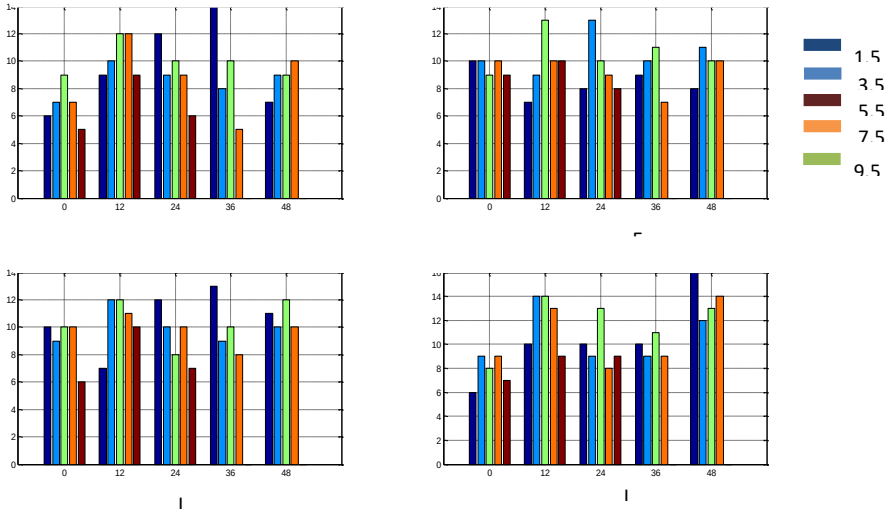

Fig.4: Influence of NaCl on Bacteriocin Activity of *Lactobacillus plantarum* subsp. *Plantarum*

3.1.3. Effect of Temperature

Temperature effects on growth of *Lactobacillus plantarum* subsp *plantarum* is shown in Fig.5. Monitoring over a period of 48h observed that the different temperature adjustments had effect on the growth of *Lactobacillus plantarum* subsp *plantarum*.

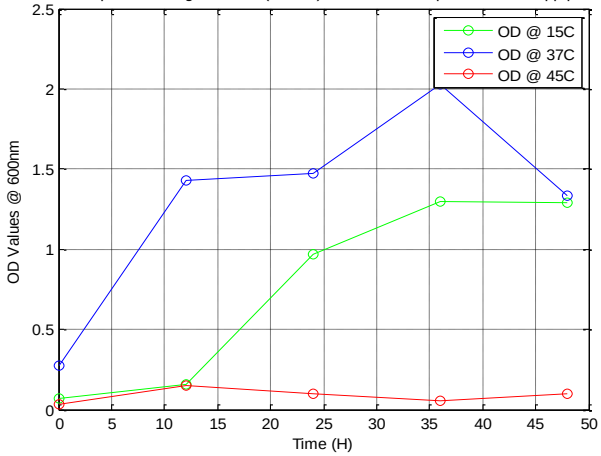

Fig.5: Influence of Incubation Temperature on Growth of *Lactobacillus plantarum* subsp *plantarum*.

At 45ºC the growth of *Lactobacillus plantarum* subsp *plantarum* was retarded while at 37ºC *Lactobacillus plantarum* subsp *plantarum* grew optimally. Effect of temperature on bacteriocin activity of *Lactobacillus plantarum* subsp *plantarum* monitored over a period of 48h Fig.6. Temperature did not have much effect on bacteriocin activity as *Lactobacillus plantarum* subsp *plantarum* was able to inhibit the indicator strains. Variation in optimal activity was observed for the different indicator strains. An increased inhibitory activity was noted at temperature 37ºC after 36h incubation for all indicator strains.

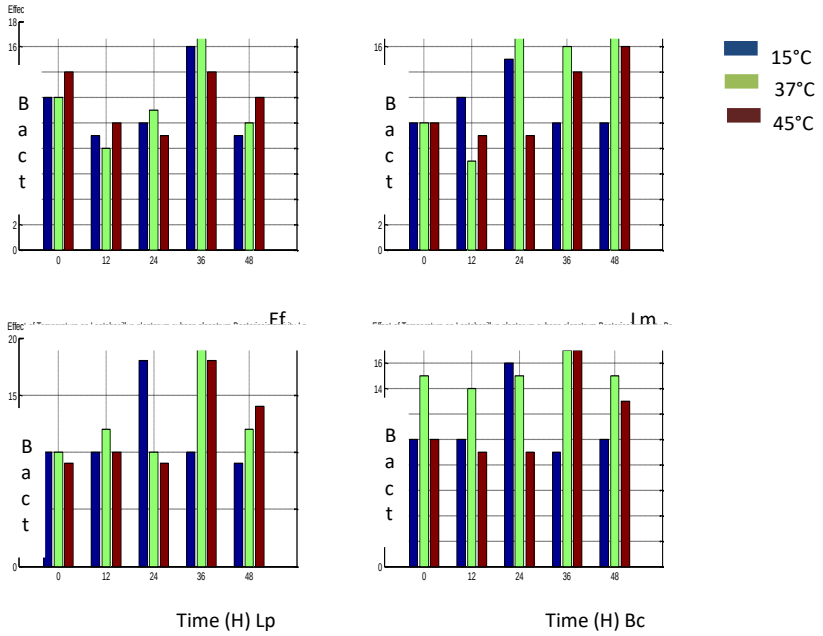

Fig.6: Influence of Incubation Temperature on Bacteriocin Activity of *Lactobacillus plantarum* subsp *plantarum*.

3.2. Characterization of Crude Bacteriocin

3.2.1. Heat Stability

Exposure of the crude bacteriocin to different heat treatment is presented in Table.1. The crude bacteriocin retained its inhibitory activity after treatment to the different heat range although no activity was detected against *Lactobacillus plantarum* used as indicator.

Table 1: Influence of Heat Treatment on Crude Bacteriocin

Heat (ºC)	Diameter of Inhibition (mm)			
	Bacillus cereus	***Enterococcus faecalis***	***Listeria monocytogene***	***Lactobacillus plantarum***
60	22	22	21	0
70	18	20	18	0
80	18	21	17	0
90	19	18	12	0
100	19	12	9	0
121	18	11	12	0
LSD	6.380	7.067	4.752	0
F.pr	<0.001	<0.001	<0.001	<0.001

3.2.2. Effect of pH

Assay of the effect of pH on crude bacteriocin detected pH effect on inhibitory activity of crude bacteriocin Table 2. pH adjustment from 2 to 7 did not affect the inhibitory property of crude bacteriocin against indicator strains. While at 8, 9 and 10 no inhibitory activity was detected against indicator strains.

Table 2: Influence of pH adjustment on Crude Bacteriocin

pH	Diameter of Inhibition (mm)			
	Bacillus cereus	***Enterococcus faecalis***	***Listeria monocytogene***	***Lactobacillus plantarum***
2	19	20	18	17
3	17	16	14	15
4	13	13	13	14
5	13	13	11	14
6	12	11	12	13
7	10	10	9	9
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
LSD	4.528	3.346	2.307	5.644
F.pr	<0.001	<0.001	<0.001	<0.001

3.2.3. Effect of Enzymes

The crude bacteriocin was subjected to treatment with different enzyme Table 3. Complete inactivation of the crude bacteriocin was observed after treatment with trypsin. Treatment with lysozyme and pepsin recorded partial inactivation of the crude bacteriocin. No effect on inhibitory activity was observed after treatment with catalase.

Table 3: Influence of Enzyme on Crude Bacteriocin

Enzyme	Diameter of Inhibition (mm)			
	Bacillus cereus	***Enterococcus faecalis***	***Listeria monocytogene***	***Lactobacillus plantarum***
Catalase	16	18	12	15
Lysozyme	14	0	0	0
Pepsin	16	0	11	0
Trypsin	0	0	0	0
LSD	3.346	2.079	3.379	4.995
F.pr	<0.001	<0.001	<0.001	<0.001

3.2.4. Effect of Organic Solvent

The crude bacteriocin was subjected to treatment with various organic solvent. Crude bacteriocin retained its inhibitory activity after treatment with ethanol, butanol, methanol and propanol-2 but complete inactivation of crude bacteriocin was observed after treatment with acetone and chloroform Table 4.

Table 4: Influence of Organic Solvent on Crude Bacteriocin

Organic Solvent	Diameter of Inhibition (mm)			
	Bacillus cereus	***Enterococcus faecalis***	***Listeria monocytogene***	***Lactobacillus plantarum***
Ethanol	12	14	14	12
Acetone	0	0	0	0
Chloroform	0	0	0	0
Butanol	15	15	14	14
Methanol	13	12	11	12
Propanol-2	15	12	16	11
LSD	7.405	7.630	5.721	5.468
F.pr	<0.001	<0.001	<0.001	<0.001

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3.2.5. Effect of Surfactants

Effect of surfactants on crude bacteriocin was evaluated Table 5. EDTA, SDS, Triton-114, Triton-100, Tween-20 and Tween-80 did not affect the inhibitory activity of the crude bacteriocin but complete inactivation was observed for urea.

Table 5: Influence of Surfactants on Crude Bacteriocin

Surfactants	Diameter of Inhibition (mm)			
	Bacillus cereus	***Enterococcus faecalis***	***Listeria monocytogene***	***Lactobacillus plantarum***
EDTA	17	17	16	14
SDS	22	22	20	20
Triton-114	14	18	15	18
Triton-100	11	10	12	11
Tween-20	22	14	13	0
Tween-80	12	11	11	0
Urea	0	0	0	0
LSD	5.273	3.461	5.346	5.004
F.pr	<0.001	<0.001	<0.001	<0.001

3.2.6. Stability of Crude Bacteriocin

Effect of storage temperature and time on bacteriocin activity is shown in Fig.7. Storage temperature and time were shown to have affect on the stability of the crude bacteriocin. At 37°C and 4°C the crude bacteriocin lost its activity after 30days and 45days of storage respectively while at -20°C it was stable retaining its activity for 60days.

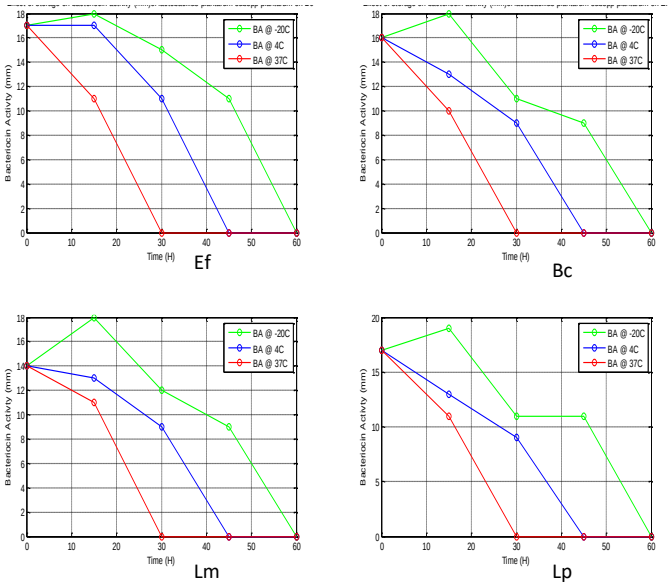

Fig.7 Influence of Storage on Crude Bacteriocin.

Discussion

Influence of different factors on growth and bacteriocin activity of *Lb plantarum* subsp *plantarum* was evaluated. According to a report by Mataragas *et al*. (2003), production of bacteriocin in situ require an in depth understanding of the influence of different factors on growth and bacteriocin activity to achieve optimal performance in food preservation. In this study, it was observed that initial pH had an influence on growth and bacteriocin production *Lb plantarum* subsp *plantarum* this agreed with an early study by Altuntas *et al*. (2010) who reported an influence of environmental factors on bacteriocin titer. Delgado *et al*. (2007) in a study on *Lb plantarum* 17.2b reported that maximum specific bacteriocin production was obtained at initial pH ranges 4.5-5.5. Also Altuntas *et*

al. (2010) in a study on *Pediococcus acidilactici* 13 reported initial pH range 6 as the optimal pH for bacteriocin production which is consistent with results of this study. Bacteriocin production was also observed to be growth associated. This could be attributed to the energy demand on metabolically active cells during bacteriocin excretion through specific ABC transporters (Delgado *et al*., 2005). Altuntas *et al*. (2010) reported that lower concentration of NaCl enhanced the growth of LAB while concentrations above 3% retarded it. Similarly in this present study optimal growth was obtained at NaCl concentration 1.5% while it was retarded at values above 3.5%. Furthermore, the concentration of NaCl had no effect on bacteriocin activity and it agreed with studies by Uguen *et al*. (1999) and Delgado *et al*. (2005). Although there are contradicting reports linking bacteriocin production to strain dependency (Soumya *et al*., 2012). Optimal temperature for growth of *Lb plantarum* subsp *plantarum* was achieved at 37ºC which agreed with studies by Altuntas *et al*., (2010). No effect of temperature on bacteriocin activity was observed; although studies by Mataragas *et al*., (2003) had reported that decrease of temperature below optimum for growth favoured bacteriocin production due to maximum utilization of energy and essential metabolites.

Castro *et al*., (2011) reported the need to consider different factors when choosing bacteriogenic strains for bacteriocin production in or ex situ. Inhibitory activity of crude bacteriocin was observed to be thermostable and the same trend has been reported by Todorov and Dicks (2005; 2006). De Kwaadsteniet *et al*. (2005); Sivakumar *et al*. (2010); Kaur and Garg (2013) have also reported the loss of activity after exposure of bacteriocin at 121ºC for 15min. Production of thermostable bacteriocins by *Lactobacillus* sp have been proven since it's an important attribute for the bacteriocin to be applied in biopreservation (Ogunbanwo *et al*., 2003). No inhibitory activity was observed against *Lactobacillus plantarum* used as indicator strain. The crude bacteriocin retained its activity in acidic conditions, this confirmed that its activity was pH dependent. It was stable within pH 2-7 but was inactivated at pH 8-10. Ogunbanwo *et al*. (2003) and Sivakumar *et al*. (2010) had reported the same trend for bacteriocin produced by *Lb plantarum* F1, *Lb bervis* OGI and *Lb acidophilus*. A contradictory report by Todorov and Dicks (2005; 2006) observed a bacteriocin with activity at acidic and alkaline conditions against pathogenic and spoilage organisms. Sivakumar *et al*. (2010) attributed the variation in response to different pH ranges to consistency of small molecular weight bacteriocins.

Partial inactivation was recorded after treatment of crude bacteriocin to pepsin. No effect on activity was noted after treatment with catalase while partial and complete inactivation was observed for lysozyme and trypsin and it agreed with a similar study, Ogunbanwo *et* al. (2003) who attributed the pattern of response to the presence of non-proteinaceous activity moiety in the bacteriocin. Although it contradicted studies by Kaur and Garg (2013); Todorov and Dicks (2006) who observed complete inactivation on treatment with proteolytic enzymes due to its proteinaceous nature. Complete inactivation was observed after treatment with acetone and chloroform and it was attributed to the lipid content of the bacteriocin (Soumya *et al*., 2012) where as the inhibitory activity was not affected by the addition of organic solvents like ethanol, butanol, methanol, propanol-2 although partial inactivation have been reported by Kaur and Garg, 2013. Influence of the addition of surfactants of the crude bacteriocin resulted in resistance to treatment with SDS, Tween-20, Tween-80, Triton-100, Triton-114 and EDTA while its activity was completely inactivated by urea. Previous studies De Kwaadsteniet *et al*. (2005) reported resistance of bacteriocin ST15 to treatment with urea and EDTA and it contradicted reports by Kaur and Garg (2013) who reported a strong inhibition of bacteriocin BA28 by urea and EDTA. Todorov and Dicks (2004) observed resistance to treatment with SDS, Tween-20, Tween-80, urea, EDTA and sensitivity to Triton-100 by bacteriocins ST11BR, ST13BR, St151BR and St34BR. Similar results have also been reported by Todorov and Dicks (2006) and it was attributed to the differences in molecular mass and structure of the peptides of the various bacteriocins (Todorov and Dicks, 2004, 2006) .Crude bacteriocin retained its activity at -20ºC for up to 60days while it became inactivated after 30days and 45days at 37ºC and 4ºC respectively. And it corresponded with results by Sivakumar *et al*. (2010) and Ogunbanwo *et al*. (2003). A different trend was reported by Kaur and Garg, 2013 who observed a decline in activity after 30days of storage at -20ºC. The loss of activity was attributed to the oxidation of the methionine group present in the bacteriocin or the action of proteolytic enzymes present in the supernatant fluid (Sivakumar *et al*., 2010; Kaur and Garg, 2013).

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