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ORIGINAL RESEARCH

Antimicrobial activity and anti-aflatoxigenic activity of bacteriocin isolated from *Pediococcus acidilactici* from fish wastes

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ABSTRACT
A study was carried out with an objective of isolating lactic acid bacteria associated with fresh water fish viscera and characterizing them in order to explore the possibilities of utilizing bacteriocins. Fish processing generates considerable quantities of waste in the form of head, viscera, scale, skin, fins, frame bones etc. These inedible byproducts harbor various types of microorganisms both harmful as well as beneficial types. The study included enumeration of LAB count in fresh and marine water fish wastes. The isolated LAB was identified by different biochemical tests and sugar fermentation tests. LAB was also tested for their antimicrobial action against selected human pathogens, fungus and aflatoxins. The bacteriocin of various isolates inhibits the growth pathogenic organisms.

KEY WORDS: *Bacteriocin, aflatoxin, LAB, Pediococcus acidilactici*

INTRODUCTION

Lactic acid bacteria (LAB) are the beneficial organisms present in most of the natural products and fish waste is not an exception (Min Tian Goa *et al*,2006). Lactic acid bacteria are characterized as gram positive cocci or rods, non-aerobic but aerotolerant, able to ferment carbohydrates for energy and acid production, also able to produce small antibacterial and antifungal materials preventing pathogenic bacteria and fungus from getting into an organism has been proven (Kwaadsteniet *et al*, 2005). Fish processing industry generates considerable quantities of by-products as wastes that includes viscera, shell (from crustacean and molluscan processing), scales, fins and frame bones. India alone generates >2 mmt of byproducts due to fish processing activities. Unlike the seafood processing sector, fresh water fish or the inland fisheries sector is un-organized and hence poses a different level of waste disposal problems. These by-products are rich in protein and fat which make them more perishable. As per one estimate visceral waste alone contributes to the total of 3, 00, 000 tones (Mahendrakar, 2000).

Gastrointestinal bacteria take part in the decomposition of nutrients and also provide the macro organism with physiologically active materials like enzymes, amino acids and vitamins (Genckal, 2006). Many studies have dealt with the preservation of fish products with LAB starters and interest has been focused on strains producing bacteriocins (Khalil *et al*, 2009). Lactic acid bacteria have wide uses in the food industry where they used for turning milk into cheese or yoghurt, cabbage into sauerkraut or kimchi, and even improving the quality of wine (Diop *et al*, 2007). They

are also consumed in probiotic products for their health-promoting effects. Lactic acid bacteria inhibit or kill undesirable bacteria in food and in the human body by a number of mechanisms including the production of lactic acid and antimicrobial peptides (Abo-Amer, 2007). Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium (Lade *et al*, 2006). Balcazar et al in the year 2008examined the antagonistic activity of strains of lactic acid bacteria, isolated from chilled fish products, against spoilage bacteria and pathogens. As LAB enjoy Generally Recognized As Safe (GRAS) status through long history of safe use in fermented food production, their bacteriocins have been studied most intensively (Garneau *et al*, 2002). Bacteriocin production could be considered as an advantage for food and feed producers, since these peptides can kill or inhibit pathogenic bacteria (Deegan *et al*, 2006). Bacteriocins are involved in protecting the host from infection, ensuring a proper balance of nutrients is extracted from the diet and even affect such complex properties as development and behavior (Moghaddam and Sattari, 2006). Bacteriocins are used in the prevention and treatment of gastro-intestinal infections and diarrhea, and in improvement of immune responses (Tagg, 2004).

Lab will help the decomposition process of organic materials, and during fermentation will produce normally unavailable organic acids, such as lactic acid, acetic acid, amino acid, malic acid and bioactive substances and vitamins. A key ingredient in this process is organic matter which is supplied by pasture residuals, (dead matter) recycling crop residues, green manures and animal manure. In addition, this process leads to increased humus in the soil. This makes lactic acid bacteria act like an organic fertilizer, will suppress pathogenic microbes both directly, and indirectly. Bacteriocins also produce an antioxidant effect which improves the immune system of plants and animals. Bacteriocins used in effluent systems will improve the efficiency of biological systems, and in the process, reduce smell, reduce sludge, and compete against harmful pathogens in the effluent and application sites.

Against this background, the present investigation was undertaken to evaluate the bacteriocin producing microbial load of processing waste of representative species from fresh water and marine environs. The bacteriocin producing organisms associated with fish wastes were identified based on several biochemical tests. An attempt was also made to evaluate the effect of pH, temperature and incubation time on the growth of bacteriocin producing organisms and the bacteriocin activity of the organisms.

RESULTS

The general microbial characteristics of various fish processing wastes of Thilapia, Rohu, Indian cat fish, Catla, Common carp, Shrimp is evaluated in this study are presented in Table 1. The isolated bacterial culture of various volume such as 0,01ml, 0.02ml, 003 ml inoculated on the plate containing *Escherichia coli.*The diameter of the zone of clearance increases with the increase in the volume of the bacterial cultures isolated from the fish processing waste as depicted on Figure 1. Based on various morphological/staining characteristics (Table 2), physiological characteristics (Table 3), biochemical characteristics (Table 4) and antibiotic sensitivity characteristics (Table 5). The isolates from different fish wastes were identified as *Lactococcus lactis*, *Pediococcus acidilactici*, *Streptococcus raffinolactis* and *Pediococcus pentosaceus*. *Lactococcus lactis* is associated with Thilapia and Catla, *Pediococcus acidilactici* is associated with all fish wastes except rohu and shrimp. *Streptococcus raffinolactis* is associated with rohu and catla. *Pediococcus pentosaceus* is associated with Indian cat fish and catla. *Pediococcus acidilactici* is sensitive to most of the antibiotics expect optochin, bacitracin etc as given in the Table 5.

The effect of medium pH, incubation time, temperature and agitation speed on the activity of bacteriocin produced by *Streptococcus raffinolactis* SNGC2011 grown in nutrient broth is presented in Figures 3 through 6. The cell growth and bacteriocin activity measured at varying temperature ranging from 10°C to 100°C, incubation period of 24 hrs, temperature 37° (Figure 3) indicated 4 to be the optimum incubation pH for cell growth is at 4 and starts declines. But the bacteriocin production is optimum at the pH ranging from 3-5 and then declines. The cell growth and bacteriocin production across incubation time ranging from 10 to 100 hrs is presented in Figure 4. The optimum incubation time for cell growth is between 30-60 hrs and bacteriocin activity is at 40 hrs. The effect of agitation speed ranging from 25 to175 rpm, at constant conditions as mentioned before is

Table 1: Microbiological characteristics of fish wastes

	Fish wastes					
Parameter (log cfu g^{-1})	**Thilapia**	**Rohu**	**Indian cat fish**	**Catla**	**Common carp**	**Shrimp**
Total plate count	5.8±1.7	5.9±1.6	5.8±1.4	5.4±1.2	5.2±1.8	5.6±1.6
Total coliform count	2.9±0.5	2.8±0.2	2.4±0.1	2.1±0.4	2.6±0.6	2.7±0.4
Total yeasts and molds count	3.0±0.9	2.7±0.5	2.8±0.9	2.4±0.9	2.8±1.2	2.6±0.2
Total LAB count	1.9±0.9	2.0±0.9	2.0±0.9	2.1±0.9	2.1±0.9	2.1±0.9

Table 2: Morphological characteristics of the isolates

Parameter	**Isolate@ number**									
	LABTI - 1	LABTI – 2	LABRO - 1	LABIC - 1	LABIC – 2	LABCT - 1	LABCT – 2	LABCT – 3	LABCT – 4	LABCC - 1
Gram's stain	+,Cocci	+,Cocci	+,Cocci	+, Cocci	+,Cocci	+, Cocci	+,Cocci	+,Cocci	+,Cocci	+, Cocci
Endospore stain	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-
Pigmentation	-	-	-	-	-	-	-	-	-	-

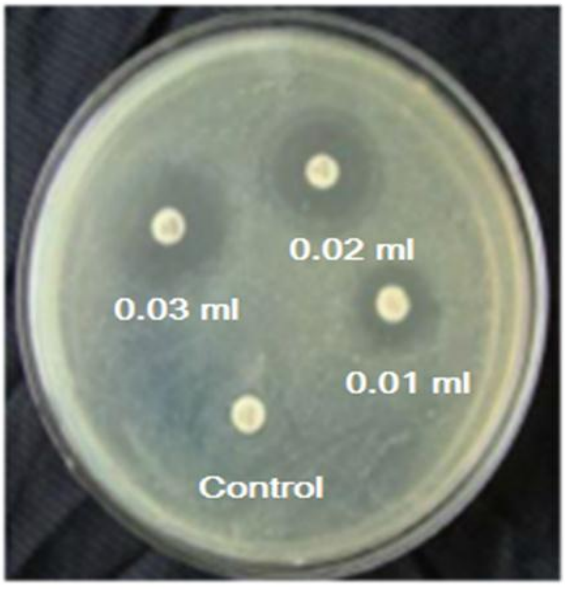


Figure 1: Nutrient agar plates showing the antagonistic activity, of different volumes of the bacterial culture isolated from fish wastes, against *Escherichia coli*

Table 3: Physiological characteristics of the isolates

Isolates	Temperature (Degree Celsius)						pH						NaCl Concentration (%)					
	10	20	30	40	50	60	3	4	5	6	7	8	2	3	4	5	6	7
LABT1	+	+	++	-	-	-	+	+	+	+	-	-	++	++	++	+	+	-
LABT2	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+
LABRO1	+	+	++	+	-	-	+	++	+	-	-	-	+	+	+	+	-	-
LABIC1	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+
LABIC2	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+
LABCT1	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+
LABCT2	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+
LABCT3	+	+	++	+	-	-	+	++	+	-	-	-	+	+	+	+	-	-
LABCT4	+	+	++	-	-	-	+	+	+	+	-	-	++	++	++	+	+	-
LABCC1	+	+	++	+	-	-	+	++	++	++	+	+	+	++	++	++	+	+

Table 4: Biochemical characteristics of the isolates

Test	Isolate[@] number									
	LABTI - 1	LABTI – 2	LABRO - 1	LABIC - 1	LABIC – 2	LABCT - 1	LABCT – 2	LABCT – 3	LABCT – 4	LABCC – 1
Indole	-	-	-	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+
CO2 Production	-	-	-	-	-	-	-	-	-	-
Casein hydrolysis	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	-	+	+	+	+	-	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-
Potassium tellurite hydrolysis	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Nitrite reduction	-	-	-	-	-	-	-	-	-	-
H2S production	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-
Phosphatase test	-	-	-	-	-	-	-	-	-	-
Hydrogen peroxide production test	+	+	-	+	+	+	+	-	+	+
Acid from carbohydrates	-	-	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-	-	-

(Contd)

from carbohydrates										
Fermentation type	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo
Fermentation of :										
Pyruvate	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	-	+	+	+	+	-	+	+
Arabinose	+	+	-	+	+	+	+	-	+	+
Mannitol	+	-	+	-	-	-	-	+	+	-
Sorbitol	-	-	+	-	-	-	-	+	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-
Sucrose	+	-	+	-	-	-	-	+	+	-
Lactose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	+	-	-	-	-	+	-	-
Inulin	-	-	+	-	-	-	-	+	-	-
Fructose	-	-	+	-	-	-	-	+	-	-
Xylose	+	-	+	-	-	-	-	+	+	-
Cellibiose	+	+	-	-	+	+	-	-	+	+
Mellibiose	+	-	-	-	-	-	-	-	+	-
Haemolysis	α	α	α	α	α	α	α	α	α	α

Table 5: Antibiotic sensitivity test of the isolates

Isolates	Resistant Antibiotics
LABTI - 1	Optochin, Bacitracin
LABTI – 2	Optochin, Bacitracin
LABRO – 1	Optochin, Metronidazole, Bacitracin
LABIC - 1	Optochin, Bacitracin
LABIC – 2	Optochin, Bacitracin
LABCT - 1	Optochin, Bacitracin
LABCT – 2	Optochin, Bacitracin
LABCT – 3	Optochin, Metronidazole, Bacitracin
LABCT – 4	Optochin, Bacitracin
LABCC – 1	Optochin, Bacitracin

Identification of the isolates based on schemes outlined in Gerhard et al (1994) and Bergy et al (2002).
LABTI-1: *Lactococcus lactis* SNGC2014, LABTI-2 : *Pediococcus acidilactici* SNGC2013, LABRO-1: *Streptococcus raffinolactis* SNGC2011, LABIC-1: *Pediococcus acidilactici* SNGC2013, LABIC-2: *Pediococcus pentosaceus* SNGC2012, LABCT-1: *Pediococcus acidilactici* SNGC2013, LABCT-2: *Pediococcus pentosaceus* SNGC2012, LABCT-3: *Streptococcus raffinolactis* SNGC2011, LABCT-4: *Lactococcus lactis* SNGC2014, LABCC-1: *Pediococcus acidilactici* SNGC2013.

presented in the Figure 5. The optimum agitation speed was found to be 100 rpm for both growth and the bacteriocin activity. Similarly effect of varying temperature (Figure 6) on growth and bacteriocin activity revealed 40-60 hrs to be the optimum.

The effect of medium pH, incubation time, temperature and agitation speed on the activity of bacteriocin produced by *Pediococcus pentosaceus* SNGC2012 grown in nutrient broth is presented in Figures 7 through 10. The cell growth and bacteriocin activity measured at varying temperature ranging from 10°C to 100°C, incubation period of 24 hrs, temperature 37°C (Figure 7) indicated 6 to be the optimum incubation pH for cell growth and bacteriocin production. The cell growth and bacteriocin production across incubation time

Fig 2

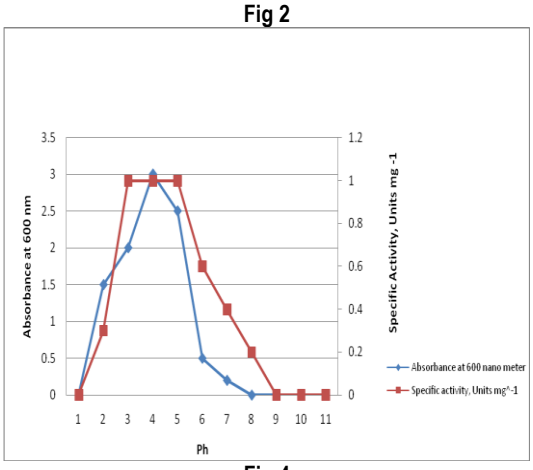


Fig 3

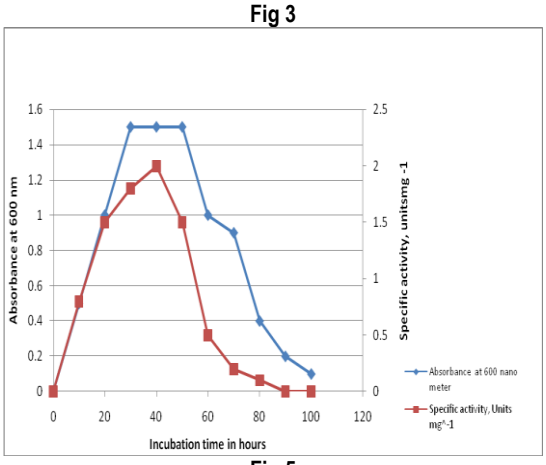


Fig 4

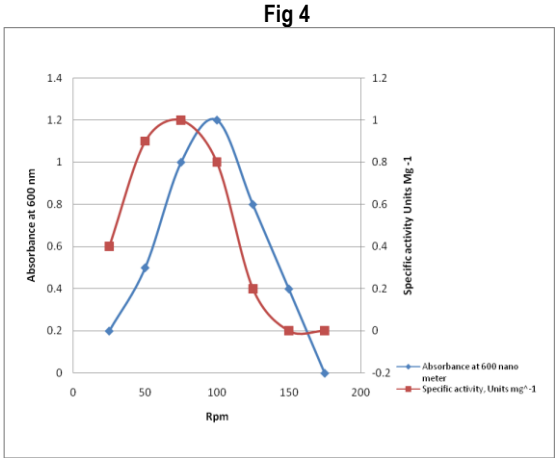
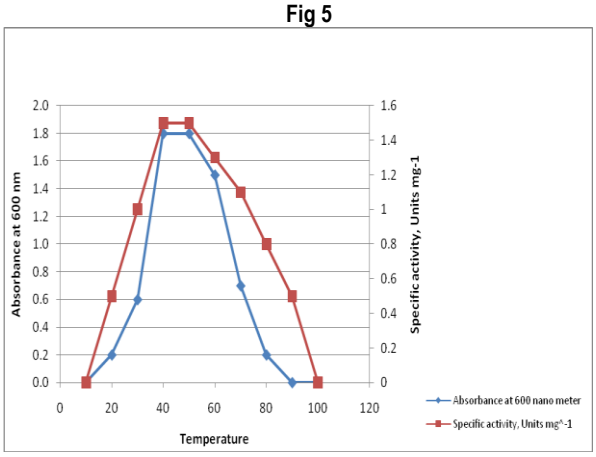


Fig 5



Effect of pH (Fig 2), incubation time (Fig 3), agitation speed (Fig 4), temperature (Fig 5) (A) the growth (as indicated by absorbance at 600 nm) and (B) bacteriocin activity of *Streptococcus raffinolactis* CFR201 cultured in nutrient broth (pH 4, Incubation period of 24 hrs, Temperature - 37°C, Agitation speed – 100 rpm)

Table 6: Activity of lyophilized culture filtrates to various enzymes for antibacterial activity against *Listeria monocytogenes*

Isolates	**Lyophilized culture filtrate treated with pepsin (zone diameter in mm)**	**Lyophilized culture filtrate treated with protenase K(zone diameter in mm)**	**Lyophilized culture filtrate treated with trypsin(zone diameter in mm)**
Streptococcus raffinolactis SNGC2011	Negative result	Negative result	Negative result
Pediococcus pentosaceus SNGC2012	Positive result – 6 mm	Positive result – 4 mm	Positive result – 2mm
Lactococcus lactis SNGC2014	Positive result - 18 mm	Positive result - 16 mm	Positive result - 11 mm
Pediococcus acidilactici SNGC2013	Positive result – 20 mm	Positive result – 16 mm	Positive result – 11mm

ranging from 10 to 100 hrs is presented in Figure 8. The optimum incubation time for cell growth is 50 hrs and bacteriocin activity is at 40 hrs and after it both declines. The effect of agitation speed ranging from 25 to175 rpm, at constant conditions as mentioned before is presented in the Figure 9. The optimum agitation speed was found to be 75 rpm for both growth and the bacteriocin activity declines only after 100 rpm. Similarly effect of varying temperature (Figure 10) on growth and bacteriocin activity revealed 50 hrs to be the optimum.

Fig 6

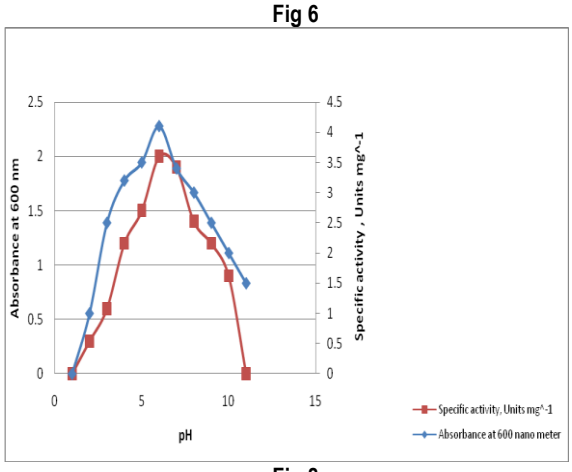


Fig 7

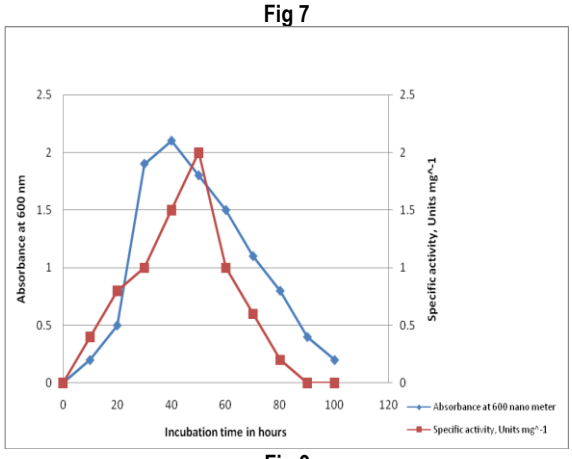


Fig 8

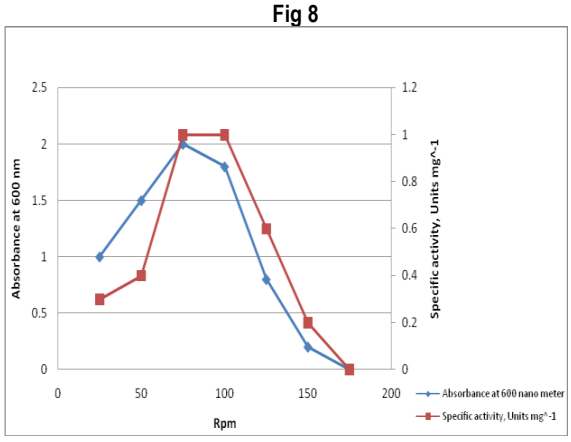
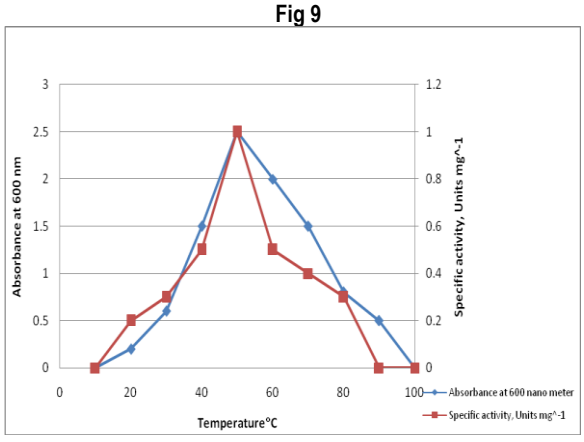


Fig 9



Effect of pH (Fig 6), incubation time (Fig 7), agitation speed (Fig 8), temperature (Fig 9)
(A) the growth (as indicated by absorbance at 600 nm) and
(B) bacteriocin activity of *Pediococcus pentosaceus* CFR2012
cultured in nutrient broth (pH 4, Incubation period of 24 hrs, Temperature -37°C, Agitation speed – 100 rpm)

The effect of medium pH, incubation time, temperature and agitation speed on the activity of bacteriocin produced by *Pediococcus acidilactici* SNGC2013 grown in nutrient broth is presented in Figures 11 through 14. The cell growth and bacteriocin activity measured at varying temperature ranging from 10°C to 100°C, incubation period of 24 hrs, temperature 37°C (Figure 11) indicated 6 to be the optimum incubation pH for cell growth and pH 4 bacteriocin productions.

The cell growth and bacteriocin production across incubation time ranging from 10 to 100 hrs is presented in Figure 12. The optimum incubation time for cell growth is 30-60 hrs and bacteriocin activity is at 40-60 hours and after it both declines. The effect of agitation speed ranging from 25 to175 rpm, at constant conditions as mentioned before is presented in the Figure 13. The optimum agitation speed was found to be 75 rpm for growth and 75- 100 rpm for the bacteriocin activity. Similarly effect of varying temperature (Figure 14) on growth and bacteriocin activity. It is revealed that 30-50 hrs to be the optimum for growth and 60 hrs for bacteriocin activity.

Table 7: Antimicrobial activity of bacteriocin produced by *Pediococcus acidilactici* SNGC2013 as affected by pH, temperature against *Listeria monocytogenes*

Isolates	**Effect of pH**		**Effect of temperature**		
	pH	**Activity**	**Temperature (°C)**	**Time (in minutes)**	**Activity**
Control	Control	3200	-	-	3200
Pediococcus acidilactici CRF2013	2.0	-	-	-	3200
	3.0	1600	60	15 mins	3200
	4.0	3200	60	15 mins	3200
	5.0	3200	60	15 mins	3200
	6.0	3200	60	30 mins	3200
	7.0	3200	60	30 mins	3200
	8.0	3200	100,	15 mins	3200
	9.0	3200	121	20 mins	3200
	10.0	3200	150	25 mins	3200

Table 8: Antimicrobial activity of isolates on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* on different incubation periods

Isolates	**Mycelial growth of *Aspergillus flavus*** **Incubation time (days)**						**Mycelial growth of *Aspergillus parasiticus*** **Incubation time (days)**					
	2	3	4	5	6	7	2	3	4	5	6	7
Lactococcus lactis SNGC2014	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus raffinolactis SNGC2011	-	-	-	+	+	+	-	-	-	+	+	+
Pediococcus acidilactici SNGC2013	-	-	-	-	-	-	-	-	-	-	-	-

(Contd)

Pediococcus pentosaceus SNGC2012	-	-	+	++	++	+++	-	-	-	+	++	+++
Control	+	+	++	+++	+++	+++	_	+	+	++	+++	+++

T**able 9:** Effect of various isolates on fungal growth at aflatoxin production level

Isolates	**Target fungi**	**Mycelial weight (g)**	**Growth inhibition(%) relative to that of control**	**Aflatoxin production (µg/ml)**	**Aflatoxin inhibition (%) relative to that of control**
Streptococcus raffinolactis SNGC2011	*A.flavus*	0.2343	22.1	5.512	74.05
	A.parasiticus	0.0625	80.9	4.316	43.9
Pediococcus pentosaceus SNGC2012	*A.flavus*	No growth	100	Nil	100
	A.parasiticus	No growth	100	NIL	100
Lactococcus lactis SNGC2014	*A.flavus*	0.1102	63.4	4.817	77.32
	A.parasiticus	0.2676	18.24	3.71	51.78
Pediococcus acidilactici SNGC2013	*A.flavus*	No growth	100	Nil	100
	A.parasiticus	No growth	100	Nil	100
Control	*A.flavus*	0.3011	-	21.241	-
	A.parasiticus	0.3273	-	7.694	-

The effect of medium pH, incubation time, temperature and agitation speed on the activity of bacteriocin produced by *Lactococcus lactis* SNGC2014 grown in nutrient broth is presented in Figures 15 through 18. The cell growth and bacteriocin activity measured at varying temperature ranging from 10°C to 100°C, incubation period of 24 hrs, temperature 37°C (Figure 15) indicated 4 to be the optimum incubation pH for cell growth and pH 6 bacteriocin production. The cell growth and bacteriocin production across incubation time ranging from 10 to 100 hrs is presented in Figure 16. The optimum incubation time for cell growth is 30 hrs and bacteriocin activity is at 40 hrs. The effect of agitation speed ranging from 25 to175 hours, at constant conditions as mentioned before is presented in the Figure 17. The optimum agitation speed was found to be 75- 100 rpm for growth and 100 hrs for the bacteriocin activity. Similarly effect of varying temperature (Figure 18) on growth and bacteriocin activity. It is revealed that 40 hrs to be the optimum for growth and 50-70 hrs for bacteriocin activity.The sensitivity of the indicator strains was estimated based on the diameter (mm) of the inhibition zones. The range of inhibitory spectrum activity in terms of Au/ml of lyophilized culture filtrate of bacteriocin against *Escherichia coli* is in the Figure 20 The antimicrobial spectrum of the bacteriocin

Fig 10

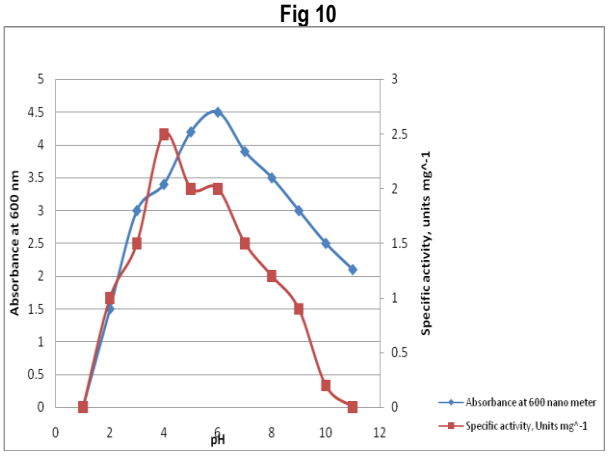


Fig 11

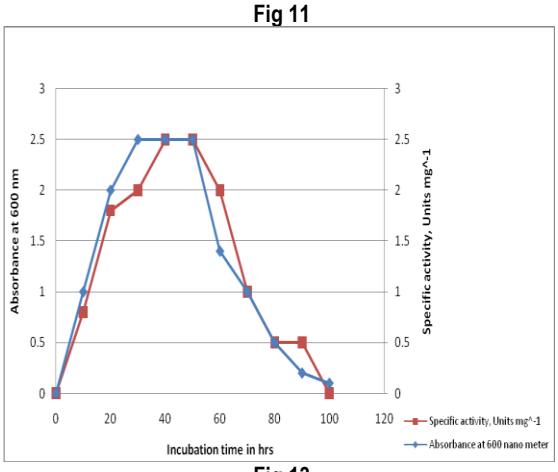


Fig 12

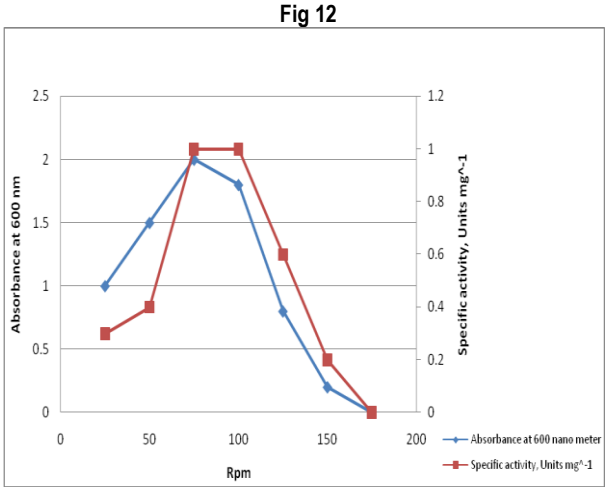
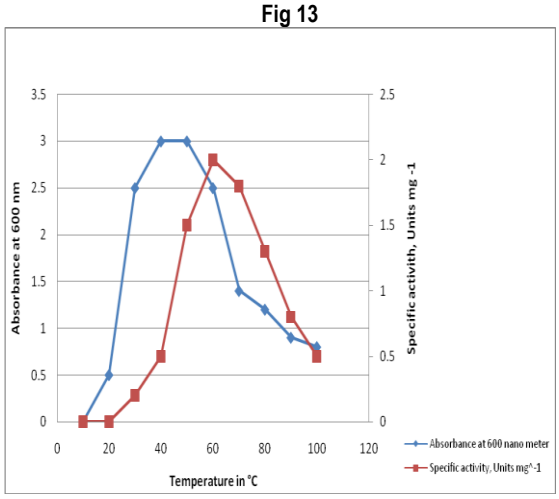


Fig 13



Effect of pH (Fig 11), incubation time (Fig 12), agitation speed (Fig 13), temperature (Fig 14)
(A) the growth (as indicated by absorbance at 600 nm) and
(B) bacteriocin activity of *Pediococcus acidilactici* CFR2013
cultured in nutrient broth (pH 4, Incubation period of 24 hrs, Temperature -37°C, Agitation speed – 100 rpm)

produced by *Streptococcus raffinolactis* SNGC2011, *Pediococcus pentosaceus* SNGC2012, *Pediococcus acidilactici* SNGC2013 and *Lactococcus lactis* SNGC2014 aganist *Escherichia coli*, *Listeria monocytogenes, Bacillus cereus, Pseudomonas aerogenes, Streptococcus aureus, Bacillus subtilis* and *Yersinia enterocolytica* are presented on the Figure 21. The effects of enzyme treatment on the antibacterial activity of different lyophilized culture filtarates presented in Table 6. The loss of antibacterial activity was observed with *Pediococcus pentosaceus* SNGC2012 and *Pediococcus acidilactici* SNGC2013 when treated with pepsin and trypsin indicating the proteinaceous nature of the active principles elaborated by them (Table 6). The effect of pH and temperature of the bacteriocin produced by *Pediococcus acidilactici* SNGC2013 against pathogen *Listeria monocytogenes* is presented in the Table 7. The potential of the antimicrobial substances elaborated by the isolates of LAB to inhibit the growth of aflatoxin production in *Aspergillus flavus* and *Aspergillus parasiticus* was evaluated and the results are presented on Table 8.

The ammonium sulphate precipitated protease subjected to gel filteration chromatography exhibited only one peak between the elution volume of 280 and 320ml and Ve/Vo of 4 (Figure 19A). Electrophoretic pattern of alkaline protease obtained by pooling and lyophilizing the fractions of gel filtration, is shown in Figure 19B. It indicates the homogeneity of the enzyme protein isolated. There was a single band which and was found to correspond to a molecular weight of <3 kD as indicated in the electrophoretic pattern.

Fig 14

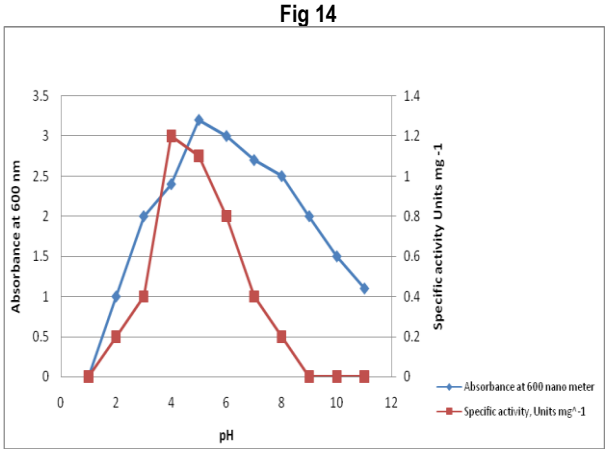


Fig 15

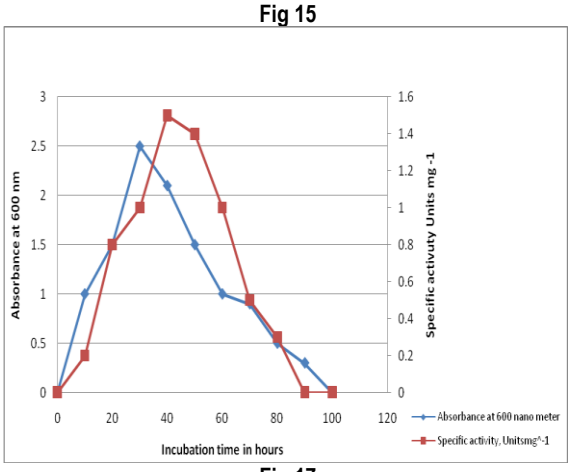


Fig 17

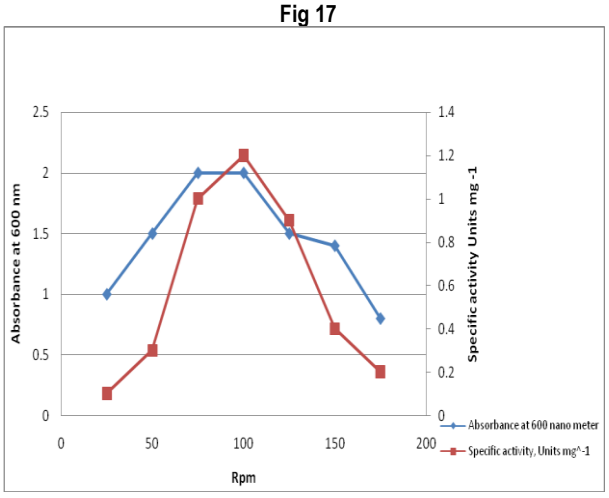
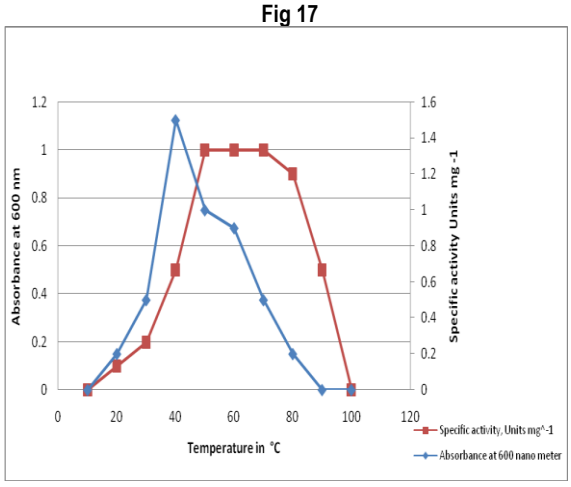
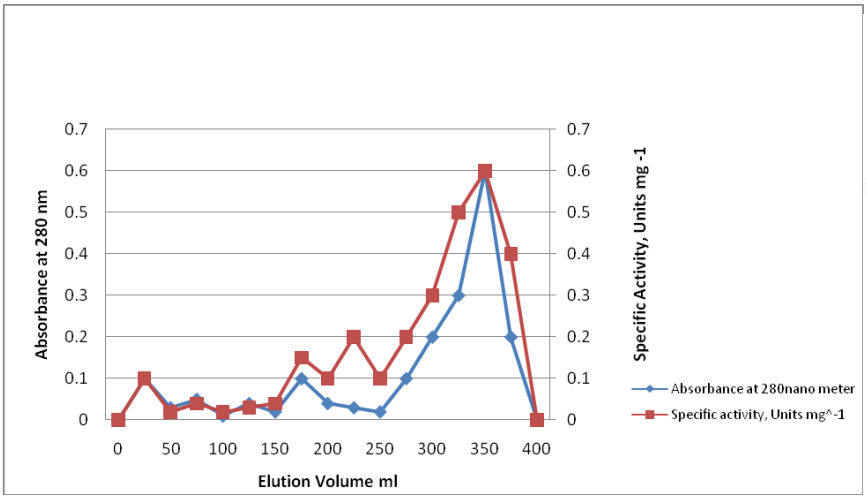


Fig 17



Effect of pH (Fig 14), incubation time (Fig 15), agitation speed (Fig 16), temperature (Fig 17)
(A) the growth (as indicated by absorbance at 600 nm) and
(B) bacteriocin activity of *Lactococcus lactis* CFR2014
cultured in nutrient broth (pH 4, Incubation period of 24 hrs, Temperature -37°C, Agitation speed – 100 rpm)



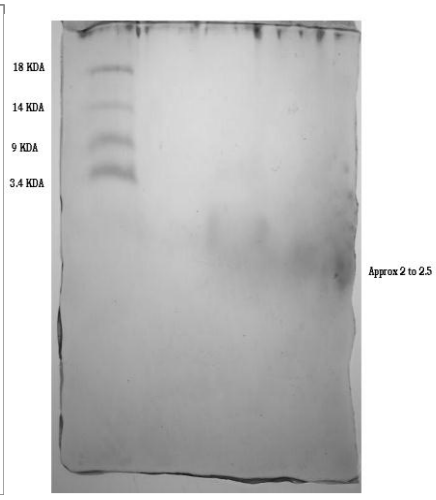


Figure 18: (A) Gel filtration (on Sephadex-G100) profile and (B) homogeneity, as indicated by electrophoresis, of the bacteriocin isolated isolated from *Pediococcus acidilactici* CFR2013. (M - Marker Proteins (Insulin – 3.4 KDa; Aprotein – 9 KDa; Lyzozyme – 14 KDa; Beta lacto globulin – 18 KDa; S 1 to 3 – sample proteins).

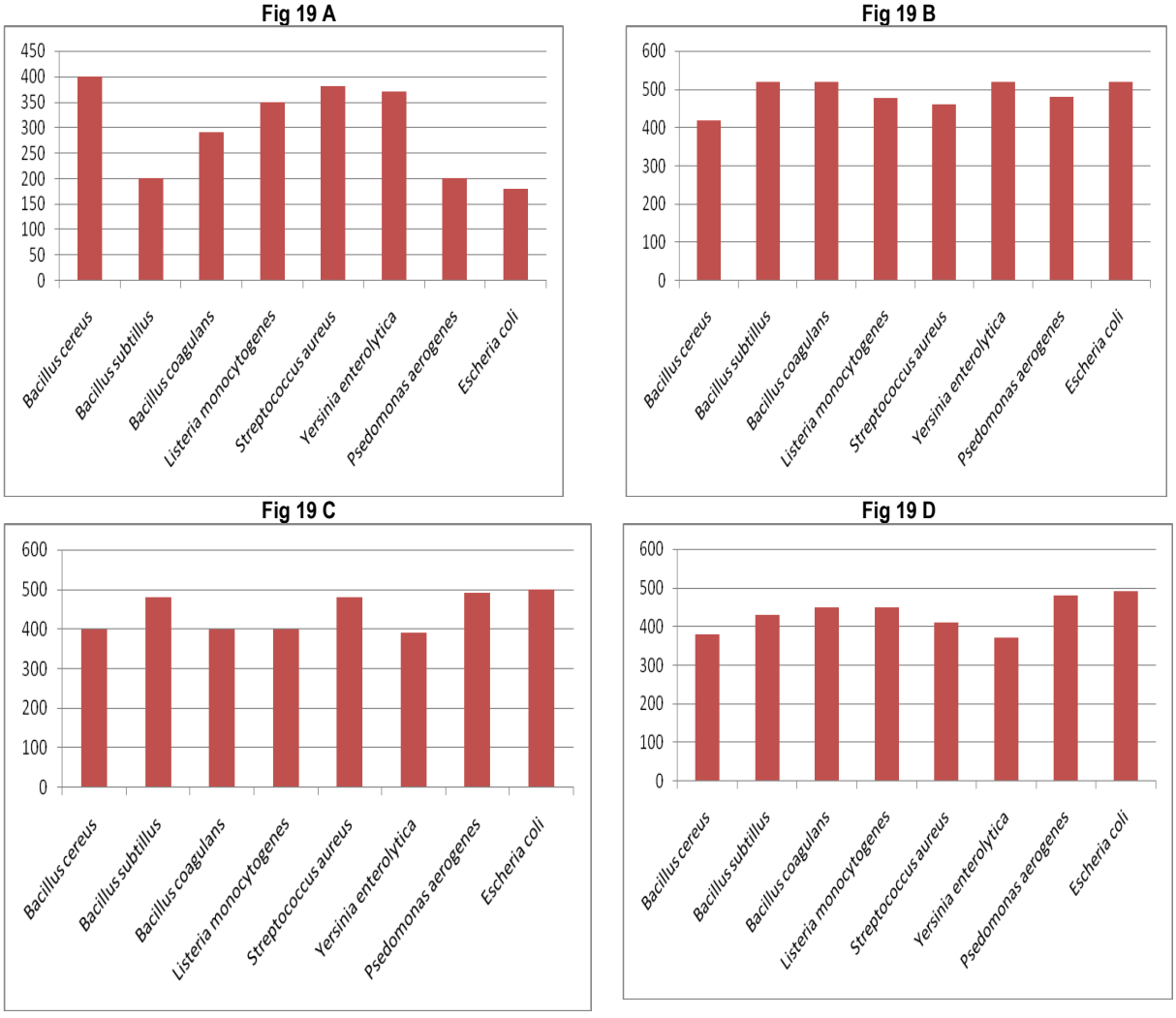


Figure 19: Effect of bacteriocin isolated from *Streptococcus raffinolactis* CFR2011 (Fig 21A), *Pediococcus acidilactici* CFR2013 (Fig 21B), *Lactococcus lactis* CFR2014 (Fig 21C), *Pediococcus pentosaceus* CFR2012 (Fig 21D) against the indicator organisms

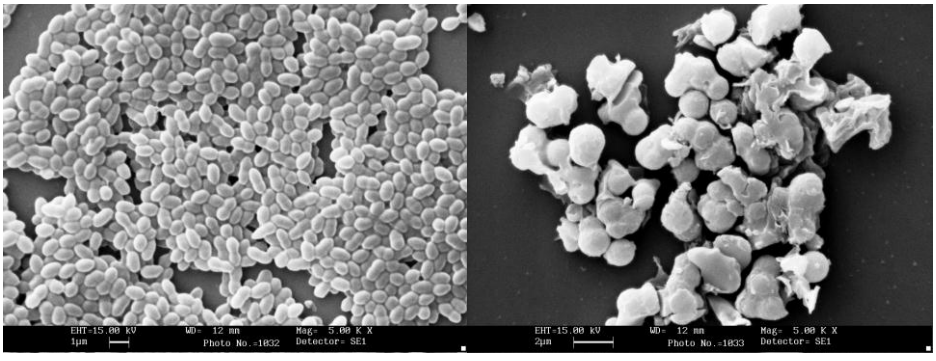


Figure 20: Scanning Electron Microscopy (SEM) plates showing the antagonistic activity of the bacteriocin obtained from *Pediococcus acidilactici* CFR 2013, isolated from fish processing, against *Escherichia coli*

Table 10: Microbiological characteristics of milk sweet along with the contaminants and bacteriocin

Days	Milk sweet (number of colonies (cfu/ml)).	Milk sweet + *Staphyloccus aureus* and *Escherichia coli* (number of colonies (cfu/ml)).	Milk sweet, *Staphyloccus aureus* and *Escherichia coli and bacteriocin* (number of colonies (cfu/ml)).
24hrs	Negative	2×10^6	0.5×10^4
48 hrs	Negative	3.8×10^6	1.1×10^4
72 hrs	Negative	4.2×10^6	2.3×10^4
96 hrs	Negative	5.9×10^6	3.4×10^4
120 hrs	Negative	7.2×10^6	4.2×10^4
144 hrs	Negative	10.8×10^6	5.1×10^4
168 hrs	Negative	10.2×10^6	4.9×10^4

DISCUSSION

As shown in the Table 1, the total bacterial load (log cfu g^{-1}) on the visceral waste varied from 5.9 to 5.2; while the total coli count (log cfu g^{-1}) varied between 2.9 and 2.1 and yeasts and mold count (log cfu g^{-1}) varied between 3.0 to 2.4. The LAB load was highest in the case of Catla, common carp and shrimp wastes (2.1 log cfu g^{-1}) while it was low in the case of Tilapia (1.9 log cfu g^{-1}). The amount of LAB isolates of the total isolates obtained was found to be considerably higher than the one reported in a related to be highest in case of visceral wastes of Mrigal (5.88 log cfu/g) and lowest in that of tilapia (4.22 log cfu/g) (Jini *et al*, 2011).

Based on various morphological/staining characteristics was on Table 2, physiological characteristics on Table 3, biochemical tests on Table 4 and antibiotic sensitivity tests on Table 5. The LAB isolated from fish wastes was found to be *Lactococcus lacti* SNGC2013, *Pediococcus acidilactici* SNGC2014, *Streptococcus raffinolactis* SNGC 2011 and *Pediococcus pentosaceus* SNGC2012. However, several researchers have reported Lactobacillus to be the major LAB present in fish viscera (Academic Press et al, 2006, Askarian *et al*, 2012 a and b, Bairagi 2002 a and b, Elsevier, 2010). It has been proved that microflora in the digestive tract of fish play an important role by producing antibacterial substances (Vijay bhasker and Somasundram, 2008). Several species of Lab have been reported to be predominant bacterioin producing bacteria and are commercially used for their applications (Nirunya *et al*, 2008).

The optimum pH by *Streptococcus raffinolactis* SNGC2011 for cell growth was at 4, from there it enters into stationary phase and the maximum extent of growth was observed up to pH 11 as shown in the Figure 3 but the bacteriocin activity was remains constant from pH 9. It has been reported that the culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane (Todorov and Dicks, 2004). The cell growth and bacteriocin production was optimum at 40 hours, as shown in the Figure 4. Growth was increasing with increasing agitation speed but specific activity of the bacteriocin was more at 100 rpm after that it declined as shown in the Figure 5. The optimum incubation temperature for cell growth and bacteriocin production was at 40C as shown in the Figure 6. The production of bacteriocins during the stationary phase of growth is characteristic of many bacterial species (Hyronimus, 2000). In other cases, the synthesis and secretion of the bacteriocin was initiated during the exponential growth phase, with a substantial increase near the end of the growth phase and with maximum amounts of bacteriocin produced in the stationary growth phase (Beg and Gupta, 2003; Cladera-Olivera, *et al*,2004 and Da Silva, 2007).

During optimization of pH, incubation temperature, and agitation speed and incubation time of *Pediococcus pentosaceus* SNGC2012 cell growth was maximum at pH 6.0, 50 °C, 60 rpm and 40 hrs . *Pediococcus pentosaceus* SNGC2012 showed maximum bacteriocin production at 6 pH, 40 °C, 60-100 rm after 50 hrs during the later growth stage after. Maximum bacteriocin production occurred in exponential growth phase in the case of *Enterococcus. faecium* IJ-21, IJ-31 as is typical for bacteriocin production by most LAB (Esakkiraj, 2009, Acadamic press, 2000) therefore, displayed primary metabolite kinetics. Decreasing bacteriocin activity after 48 hrs incubation may be explained by bacteriocin degradation due to culture enzymes, or low culture pH. In addition, readsorption of bacteriocin to the producer cell surface at low pH may contribute to the observed bacteriocin decrease in the culture medium.

Optimization of pH, incubation temperature, and agitation speed and incubation time of *Pediococcus acidolactici* SNGC2013, cell growth was maximum at pH 6.0, 60 - 50 °C, 60 rpm and 40- 50 `hrs. *Pediococcus acidolactici* SNGC2013 showed maximum bacteriocin production at 4 pH, 60 °C, 60-100 rpm after 30- 50 hrs during the later growth stage after. Bacterion has been produced from pH 4-10, when the cells are undergoing decline phase, the bacteriocins production are also at the same rate .In case of temperature, the bacteriocin production is maximum at the stationary phase. Activity levels do not always correlate with cell mass or growth rate. (Godin *et al*, 2007). Increased levels of bacteriocin production are often noticed just below the optimal growth conditions (Todorov and Dicks, 2004). Similar behavior has been observed in relation to other bacteriocins, such that the optimum pH for cell growth did not correlate with the temperature or pH requirements for maximum bacteriocin activity (Pringsulaka *et al*, 2012, Udandi Boominadhan, 2009).

The optimum pH by *Lactococcus lactis* SNGC2014 for cell growth was at 4.5, from there it enters into decline as shown in the Figure 15 but the bacteriocin activity was maximum at 4 pH. The cell growth and bacteriocin production was optimum at 30 hours and 40 hrs and steadily declines after that, as shown in the Figure 16. Growth was increasing with increasing agitation speed and was more at 100 rpm after that it declines as shown in the Figure17. The bacteriocin production was maximum at 100 rpm and declines. The optimum incubation temperature for cell growth was at 40°C and bacteriocin production was at 50 – 70 °C and declines as shown in the Figure 18. Optimum bacteriocin production levels were observed in exponential growth phase attaining maximum level in late exponential phase. Growth beyond stationary phase resulted in decreased bacteriocin production. Shin *et al*., (2008) reported that 37 °C and pH 5-7 as optimum for the bacteriocin production from *Pediococcus pentosaceus* K23-2. Hu *et al*., (2008) reported enterocin from *Enterococcus duran* over a temperature range 20-43 °C. Similarly, *Bacillus firmus* exhibited maximum enzyme yields at an aeration rate of 7.0 l min^{-1} and an agitation rate of 360 rpm. However, lowering the aeration rate to 0.1 l min^{-1} caused a drastic reduction in the enzyme yields(Askarian *et al*, 2011). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as bacteriocin synthesis. It has been reported that the synthesis of bacteriocin in several species is constitutive or partially inducible and is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phase (Askarian *et al*, 2012 a; Askarian *et al*, 2012 b).

The antibacterial activity of of lyophilized culture filterates of *Pediococcus pentosaceus* SNGC 2012*, Lactococcus lactis* SNGC 2013 and *Pediococcus acidilactici* SNGC 2014 was not affected by treatment with pepsin, trypsin and proteinase K; however for, *Streptococcus raffinolactis* SNGC 2013, resulted in substantial decreases in antibacterial activity (Table 6). These results indicate that the antibacterial compound produced by *Pediococcus pentosaceus* SNGC 2012*, Lactococcus lactis* SNGC 2013 and *Pediococcus acidilactici* SNGC 2014 have a proteinaceous nature and could be classified as bacteriocin (Drenkard, 2003).

In our study, the inhibition zone is about 2 mm- 20 mm in diameter by various isolates against *Listeria monocytogenes* presented in Table 7. The inhibition zones are between 0.5-13.0 mm in diameter by the bacteriocin producing isolates against the indicator organisms, as reported by Corsini et al, 2010. The interest in bacteriocins produced by LAB has grown because many bacteriocins inhibit food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, a

common contaminant of raw foods, milk, meat and vegetables which are recalcitrant to traditional food preservation method (Cleveland *et al*., 2001).

The antibacterial activity of culture filterates of *Streptococcus raffinolactis* SNGC2011, *Pediococcus pentosaceus* SNGC 2012*, Lactococcus lactis* SNGC 2012 and *Pediococcus acidilactici* SNGC2013 against *Aspergillus flavus and Aspergillus parasiticus* strains as described in Table 8. *Lactococcus lactis* SNGC2012 and *Pediococcus acidilactici* SNGC2013 inhibits fungal growth of incubation of 7 days. A few LAB bacteriocins such as bulgaricin or acidophilin are active against a broad spectrum of gram-positive, or Gram negative bacteria, as well as yeasts and molds (Magnusson *et al* 2003).The isolates affects the aflatoxin production ranging from 43.9 to 100%, which is presented on Table 9. The *Streptococcus raffinolactis* SNGC2011 against *Aspergillus parasiticus* is the lowest and is about 43.9%and the highest one is *Pediococcus acidilactici* SNGC2013. It has been established that inhibition of mould and yeast growth by *Lactobacillus buchneri* is due to production of acetic acid (Holzer *et al.*, 2003). Antifungal activities by a *Lactobacillus casei* strain that inhibited both the growth and the aflatoxins production of *Aspergillus parasiticus* have reported (Vanne *et al*., 2000, Lavermicocca *et al*., 2000). Several studies reported a *Lactobacillus plantarum* that was able to inhibit the growth of *Aspergillus flavus* but felt the effect was due to a combination of acidity and microbial competition. From the results, it could be safely concluded that the action of the suspensions of lactic acid bacteria used in this work is broad all being active against more than one. *Aspergillus* sp.

Bacteriocin was further purified (Lavermicocca *et al*, 2000) by gel filteration chromatography exhibited as one peak between the elution volume of 280 and 320ml and Ve/Vo of 4 as in the Figure 19 A. A protocol similar to this one but without gel filtration was used for purification of diploccocin and other protocols have been also used by several authors for purification of bacteriocins produced by other *Lactobacillus plantarum* strains (Gong *et al*., 2010). The fractions collected were pooled, lyophilized for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. There was a single band which was found to be Rf corresponding to a molecular weight of below 20,000D as indicated in the electrophoretic pattern, as expressed in the Figure 19 B which indicated the homogeneity of the enzyme The molecular mass of the *Lactobacillus acidophilus* 30SC bacteriocin was estimated at 3.5 kDa (Oh *et al*, 2000). Previous studies showed that the molecular weight of nisin extracted from growth media was about 3.5 kDa. Dehydroalanine, lanthionine and B-methyl lanthionine, and has a molecular weight of 3510 Da, which is inactivated by a-chymotrypsin, but is resistant to treatments with pronase, trypsin, and heat under acidic conditions.

Because of the heterogeneous nature of the bacterocins, a variety of combination of procedures has been suggested for their purification with varying success. The size of bacteriocins ranges from small peptides to large protein complexes with lipid and/or carbohydrate moieties. The majority are small cationic and hydrophobic molecules. The hydrophobic nature of the bacteriocins has complicated the purification process because bacteriocins tend to aggregate and sometimes nonspecifically adhere to materials that are employed during purification procedures. In some cases, bacteriocins are co purified with other cellular proteins (Giacometti *et al* 2000).

The inhibitory activity exerted by these strains appears to be the result of a low molecular weight protein as purified fraction showed substantial antibacterial activity against indicator organisms, and firmly suggests being a bacteriocin. LAB produces bacteriocins either spontaneously or by induction. The genetic determinants for most bacteriocins are located on plasmids, with a few exceptions of chromosomally encoded ones. Bacteriocins from Grampositive bacteria, do not bind to specific receptors for adsorption, are generally of lower molecular weight and demonstrate a broader spectrum of antimicrobial activity. The bacteriocins that are released are species specific. The majority of bacteriocins produced by LAB have been characterized according to their activity as a proteinaceous inhibition, on the estimation of their molecular mass, and on the determination of their spectrum of inhibition. (Ahire and Dicks, 2015).

As exhibited the maximum antimicrobial activity against *Listeria monocytogenes,* only it was taken up as in the Table 7. Inhibitory activity of bacteriocin produced by *Pediococcus acidilactici* SNGC 2013 did not decrease by heating, the

activity was stable at 150ºC for 25 min. The activity remained the same even though there is variation in pH ranging from 2.0 to 10.0 (3200). Begde *et al* 2011 showed that nisin was remained stable after autoclaving at 115.6ºC at pH 2.0, but loses 40% of its activity at pH 5.0 and more than 90% at neutral pH (6.8). Heat stability is a very useful characteristic in case of using bacteriocin as a food preservative, because many food-processing procedures involve a heating step.

Pediococcus acidilactici SNGC2013 displayed broad spectrum of antibacterial activity, as it inhibited *Escherichia coli, Listeria monocytogenes, Bacillus cereus, Pseudomonas aerogenes, Staphyllococcus aureus, Bacillus subtilis* and *Yersinia enterocolytica* indicator strains (Figure 21) (Guerra and Castro, 2002). Bacteriocins inhibit closely related bacteria can be included in the category of bacteriocins . Because molecular characterization of the compounds has not yet been done, they will be referred to as bacteriocin-like substances. The insensitivity to lipase A and amylase suggests the absence of a quaternary structure in which a lipidic or glucosidic moiety could be present. The antagonistic effects of bacteriocins against food spoilage, which is usually achieved by inhibition of *Pseudomonas, Staphylococcus aureus, Salmonella typhi and Listeria monocytogenes* (Chiang *et al*., 2000, Leroy *et al*, 2003) and they have great potential as biopreservatives for food (Gravesen *et al*., 2004).They are generally considered to act at the cytoplasmic membrane and disrupt the proton motive force through formation of pores in the phospholipid bilayer of microbes.

The diameter of the inhibition zone of various aliquots of the dialyzed protease were compared and presented in the Figure 2 and the inhibition zone of 0.0657 mg/ml bacteriocin was found to be predominant against pathogens such as as *Escherichia coli, Listeria monocytogenes, Bacillus cereus* and *Yersinia enterocolytica* as shown in the Figure 22. The inhibition of the pathogens was further studied by SEM as shown in the Figures 23. For nisin, it was reported that the cytoplasmic membrane is the main target, because treatment with nisin causes rapid, nonspecific efflux of amino acids and cations, and rupture of the cell membrane, resulting in the death of sensitive cells. The phospholipid composition of the membrane may be influential in the effectiveness of nisin. The combined results obtained in cells, vesicles and liposomes, suggest that the specificity of lactococcin A from *Lactococcus lactis* may be mediated by a receptor protein associated with the cytoplasmic membrane (Ana Maria and Letícia, 2013). The treatment of the cell walls to remove lipoteichoic acid prevented the binding of pediocin AcH from *Pediococcus. acidilactici*. It has also been suggested that lipoteichoic acid molecules, that are present only in Gram-positive organisms may be one of the binding sites for pediocin AcH. This may be the reason why LAB bacteriocins are adsorbed to Gram-positive bacteria and not to Gram-negative bacteria. The cell lysis is associated with cell death, and may depend on the strains of sensitive bacteria, presence of nonspecific receptor sites, such as lipoteichoic acid and specific receptors.

The foregone discussion concludes that the bacteriocin producing bacteria discussed so far are a part of the natural flora of both marine and fresh water fishes. Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium. *Pediococcus acidilactici* play an important role as probiotics in human and animal health (Haakensen *et al*, 2009). It can be used as probiotic bacteria, so many studies are undergoing. Probiotic must fulfill the following conditions: a normal inhabitant of the gut; ability to adhere to the intestinal epithelium, thus overcoming potential hurdles such as the low pH of the stomach; the presence of bile acids in the intestines; and competition with other microorganisms in the gastrointestinal tract **(**Dani *et al* 2002**).** When dogs with digestive disorders were treated by antibiotics together with *Pediococcus acidilactici* probiotic products, the surviving millions of *Pediococcus* product are able to alleviate the disruptive balance of microorganisms in dogs' GI tracts caused by antibiotics treatment and to normalize the intestinal microflora. Probiotic to influence the ecology of the gut. It has been postulated that certain gut microorganisms provide health benefits that include stimulation of the immune system, inactivation of potentially carcinogenic compounds, and reduction of serum cholesterol. Bacteriocins might enhance the ability of these organisms to colonize and compete with indigenous as well as potentially pathogenic gut micro flora. The inhibitory effect of Lactobacillus on growth of *Shigella sonnei* were studied Zhang et al, 2010. Shanmugam *et al*, 2001 studied the *Lactobacillus sp* in the production of chitin and chitosan from

shrimp (*Penaeus indicus)* shell waste by fermentation and their quality characteristics were analyzed. Researchers provided evidence that *Pediococcus* inoculants or purified pediocin can function as biopreservatives to eliminate Gram-positive pathogenic bacteria in cooked fish and meat during extended refrigerated storage. The inhibition of *Clostridium perifringes* by the bacteriocin plantaricin in meat samples is studied by Wijnker *et al*, 2011. The potential for structural manipulation with a ribosomally synthesized compound is great. This has yet to become a major emphasis of bacteriocin research, but with a gene replacement strategy such as that developed for nisin, the opportunity to develop genetically engineered variants of bacteriocins is greatly enhanced.

Bacteriocins are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Further, strain improvement using mutagenesis and/or recombinant DNA technology can be applied to augment the efficiency of the producer strain to a commercial status. The various nutritional and environmental parameters affecting the production of bacteriocins are delineated.

MATERIALS AND METHODS

The fish wastes were collected from freshly harvested fish procured from local market and brought under iced conditions. The fish wastes analyzed in the study included Tilapia, Rohu, Indian cat fish, Catla, Common carp and Tiger shrimp. All the chemicals, enzymes and microbiological media were dehydrated media (Hi-Media, Mumbai), were of analytical grade. All microbiological media used requisite quantities were prepared and sterilized before use at 121^0 C for 15 min at 15 lbs. The bacterial cultures such as *Bacillus cereus, Escherichia coli, Bacillus subtilis, Bacillus coagulans, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes, Yersinia enterocolitica, Aspergillus flavus* and *Aspergillus parasiticus* (Do you have genbank accession nos of this organisms.. or if ATCC mention ATCC number) were procured from Department of Microbiology, Sree Narayana Guru Collage, K.G. Chavadi, Coimbatore. The bacterial cultures were maintained at 6°C on Nutrient agar slants and fungal cultures on Potato Dextrose agar slants. The chemicals, enzymes and microbiological media used in the present study were of acceptable quality.

Fish wastes were homogenized under aseptic conditions in a top driven homogenizer (Polytron, Sweden) and taken for further analysis. The total plate count (TPC), total yeasts and mold count, total coliform count (TCC) and total LAB count of each sample were determined using standard methods (APHA, 2001). TPC was enumerated by employing plate count agar; while, total coliform count (TCC) was enumerated using eosin methylene blue (EMB) agar. Yeast and mold count was enumerated using potato dextrose agar (PDA). Lactic acid bacteria were isolated (Ammor *et. al*, 2005) using MRS agar (Hi-media- Deman Rogosa and sharp, 2003).

Effect of pH, temperature, salt concentration and incubation time of the isolates

The isolates were then characterized by their growth at various temperatures (10, 20, 30, 40, 50 and 60°C), tolerance of different salt levels (2, 3, 4, 5, 6 and 7% Nacl) and different pH (3, 4, 5, 6, 7 and 8 pH) and production of gas from sugars (Harrigan and McCance, 1990).

Identification and characterization of lactic acid bacteria

The distinct colonies of LAB were further picked for further characterization and identification. The isolates were characterized based on colony morphology, staining and biochemical tests to identify them up to the genus level by employing the schemes outlined in (Gerhard *et al*, 1994) and Bergey's Manual of Determinative Bacteriology (Bergey's *et. al*, 2002).

Effect of pH, temperature and incubation time of the isolates

The isolates that were exhibited maximum bacteriocin production were identified as *Streptococcus raffinolactis* SNGC2011*, Pediococcus pentosaceus* SNGC2012*, Pediococcus acidilactici* SNGC2013 and *Lactococcus lactis* SNGC2014, were cultured on MRS agar plates. MRS agar plates with different pH ranging from 3-11 were prepared using appropriate buffers (acetate buffer – 4.0; phosphate buffer – pH 7.0; tris-hydrochloric acid – pH 9.0 and Glycine-sodium hydroxide buffer – pH 11.0) and inoculated with the cultured isolate for determining the effect of pH on the bacteriocin production of the isolate. Further, MRS agar plates with pH that showed maximum bacteriocin production were prepared and inoculated with the isolate followed by incubation at different temperatures ranging from 10°C to 45°C to evaluate the effect of temperature on the bacteriocin production exhibited by the isolate. Similarly, the bacteriocin producing isolate was inoculated on to MRS

agar plates (with pH that gave maximum bacteriocin production) and incubated for varying incubation time ranging from 10 hours to 50 hours for evaluating the effect of incubation time on the bacteriocin production exhibited by the isolate. Growth was measured spectrophotometrically (Spectronic GENESYS 5, USA) at 600 nm in each case. As the maximum growth was shown at pH 4, 37°C and 24 hours, for measuring specific activity, the isolates were grown using these conditions in MRS broth in shaker incubator (Alpha scientific co, India).

Antibiotic sensitivity test

The 24 hour old cultures of *Streptococcus raffinolactis* SNGC2011*, Pediococcus pentosaceus* SNGC2012*, Pediococcus acidilactici* SNGC2013 and *Lactococcus lactis* SNGC2014, (from where these organisms procured, its not mentioned above.mention the source and genbank accession number ,if any) isolated from the fish wastes were seeded into the agar plates and allowed to dry. The antibiotic sensitive discs were placed on to the plates and incubated at 37ºC for 24 hours. The incubated plates were examined for zone of clearance around the antibiotic discs.

Optimizing the conditions for the activity of bacteriocin and cell growth of the isolates

About 10 ml of 24 hours old cultures of *Streptococcus raffinolactis* SNGC2011*, Pediococcus pentosaceus* SNGC2012*, Pediococcus acidilactici* SNGC2013 and *Lactococcus acidilactici* SNGC2014 cultures were taken and inoculated into 100 ml of nutrient broth, grown in shaker incubator (Alpha scientific) and (1) incubated at different temperatures ranging from 10ºC - 100ºC, keeping medium pH at 4/, incubation period of 24 hours, and agitation speed at 100 rpm. (2) Then keeping temperature/, at 37ºC, incubation periods of 24 hours, agitation speed at 100 rpm/, medium pH was varied ranging from 3 – 11/, (3) Incubation period was varied from 10 hours – 100 hours, keeping temperature at 37ºC, medium pH 4 and agitation speed at 100 rpm. (4) Agitation speed/, was varied from 25 rpm – 175 rpm, keeping temperature at 37ºC, medium pH 4 and incubation period at 24 hours. Growth was measured spectrophotometrically (Spectronic GENESYS 5, USA) at 600 nm in each case.

For determining the effect of medium pH, temperature, incubation period and agitation the bacteria were grown in MRS broth as mentioned in the procedure before. The cells were harvested by centrifuging at 10,000 rpm for 10min at 4^{o}C (Rota 4R – V/F M, Plato crafts, USA). The bacteriocin activity of lactic acid bacteria in the supernatant was assayed across different pH values ranging from 4 to 11 using the following buffer systems, as per the method of Chong *et al* (2000) with some modification; using buffers such as Citrate phosphate buffer (pH 4 & 5), Phosphate buffer (pH 6 - 8), Glycine-NaOH buffer (pH9 – 11) of (Chong *et al*, 2002). Briefly, the assay mixture consisted of 1.25ml of respective buffers of different pH, 0.5ml of 1% lactose, which acts as the substrate and 0.25ml of enzyme extract. The reaction was stopped by adding 3ml of 5% TCA (w/v) after holding the reaction mixture at 37^{o}C for 30mins. After standing at 4^{o}C for 5 mints, the solution was filtered and the TCA soluble peptides in the filtrate were quantitated by measuring the absorbance at 660nm using Lowry' method (Lowry *et al*, 1951). Blanks were obtained by adding TCA solution to the substrate prior to the addition of enzyme extract. The absorbance was converted into µg tyrosine using a standard curve. One unit of bacteriocin activity (U) was defined as µg tyrosine liberated per ml per min of the enzyme extract. Specific activity was expressed as units per mg protein (U mg^{-1} protein) of the enzyme extract. Since the effect of factors on growth and bacteriocin production was determined, *Pediococcus acidilactici* SNGC2013 was grown with the temperature 37ºC, medium pH 4 and agitation speed at 100 rpm.

Partial purification and characterization of the bacteriocin of *Pediococcus acidilactici* SNGC2013-A

The *Pediococcus acidilactici* SNGC2013 was grown and cells were harvested as mentioned before. The cell precipitates were discarded and the supernatant used for extraction and partial purification of the bacteriocin. The supernatants were precipitated with 70% ammonium sulphate and allowed to stand at 4^{o}C overnight. The resulting precipitates were collected by centrifugation at 10,000 rpm for 20min at 4^{o}C. The precipitates were dissolved in 0.9% NaCl and kept for dialysis at 4^{o}C in the refrigerator. The activities of bacteriocins obtained were assayed as mentioned before. The concentrated bacteriocins were lyophilized (Heto Dry Winner, Denmark) and used for further studies such as for the determination of the molecular weight and antibacterial activity.

Gel-filtration chromatography

Further purification of the bacteriocin was carried out by gel filtration using Sephadex G-100-120 (Sigma, MO, USA; Molecular weight above 100,000 Da are excluded) as column matrix. The column was packed to a height of 80 cm in a glass column having an internal diameter of 1 cm and equilibrated with 20 Mm Tris- HCl, pH 8.5

buffer. The flow rate was maintained at 20ml^{-1} hour. Fractions of 5.0ml each was collected from 1- 120 tubes. The absorbance at 280 nm was recorded and the activity bacteriocin determined as mentioned as previous section.

Determination of molecular weight and homogeneity by electrophoresis

The lyophilized bacteriocin obtained from the ammonium sulphate precipitate and dialysis was subjected to SDS PAGE for ascertaining the homogeneity and also to further purification as well as also to estimate the molecular weight of the protein. SDS PAGE was performed on a slab gel containing 10% polyacrylamide, which was employed in order to achieve separation of proteins in the range from 1,000 to 100,000 Da (Laemmli, 1970). To 50µl of bacteriocin sample 50µl of sample buffer was added and boiled in boiling water bath (Siskin) for 3-5 mins and after cooling was loaded into the gel. Proteins were detected using Coomassie Brilliant Blue G. The molecular weight of the purified bacteriocin was determined in comparison with standard molecular weight markers (Sigma, Molecular weight kit).

Effect of heat, pH and enzymes on antibacterial activity

To determine the thermal stability of bacteriocin, 1 ml (1mg/ml) of the crude bacteriocin was boiled for 15 minutes, cooled and assayed for activity. Another sample was autoclaved (121°C) for 15 minutes which acts as the control, cooled and assayed for activity.The sensitivity of bacteriocin to enzymes was tested. The enzymes such as trypsin, pepsin and proteiase K respectively were dissolved in sterile double distilled water, pH 4 at a concentration of 1 mg/ml stock. The working concentration was 200µg/ml. Freeze dried crude bacteriocins were added to enzyme solutions at a concentration of 1 mg/ml and the samples were incubated 37°C for 24 hours. The treated samples were tested for antibacterial activity by agar well diffusion method.

Freeze dried bacteriocin was dissolved in sterile double distilled water and was adjusted to various pH using 10 mmol/NaoH or 10 mmol/Hcl. The samples were then assayed for activity with appropriate controls.

Effect of lyophilized lactic acid bacteria culture filtrate against fungal growth and aflatoxin production

Preparation of spore suspension

The spore suspension of *Aspergillus flavus* and *Aspergiluus parasiticus* were grown on PDA slants for 7 to 10 days at 25 ± 3°C until well sporulated and stored at 5°C (Fan and Chen, 1999). Adding 10 ml of sterile water and aseptically dislodging the spores with a sterile inoculating loop harvested the spores. This was serially diluted to obtain a final spore suspension containing approximately 3.0×10^4 spores/ml of *Aspergillus flavus* and 2.6×10^4 spores/ml of *Aspergillus parasiticus* and the same were used for all experiments.

Medium and growth conditions

To the aliquots of 10ml Yeast extract sucrose (YES) broth, 100 µl of the spore suspension containing approximately 3.0×10^4 spores/ml of *Aspergillus flavus* and 2.6×10^4 spores/ml of *Aspergillus parasiticus,* individually were added. To this, lyophilized preparation of culture filtrate (1.25ml in sterile distilled water) was added and mixed well. This was incubated at 25°C ± 2°C in dark for 90 hours. A control containing 10 ml YES broth (without culture filtrate) was used. Mold growth was observed visually throughout the incubation period and mycelial weights were determined at the end of the incubation period. After incubation, the cultures were given a brief heat treatment (121^{o}C for 30 seconds) to kill the spores and vegetative mycelia. The mycelial mat were separated from the residual culture broth, washed with distilled water and dried at 85^{o}C for 24 hours. The weight of the dried mycelial mat was then determined. Throughout the entire study duplicate samples were taken and experiments were replicated three times to control variability percentage of inhibition of growth (%) was determined as follows = (1-T/C) × 100, where in T was weight of mycelial treated sample and C was weight of untreated mycelia (control).

Aflatoxin quantification

The aflatoxins were extracted from the YES broth with 5 ml of chloroform by liquid extraction. Total aflatoxin content was then determined by measuring UV absorption at 362 nm in an UV-VIS Spectrophotometer (Hitachi-Perkin-Elmer 139 UV–VIS spectrophotometer), and calculating total aflatoxin content using the molar extinction coefficient of 21,800 reported for aflatoxin B (Asao *et al*., 1963). Since the technique was designed to follow the general trend of aflatoxin production, any error introduced by assuming all of the absorption at 362nm to be aflatoxin B did not affect the final interpretation of the results. Thin-layer chromatographic examination of selected extracts indicated that the fluorescence intensities of the four aflatoxins were in general agreement with the absorption readings and reinforced this conclusion. Aflatoxin inhibition was

calculated as follows, % Inhibition = (1-t/c) × 100 where in t is concentration (µg/ml) of aflatoxin in treated sample and c is the concentration (µg/ml) of aflatoxin in control.

Assay for anti-microbial activity against pathogens – plate studies

Antimicrobial activity of bacteriocin produced by *Streptococcus raffinolactis* SNGC2011*, Pediococcus pentosaceus* SNGC2012*, Pediococcus acidilactici* SNGC2013 and *Lactococcus lactis* SNGC2014 were assayed as per the methods (Cappuccino and Sherman, 2004). For this purpose *Escheria coli*, *Listeria monocytogenes, Bacillus cereus, Pseudomonas aerogenes, Staphyllococcus aureus, Bacillus subtilis* and *Yersinia enterocolytica* were taken as the indicator organisms. These organisms were grown individually in nutrient broth for 24 hours at 37°C. The culture broth at 1% level was added into melted plate count agar, mixed uniformly and poured into sterile petri plates. Appropriate numbers of wells, each of 5 mm was made in the solidified agar. Aliquots (5µl, 10µl, 15 µl) of the partially purified bacteriocin was added into the wells and pre-incubated at 4°C for 3 hours to allow the test material to diffuse into the agar and plates were further incubated at 37°C for 18 hours. The incubated plates were examined for zone of clearance around the individual wells. The diameter of the zone of clearance, was measured and zones were compared with different amount of the samples and expressed as arbitrary units per ml (AU/ML) and was calculated as

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AU/ml = Diameter of the zone of clearance (mm) x1000 / Volume taken in the well (µl)

Anti-microbial activity of bacteriocins – Scanning electron microscopy (SEM) studies.

Partially purified bacteriocin of was added to 10^{-4}dilution of cells of *Escherichia coli* and was incubated at 37°C for 24 hours. After incubation, the cells were centrifuged at 6000 rpm for 15 minutes and washed twice with 0.01 M Potassium phosphate buffer pH 7.0. The samples obtained in the form of pellets after centrifugation at 6000 rpm were fixed with 2% glutaraldehyde for 2 hours at 4°C. The pellets thus obtained were dehydrated in a gradient ethanol (10-100%). Upto 40% ethanol centrifugation carried out after which the cells were transferred onto the slide and treated upto 100% ethanol. The slides were then dried in desiccators (Mc Dougall *et al*, 1994).

A carbon-coated membrane was placed on an Aluminum disk and the sample was placed on the lining. An inert metal like gold was coated on the sample and this was placed inside the scanning electron microscope (LEO 435VP, U K) at 20 KV attached to Mitsubishi Video copy processor. The amplified image was obtained by a 35 mm Ricoh camera that is connected to a monitor optically through fiber optics.

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