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Bacteriocin and Cellulose Production by Lactic Acid Bacteria from Raw Cow Milk

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Bacteriocin producing *Lactobacillus brevis* strain isolated from raw cow milk showed high range of antimicrobial activity against some food borne pathogens such as *Brevibacillus brevis, Proteus vulgaricus, Salmonella typhi and Pseudomonas sp.* Maximum growth of *Lactobacillus brevis* strain was found in 36th hour culture. The bacteriocin has purified by column chromatography (DEAE cellulose). The purified Bacteriocin like substances was identified as 29 K Da peptide by SDS PAGE. The study revealed the possibility of using bacteriocin as a food preservative and the *Lactobacillus brevis* strain as a probiotic. Cellulose production was estimated by Upedegraff method.

Key words: Bacteriocin, Cellulose production, Lactic acid bacteri, Raw cow milk.

Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Tagg *et al.,* 1976). Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which the important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, and plantaricins (Nettles and Barefoot, 1993).

Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliand, 1990). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990). Lactic acid bacteria are useful in the food industry. They reduce the pH in food, low enough to inhibit the growth of most of other microorganisms including common human pathogens, thus increasing the shelf life of fermented food (Ivanova *et al.,* 2000).

Cow milk acts as an effective prebiotic (i.e., a food that selectively stimulates the growth of beneficial bacteria in the colon). The high concentrations of lactose and non digestable oligosaccharides found in cow milk promote the colony formation of *Bifidobacteria* and *Lactobacillus* spp. (Yoshioka et al., 1983). As a result intestinal colonization with *Bifidobacterium* and *Lactobacillus* spp. discourages the growth of *Clostridium* sp. and other pathogens.

Cellulose is an exopolysaccharide produced by microbial cultures and are involved in cell adhesion and biofilm formation. Lignin, hemicellulose and xylosans are other products from a microbial culture which can be extracted with acetic-ntiric acid reagent. Cellulose remains dissolved in H_2SO_4 and is determined by anthrone

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reagent (Updegraff, 1969). Enhancement of cellulose production in a co-culture with various *Lactobacillus mali* strains showed that cell-cell interaction assisted by exopolysaccharide producing *Lactobacillus mali* promotes cellulose production in st-60-12 (cellulose producing acetic-acid bacterium such as *Glucoacetobacter xylinus*) (Seto *et al.,* 2006).

Hence, the production of bacteriocin and cellulose was much useful in food industry. The production was extra cellularly got from lactic acid bacteria which were isolated from raw cow milk.

MATERIAL AND METHODS

Isolation of Lactobacillus from raw cow milk

The milk sample was collected from healthy cow and it was stored in cool Temperature for further studies. Then the sample was serially diluted and 1 ml of each dilution were plated on Nutrient agar and incubated for 24 hours. The different colony types were taken and Gram staining was performed for identification of Gram-positive lactic acid bacteria.

Identification of bacteriocin producers

Physiological and biochemical tests were done to identify the lactic acid bacteria that produce Bacteriocin. Identified organism was subjected to isolation by pure culture technique.

Time kinetics determination

The test organisms were inoculated into Nutrient broth and kept in shaker at 150 rpm at 38^0C. Samples were taken at different time points such as 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs, 72 hrs to study the time kinetics of the strain of interest. The samples at different time point were processed separately and centrifuged at 12000 rpm for 10 minutes at 4^0 C. The supernatant were transformed into sterile tubes. The supernatants were precipitated in 70% methanol and 10 %TCA (Trichloro acetic acid). The above obtained precipitates were subjected to antibacterial assay.

Partial purification of crude protein

Partial purification of the crude extract was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure of Stempion *et al.,* (1970).

Protein estimation

Protein estimation was done as described by Lowry *et al.,* (1946), using Bovine serum Albumin at the rate of 1mg/ml as the standard. Different concentrations of the standard ranging from 0.1 to 1mg/ml were taken and made up to 1 mg/ml. Then 5ml of alkaline copper reagent was added, mixed well and allowed to stand for 10 minutes at room temperature. Then 0.5ml of diluted Folin's phenol reagent was added and mixed well. The mixture was incubated for 30 minutes at room temperature. The absorbance at 650nm was read spectrophotometrically. The protein concentrations of Bacteriocin extracts were estimated.

Antibacterial assay by disc diffusion method (Tramer and Fowler, 1964)

The experiment was conducted to determine the antibacterial activity of Bacteriocin produced. Muller Hinton agar poured to sterile Petri dishes and different pathogens (*Brevibacillus brevis*, *Proteus vulgaricus*, *Lactobacillus bulgaricus* , *Lactococcus lactis* , *Escherichia coli, Salmonella typhi*, *Psuedomonas spp.*) were swabbed by using sterile cotton swabs. Sterile filter paper disc impregnated with 12 µl of bacteriocin were placed in the agar plate. The plates were incubated at 37°C for 24-36 hrs. The zone of inhibition was then measured.

Molecular weight determination

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS -PAGE)

Sodium dodecyl sulphate poly acrylamide gel electrophoresis was carried out using treatment of Sodium dodecyl sulphate (SDS), by the method of Tomoeda *et al.,* (1968).

Cellulose production

Mass production of *Lactobacillus brevis* was done to derive high quantity of cellulose. Cellulose was assayed using a method described by Updegraff in 1969, where the fiber is dissolved in acetic acid and nitric acid to remove lignin, hemicellulose, and xylosans. The resulting cellulose is allowed to react with anthrone in sulfuric acid. The resulting coloured compound is assayed using spectrometer.

Estimation of Cellulose

Cellulose estimation was done by taking optical density at 630nm wavelength against a reagent blank (Capaldo-Kimpal and Barbour, 1971).

RESULTS

Isolation and Identification of *Lactobacillus brevis*

The organism isolated from pure culture methods was identified as *Lactobacillus brevis* by microscopically, colony morphology and standard biochemical tests.

Time kinetics determination

The test organism were inoculated into nutrient broth and kept in shaker, at 150 rpm at 38^0C for time kinetics determination. The samples were taken at different time points such as 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs. The OD values of the samples were recorded and plotted on a graph. Later it was determined at 36^{th} hrs incubation the growth level attained stationary phase.

Protein estimation

The protein content in bacteriocin from milk sample was found to be 2.09mg/ml

Isolation of bacteriocin for antibacterial assay (Tramer and Fowler, 1964)

The samples at different time point (as showed above) were processed separately and centrifuged at 12000 rpm for 10 minutes at 4^oC. The polypeptides in the supernatants were precipitated in 70% methanol and 10% Trichloro acetic acid (TCA) in separate tubes. The TCA and methanol precipitates were dissolved in phosphate buffered saline (PBS) and subjected to antibacterial assay. sample was screened for bacteriocin activity against different pathogenic bacteria *(Brevibacillus brevis*, *Proteus vulgaricus Lactobacillus bulcaricus*, *Lactobacillus lactics*, *Escherchia coli*. *Salmonella typhi*, and *Pseudomonas spp)*. Among the seven pathogens tested bacteriocin activity was noticed against only four pathogens such as, *Brevibacillus brevis*, *Proteus vulgaricus*, *Salmonella typhi and*

Table 1. Biochemical test for *Lactobacillus brevis*

S. No	Organisms	Test						
		Gm	mot	Ind	MR	VP	Cit	Cat
1	*Lactobacillus brevis*	+	-	-	-	-	-	+

Gm – Gram Staining, mot – Motility, Ind – Indole, MR – Methyl Red,
VP – Voges Proskauer, Cit – Citrate, Cat – Catalase.

Table 2. Time kinetics determination

S.No.	Time Intervals (hrs)	OD value (750 nm)
1	12	0.8
2	24	1.6
3	36	2.3
4	48	1.8
5	60	1.6
6	72	1.0

Table 3. Antimicrobial activity Zone of inhibition

S. No	Pathogens	Zone of inhibition(mm) at different Time Intervals (hrs)					
		12	24	36	48	60	72
1.	*Brevibacillus brevis*	-	-	1.62	0.9	-	-
2.	*Proteus vulgaris*	-	0.8	1.0	0.8	-	-
3.	*Lactobacillus bulgaricus*	-	-	-	-	-	-
4.	*Lactococcus lactics*	-	-	-	-	-	-
5.	*Escherichia coli*	-	-	-	-	-	-
6.	*Salmonella typhi*	-	0.9	0.2	0.7	-	-
7.	*Pseudomonas spp.*	-	-	1.2	0.8	-	-

Pseudomonas spp. In the sample tested 36th hr and 48th hr sample showed activity against four positive pathogens. 24th hr cultures precipitate shows activity against only *Protease vulgaricus and salmonella typhi*. Where as, 36th, and 48th hr cultures precipitate shows activity against *Brevibacillus brevis, Proteus vulgaricus, salmonella typhi and Pseudomonas spp.* 12th, 60th and 72th hr cultures does not show any activity for all seven pathogens.

Among them, TCA precipitate of 36th hr cultures showed largest zone of growth inhibition (**1.62 cm**) against *Brevibacillus brevis*. It was selected for further studies

Estimation of cellulose

The cellulose content of the sample was analyzed and estimated as 0.52 U/gm

Molecular weight analysis by SDS-PAGE

For this experiment, samples were collected before and after heat shock treatment of the bacteria *Lactobacillus brevis*. The crude supernatant and the intracellular proteins were run on SDS-PAGE along with the molecular weight marker. While comparing the lane 1 with lane 3, the intracellular protein profile showed the presence of stress protein, but a better expression of the 29 KDa heat shock protein was found only after the heat shock treatment of the same 36 hr culture.

DISSCUSSION

The present study was primarily aimed at bacteriocin and cellulose production from *Lactobacillus brevis* where bacteriocin had a wide inhibitory spectrum towards both Gram-negative and Gram-positive food spoilage and pathogenic bacteria. Graciela in 1995 produced bacteriocin in a mixed fermentation environment, which proved advantageous for a producer organism to dominate the microbial population.

In this work *Lactobacillus brevis* was selectively isolated and identified from the sample. A similar kind of work was done by S.T Ogunbanwo *et al.*, 2003 from the sample wheat maize. One of the LABs isolated in his study (*Lactococcus lactis)* has also been found as one of the dominant flora of Beyaz cheese by Durlu-Ozkaya *et al.*, 2001; Erdogan and Gurses, 2005.

The production of bacteriocin by *Lactobacillus brevis* at the time intervals of 12hrs, 24hrs, 36hrs, 48hrs, 60hrs and 72hrs were estimated and the OD values are 0.8, 1.6, 2.3, 1.8, 1.6, 1.0 respectively at 750nm in the present study. A similar work was carried out by Balasubramanyan and Varadaraj in 1998 at the absorbance value of 580nm.

Partial purification of bacteriocin was done by Column chromatography using DEAE cellulose. A similar method was followed by Kato *et al.*, in 1994 for partial purification of bacteriocin.

Protein estimation was done based on Lowry *et al.*, 1969. The amount of protein was found to be 2.09mg/ml.Bacteriocin is encoded by a plasmid of approximately 5.5 kbp in size. Pediocin like bacteriocins may be either plasmid encoded (Gonzalez and Kunka, 1987) or genomically encoded (Holck *et al.*, 1994).

Antimicrobial activity test was done with seven pathogens in which four species showed zone of inhibition *Proteus vulgaricus, Salmonella typhi, Brevibacillus brevis, Pseudomonas spp., Brevibacillus brevis* showed highest antimicrobial activity which was evident from the zone of 1.62cm in diameter. Antimicrobial activity of bacteriocin work was done by Nettles and Barefoot in 1993 in which the highest inhibitory activity was against *Proteus vulgaricus.*

Cellulose production was estimated to be 0.52U/g using Updegraff 1969. This cellulose production which is a virulence factor in pathogenic organisms has been reported (Reed *et al.*, 1988; Davies *et al.*, 1993; Gulsun *et al.*, 2005) and is probably the reason for the potency of the bacteriocin like substances produced by *Lactobacillus plantarum* and *Lactococcus lactis* strains in this study which is nonpathogenic.

CONCLUSION

Bacteriocins and cellulose produced by lactic acid bacteria are of biological source, biodegradable and will not result in any side effects. Bacteriocins may serve as alternative preservative agents for the functional foods and can be applied in food technology. Bacteriocin and cellulose production were of commercially valuable and developing production now a days.

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