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Characterization of Bacteriocin Produced by Bacillus Megaterium Isolated from Exotic Fruits.

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Abstract: In this study bacteriocin producing bacteria were isolated and enumerated from exotic fruits like dragon fruit (Hylocereus undatus), hog plum (Spondias mombin), Thailand litchi (Lychee chinensis), kiwi (Actinidia deliciosa), Thailand green apple (Ziziphus mauritiana) and were screened for production of bacteriocin. These fruits are imported from countries from Thailand Malaysia, Vietnam and some are also locally grown in India. Characterization and In vitro evaluation of the isolated bacteria were carried out to assess their in antimicrobial activity. Comparison of the Gram positive and Gram negative bacteria on these fruits were made. The bacteriocin producing microbes were tested for their antimicrobial activity against six indicator bacteria, namely Bacillus subtilis, Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella and Escherichia coli by agar diffusion assay. Finally one of the bacterial strain B4 showing inhibition against all the six indicator strains was selected for bacteriocin production. The biochemical tests of the strain indicated that it belongs to Bacillus megaterium. Physical parameters indicated that the optimum pH-8 and optimum temperature of 30° C. Maximum production of bacteriocin was observed in presence of ammonium chloride as a source of nitrogen and the molecular weight of bacteriocin was found to be 15kDa by SDS-PAGE.

Key words: Bacteriocin, Bacillus, molecular weight, antimicrobial activity.

I. INTRODUCTION

Bacteriocins are bactericidal agents produced by one strain of bacteria to inhibit the other strain of a closely related species. They are proteinaceous compounds synthesized ribosomally by different eubacteria. They are heterogeneous in nature and display variable molecular weights, biochemical properties, mode of action and inhibitory spectra [1]. Gratia discovered bacteriocins in 1925. Both Gram-negative and Gram-positive bacteria produce bacteriocins that have diverse ecological and evolutionary significance. The biosynthesis of bacteriocins is a self-regulated activity and released by a controlled specific mechanism. Bacteriocins produced by Gram positive bacteria have broader spectrum of antimicrobial activity than the ones produced by Gram negative bacteria [2].

Bacteriocins are peptides ranging from only 19 amino acids to as large 90 kDa proteins. The spectrum of activity can be either narrow and confined to inhibition of closely related species, or it can be relatively broad. Bacteriocins are natural agents present in several of the foods. Many bacteriocins are active at small concentrations, and exhibit both bactericidal and bacteriostatic activity toward target organism. Their mode of action ranges from pore formation in cytoplasmic membrane to the inhibition of cell wall biosynthesis and enzyme activities in target cells [3].

Bacteriocins are classified into different groups [4]. Class I bacteriocins(lantibiotics) are small peptides that undergo extensive post-translational modification to produce the active peptide. Class II bacteriocins are thermo-stable, membrane active peptides with low molecular weight. Members of class III are large heat labile proteins, and a fourth class (complex bacteriocins) require non-protein moieties for activity.

Currently, bacteriocins produced from lactic acid bacteria that are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) are extensively studied [5]. Nisin from Lactococcus lactis is the most studied and is used as food preservative in dairy and meat products. Bacteriocins can be introduced into food in three different ways: bacteriocins can be produced in situ in fermented food by bacterial cultures that substitutes for all or part of the starter culture; purified or semi-purified bacteriocins can be added directly to food; or added as an ingredient [6].

The bacteriocins from Escherichia coli called colicins, are the longest studied bacteriocins. One of the oldest colicins, called colicin V and is now known as microcin V, is a much smaller peptide, being produced and secreted in a different manner than the classic colicins [7]. Bacteriocins produced by Enterococci are termed as enterocins. Bacillus sp. also produce a large number of bacteriocins like the subtilosin, bacillocin 490, cerein, haloduracin, thuricin, megacin and bacteriocin like inhibitory substance (BLIS) by B. subtilis [8], B. licheniformis [9], B. cereus [10], B. halodurans [11], B. thuringiensis [12], B. megaterium [13], B. amyloliquefaciens [14] respectively.

However, resistance to the bacteriocins already in use has been well documented, nisin-resistance phenotype has already been demonstrated in some Gram-positive bacteria, including Staphylococcus aureus, Streptococcus bovis and Listeria

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monocytogens [15]. Resistance to other antibiotics like Lacticin3147, pediocin-like bacteriocin and lysostaphin [16] and there is a need to discover new strain of bacteriocin producing microbes from other potential sources.
In this study a comparison on the bacteria producing bacteriocin on these different exotic fruits was attempted. Understanding the prevalence of the bacteria producing bacteriocin on these exotic fruits and may act as a potential source for the discovery of novel bacteriocin producing microbe. This is a preliminary report of the study undertaken on these fruits and needs further analysis.

II. MATERIAL AND METHODS

A. Sample collection, isolation, identification, and purification of bacteriocin producing bacteria:

Dragon fruit, hog plum, Thailand litchi, kiwi fruit and Thailand green apple were collected from local markets of Bangalore, India. The fruits were washed with sterile distilled water and dried. They were transported at appropriate storage conditions and 5g of samples was weighed and crushed and homogenized in separate sterilized mortar and pestle using 15ml of phosphate saline. The sample was centrifuged at 6000rpm for 10 minutes, 200µl of this supernatant was used as inoculum on MRS agar by spread plate technique. The cultures were grown at 37°C for 24-48h. Twenty-five well isolated colonies from each sample were picked up and transferred to MRS broth. They were propagated twice and streaked on MRS agar to check the purity of the isolates. Biochemical tests IMViC, Gelatin liquefaction, Hydrogen Sulphide production, Casein hydrolysis, Lipid hydrolysis, Starch hydrolysis, Carbohydrate fermentation, Urease, Triple sugar iron agar (TSI), Catalase, Oxidase and Nitrate reduction tests were performed [18].

B. Antibacterial activity of bacteriocin producing bacteria:

The agar diffusion bioassay described by Herreros et al. [17] was used to screen for bacteriocin producing bacteria using cell free supernatants. The organisms were grown on MRS broth and after an incubation of 48h, the broth was centrifuged at 6000rpm for 10min at 4°C. The efficacy of cell free supernatants (CFS) was tested by well diffusion assay against pathogens. The plates were incubated at 37°C for 24 h and examined for lysis around the wells. A direct comparison was made between the diameters of the zone of inhibition produced by different strains.

C. Effect of pH, temperature, and nitrogen source on bacteriocin production

Effect of pH was determined at 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and incubated for 24h at 37℃. Effect of temperature was determined at different temperatures 30℃, 35℃, 37℃, 40℃, 45℃. Nitrogen sources such as gelatine, peptone, ammonium nitrate, sodium nitrate and ammonium chloride at 1% concentration, and Nitrogen source with maximum diameter further assayed at different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%). The cultures were incubated for 24 hrs and the broth was centrifuged at 6000rpm for 10min at 4°C and CFS was assayed by agar well diffusion

D. Production and purification of bacteriocin

Bacteriocin was produced under optimized conditions in 200ml MRS broth for 16hrs, cell free supernatant was obtained by centrifugation at 8500xg for 15min at 4°C. The resulting supernatant was subjected to ammonium sulphate precipitation at 40%, 60% and 80% saturation. The resulting pellets were resuspended in 10mM Tris HCl solution. A sephadex G50 column was equilibrated with phosphate buffer (pH 6.0) 3ml of the extracted samples was eluted at a flow rate of 0.5ml/min with phosphate buffer (pH 6.0). The purified bacteriocin sample (100µg) was loaded onto a polyacrylamide gel and electrophoresed at 100V for 5h. After electrophoresis, half of the gel was stained with Coomassie blue R-250.

III. RESULTS AND DISCUSSION:

A. Isolation and identification of bacteria producing bacteriocin

The organisms from the exotic fruit samples were isolated on MRS media and enumerated. The number of cultivable bacteria ranged from $2.7x10^6$ to $4.6x10^6$ in all the different samples. Well-developed colonies were streaked onto MRS media and five well isolated colonies were selected randomly from each sample for screening of bacteriocin. Among the twenty five isolates B1, B4, B5, B7, B9, B14 tested positive for bacteriocin production against all six indicator organisms and zone of inhibition and is represented in table-1. Organism B4 was selected for further studies since it showed maximum zone of inhibition against all indicator organisms. Biochemical tests were performed for B4 to identify the bacteria genera. Biochemical tests were performed. The results of the tests are shown in table-2. The tests concluded that the bacterium is Bacillus megaterium which is concordant with the biochemical tests of Bacillus megaterium isolated from infected fruits [19] and should be further confirmed by molecular methods. Bacteriocins produced from Bacillus megaterium, megacin A-216 and megacin A-19213 have been well characterized [20]. Bacillus species is the second most studied genera for the production of bacteriocin and are generally regarded as safe bacteria e.g. Bacillus subtilis and

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Bacillus licheniformis [21]. Bacteriocins from Bacillus are becoming more important due to their broad spectrum of activity towards Gram-negative, Gram-positive, yeast and fungi, compared to other bacteriocins [22].

Optimization of Bacteriocin production at different pH, temperature and Nitrogen source

Optimum pH for the production of bacteriocin was found to between 7 and 8. Studies have shown that the optimal pH for the bacteriocin producing microbes were pH 7.4 and 8.5 [23]. The optimum temperature for bacteriocin production in most strains isolated to date ranged from 30°C to 37°C [24].The optimum incubation temperature for bacteriocin production must be evaluated on individual basis and is strain-dependent. Optimum temperature was found to be 30℃ and 35℃ for the production of bacteriocin. Zhou et al. [25] found that the optimal temperature for cell growth is 35°C, and the optimal bacteriocin production condition is a range dependent phenomenon in Lactobacillus, where temperature is from 27 to 34°C. Nitrogen is an important source for the production of proteins. Studies have shown that organic nitrogen sources like peptone and yeast extract increase the production of bacteriocin [26]. In this study optimum production of bacteriocin was observed in presence of inorganic nitrogen source i.e. ammonium chloride. Production of bacteriocin by LAB is greatly influenced by medium formulation and culture conditions. Growth of LAB and bacteriocins production is not only affected by the type of carbon (C) and nitrogen (N) sources but also by their concentrations and ratios [27]. 0.5% ammonium chloride was found to be optimum for the production of bacteriocin by Bacillus megaterium.

B. Production of Bacteriocin

Bacteriocin was precipitated from culture media with different percent of ammonium sulphate This technique is commonly used for proteins of 15–17 kDa or higher [28]. Maximum yield of protein was obtained in 80% ammonium sulphate where 281mg/ml of protein was obtained which indicated that 80% ammonium sulphate precipitation could be used for protein purification from culture media consistent with other research works [29, 30]. An experiment showed that B. subtilis BSX derived bacteriocin retained almost 100% activity after centrifugation at a saturation degree of 50–60% with ammonium sulphate [31]. Lv et al. [32] purified bacteriocin produced by Lb. plantarum JY22 by Sephadex G50 gel filtration chromatography. Similar procedure was followed using Sephadex G-25 Gel to purify the bacteriocin from B. megaterium**.** The molecular weight of the proteins was determined by recording the distance travelled by the tracking dye and the proteins of the standard. The standard marker used was Novex sharp pre-stained protein standard. The molecular weight of sample was found to be 15 kDa.

CONCLUSION

In this study, a bacteriocin producing strain of Bacillus megaterium B4 was screened and was determined to have broad antibacterial activity against both Gram positive bacteria (Bacillus subtilis and staphylococcus aureus) and Gram-negative bacteria (Pseudomonas aeruginosa and Escherichia coli). The maximum antibacterial activity was displayed between pH 7 and pH 8 and 30℃, and production of bacteriocin was more in presence of 0.5% ammonium chloride when used as nitrogen source. Bacteriocin was purified in three steps and its molecular weight was determined to be approximately 15 kDa by SDS-PAGE. Further identification and characterization of the bacteriocin will indicate its prospects of application in food industry.

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Table-1 Diameter showing zone of inhibition against indicator organisms with organisms.

Organism / Isolate	E. coli	B. subtilis	P. aeruginosa	S. aureus	S. epidermidis	Salmonella
	Zone of inhibition (mm)					
B1	+	++	++	++	+	+
B4	+++	+++	+++	+++	++	+++
B5	+	+++	+++	++	++	+
B7	+	++	++	++	++	+
B9	+	+	+	+	+	+
B14	+	+	+	++	+	+

"+++" is inhibition zone >11.00 mm; "++" is inhibition zone from 9.00 to 11.00 mm and "+" is inhibition zone from 7.00 to 9.00 mm.

Table-2: Biochemical test of B4 isolate:

Sl.no.	Biochemical tests	Results
1	Indole test	-
2	methyl red	+
3	VP test	-
4	Citrate	-
5	Gelatin liquification	-
6	H_2S production	-
7	Casein hydrolysis	+
8	Lipid hydrolysis	+
9	Starch hydrolysis	-
10	Cellulose degradation	-
11	Fermentation of glucose	+
12	Fermentation of sucrose	+
13	Fermentation of mannitol	+
14	Fermentation of lactose	+
15	Urease test	+
16	Triple Sugar Iron Agar test	+
17	Catalase test	+
18	Oxidase test	+
19	Nitrate reduction	-

Table-3 Diameter showing zone of inhibition against indicator organisms with organisms in presence of nitrogen source and % of ammonium chloride

Nitrogen Source	Zone of inhibition (mm)	Ammonium Chloride (%)	Zone of inhibition (mm)
Gelatin	++	**0.5**	+++
Peptone	++	**1.0**	++
Ammonium chloride	+++	**1.5**	++
Ammonium nitrate	+	**2.0**	+
Sodium nitrate	+	**2.5**	+

"+++" is inhibition zone >11.00 mm; "++" is inhibition zone from 9.00 to 11.00 mm and "+" is inhibition zone from 7.00 to 9.00 mm.

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Graph 1 and 2: Effect of pH and temperature on the production of bacteriocin.

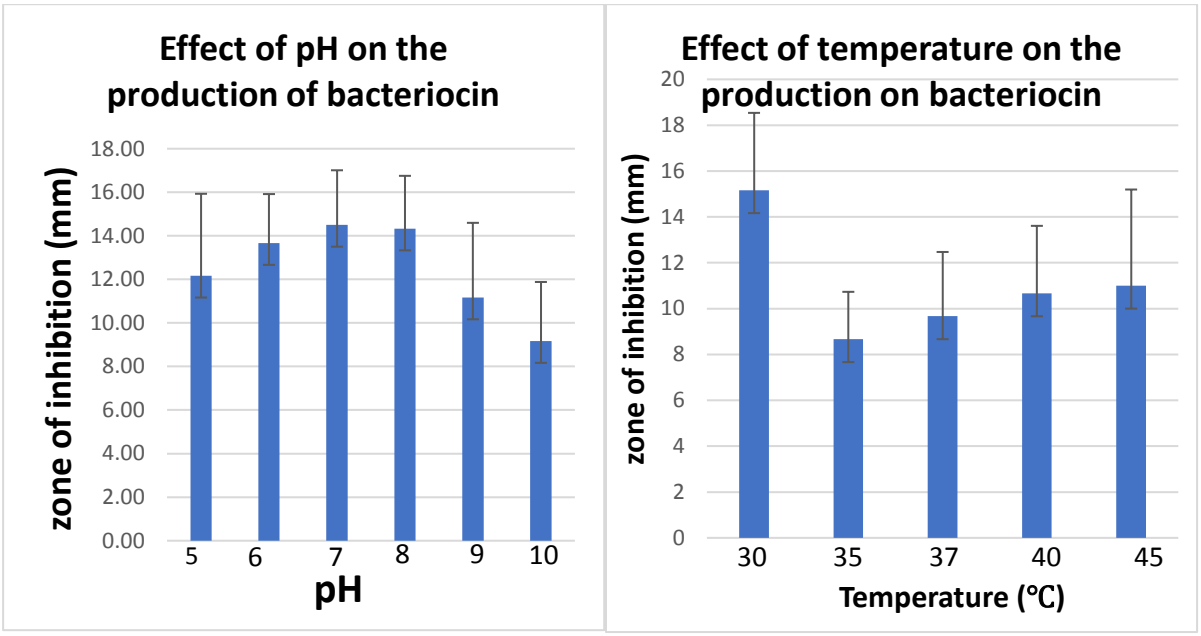

Table-3: Protein content in CFS after ammonium precipitation

Ammonium sulphate precipitation (%)	Protein content (mg/ml)
40	262
60	266
80	281

Plate 1 & 2: Zone of inhibition of culture 4,7 &9 against Bacillus Subtilis and Pseudomonas

Plate 5 & 6 showing effect of pH and temperature on production of bacteriocin and its effect on zone of inhibition.

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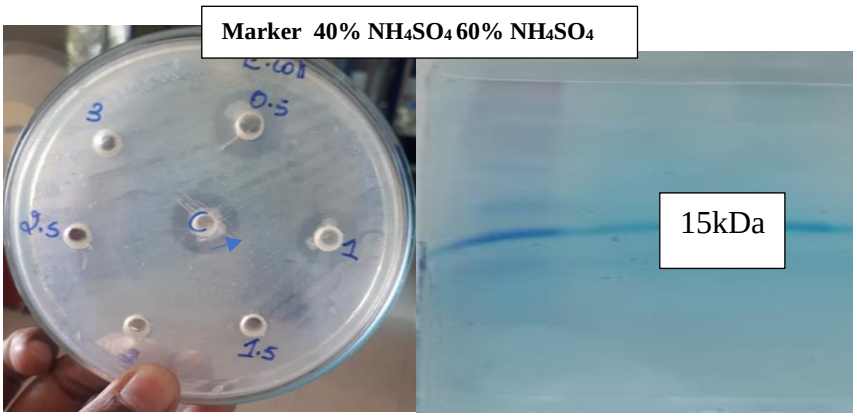

Plate 7 & Fig 1: Effect of different % of Ammonium chloride on bacteriocin production and their effect on E.coli &SDS PAGE showing molecular weight of bacteriocin to be 15KDa