Screening and Isolation of Lactobacillus from Novel Sources for Production of Bacteriocins

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Abstract—Bacteriocins are primary metabolites which are synthesized in the ribosome of bacteria. These antimicrobial bioactive peptide substances kill or inhibit the growth of other closely related bacteria. The antimicrobial property of bacteriocins is used in the food industry, treatment of pathogen associated diseases, cancer therapy etc. Along with these applications of bacteriocins, they are also referred as next wave of conventional antibiotics. Bacteriocins fall under 'GRAS' (Generally Recognised as Safe) category as they usually have no side effects on the humans. In this study, bacteriocin producing bacteria were isolated with an aim of identifying a novel bacteriocin. The sources selected were paneer (cottage cheese) and Sauerkraut as they are rich in Lactic Acid Bacteria (LAB). The bacteriocin produced from these sources was tested for their inhibitory activity against food spoiling pathogens like *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Staphylococcus aureus* using Agar Well Diffusion Technique. Properties of the obtained bacteriocin were also tested. Future studies include extraction and SDS PAGE, HPLC of the extracted bacteriocin.

Index Terms— antimicrobial peptide, bacteriocins, bacteriocin like inhibitory substance, BLIS, lactic acid bacteria, natural antibiotics, primary metabolites.

1 Introduction

THE term Bacteriocin was introduced to denote toxic protein that is produced in the ribosome of bacteria [1]. They are bioactive peptides do not harm the producing microbe but kill or inhibit the growth of other bacteria [1]. These antimicrobial peptides display antimicrobial activity against narrow spectrum as well as broad spectrum bacteria [2]. Bacteriocins have a very versatile nature as they have wide range of applications ranging from food industry to cancer therapy. Food preservation is carried out by the addition of chemical additives is a common phenomenon but they are in turn harmful to the humans; hence bacteriocins being GRAS products have an upper hand and are widely used in the food industry [3]. They also have wide scope in areas like probiotics, cancer therapy and treatment of pathogen associated diseases. Bacteriocins are often referred to as next generation of conventional antibiotics [3]

1.1 Classification

Bacteriocins are produced from Gram positive as well as Gram negative bacteria.

A. Gram Positive – From LAB

Lactic Acid Bacteria are primary bacteria which produce bacteriocin. Classification of bacteriocins synthesized by LAB is done on the basis of their biochemical characteristics and is divided in three groups namely:

a) Class I: Lantibiotics

These are small membrane-active, heat-stable peptides which contain unusual thioether amino acids. Nisin is the model bacteriocin of this group which is isolated from Lactococcus lactic [4].

b) Class II:

According to recent studies, this class of bacteriocins display overall better performance in terms of physiochemical properties and biological activities as compared to other classes; hence have emerged as the most promising candidates in the food industry [4]. Further Class II bacteriocins are divided into:

- Class IIa
- Class IIb
- Class IIc

c) Class III:

These types of bacteriocins are large (>30kDa), heat-labile proteins and are secreted by the bacterial preprotein translocase [4].

B. Gram Negative – From E. coli

Colicins or Microcins are very common bacteriocins which are produced by Gram negative bacteria like E. coli. Microcins are much smaller and are produced and secreted in a different manner as compared to classic colicins [4].

2 Methods and Materials

2.1 Isolation of Lactic Acid Bacteria (LAB)

Lactic acid bacteria where isolated from samples collected from Pune, India. Cottage Cheese (paneer) was purchased from a local vendor while Sauerkraut was prepared in the laboratory by finely chopping 500 grams of cabbage and adding 2.5% NaCl. Later it was allowed to ferment over a period of one month in an air tight environment. Samples were collected every week and were labelled according to the week; S1 meaning sample of week 1. MRS media, de-Mann, Rogosa and Sharpe (MRS) agar plates (peptone 1% , beef extract 1%, Yeast extract 0.4%, glucose 2%, agar 1%, sodium polysorbate 0.1%,

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acetate trihydrate 0.5%, tri-ammonium citrate 0.2%, magnesium sulphatehepta-hydrate 0.02%, manganese sulphate tetrahydrate 0.005%, di-potassium hydrogen phosphate 0.2%, pH 6.2), was used to promote specific growth of LAB. The samples were diluted using serial dilution in sterile saline till the power 1013. These diluted samples were spread onto the MRS agar plates and were incubated for 24 hours at 37°C. Single colonies according to their morphologies were picked and were further sub-cultured twice using 4 Streak technique and incubated at 37°C for 24 hours to obtain pure cultures. These pure colonies were transferred onto MRS slants and preserved at 4°C.

2.2 Preliminary Screening of Lactobacillus

Preliminary screening of the isolated Lactobacillus strains was carried out to check their antimicrobial activity against indicator strain L. monocytogenes (MTCC 657/ATCC 19111) using Cross-Streak Technique on Nutrient Agar.

2.3 Test of Acid Production

Isolated strains were subjected to fermentation for 24 hours and the collected spent media was centrifuged at 1000 rpm for 20 minutes at room temperature to get cell free supernatant (CFS). Bromothymol blue solution was used for the analysis of acid production. 200 µL Bromothymol blue solution was added to 1 mL of CFS. Acid producing cultures showed colour change from blue to yellow while non-acid producing cultures did not show any colour change.

2.4 Agar Well Diffusion Technique

Qualitative analysis of the bacteriocin producing strains that were obtained after acid production was carried out using Agar Well Diffusion Technique. Selected strains were inoculated in 100 ml Nutrient Broth and were incubated for 24hours at 37°C. Cell free supernatant (CFS) was collected after centrifugation at 5000 rpm for 30 minutes. Nutrient Agar plates were prepared and were seeded with indicator strains viz. L. monocytogenes, S. aureus and P. aeruginosa. 50µl of the CFS was aliquoted in the wells bored in the above mentioned plates. The procedure was carried out in triplicates and the zone of inhibition of each plate was noted for further analysis.

2.5 Gram Staining

Gram Staining of the selected strains was carried out in order to characterize bacteriocin on the basis of Gram positive and Gram negative.

2.6 Temperature Stability

Strains producing bacteriocin were fermented for 24hours at room temperature and were further centrifuged at 10000 rpm 20 mins at room temperature. The CFS of every strain was exposed to a variety of temperatures viz. 4°C, 37°C and 60°C for 24 hours. 50 µl was aliqouted in pre-prepared Nutrient Agar plates seeded with indicator strain S. aureus.

2.7 Extraction

Extraction of bacteriocin produced by strain P4 was carried out using Chloroform extraction method. Strain P4 was inoculated in 500 ml Nutrient Broth and incubated for 24 hours at room temperature. The spent media was centrifuged at 3500 rpm for 40mins and the CFS was collected. 250 ml of chloroform was added to the CFS and stirred vigorously on a magnetic stirrer for 20 minutes. The mixture was further subjected to centrifugation at 3500 rpm for 40 minutes at 12°C. The intermediate precipitated layer was collected and suspended in 0.5 ml of Tris Buffer (0.1 mol, pH 7.0). The combined mixture was again subjected to centrifugation at 10000 rpm for 20minutes

3 Results

3.1 Isolation of LAB

11 strains were isolated from the sources mentioned above. The strains were named numerically for convenience:

TABLE 1
STRAINS ISOLATED FROM SAMPLES

Source	Strain number
Cottage Cheese	$P_1,P_2,P_3,P_4,P_5,P_6,P_7$
Sauerkraut	S_1,S_2,S_3,S_4

3.2 Preliminary Screening

6 strains were selected for further analysis while the other strains were discarded

TABLE 2
SELECTED STRAINS AFTER PRELIMINARY ANALYSIS

Source	Total Strains	Strain number
Cottage Cheese	$P_1,P_2,P_3,P_4,P_5,P_6,P_7$	P_2,P_3,P_4,P_6
Sauerkraut	S_1,S_2,S_3,S_4	S_2,S_3

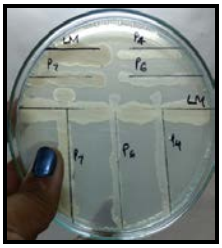

Fig. 1. Cross Streak Technique.

3.3 Test of Acid Production

The acid producing strains were eliminated as they showed a colour changed when exposed to bromothymol blue.

TABLE 3
ACID PRODUCTION

Source	Total Strains	Strain number
Cottage Cheese	P_2,P_3,P_4,P_6	P_3
Sauerkraut	S_2,S_3	S_2,

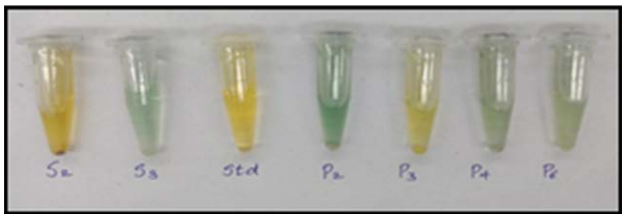

Fig. 2. Test for Acid Production.

3.4 Agar Well Diffusion

Quantitative analysis of the strains was carried out to check inhibition against the indicator strains.

TABLE 4
INHIBITION AGAINST INDICATOR STRAINS USING AGAR WELL DIFFUSION

Strains	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
P_2	+++	++	+
P_4	+++	+++	+++
P_6	+	++	
S_3	++	+++	+++

The experiment was performed in triplicates. Hence, '+' denotes number of plates showing inhibition against particular indicator strain.

Fig. 3. Agar Well Diffusion.

3.5 Gram Staining

Gram Staining was performed.

Fig. 4. Gram Staining of strain P4.

3.5 Temperature Stability

All strains showed temperature stability at the three specified temperatures viz. 4°C, 37°C and 60°C. The strains retained their activity against the indicator strain.

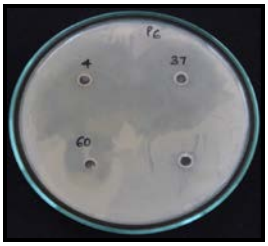

Fig. 5. Temperature Stability.

3.7 Extraction

Extraction was carried out and the protein was collected and stored for further analysis.

4 CONCLUSION

Screening of isolated microorganisms showing antimicrobial activity was successfully carried out. This inhibitory action of the microorganisms plays a crucial role in the applications of bacteriocins producing strains. The indicator strains used were major food spoiling organisms hence these bacteriocin producing strains have a potential application in the food industry. Extraction of the remaining strains is currently being carried out. Future studies will cater to SDS PAGE analysis and HPLC of the extracted product.

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