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Effect of Media on Bacteriocin Production by *Lactobacillus brevis* and Evaluation of Anti-Bacterial Activity.

S Karthick Raja Namasivayam*, J Ceely Rebaca Angel, RS Arvind Bharani, and MY Karthik.

Department of Biotechnology, Sathyabama University, Chennai 600119, Tamilnadu, India

ABSTRACT

Bacteriocins are peptides or proteins, produced by *Lactobacillus* species which have an antimicrobial activity against microorganisms and extensively used as food preservative. The present study aimed to study the effect of media on bacteriocin production by *Lactobacillus brevis* isolated from curd, cabbage and meat and its antibacterial activity against common food spoiling bacteria. In this study, MRS medium with different carbon sources such as sucrose, lactose and maltose nitrogen sources such as tryptone, glycine, cysteine, histidine, ammonium nitrate, urea and soyabean meal. Maximum bacteriocin production was recorded in MRS media with maltose and soyabean meal. The ammonium sulfate precipitation and dialysis improved bacteriocin activity of bacteriocin derived from all the media. Effect of temperature revealed bacteriocin could retain the activity at 100^0C. From this study it is cleared that the bacteriocin from *Lactobacillus brevis* can be recommended as antibacterial agent against possible food borne pathogenic bacteria.

Keywords: *Lactobacillus brevis*,bacteriocin,media,purification

Corresponding author

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INTRODUCTION

One of the concerns in food industry is the contamination by pathogens, which are frequent cause of food borne diseases. Over the past decade, recurrent outbreaks of diarrhea, combined with the natural resistance of the causative agents, contributed to its status as hazard.In the recent years the food industry faced the need of increasing the possibilities for better conservation and of the food products [1].Today, the conservation is commonly performed by sterilization or by adding sugar, salt, organic acids or by smoking. However, some of these compounds change the taste quality and the appliance of others is not healthy. For improving the quality of the products the approach by which chemicals are added must be ceased and the sterilization must be avoided as for as possible. A new protection is required, which is healthy and natural [2].Biotechnology in the food-processing sector targets the selection, production and improvement of useful microorganisms and their products, as well as their technical application in food quality.The use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods is defined as biopreservation [3].Antagonistic properties of lactic acid bacteria (LAB) allied to their safe history of use in traditional food fermented products make them very attractive to be used as biopreservatives [4].Antibiotics are at present restricted for use in foods and feeds, and bacteriocins are an interesting group of biomolecules with antimicrobial properties that may represent a good alternative[5,6]. Bacteriocins are peptides or proteins, which have an antimicrobial activity against closely related microorganisms. Many of the bacteriocins produced by lactic acid bacteria are proved to inhibit or eliminate the growth of food borne pathogens such as *Listeria monocytogenes, clostridium perfringens, Bacillus cereus,* and *Staphylococcus aureus*. Bacteriocinogenic lactic acid bacteria and/or their isolated bacteriocins are considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed. Bacteriocin production could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources [7,8].In the present study, effect of media on bacteriocin production by *Lactobacillus brevis* isolated from different samples has been carried out.

MATERIALS AND METHODS

Isolation of *lactobacillus*

Curd, cabbage and meat were used for the isolation.

Isolation from curd

The curd sample was collected from home in sterile screw cap vials and brought to the laboratory and stored at 4˚c in refrigerator till isolation. 1ml of the sample was added to 9ml of sterile distilled water and serially diluted. .1ml of the sample was spread plated on sterile deMan Ragosa and Sharpe (MRS) agar medium. The seeded plates were incubated at 37˚c for 24-48 hours. The individual colonies were developed and it was counted and used for further studies.

Isolation from cabbage

Fresh healthy cabbage was purchased from retail market in Tondiarpet and brought to the laboratory in ice box. 25g of fresh cabbage was homogenized in 225 ml of quarter strength phosphate buffer and 1ml of this homogenized suspension was serially diluted and plated on MRS agar media as described earlier.

Isolation from meat

Fresh healthy meat was purchased from retail market in Tondiarpet and brought to the laboratory in a sterile polythene bag and kept in ice box. 25 gm of fresh meat was taken and homogenized in 225ml of quarter strength phosphate buffer and 1ml of the homogenized suspension was serially diluted and plated on MRS agar media as described earlier.

Identification

The respective bacterial isolates stored on MRS slants as pure culture were subjected to morphological, physiological and biochemical characterization according to Bergeys manual of systemic bacteriology. Majority of *Lactobacilli* obtained belong to *Lactobacillus brevis. Lactobacillus brevis* was maintained on MRS slants as pure culture.

Bacteriocin production

Isolated *Lactobacillus brevis* was inoculated in MRS broth and incubated at 37˚c for 24 hours. After incubation, the broth was centrifuged at 10000 rpm for 10 minutes and the cells were separated out. The active cell free supernatant was used for crude bacteriocin assay and further steps were done based upon the purification method such as ammonium sulfate precipitation , dialysis and assay was done.

Bacteriocin assay

The centrifuged or partially purified proteinaceous compound from *Lactobacillus brevis* was subjected for its bactericidal activity against common pathogens involved in food spoilage. The organisms selected to test the inhibitory effect of Bacteriocin are

- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Serratia marcescens*

Anti-Bacterial assay

Preparation of Test Cultures

All the test cultures are grown in nutrient broth medium, and incubated at 37˚c for 12-24 hours.

Agar well diffusion technique

Antimicrobial activity of the bacterial isolates against pathogenic microorganisms was determined by well diffusion method under aerobic conditions. Muller Hinton Agar was prepared and swabbed with test culture individually. Wells (6mm) were cut into the plates and 100µl of cell free culture supernatant fluid of the *Lactobacillus brevis* was placed into each well. Plates were kept at cool temperature for 2 h and then incubated at 37˚c for 24 hours. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

Optimisation for bacteriocin production

Effect of nutrient factors on bacteriocin production

Effect on carbon sources

MRS media of 100ml was prepared and filter sterilized carbon sources (sucrose or lactose or maltose) were added aseptically in the amount of MRS + Sucrose (0.01%,)MRS +Lactose (0.01%),MRS + Maltose (0.01%). MRS media of 100ml was prepared and inoculated with *Lactobacillus brevis* culture and incubated at 37˚c for 24 hours. Purification process such as ammonium sulfate precipitation and dialysis was done as described later and bacteriocin assay was done as described earlier.

Effect on nitrogen sources

MRS media of 100 ml was prepared and nitrogen sources such as aminoacids and other compounds were added in the amount of MRS + Histidine (0.01%),MRS + Glycine (0.01%),MRS + cysteine (0.01%), MRS + Tryptone (0.01%), MRS + Ammonium nitrate (0.01%),MRS + Urea (0.01%), MRS + Soyabean meal (0.01%)

MRS media of 100ml was prepared and inoculated with *Lactobacillus brevis* culture and incubated at 37˚c for 24 hours. Centrifugation and further purification process such as ammonium sulfate precipitation and purification was done. After purification, bacteriocin assay was done as described earlier.

Partial purification

Ammonium Sulfate Precipitation

The charges on a protein solution can be neutralized by addition of salts and this has been used in purification of proteins.

Lactobacillus brevis was isolated and was inoculated and allowed to grown in MRS broth at 37˚c for 24 h. After incubation, the broth was centrifuged at 5000 rpm for 10 minutes and the cells were separated out. Different concentrations of ammonium sulfate were added to the supernatant. After stirrig on a magnetic stirrer, it was kept undisturbed at 4˚c overnight. Precipitates formed were collected by centrifugation at 10,00 rpm for 10 min and redissoloved in 20mmol sodium phosphate buffer with pH=6.0. And the bacteriocin assay was done as described earlier:

Dialysis

Dialysis is a process that is based on the principle of osmosis. Moving from an area of high concentration to lower concentration. Dialysis was done with the semipermeable membrane that has pore of varying molecular weight cut off(MWCO). These pores allow smaller substance or compound to flow between them.

Activation of membrane

Membrane is boiled with 2% sodium carbonate and 0.03% EDTA for 10 minutes to remove sulphides and heavy metals. Then the mrmbrane was placed in 1%sucrose solution for 30 minutes to remove water. Dialysis is commonly used for removing salts from proteins. The presence of salts in proteins interferes in many ways. Hence semipermeable membranes called dialysis tubes that have property to allow compounds with small molecular weight.

The protein solution to be desalted is taken inside a dialysis bag and the two ends secured tightly to prevent leakage.The bag is now suspended in a large beaker containing 500 ml of 50Mm phosphate buffer at pH 7.The salt molecule passes freely and gets diluted by the large volume of phosphate buffer.To concentrate the protein sample, dialysis bag was suspended in sucrose solution. Where the water inside will move out, get absorbed by sucrose. Sucrose being impermeable remains in the solution. The bag contains desalted partially purified protein.

Bacteriocin yield

Bacteriocin yield was calculated by purifying the known volume of bacteriocin through ammonium sulfate precipitation and dialysis techniques. The final volume of bacteriocin was dried under hot air oven at 40˚c for 2 hours. The yield of bacteriocin was calculated from the following formula,

$$\text{Percentage} = \frac{\text{Weight of the sample before Dialysis}}{\text{Volume of the sample used for Dialysis}} \times 100$$

Estimation of protein

The partially purified proteins are estimated quantitatively by lowry *et al* . method.

Effect of parameter on bacteriocin activity

Temperature

A volume of 5mL of bacteriocin were used for assay which was obtained after centrifugation, ammonium sulfate precipitation, dialysis. 5mL was taken in different test tubes was overlaid with paraffin oil to prevent evaporation and then heated at 50˚c, 60˚c and 70˚c for 15 minutes. The heat treated bacteriocin samples were then assayed for antimicrobial activity as described earlier.

RESULTS AND DISCUSSION

Isolation and identification of *L.brevis*

A total of 396 isolates from 20 curd,300 isolates from 20 cabbage,74 isolates from 5 meat samples were obtained.(Table 1).Identification of *L.brevis* was carried out by morphological and biochemical methods.Ogunbanwo *et al* [9] isolated *Lactobacillus brevis* from ogi and cassava (African fermented food). Bromberg *et al* [10] isolated bacteriocins producing lactic acid bacteria such as *Pediococcus, Leuconostoc, Carnobacterium* and *Lactobacillus* species from meat and meat products. Callewaert *et al* [11] isolated *Lactobacillus amylovorus* DCE 471 from corn steep liquor.Joshi *et* al [12] isolated *Lactobacillus plantarum* and *Lactococcus lactis* from fermented vegetables.

Effect of media on bacteriocin production

Modification of nutrients of cultivation media should be considered for maximal production of bacteriocin that has potential use as a food biopreservative [13]. In the present study, bacteriocin activity was evaluated against three bacterial species in MRS media supplemented with various nitrogen and carbon sources Crude bacteriocin derived from MRS media was collected,centrifuged, and the supernatant was subjected to ammonium sulphate precipitation followed by dialysis.Partial purified bacteriocin showed distinct activity against the tested strains. did not show any distinct anti bacterial activity against all the tested strains. Among the different nitrogen sources tested, MRS media supplemented with soy bean meal recorded maximum anti bacterial activity against the tested bacterial strains.20.0,23.5 and 21.0 mm of zone of inhibition was recorded against *E.coli,K.pneumoniae* and *S.marcescens* (Figure 1,2).Media with cysteine and histidine revealed 15.0,14.0,17.0mm and 18.0,19.0,14.5mm of zone of inhibition against the respective bacterial strains. Similar finding could observed in tryptone (Table2).Media with urea and ammonium nitrate did not show any improved activity. Effect of nitrogen sources on bacteriocin production was reported by Daba et al [14] Biswas *et al* [15], Parada *et al* [16].Among the different carbon sources, MRS media with maltose derived bacteriocin recorded maximum anti bacterial activity against all the tested strains.23.0,20.0 and 21.5mm of zone of inhibition was recorded (Table 3).

Partial purification

Partial purification was carried out by ammonium sulphate precipitation at 40,60 and 80 %.Bacteriocin activity was found to be maximum against all the tested bacteria fractionated with 60 and 80 % ammonium sulphate precipitation.Bactericon yield was found to be in soya bean meal and maltose supplemented media.

Effect of temperature

Bacteriocin derived from the respective media could retained all the tested temperature and maximum activity was recorded at 100˚C and moderate activity at 60 and 80˚C. Joshi *et al* [12] also reported similar finding.Bacteriocin produced by natural fermentation of carrot,radish and cucumber retained activity at 100˚C.

In conclusion, bacteiocin from *Lactobacillus brevis* isolated from curd, cabbage and meat samples possesses a distinct inhibitory activity against *Escherichia coli, Klebsiella pneumoniae* and *Serratia marcescens.* It also revealed that *Lactobacillus brevis* is thermostable. It can withstand temperature upto 100˚C. Therefore it has a potential for application as a biopreservative in different food products. Since lactic acid fermentation is employed mostly for development of products, especially for flavor and taste of fermented products, the production of bacteriocin in such products assumes more significance as biopreservative apart from imparting probiotic effect to the product.

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Table 1: Samples selected for isolation *L.brevis*

S.No	Sample	No.of samples	N.o of isolates
1	Curd	20	396
2	Cabbage	20	300
3	Meat	5	74

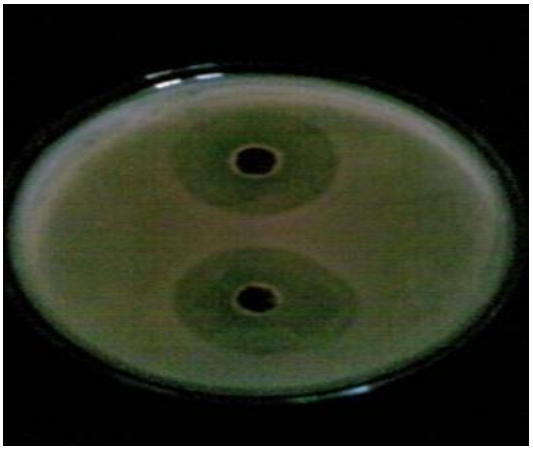
Table 2: Effect of carbon and nitrogen source on the anti-bacterial activity of bacteriocin against tested bacteria

S.No	Media	Zone of inhibition (mm)		
		E.coli	*K.pneumoniae*	*S.marcescens*
1	MRS broth	10.0	12.5	14.0
2	MRS broth +sucrose	15.5	19.0	16.0
3	MRS broth +lactose	19.5	17.0	17.5
4	MRS broth+maltose	23.0	20.0	21.5
5	MRS broth+histidine	18.0	19.0	14.5
6	MRS broth+glycine	17.0	18.4	15.0
7	MRS broth+cysteine	15.0	14.0	17.0
8	MRS broth+urea	11.2	12.3	14.5
9	MRSbroth+ammonium nitrate	13.4	14.0	16.0
10	MRS broth+soyabean meal	20.0	23.5	21.0

Figure 1: Zone of inhibition (mm} of bacteriocin derived from media supplemented with soya bean meal against pathogenic bacteria

E.coli

K.pneumoniae



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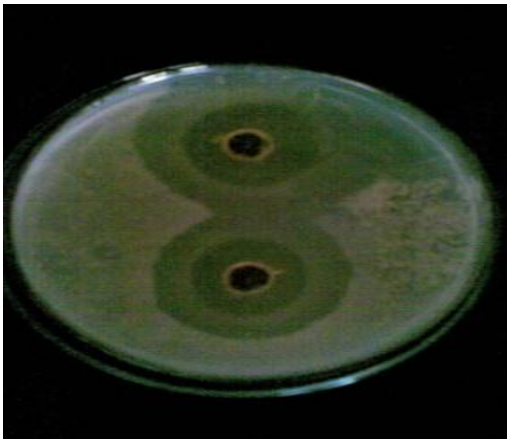
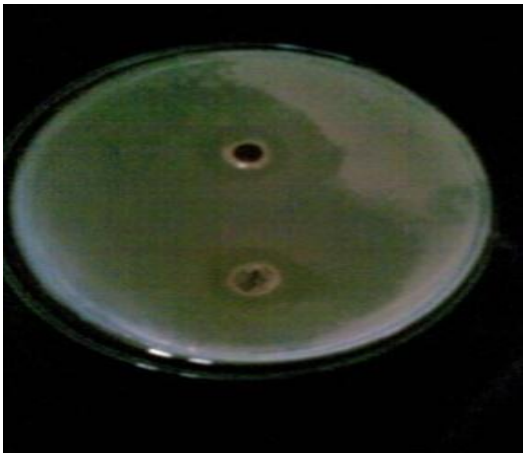
S.marcescens

Figure 2: Figure 1.Zone of inhibition (mm} of bacteriocin derived from media supplemented with maltose l against pathogenic bacteria

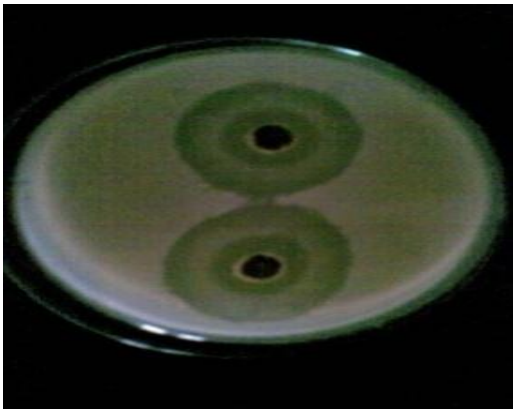
E.coli



K.pneumoniae



S.marcescens



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