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Research Article

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Production, Purification and Characterization of Bacteriocins from Lactic Acid Bacteria

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ABSTRACT

Antimicrobial peptides (AMPs) or bacteriocins consist of molecules that act on the defence systems of numerous organisms. These compounds have become extremely significant due to the increasing resistance of microorganisms to common antibiotics. In this work, selective media was used for isolation of Lactobacillus species producing bacteriocins. Optimization was carried out for physical and media conditions. Optimized conditions were found to be temperature 37ºC, neutral pH, lactose at 2.0% concentration and ammonium nitrate at 2.5%. Under these optimized conditions enhanced production of bacteriocin was observed. Further produced bacteriocin was subjected to mass spectroscopy and presence of multiple bacteriocins was observed.

Keywords:*Antimicrobial, Bacteriocin, Lactobacillus, Optimization.*

INTRODUCTION

Bacteriocins are antimicrobial peptides [AMP] secreted by every bacterium to inhibit the growth of similar or closely related bacterial strains in a bacterial pool which has competition for obtaining nutrients[1]. They consist of molecules which act on the defence system of other bacteria, fungi, parasites and viruses. These compounds have gained importance in the fields of health care and food preservation as microbes are showing increasing resistance towards commonly used antibiotics and preservatives. However, the low quantity of peptides obtained from direct purification is, to date, still a remarkable bottleneck for scientific and industrial research development. These compounds can protect against broad array of infectious microbes. AMPs have a very good future in pharmaceutical and food industries.

One traditionally sidestepped but ever-present issue is that of defining what constitutes a bacteriocin. Bacteriocins comprise a rather ill-defined potpourri of proteinaceous molecules that typically first attracted the attentions of researchers because of their physiological capability of interfering with the growth on agar media of certain other, generally closely related, bacteria. Until relatively recently, most of the significant progress in bacteriocin research stemmed from investigations of the colicins, those prototype bacteriocins produced by various members of the family *Enterobacteriaceae*, and this resulted in considerable in-depth knowledge of the genetic basis, domain structure, mode of formation, and killing action of these molecules. However, there now appears to be a remarkable renaissance of research activity centred upon the bacteriocin-like activities of gram-positive bacteria, particularly lactic acid bacteria[2,3].

Understanding the antibiotic properties of these molecules has taken on increased importance in recent years due to the alarming spread of antibiotic resistance among nosocomial strains of bacteria. There is a pressing need to develop novel effective classes of antimicrobial drugs with mechanisms of action based

on cellular targets different from those on which existing antibiotics act. It is also very important to make sure that these bacteriocins should cause no harm to the host cells.

Hence non-pathogenic bacteria are been focused for obtaining these bacteriocins for large scale applications like Lactic acid bacteria and *Bifidobacteria*.These are the most common types of microbes used as probiotics. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures such as in yogurt, soy yogurt, or as dietary supplements[4].

Indeed, the majority of current reports of bacteriocin-like activities relate to those produced by gram-positive bacteria. It seems that much of the renewed interest in these substances is a direct response to the perceived potential practical applications of these agents either to preservation of foods or to the prevention and treatment of bacterial infections[5].

MATERIALS AND METHODS

Samples of pasteurized milk and yogurt were collected from a commercial outlet and the other sample was curd prepared at home. The samples were subjected to serial dilution (10^{-4}) and then cultured on MRS, selective media specific for *Lactobacillus species*[6]. Obtained pure cultures were swabbed on nutrient agar plates and incubated for 24, 48, 72 and 96 hours. Bacteriocin production was assessed for each set of samples.

Identification of the isolates was carried out by grams staining, endospore staining and biochemical tests.

Antibiogram test was performed to check the bacteriocin titre of the bacterial species (against *E.coli*) by well diffusion method[7].

The activity of organism is influenced for maximum production by various factors which include different carbon sources, different nitrogen sources, different pH & different temperature. Growth and bacteriocin production were estimated at various temperature 25, 30, 37, 40ºC, at various pH 4.0, 5.0, 6.0, 7.0, 8.0 and various carbon sources like dextrose, starch, lactose, cellulose, and maltose. The source which gave better result was further optimized for various concentrations from 0.5% to 3.0%.Bacteriocin production was also optimized for various nitrogen sources liketryptone, peptone, yeast extract, sodium, potassium and ammonium nitrates. The source with better results was further optimized for various concentrations from 0.5% to 3.0%[8].

Mass production of bacteriocin: Mass production of the bacteriocin was done by submerged fermentation. In submerged fermentation, the organism was inoculated in nutrient broth in conical flasks and incubated for 72 hours at37°C in a shaker at 75rpm.

Differential salt precipitation with ammonium sulphate is carried out to extract the bacteriocins.First 50% saturation was considered and the salt was added slowly to the supernatant with continuous stirring with the help of magnetic stirrer. This solution was centrifuged at 10,000rpm for 10 min. Pellet and supernatant were collected. Pellet was re-suspended in 10ml buffer and supernatant was taken for second salt precipitation.For second salt precipitation, 20% of salt concentration was considered and the process was repeated. After centrifugation pellet and supernatant was separated and stored at 4°C.Anti-microbial activity was checked for all three samples(1^{st} pellet, 2^{nd} pellet and 2^{nd} supernatant) and the one with maximum zone of inhibition was purified.

Gel filtration: The protein sample showing maximum zone of inhibition after ammonium salt precipitation was purified by gel filtration chromatography using Sephadex G-75(sigma). Column was equilibrated by phosphate buffer of 0.1M (pH 7.0) for 30min and proteins were eluted with the same buffer at a flow rate of 2ml min^{-1}. Concentration of protein of each fraction was determined at Lowry's method using standards.

Further antimicrobial activity of the extracted bacteriocin against pathogenic microorganisms like *Klebsiella, Proteus, E.coli,* E*nterococci, Bacillus cereus* was studied[9].

RESULTS AND DISCUSSION

Samples were subjected to selective isolation of *Lactobacillus* species on MRS media. Zone of inhibition was verified against *E.coli.*Only isolates from yogurt and homemade curd showed significant zone of

inhibition. Maximum zone of inhibition was observed in agar plates of homemade curd & yogurt which were incubated for 72 hours. There were 4 isolates obtained from 2 sources.

Isolate 1 & Isolate 2 were isolated from homemade curd.Isolate 3 & isolate 4 were isolated from yogurt.

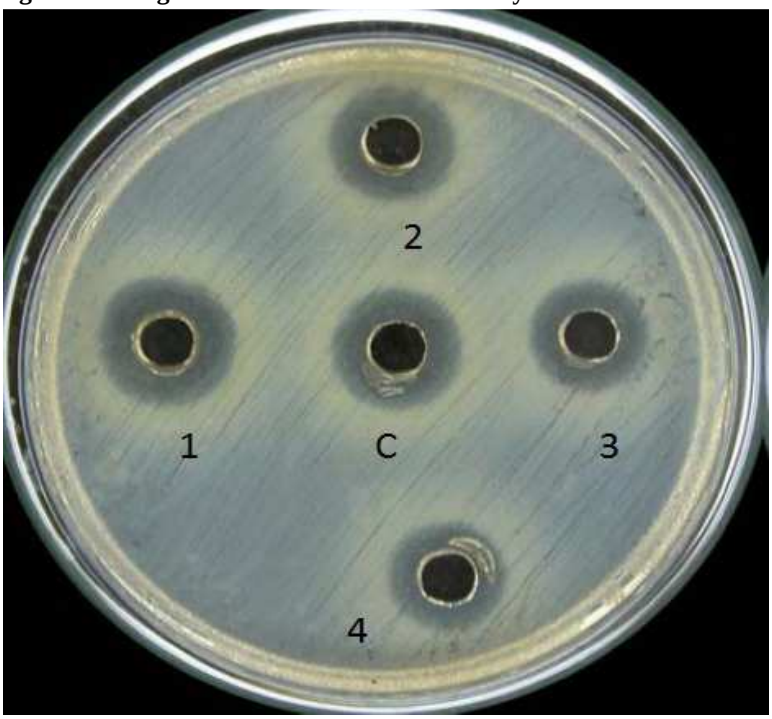
Identification: All the four isolates were found to be gram positive and showed terminal spores. Based on the bergely's manual& standard biochemical test and literature review the isolates were anticipated to be genus Lactobacillus.

Table 1: Biochemical test results for the isolates

Test	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Indole Test	-ve	-ve	-ve	-ve
Methyl Red Test	+ve	+ve	+ve	-ve
Vogues Proskuer Test	+ve	+ve	-ve	+ve
Citrate Utilization Test	+ve	+ve	-ve	-ve
Catalase Test	+ve	-ve	+ve	+ve
Lactose Fermentation Test	+ve	+ve	+ve	-ve
Starch hydrolysis Test	-ve	-ve	-ve	+ve
Casein Hydrolysis Test	-ve	-ve	+ve	-ve
Gelatinase Test	-ve	-ve	+ve	+ve
Hydrogen Sulphide Test	-ve	-ve	-ve	+ve
Carbohydrate Fermentation Test	+ve	+ve	-ve	+ve
Oxidase Test	-ve	+ve	+-ve	-ve

Antibiogram: Initial antibiogram studies of these isolates showed that all are effective against *E.coli*(Fig1). But the best among was chosen based on highest antibiotic titre, i.e. larger diameter of zone of inhibition,i,e Isolate 1 was chosen for further optimization and mass production.

Fig. 1: Antibiogram test for obtained isolates by well diffusion method



1-isolate1(18mm), 2-isolate2(17mm), 3-isolate3 (15mm), 4-isolate4(9mm), c-control (sterptomycin)

Optimization:

Optimization conditions considered for enhancing bacteriocin production from isolate 1 include, temperature, pH, carbon and nitrogen sources.

Table 2: Inhibition zone at various temperature, pH, carbon and nitrogen sources

Temperature	Inhibition zone in mm	pH	Inhibition zone in mm	Carbon source	Inhibition zone in mm	Nitrogen source	Inhibition zone in mm
25ºC	12	4	14	Dextrose	17	Tryptone	18
30 ºC	15	5	15	Lactose	18	Peptone	18
37ºC	17.5	6	16	Maltose	15	Yeast Extract	18.5
40ºC	16	7	17.5	Cellulose	12	Sodium Nitrate	17
		8	16	Starch	12	Potassium Nitrate	17
						Ammonium Nitrate	19

Among the carbon sources lactose was found to be best and further its concentration was optimized. Among the nitrogen sources ammonium nitrate was found to be best and further its concentration was optimized.

Results show optimum temperature of 37ºC, optimum pH of 7.0, optimum carbon source being lactose at 2% and optimum nitrogen source being ammonium nitrate at 2.5%.

Protein Estimation: Protein extracted from the mass production culture filtrateby salt precipitation was subjected to estimation by Lowry's method.Maximum absorbance was observed in 12^{th} and 15^{th} fraction. 15^{th} fraction was chosen for further assessment.

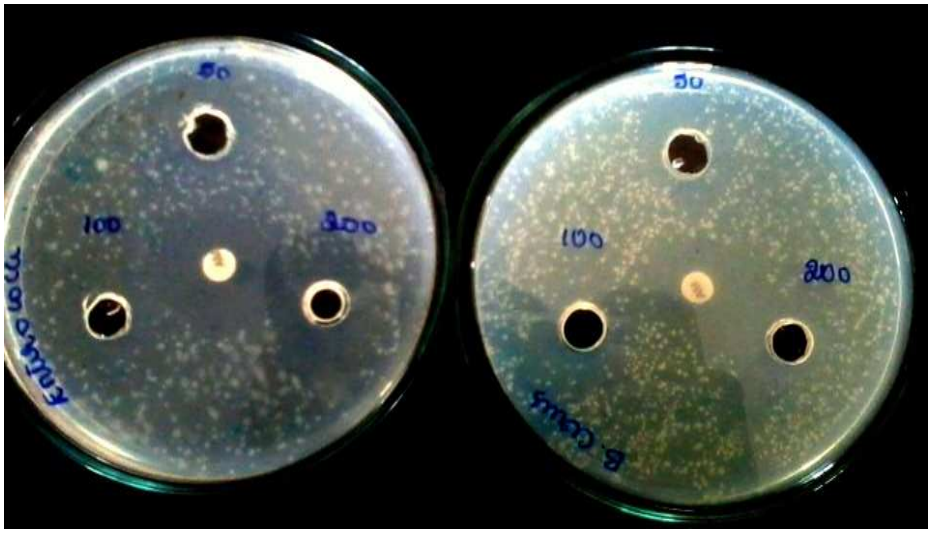
Effect of Bacteriocin against pathogenic microorganisms:

Antimicrobial activity of bacteriocin against pathogenic microorganisms like *Klebsiella, Proteus, E.coli*, E*nterococci, Bacillus cereus* was checked in terms of inhibiton zone (table 3). Highest inhibition was shown against *Enterococci* and *Bacillus cereus* (Fig2).

Table 3: Effect of bacteriocin against different pathogenic microbes

Organism	Diameter of inhibition zone (mm)		
	50µl	100µl	200µl
Proteus vulgaris	11	13	15
Klebsiellapneumoniae	9	11	15
E.coli	11	13	13
Enterococci	13	18	20
B.cereus	13	16	18

Fig 2: Zone of inhibition against E*nterococci* and *Bacillus cereus*



Bacteriocin production is greatly influenced by the physicochemical conditions in which bacterium is cultured. In our work we observed that the bacteriocin production was enhanced under optimum conditions. This may be further used for large scale production at industrial levels.

CONCLUSION

*Lactobacillusspp*are non-pathogenic, useful lactic acid bacteria. They produce secondary metabolites termed bacteriocins which are used as anti-microbial agents.Optimum conditions of temperature, pH and concentration of carbon, nitrogen sources enhance the production quantity or titre of bacteriocin. This would provide a good lead for medical applications, i.e. use of these bacteriocins as treatment for various microbial infections. Further research can be carried out on utilization of these proteins for probiotic applications and as preservatives in food industries.

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