

CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF BACTERIOCIN PRODUCING *BACILLUS SUBTILIS* ISOLATED FROM RAW MILK

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Abstract—

This study presents bacteriocin (antimicrobial compound), produced from *Bacillus subtilis* isolated from dairy products. The strains from unpasteurized milk that showed the largest zone of growth inhibition against the indicator strain, *Micrococcus luteus* was selected for the study. The selected strain was identified as *Bacillus subtilis* based on its morphological, physiological and biochemical characteristics. The antibiotic susceptibility tests for *B. subtilis* against various antibiotics demonstrated high sensitivity to Tetracycline, Moderate and less sensitivity towards Streptomycin and Chloramphenicol respectively while being resistant to penicillin. Culture filtrate containing bacteriocin showed the nature of its thermostability (up to 100°C), expressed a pH tolerance (2.0-10.0), and emerged resistance towards trypsin and pepsin. Partial purification of bacteriocin was performed using ammonium sulphate precipitation and the dissolved crude antimicrobial material was then purified by passing into a column of Silica gel. In SDS-PAGE analysis, purified bacteriocin isolated from *Bacillus subtilis* showed the molecular weight to be approximately 3.4 KDa. The isolate Bacteriocin was tested finally for antibacterial activity against *E. coli*, associated with food borne illnesses. The zone of inhibition clearly proved that bacteriocin has inhibitory effect on the food pathogen *E.coli*, thus confirming its bactericidal action in food.

Key words: Bacteriocin, *Bacillus subtilis*, *Micrococcus luteus*, *E.coli*, antibacterial agent

I INTRODUCTION

Most bacteria produce antimicrobial compounds such as broad spectrum classical antibiotics, metabolic products viz, organic acids and lytic agents such as lysozyme, in addition to several types of protein exotoxin and bacteriocins, which are biologically active compounds with bactericidal action. Bacteriocin (antimicrobial peptides) family is the most abundant and diverse group of bacterial defence systems. Bacteriocins are microbially produced peptides that are usually active against bacteria closely related to the producer organism [5]. Some bacteriocins are active against pathogenic and food spoilage bacteria, and much research has focused on their potential as antimicrobials in food preservation. In contrast to the currently used antibiotics, bacteriocins are often considered more natural so that they are thought to have been present in many of foods eaten since ancient times [1]. Most of the research is carried out on the bacteriocins produced by lactic acid bacteria but they have the disadvantage that they are not suitable for use as probiotics in the agricultural industry since they are sensitive to heat and effect of salts.

Similar to LAB, some representatives of *Bacillus* species such as *B. subtilis*, *B. licheniformis* are generally recognized as safe (GRAS) bacteria [7]. *Bacillus subtilis* is a known producer of many antibiotic and antimicrobial compounds including the bacteriocins [8]. In particular, bacteriocins from *Bacillus subtilis* are found to exhibit antimicrobial activity against several pathogenic organisms like *Micrococcus luteus* and *Escherichia coli*. This distinctive property of *Bacillus subtilis* enhances its utilization as a probiotics and hence used up in **bio preservation**.

Therefore, in the present study, our primary objective was to evaluate the use of *Bacillus* bacteriocins in food preservation with an attempt to isolate and characterise the bacteriocin from *Bacillus subtilis* that has been isolated from raw milk, to get a better insight into its bacteriocidal properties against food borne pathogen such as *E.coli*.

II MATERIALS AND METHODS

A. Selective Isolation of *Bacillus Subtilis*

Samples from dairy product such as raw unpasteurized milk and curd were processed for isolation

of *Bacillus subtilis*. Spore forming *Bacillus subtilis* was identified by ethanol treatment and heat treatment. In ethanol treatment, the sample was treated with ethanol for about 30 minutes where as in case of heat treatment, the sample was subjected to heat treatment at 80°C for 20 minutes. The treated samples were then overlaid with the indicator strain, (*Micrococcus luteus*) to isolate spore forming bacteriocin producing *Bacillus subtilis*. The isolated colonies showing zone of inhibition were selected for further studies. The suspected colonies were Gram positive bacteria and identified as *Bacillus subtilis* as per Microbial tests (Gram staining, endospore staining), Biochemical tests (IMViC tests, Catalase test, Starch hydrolysis, Casein hydrolysis, Urease test, Nitrate reductase test, Lipid hydrolysis test, Fermentation test, Oxidase test) and Physiological Tests (Growth at different pH).

B. Antibiotic Assay

Antibiotic assay was a modification of the agar overlay diffusion method. Cells were grown in LB broth at 37°C for 16hrs. Formerly prepared nutrient agar plates containing 15ml of nutrient agar were overlaid with 4ml of soft agar (1%) containing 200µl of freshly grown cells. Antibiotic discs were prepared and dispensed on to the solid media and incubated at anaerobic conditions at 37°C for 24 hours. Inhibition zones were measured and susceptibility was expressed.

C. Testing of Culture Filtrate of isolated cultures for Antimicrobial Activity

Agar well diffusion is performed to assay for the culture filtrate activity of the isolates against the indicator strain *Micrococcus luteus* [3]. To determine antimicrobial activity, the isolates grown overnight in LB broth by continuous shaking were taken. It was then centrifuged twice at 10,000rpm at 4°C for 10 minutes. The culture filtrate was taken and tests its antimicrobial activity. The NA was poured onto petriplates and allowed to solidify. It was then overlaid with LB soft agar seeded with the indicator strain. Wells were then made with the help of gel puncture and about 50µl of culture filtrates were added to the well. The plates were incubated at 37°C for overnight. Appearance of zone around the well determines the antimicrobial activity of the isolates.

D. Thermostability and pH optimization of the culture filtrate

Thermostability and pH optimization was done to determine the ability of the cell free supernatant to exhibit antimicrobial compound at different temperatures and also at different pH range. For this purpose, the overnight grown culture was taken and the cells were deleted down by centrifuging at 10,000rpm for 10 minutes. The culture filtrate pH was adjusted to pH range of 2, 4, 6, 8, and 10. Simultaneously the culture filtrate was also adjusted to heat treatment at varying temperature (40°C, 60°C, 80°C, 100°C and 120°C) and incubated at 37°C for 10 minutes. Subsequently the pH was neutralized and the activity of both pH and heat treated culture filtrate were checked by gel diffusion assay.

E. Proteolytic Inactivation of Culture Filtrate

To determine the effect of proteolytic enzyme on culture filtrate, culture was grown and centrifuged at 10,000rpm for 10 minutes and the culture filtrate was collected. 10µl of enzyme stock solution was added to 20µl of culture filtrate and incubated at 37°C for 6 hrs. NA plates overlaid with LB soft agar along with the indicator strain were prepared. The wells were made with the help of gel puncture and the enzyme treated Culture Filtrate was added to the wells. The plates are then incubated at 37°C and observed for zone of inhibition. If the enzyme treated samples show negative results compared to that of the positive control, it means the activity was due to protein, possibly bacteriocin.

F. Extraction of Crude Bacteriocin Ammonium sulphate precipitation method

In this method, 50ml of LB broth was inoculated with the test culture and kept for overnight incubation. The culture broth was then transferred to sterile polypropylene tubes and centrifuged at 10,000rpm for 10 minutes at 4°C. The cell free extract was collected by centrifugation and the supernatant was transferred into a beaker. The solution was stirred in magnetic stirrer using magnetic bead and ammonium sulphate (50%) was added slowly till its final saturation. The proteinaceous bacteriocin tends to precipitate by the slow addition of ammonium sulphate. This was kept at 4°C for overnight. The solution was then centrifuged at 10,000rpm for 15 minutes. The

supernatant was decanted and the pellet was resuspended in sterile distilled water.

G. Purification Column Chromatography

For purification, 1.0 gm of Silica gel 100- 200 Mesh was added to 50 ml sterile distilled water and kept for overnight soaking. To this 10 ml of crude bacteriocin sample was added carefully to the top of the gel and allowed to pass into the gel by running the column. After 30 minutes the samples were eluted and the fractions were collected at 15 minutes interval each in 5 eppendorf tubes and stored at -20°C.

H. Characterisation of Crude Bacteriocin SDS-PAGE

Sample subjected to SDS-PAGE was mixed with 5X sample buffer. The sample solution was heated in boiling water for 2 to 3 minutes to ensure complete interaction between protein and SDS. The sample was loaded in the respective wells and electric field was applied to the buffer tank setup. 50V was applied to the stacking gel & 100V to the resolving gel. Once the solvent front has reached the bottom of the gel & the current was switched off. Polypeptides separated by SDS-PAGE were simultaneously fixed with methanol, glacial acetic acid & stained with CBB R-250. The gel was transferred to plastic tray containing a minimum of 5 gel volumes of CBB stain & left over for a night. Replaced the stain with destain with fresh one till protein bands were closely visible with low background.

I. Protective Effect of Bacteriocin on *E.Coli*

Escherichia coli are a gram-positive, rod shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E.coli* strains are harmless, but some serotypes are pathogenic and can cause serious food poisoning in humans.

35% of food was contaminated by *E.coli* while 57.5% of water used by vendors was contaminated by coli forms [6]. Holding the food for longer time creates favourable conditions for the growth of food borne pathogens. In such foods, the counts of *E.coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium perfringens* are reported to be high.

J. Assesment of Antimicrobial Activity

To perform the action, Purified antimicrobial compound was taken and its activity was tested. The NA was poured on to petriplates and allowed to solidify. It was overlaid with LB soft agar seeded with the pathogenic strain namely *E.coli*. Wells were then made with a diameter of 6mm and 30µl of antimicrobial compound were added to the well. The plates were then incubated at 37°C for overnight. Appearance of zone around the well determines the antimicrobial activity of the Bacteriocin on *E.coli*.

III RESULTS AND DISCUSSION

A. Screening and Isolation of Bacteriocin Producing *Bacillus* Cultures from Various Sources

Bacillus subtilis, spore formers are commonly found in fermented food and soil. Cultures were isolated selectively by ethanol treatment and heat treatment from raw milk and curd. Colony producing bacteriocin exhibits zone of inhibition against the indicator organism, *Micrococcus luteus*. The following table shows the zone produced by the cultures against the lawn of the indicator organism.

Table 1. Zone of inhibition of the cultures

S.No	1	2
Isolates	<i>Bacillus subtilis</i>	<i>Bacillus species</i>
Sources	Raw milk	Curd
Antimicrobial activity	+++	—



+++ : maximum zone — : no zone

Fig.1. Isolated colonies showing zone of inhibition against the indicator organism *Micrococcus luteus*

B. Characterization of Bacteriocinogenic *Bacillus Subtilis*

Table 2. Biochemical properties of the *Bacillus subtilis*

Biochemical Tests	Results
Indole	—
Methyl red	—
Voges proskauer	+
Citrate	+
Catalase	+
Starch hydrolysis	+
Casein hydrolysis	+
Urease	+
Nitrate reduction	+
Lipid hydrolysis	+
Triple Sugar Iron	Yellow /Yellow without gas production (acid slant/acid butt)
Oxidase	—

+: positive, —: negative Yellow/yellow: glucose and lactose or /+ sucrose fermentation has occurred

Identification of Physiological Properties

The growth of the selected isolate was tested at different pH (2, 4, 6, 7, 8, 10, 12 and 14).

Table 3. Physiological properties of the isolates

Properties	Morphology	Growth at different pH	2	4	6	7	8	10	12	14
<i>Bacillus subtilis</i>	Rod		-	+	++	+++	+++	++	+	-

+++ : maximum growth ++: minimum growth —: no growth,

The isolate was able to grow in a pH range of 4 to 12.

Microbial characteristics of the isolates

The isolated culture was found to be Rod-shaped, Gram-positive and Spore bacteria from morphological analysis.

Identification of culture by Biochemical tests

The culture was identified by biochemical tests.

C. Antibiotic Susceptibility

The selected isolates were tested for its susceptibility towards various antibiotics such as streptomycin, Chloramphenicol, penicillin.

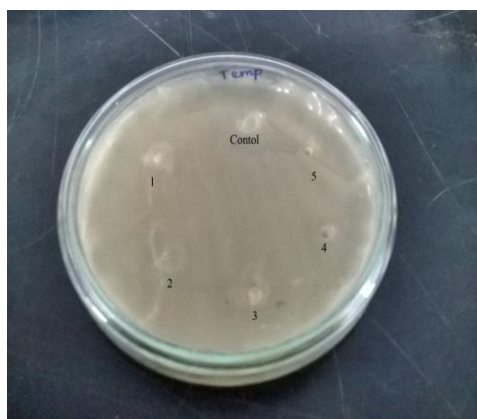


Fig.2 Antibiotic susceptibility of the culture, *Bacillus subtilis* towards various antibiotics.

The above results showed that the culture is highly sensitive to the antibiotic; Tetracycline. Moderate and less sensitive towards streptomycin and Chloramphenicol respectively. No sensitivity is shown by penicillin.

D. Thermostability and pH of Culture Filtrate

Antimicrobial protein production by culture filtrate was strongly dependent on pH and temperature. In our study, Culture filtrate exhibited antimicrobial activity at acidic, neutral and basic pH levels (2-10), but maximum activity was noticed at pH 6.0 and 6.5. Similar results were observed [4]. The activity of culture organism exhibiting antimicrobial activity observed at different growth temperatures, suggested that the temperature play an important role in antimicrobial production.



Temp (°C) 1-20°C, 2-40°C, 3-60°C, 4-80°C, 5-100°C



Control (CF) pH

Fig.3&4 Plates showing Thermostability and pH optimization of the culture filtrate

E. Sensitivity of the Culture Filtrate to Different Proteolytic Enzymes

In order to check the nature of the antimicrobial compound and to find out whether it is a protein or not, the cell free supernatant was treated with various proteolytic enzymes like pepsin, trypsin and its activity was checked against *Micrococcus luteus*.

Table 4. Proteolytic inactivation of culture filtrate by various enzymes

S.No	Isolates	Proteolytic enzymes	
		Trypsin	Pepsin
1	<i>Bacillus subtilis</i>	++	++

++: minimum activity

+++: maximum activity

Bacillus subtilis showed the resistance to trypsin and pepsin but it may be sensitivity to pronase E and protease K.

F. Purification by Column Chromatography

This method was employed for purifying a compound. Hence the protein was completely eluted from the column. That protein was utilized for protective action against *E.coli*. Antimicrobial compound was purified by silica gel-mesh 100 - 200 column chromatography. Purified compound was collected in 5 eppendorf tubes at the interval of 15 minutes each and it was stored at -20°C.

G. Characterization of Crude Bacteriocin SDS-PAGE

SDS-PAGE was done to determine the molecular weight of the desired compound.



Fig.5,Plate 4. Molecular weight of the sample by PAGE

The above result revealed the isolate *Bacillus subtilis* is found to have a molecular weight of approximately 3.4 KDa.

H. Protective effect of Bacteriocin on *E.Coli*

Testing culture filtrate and purified bacteriocin against pathogenic *E.coli*.

Agar well diffusion assay is performed to assay the antimicrobial activity against the pathogenic organism.



Fig.6.Purified bacteriocin



Fig.7.Culture filtrate

Fig.6&7, Purified bacteriocin and culture filtrate showing zone of inhibition against *E.coli* respectively

The zone of inhibition as shown in Plate 5 clearly prove that bacteriocin and culture filtrate are effective against the food pathogen *E.coli* thus confirming their antibacterial activity. Similar result was also reported [2] paving for the possibility of exploiting *Bacillus Subtilis* for bacteriocin production to be used up as bio preservative in the food Industry.

IV CONCLUSION

Thus from the present study, it is apparent that the bacteriocin produced by *Bacillus subtilis* is useful in the biological control of pathogenic and food spoilage microorganisms such as *E.coli*. However, most studies concerning food application have focused on LAB bacteriocins, mainly *nisin* and a few others. Although *nisin* is the only bacteriocins currently licensed as a preservative, its applications are restricted due to its very low activity at a neutral or an alkaline pH. Hence, bacteriocin from *Bacillus subtilis* is considered to be a

strong candidate as bio preservative in food due to its heat stability, wider pH tolerance.

V FUTURE PROSPECTS

Continued research on bacteriocins will undoubtedly lead to our increased understanding, and the inhibitory spectrum of the antimicrobial substance has a potential application to be exploited as a much preferred biopreservative in the food industry. The route of future application/commercialization of these *B.subtilis* associated bacteriocins, will be dependent on whether the application will involve the use of the peptides in a (partially) purified or concentrated form. Therefore, it is important to emphasize that it is necessary to deepen the study of bacteriocins produced by *Bacillus subtilis* spp to explore new avenues in the food and health care industry, as an effective natural biopreservative and also in the enhancement of functional food properties.

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