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PRODUCTION AND PURIFICATION OF BACTERIOCINS OF LACTIC ACID BACTERIA AND THEIR APPLICATION IN PREVENTION OF DENTAL CARIES

Dairy Technology

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ABSTRACT

Many strains of lactic acid bacteria produce bacteriocins and bacteriocin-like molecules that display antibacterial activity. Bacteriocins produced by lactic acid bacteria are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria. Major oral health problems around the world are generally considered to be dental caries. Oral health is now recognized equally important in relation to general health. In this study, bacteriocins were isolated from the species of lactic acid bacteria isolated from curd. The purified bacteriocins were then subjected for their anti-microbial effect against dental caries causing organisms isolated from teeth. Thus, it has been found that the bacteriocins of lactic acid bacteria have shown the anti- bacterial activity against strains of *Streptococcus sobrinus.* Since, these bacteriocins can inhibit the activity of dental cavity causing organisms, therefore, they can be inoculated in food products, so that the food itself becomes the weapon against cavities.

KEYWORDS

Lactic acid bacteria, dental caries, bacteriocins, *Streptococcus sobrinus*

Dental caries is the most common disease occuring in all age groups and is the major cause of oral pain and tooth loss. World Health Organization has pointed, that despite of great improvements in the oral health of population in several countries, dental caries still persists and has become a global problem (Yadav and Prakash, 2016).

A study was carried on dental caries at schools (urban and rural) of Gautam Budh Nagar, U.P. where children were divided in two groups. Group I contained age group of 7-9 years and Group II contained 10-12 years age children. Statistical analysis was done using Chi-square test. The results are shown in Figure 1. On analyzing the result of rural and urban schools, it showed that children of urban schools are more prone to dental caries than children of rural schools (Seth et al, 2016).

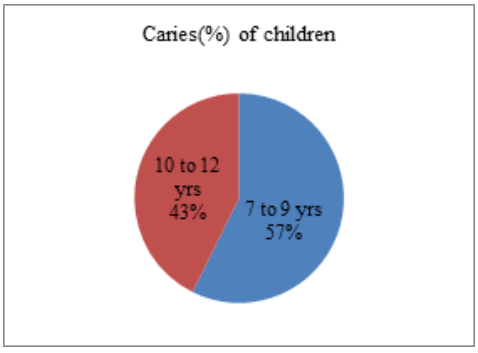


Fig. 1- Percentage of caries in children according to age

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming and non-motile bacteria which produces variety of antimicrobial compounds such as lactic acid, acetic acid, ethanol, formic acid, fatty acids, hydrogen peroxide and bacteriocins (Upendra et al, 2016). Bacteriocins are the bacterial peptides that help in regulating competitive interactions in natural microbial systems. They possess certain characteristics like narrow target range, stability, high activity and low toxicity. These properties position them as viable alternatives to existing antibiotics. Increasing antimicrobial resistance and growing awareness of microbiome for the importance of human health, underscore the need of this class of antimicrobials, as an approach for the treatment of infectious diseases (Dorit et al, 2016). These are believed to be safe for human consumption since they become inactive when comes in contact with digestive enzymes in the stomach (Upendra et al, 2016).

In a study, it has been demonstrated that nisin, pediocin, and two enterocins had antibacterial effects against several foodborne and spoilage bacteria (Turgis et al., 2012). A bacteriocin called mutacin 1140 is found to prevent dental caries by *Streptococcus mutans* (Bastos et al, 2015).

Material and methods

Collection of sample: Samples of curd were collected from different halwai shops. These were then subjected for isolation of lactic acid bacteria.

Isolation of lactic acid bacteria from curd: Suitable dilutions from curd was spread plated on selective media- MRS agar (from Hi Media company, Mumbai, India) and the plates were incubated at 37ºC for 24h. They were observed periodically for microbial growth. The pure cultures of this suspected lactic acid bacterial spp. was streaked on the slants of these selective media so as to obtain pure culture of the isolates. A lyophilized culture of *Pediococcus pentosaceus* PP34 as reference was inoculated in sterilized MRS broth (from Hi-media company, Mumbai, India) and incubated at 37ºC for 24h. The microbial growth was streaked on the plates of selective media- MRS agar so as to obtain pure culture.

Microbial and biochemical identification of isolates: Several identification tests were performed. The microbial staining include- Negative staining and Gram staining. Some confirmatory biochemical tests, selective for Lactic acid bacterial spp. were performed, which includes- catalase test: few drops of hydrogen peroxide (30% H_2O_2) were gently poured on microbial growth to observe for presence or absence of effervescence; utilization of different carbohydrate sources including arabinose, maltose, xylose, fructose and mannose.

Production of bacteriocin from the isolates of lactic acid bacteria: The isolated species of lactic acid bacteria and the reference culture were grown in sterilized MRS broth for 24 h at 37°C. The cells were removed by centrifugation at 14,000 rpm for 12 min in 1 ml sterilized Eppendorf tubes. The culture supernatant obtained was transferred to another sterilized Eppendorf tubes and cells were killed by boiling for 3-5 min.

Anti-microbial assay against *Streptococcus sobrinus*: Spot-on-lawn assay technique was done in order to determine the bacteriocin activity. The isolated strains of *Streptococcus sobrinus* from infected teeth were used as indicator organisms and plated on TYCSB agar media (composition by Wan *et al*, 2002).

Optimization of the conditions for maximum production of bacteriocin: The conditions which were used for optimizing maximum production of bacteriocins were- Medium: M-17; pH values

(5.0, 6.0 and 7.0) and temperature- 37°C, 42°C and 50°C. The samples were drawn at 0, 8,16, 24 and 36 h intervals and tested for pH and bacteriocin activity units. The results obtained were represented in the form of table and graph.

Partially purification of the bacteriocins: This was done using ammonium sulfate precipitation to achieve 60% saturation of the crude bacteriocin solutin and stirring which continued for another one hour in a cold room at 5 to 7°C. The mixture was then kept overnight in cold room. It was then centrifuged at 16000 rpm for 20 min. The precipitates were dissolved in distilled water. The supernatant was adjusted to 30, 60, 80 and 100% saturation levels by further addition of solid ammonium sulfate. The pellet in each case was dissolved in distilled water. The bacteriocin solution obtained after each ammonium sulfate fractionation step was dialyzed using a 1000 MWCO (molecular weight cut off) cellulose acetate membrane for about 18h against distilled water. The specific activity and recovery of the bacteriocin were calculated from the protein content and activity units of the samples.

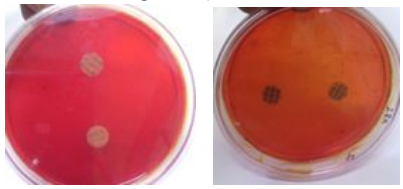
Results and discussion

Cultural characterization and microscopic identification of isolates: The total isolates obtained were 50 in number. In each culture, the colonies obtained were raised elevation, white colour, punctiform in size, entire margin and opaque appearance. Microscopic analysis showed some of the isolates in cocci shape and some in rod shape. Some of the cells were present in chains, some were mostly scattered and some in pairs. The cells of *Pediococcus pentosaceus PP34* were cocci in shape and scattered. All the cells were violet in colour i.e. all the cells were Gram positive.

The cells of *Lactobacillus plantarum* are short to medium rods occuring singly; cells of *Lactobacillus brevis* are short rods, occuring in short chains; cells of *Pediococcus pentosaceus* are cocci in shape, occuring in pairs and in tetrads and the cells of *Lactobacillus mesenteroides* are cocci or cocobacillary in shape (Fleming et al, 2007).

Biochemical examination of isolates: The 50 isolates obtained were subjected for catalase test. Only 13 isolates were catalase nagative which were subjected to further examination. Therefore, analysis for carbohydrate fermentation was done using mannose, maltose, fructose, arabinose and xylose. Out of 13 isolates, 4 isolates- 11(1), 13(3), 13(4) and 14(3) gave positive test for mannose, maltose, arabinose and 5 isolates-11(1), 13(3), 13(4),14(3) and 13(5) gave positive test for fructose fermentation. The reference culture gave positive test for fructose, maltose and mannose (Figure 2).

The species of *Lactobacillus bulgaricus* and Lactobacillus *helveticus* gave positive test for fructose and mannose and some strains of *Lactobacillus bulgaricus* gave positive test for maltose; while the species of *Lacobacillus acidophilus, Lactobacillus casei* and *Lactococcus lactis* gave positive test for maltose, fructose and mannose; the species of *Lactobacillus brevis* gave positive test for arabinose, fructose, mannose and some strains gave positive test for maltose (Nikita and Hemantgi, 2012).

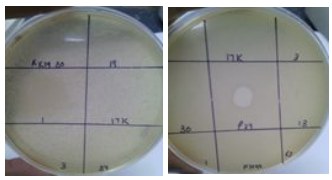


(a) Negative **(b)- Positive**

Figure 2: Sugar fermentation test

Anti-microbial assay against *Streptococcus mutans* and *Streptococcus sobrinus*: As indicator organisms, 13 isolates of *Streptococcus sobrinus* was used against 13 selected isolates of lactic acid bacterial isolates. Out of 13 isolates of bacteriocins, 5 isolates showed anti-microbial activity against *Streptococcus sobrinus* species. The result is shown in Figure 3.

The bacteriocins like Nisin A and mutacin B-Ny266 are active against a range of organisms, including species of *Actinomyces, Bacillus, Clostridium, Corynebacterim, Enterococcus, Gardnerella, Lactococcus, Listeria, Micrococcus, Mycobcterium, Propionibacte rium, Streptococcus, and Staphylococcus* (Mota et al, 2005)



(a)- Negative **(b)- Positive**

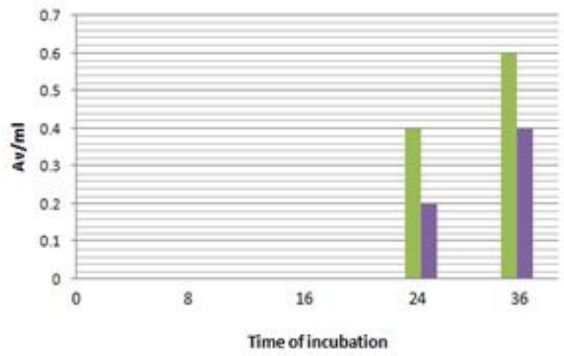
Figure 3: Anti-microbial assay

Optimization of conditions for maximum bacteriocin production: The optimization of conditions for different medium, pH and temperatures has been tabulated in Table 1, Figure 4(I), 4(II) and Figure 5(I), 5(II) respectively.

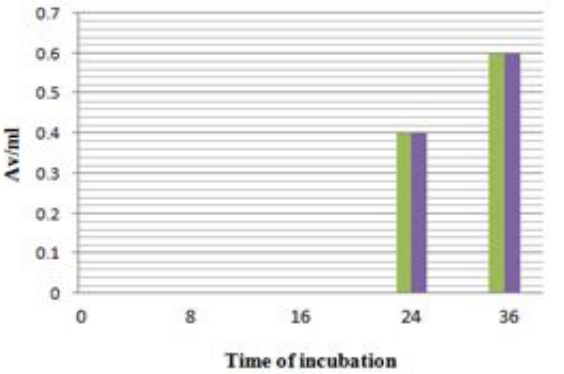
In a study, the optimization of conditions for bacteriocin producing strain of *Pediococcus pentosaceus,* the growth can be very rapid in broth and final pH in MRS broth is usually below 4.0. The growth of the bacterium is possible at pH 8.0 (Ludwig et al, 2011).

Table 1- Optimization of condition for different medium

	pH					AU/ml					Inhibition zone diameter (cm)				
	Incubation time (h)														
Isolates	**0**	**8**	**16**	**24**	**36**	**0**	**8**	**16**	**24**	**36**	**0**	**8**	**16**	**24**	**36**
11(1)	6.6	6.1	6.0	5.9	4.8	0	0	0	22(a)- 1,02,400 28(a)- 1,02,400 29(a)- 51,200 19(b)- 25,600 24(b)- 51,200	22(a)- 1,02,400 28(a)- 1,02,400 29(a)- 1,02,400 19(b)- 25,600 24(b)- 51,200	0	0	10	0.2	0.4
13(3)	6.6	6.04	5.67	5.31	3.98	0	0	0	28(a)- 12,800 24(b)- 12,800	28(a)- 12,800 24(b)- 25,600	0	0	0	0.3	0.4
13(4)	6.6	6.21	6.32	6.44	6.1	0	0	0	28(a)- 25,600 24(b)- 12,800	28(a)- 25,600 24(b)- 25,600	0	0	0	0.3	0.3
13(5)	6.6	6.07	5.38	4.7	4.82	0	0	0	28(a)- 12,800 24(b)- 51,200	28(a)- 12,800 24(b)- 25,600	0	0	0	0.2	0.3
14(3)	6.6	6.35	5.92	5.5	4.0	0	0	0	28(a)- 12,800 24(b)- 51,200	28(a)- 12,800 24(b)- 51,200	0	0	0	0.3	0.4
Pediococcus pentosaceus PP34	6.6	6.2	6.16	6.12	4.1	0	0	0	22(a)- 12,800 28(a)- 2,04,800 29(a)- 51,200 19(b)- 12,800, 24(b)- 12,800	22(a)- 12,800, 28(a)- 51,200 29(a)- 51,200 19(b)- 25,600 24(b)- 25,600	0	0	0	0.3	0.4

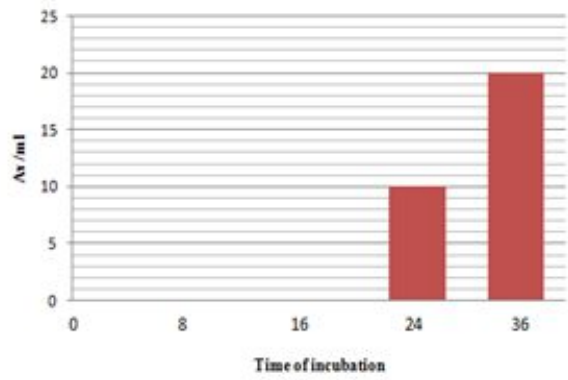


I. Influence of bacteriocin 13(3) production against organism 28(a)

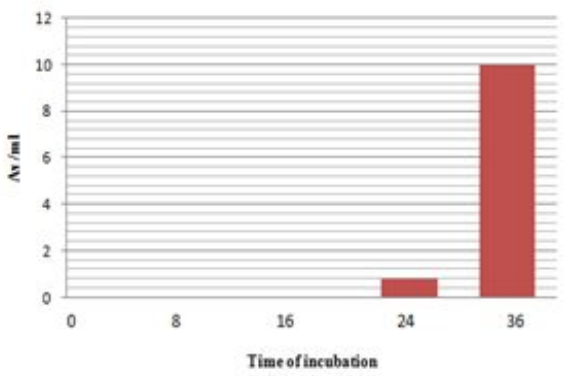


I. II. Influence of PP34 production against organism 22(a)

Figure 4: AU/ml graph for initial pH



I.Influence of bacteriocin 11(1) production against organism 28(a)



II. Influence of bacteriocin 11(1) production against organism 24(b)

Figure 5: AU/ml graph for different temperature

Partially purification of the bacteriocins: The results for the ammonium sulfate precipitation is given in Table 2.

In a study of the ammonium fractionation of the bacteriocin of lactic acid bacteria, total protein (mg)- 1920 (U), specific activity (U/mg)-19.45 and yeild- 85.71 has been recorded (Elayaraja et al, 2014). In a study of the bacteriocin Pediocin PA-I isolated from Pediococcus spp, the ammonim sulphate precipitation purification, gave, total activity (AU)-819.2 X 104, total protein concentration-498.8, specific activity-16.4 x 103 and overall yeild- 80% (Garsa et al, 2014).

Table 2- Partial purification of bacteriocin

Bacteriocins	Ammonium sulphate saturation (%)	Total bacteriocin	Total protein	Specific activity (AU/mg protein	Purification fold	Recovery (%)
11(1)	0	50,00,000	1500	3200	1	100
	0-30	1,00,000	20	5500	1.5	1.8
	30-60	10,00,000	50.5	10285.714	3.0	8.25
	60-80	90,000	21.22	5181.18	1.2	1.5
	80-100	50,000	8.56	4716.98	1.5	0.525
13(3)	0	45,00,000	1000	2700	1	100
	0-30	80,000	20	5500	1.5	1.8
	30-60	10,00,000	45.8	10000	3.0	8.25
	60-80	65,000	17.45	4658.18	1.2	1.5
	80-100	20,000	6.56	3256.98	1.5	0.525
13(4)	0	50,00,000	1500	3200	1	100
	0-30	1,00,000	20	5500	1.5	1.8
	30-60	10,00,000	50.5	10285.714	3.0	8.25
	60-80	90,000	21.22	5181.18	1.2	1.5
	80-100	50,000	8.56	4716.98	1.5	0.525
13(5)	0	50,00,000	1500	3200	1	100
	0-30	1,00,000	20	5500	1.5	1.8
	30-60	10,00,000	50.5	10285.714	3.0	8.25
	60-80	90,000	21.22	5181.18	1.2	1.5
	80-100	50,000	8.56	4716.98	1.5	0.525
14(3)	0	75,00,000	2000	4500	1	100
	0-30	4,00,000	40	6000	1.5	1.8
	30-60	30,00,000	65.5	15789.00	3.0	8.25
	60-80	1,00,000	25.22	7000	1.2	1.5
	80-100	70,000	10.56	5564.45	1.5	0.525
Pediococcus pentosaceus PP34	0	45,00,000	1200	2800	1	100
	0-30	80,000	15	5600	1.5	1.8
	30-60	10,00,000	42.8	9654.89	3.0	8.25
	60-80	65,000	16.66	4600	1.2	1.5
	80-100	20,000	9.56	4716.98	1.5	0.525

Conclusion:

Salient findings of the study suggests that 5 isolates showed anti bacterial activity against isolates of *Streptococcus sobrinus*. These five isolates could be regarded as strains of species of *Lactobacillus brevis* having different properties. Therefore, these bacteriocins from lactic acid bacteria can be applied to various food products which have tendancy to inhibit dental cavities in humans. Thus, these bacteriocins will help in preventing cavities in consumers from the bacteriocin fortified food sources.

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