WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 5, 1272-1278. Research Article ISSN 2277 – 7105

ISOLATION, CHARACTERIZATION AND ESTIMATION OF ANTIMICROBIAL ACTIVITY OF NOVEL BACTERIOCIN FROM LACTOBACILLUS CASEI

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Article Received on
25 May 2014,

Revised on 20 June 2014,
Accepted on 15 July 2014

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ABSTRACT

Food borne pathogens are continuing to become a matter of great concern in various industries like diary, egg and other food industries. Various gram positive and gram negative bacteria like Salmonella typhi, Bacillus subtilis are the main causative organisms. The present study is based on isolation, identification and screening of major food borne pathogens and novel bacteriocin producing strain of Lactobacillus casei. Investigations were conducted on 389 food samples and from this 12 different genus of bacteria were identified and isolated. From the 62 isolates of Lactobacillus sp. isolated, 9 isolates were Lactobacillus casei and of which 11% (1 isolate) were found capable of producing bacteriocin in large amounts. The isolates were grown for 96 hours under stress condition and the bacteriocin was extracted by centrifugation. Ammonium sulphate precipitation at 70% level of saturation was performed and the precipitate was centrifuged. Well diffusion assay of the extracted protein was performed on different isolated food borne pathogens and the diameter of the zones of inhibition was recorded. The study reveals the extensive scope of bacteriocin as preservatives in the field of food industry and the need for further research in it.

KEY WORDS: Bacillus subtilis, Ammonium sulphate, centrifuged.

INTRODUCTION

Contamination by micro organisms especially the food borne pathogens is becoming a threat to the food industry. This decreases the quality control of the food products and thereby decreasing consumer demand for the product in the market which brings them a great loss.

The Food and Drug Administration (FDA) have prioritized the matter as top level and have listed the food borne pathogens in Bacteriological Analytical Manual, FDA (FDA, 2012). The list include various microorganisms like Escherichia coli, Salmonella sp., Shigella sp., Listeria monocytogenes, Staphylococcus aureus, and Bacillus subtilis. The pathogens recorded previously of causing contamination in food industry are Staphylococcus and Streptococcus species.

Bacteriocins are classified as antibacterial peptides or proteins that are synthesised by bacteria as a microbial defence mechanism and they are classified into three classes mainly.Class1 consist of smaller molecular weight(nisin),class2 were molecular weight <10kDaand class 3 with molecular weight >10kDa.At the molecular level, they are synthesized by small ribosomes and they can permeabilize through membrane and are cationic in nature (Klaenhammer[a], 1993; Jack et al., 1995; Thompson et al., 1996). Previous studies have found the antimicrobial nature of the peptides. Despite of their different source, structure, mode of action and specificity, any molecule of protein that is secreted by the bacteria and has antimicrobial activity is considered to be a bacteriocin (Rammelsberg and Radler, 1990). These bacteria inhibits gram positive bacteria and food spoilage bacteria (Klaenhammer[b], 1988, Caslaet al., 1996, Ennanet al., 1996, Contreras et al., 1997, Messiet al., 2001) and gram negative bacteria (Lewuset al., 1991, Stevens et al., 1991, Messiet al., 2001).

Lactic acid bacteria (LAB) are characterized as gram positive bacteria and they are cocci or rod shaped. The genus is anaerobic but can tolerate and grow in the presence of air. These bacteria produce antagonist substances called bacteriocins which have high antimicrobial activity in low concentration (Klaenhammer[a], 1993, Moronoet al., 2006). Nisin,which is a commercially used bacteriocin produced by Lactobacillus lactis is used as a preservative in the food industry but fail to provide its full potential due to its unstable nature. The antimicrobial activity of these compounds has increased the scope of research and interest in the isolation of Lactobacillus sp. producing bacteriocin and characterization of these peptides (Derazet al., 2005).

MATERIALS AND METHODS

1. Bacterial Strains Isolation and Culture Conditions

389 samples were collected from randomly selected from various retail shops in Erode, Tiruppur, Namakkal and Coimbatore districts of Tamil Nadu, India and immediately to

laboratory conditions for isolation. The various samples procured were meat, fish products, milk, dairy products, raw vegetables, bakery products, beverage and fermented rice products. The food samples were aseptically inoculated into freshly made sterile Brain Heart Infusion broth (Hi-Media Laboratories, Mumbai) test tubes and is maintained aerobically at 30^0C-37^0C for 24 hrs. The fermented products were soaked in 10 ml distilled water tubes. The fermented material is then crushed with sterilised mortar and pestle and the mass is serially diluted from 10^{-2} per ml to 10^{-8} per ml and incubated at 30^0C-37^0C. The serially diluted fermented material and the food samples from BHI broth were inoculated into culture specific medium De man Rogosa Sharpe (Hi-media Laboratories), Tryptone Soy Agar (Hi-media Laboratories), Sheep Blood Agar (Hi-media Laboratories), MacConkey Agar (Hi-media Laboratories), Xylose lysine deoxycholate Agar (Hi-media Laboratories) and incubate at 30^0C-37^0C for 16-24 hrs in aerobic condition. The colonies with different morphological characters were selected randomly and repeated streaking in fresh agar culture was carried out each time until pure culture is obtained. The pure cultures were regrown in Nutrient Agar (Hi-media Laboratories) and preserved at 4^0C before morphological and biochemical characterisation according to Bergey's manual of determinative bacteriology (Holt et al., 1994).

2. Screening of Isolates for Bacteriocin Production

The bacteriocin produced by 11 isolates of Lactobacillus casei was checked for activity by screening against maximum amount of food borne pathogens isolated. The isolates of Lactobacillus casei were inoculated in MRS broth and maintained at 37^0C for 96 hrs anaerobically (stress provided). The culture was centrifuged at 10000xg for 15 mins and the supernatant was collected. The crude bacteriocin was tested for activity by well diffusion method. The well dimensions were maintained at 7mm in diameter and 5mm deep in each culture using gel puncture method and 30 µl of sample was added to each well produced. The diameters of the zones were recorded for maximum number of indicator bacterial isolates to select the best strains.

3. Extraction of Bacteriocin

250 ml of MRS broth was inoculated with the best strains of Lactobacillus caseiand incubated at 37^0C for 96 hrs anaerobically. The culture was centrifuged at 10000xg for 15 mins and the supernatant was collected. The proteins were precipitated by adding 70% of ammonium sulphate and incubated at 4^0C overnight. The precipitated protein was extracted by

centrifugation at 12000xg at 4^0C for 45 mins and dissolved in 0.1M phosphate buffer and the pH was checked. The protein samples were then dialysed against the same buffer for 12hrs and the purified sample was stored at 4^0C for further use.

4. Antimicrobial Assay of purified bacteriocin

Antimicrobial property of the purified bacteriocin was analyzed by swabbing 1 ml of the indicator bacteria with sterile cotton buds which was previously poured on sterile nutrient agar plates. The plates are incubated at 37^0C for 24 hrs and the zone of inhibition is recorded.

RESULTS AND DISCUSSION

1. Isolation and Identification of Bacteria

From a total of 389 samples investigated, 688 species were isolated. The suspected bacterial colonies were purified by repeated streaking in selective agar media plates until the pure cultures were obtained. The morphological and biochemical characteristics were recorded.

Table 1.1: Morphological Characteristics of isolated bacteria (Suspected)

Bacteria Investigated	Motility Test	Gram Staining	Flagella Staining	Endospore Staining
Aeromonassorbia	Motile	Gram Negative	Single Polar Flagella	No Endospore
Bacillus cereus	Motile	Gram Positive, Rod	Peritrichous Flagella	Central
Bacillus subtilis	Motile	Gram Positive, Rod	Peritrichous Flagella	Subterminal
Escherichia coli	Motile	Gram Negative, Rod	PeritrichousFlagella	No Endospore
Klebsiellaoxytoca	Non-Motile	Gram Negative, Rod	No Flagella	No Endospore
Klebsiella pneumonia	Non-Motile	Gram Negative, Rod	No Flagella	No Endospore
Listeria monocytogenes	Motile	Gram Positive, Cocci	Peritrichous Flagella	No Endospore
Salmonella enterica	Motile	Gram Negative, Rod	Peritrichous Flagella	No Endospore
Staphylococcus aureus	Non-Motile	Gram Positive, Cocci	No Flagella	No Endospore
Streptococcus agalactiae	Non-Motile	Gram Positive, Cocci	No Flagella	No Endospore

Table1.2: Percentage of Isolated Tested Positive for Biochemical Tests NR-Nitrate Reduction, SH-Starch Hydrolysis, CT-Citrate, CA-Catalase, OX-Oxidase, UR-Urease

Bacteria Investigated	No. of Isolates	NR	SH	CT	CA	OX	UR
Aeromonassorbia	34	97	-	85	-	84	-
Bacillus cereus	22	92	89	100	85	-	59
Bacillus subtilis	31	*1	86	99	90	64	-
Escherichia coli	86	100	-	-	85	-	-
Klebsiellaoxytoca	12	95	-	99	80	-	89
Klebsiella pneumonia	21	95	-	98	84	-	85
Listeria monocytogenes	82	-	-	-	99	-	-
Salmonella enterica	8	95	-	-	88	-	-
Staphylococcus aureus	11	100	-	99	85	-	-
Streptococcus agalactiae	54	-	-	-	-	-	-

2. Antimicrobial Assay

Antimicrobial assay was performed with both crude and partially purified bacteriocin. The partially purified bacteriocin displayed a large zone of inhibition in microorganisms like Staphylococcus aureus and Bacillus aureus. The results were almost similar to the study done by Dhanpathiet al., 2008. The bacteriocin produced by lactobacillus plantarumwas found to be effective against both gram positive and gram negative bacteria. The comparison of crude and purified bacteriocin revealed an increase in activity. The study reveals a positive co-relationship with the study done by Bizaniet al., 2002.

Table 2.1: Antimicrobial Activity of Crude and Partially Purified Bacteriocin

Isolated strain	Bl23(crude)	Bl23(partially purified)
Aeromonassorbia	5	6
Bacillus cereus	7	9
Bacillus subtilis	6	13
Escherichia coli	5	9
Klebsiellaoxytoca	0	0
Klebsiella pneumonia	1	4
Listeria monocytogenes	4	7
Salmonella enterica	2	4
Staphylococcus aureus	8	13
Streptococcus agalactiae	4	6

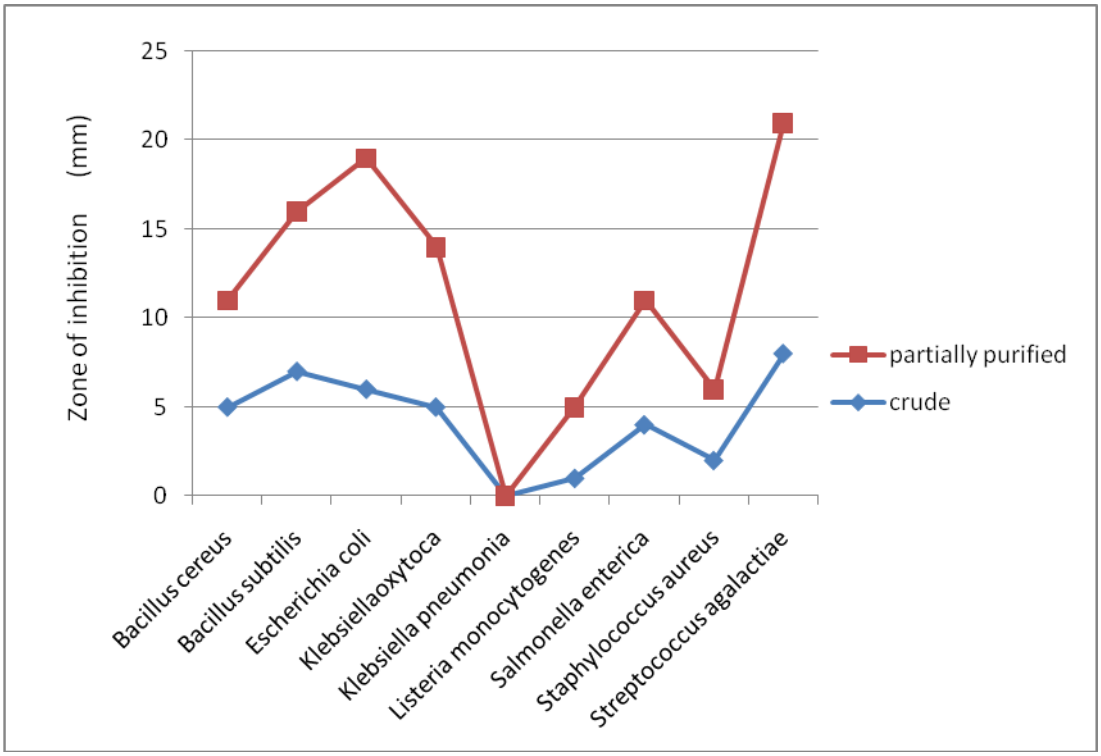

Figure 2.2: Comparison of Zones of Inhibition of Crude and Partially Purified

CONCLUSION

The presence of various microorganism like Aeromonas, Bacillus, Escherichia, Klebsiella, Listeria, Staphylococcus, Salmonella and Streptococcus was confirmed in the present study on different food, fermented and dairy products. The strains of Lactobacillus caseiwhich produces bacteriocins were isolated and the crude and partially purified bacteriocins were tested for antimicrobial screening with the indicator organisms. The inhibition was found to be more active in gram positive bacteria and the prospects of the usage of these bacteriocins in the food industry as preservatives is large. Further studies like large scale production, improving bacteriocins activity against gram negative bacteria and structural analysis can be implored on and which will result into further profound research in this particular aspect.

ACKNOWLEDGEMENT

The authors wish to acknowledge thefinancial grant with reference no.20/AICTE/RIFD/RPS POLICY-1)28/2012-13 under Research PromotionScheme of All India Council of Technical Education (AICTE), New Delhi to executethis research work as a part of the fundedproject. Authors would also like to conveythanks to Bannari Amman EducationalTrust for the kind support.

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