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STUDIES AN APPROACH TO BIOPRESERVATION EMBROIL THE DISTILLATION, CHARACTERISATION, AND ROUTINE OF BACTERIOCIN TO SPREAD THE SHELF LIFE OF NEW GROWTH.

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Abstract:

The yoghurt-isolated Lactobacillus that produced bacteriocin was tested for bacteriocin production using an agar well diffusion assay, and it was found to be a broad spectrum bacteriocin. By using a biochemical procedure and 16s rDNA sequencing, Lactobacillus delbrueckii subsp bulgaricus was found. The bacteriocin was purified using the salt precipitation and gel chromatography method, and SDS PAGE analysis revealed that its molecular weight was 20 KD. When the purified bacteriocin was characterized, it was discovered to have a shelf life of up to 20 days at 4°C and to be stable at temperatures up to 80°C for 60min, active at salinities up to 2.5% w/v, tolerate metal ions up to 0.25% concentration w/v, and improve in activity when combined with Tween 20. It was discovered that the crude, salt-precipitated, and gel-purified bacteriocin had protein concentrations of 310 g/ml, 280 g/ml, and 210 g/ml, respectively. Two types of sprouts, Cicer arietinum (Black chickpea) and Vigna radiate (Green gram), were tested for the bio preservation properties of the lyophilized culture, crude bacteriocin, and salt precipitated bacteriocin. Under 4°C, these may be kept for 10 days and 35 days, respectively.

Keywords: Lactobacillus delbrueckii subsp. bulgaricus, gel chromatography, biopreservation, characterisation, shelf-life, sprouts.

Introduction

The extension of food safety and shelf life by the employment of natural or under control microbiota and/or their antibacterial substances is known as biopreservation.1. In developed nations, bio preservation technologies are used in food processing, food safety regulations, product deterioration, and food-related sickness. Fermentation, a method based on the growth of bacteria in foods—natural or added—is one of the most popular methods of food preservation. Lactic acid bacteria, which make organic acids and other chemicals, make up the majority of the organisms. These substances provide food products distinct flavors and

textures in addition to their antibacterial effects. Bacteriocins and other antimicrobial compounds produced by lactic acid bacteria are referred to as natural preservatives or bio preservatives. The use of bacteriocins in biopreservation is very promising. There are three techniques to preserve biological material. 1. Development of the lactic acid bacteria that give food its bacteriocins. 2. The addition of biopreservatives that have been purified or partially purified. 3. Making use of products that have been pre-treated with strains that produce bacteriocin 2. Bacteriocins are protein-based toxins created by bacteria to stop the growth of other strains of bacteria that are similar to them or closely related to them. They are ribosomally produced antimicrobial peptides that act like antibiotics and are encoded in plasmids, chromosomes, or transposons. They are extracellular bioactive peptides or peptide complexes that are bactericidal against other species that are often closely related to the producer strain due to the combined action of the bacteriocin and host autolysin. 3. Given that a variety of strains are used as starter cultures (or protective cultures) in the production of dairy, meat, and vegetable products, bio preservation can be said to be one of the safest methods of preservation. Lactic acid bacteria (LAB), which have a GRAS (Generally regarded as Safe) status, play an important role in food fermentation. The main benefit of these microbes is the preservation of the nutritional value of the raw material through prolonged shelf life, suppression of rotting, and inhibition of bacteria associated with spoilage. 2. Synthetic/Chemical food preservatives do have a number of negative side effects on both the customer and the food being preserved, which is a serious worry. According to research, eating food that contains preservatives can cause behavioral changes, breathing difficulties for people who have asthma, heart problems (sulphites, benzoates, and aspartame consumption), and even cancer (nitrosamines used to preserve meat and meat products interact with stomach and gastric juices to form carcinogens). Because they are significantly safer for consumers and the food, bio preservatives should take their place. As was already noted, the substances created by LAB, such as bacteriocins, not only extend the shelf life of the food but also maintain its nutritional value.

Materials and methods

Isolation and detection of bacteriocin production by Agar well diffusion Assay

Locally available yoghurt was used for isolation, it was homogenised and about 0.1ml was serially diluted and plated on MRS agar plates[4, 5, 6.] Overnight inoculum of the isolate was inoculated onto 100ml MRS broth, incubated at 30°C for 48hours. The broth was centrifuged at 7,000rpm for 10min at 4°C. The supernatant was neutralised with 1N NaOH and used as crude bacteriocin[7]. Nutrient agar plates were pre inoculated with the test organism onto which 6mm wells were cut and about 300µl of crude bacteriocin was added, incubated at 30°C for 24 hours. The bacteriocin production was confirmed by observing the zone of inhibition against the test organisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis* and *Klebsiella pneumoniae.* The activity of bacteriocin was measured in arbitrary units

per mille litre. One arbitrary unit is defined as the reciprocal of the highest serial two fold dilution showing a clear zone of growth inhibition of the indicator strain [8, 9, 10] .

Identification of Lactic acid Bacteria

The isolate was Gram stained and subjected to biochemical tests to identify up to genus level [11, 12, 13] and 16s rDNA sequencing was carried out to identify up to species level [14, 15].

Partial purification of bacteriocin

About 250ml of MRS broth was inoculated with 1% overnight inoculum of the isolate and incubated at 30°C for 48hours. The cells were separated by centrifuging the broth at 7,000rpm for 10min at 4°C. The crude bacteriocin was partially purified by salt saturation method using ammonium sulphate to precipitate out proteins. The crude bacteriocin was stirred using a magnetic stirrer with the addition of ammonium sulphate slowly till saturation point of 70% is reached at a temperature of 4°C. The precipitated proteins were separated by centrifugation at 10,000rpm for 20min at 4°C. The protein pellet obtained was dissolved in 1M phosphate buffer of pH 7.0 [10].

Gel chromatography

Sephadex G25 was used as a column to purify (desalt) the salt precipitated protein. The gel column was pre equilibrated with 0.1M Tris HCl buffer (pH 7), 10ml of the precipitated protein dissolved in phosphate buffer previously was added onto the column without disturbing the gel. The proteins were eluted with 0.1M Tris HCl buffer (pH 7) and sample fractions of 1ml were collected in eppendoff tubes by setting the flow rate to 15ml/hour [16].

Molecular weight determination of protein by SDS PAGE

SDS PAGE was done t**o** determine the molecular weight and purity of protein. SDS PAGE was carried out using 15% and 4% concentration of acrylamide. Protein fraction with highest concentration was pooled and loaded onto the well along with a pre stained broad range marker (3.5KD- 260KD). Electrophoresis was carried out a constant current of 50mA until the tracking dye (mercaptoethanol) had migrated till the end. The gel was stained using the staining solution (Coomassie brilliant blue 250, methanol, acetic acid, distilled water) for 4hours and destained overnight using destaining solution(methanol, acetic acid, distilled water) [17].

Protein estimation

The concentration of proteins in crude bacteriocin sample, 70% ammonium sulphate salt precipitated sample was estimated by Lowry's method using FC reagent and BSA as standard [18].

Stability of bacteriocin at different temperatures and pH

The crude bacteriocin was subjected to different temperatures and pH to check its stability. The bacteriocin was heated at 30°C, 40°C, 60°C, 80°C and 100°C for a time period of 15min, 30min, 45min and 60min. In case of pH, the pH of crude bacteriocin was adjusted to 2,3,4,5,6,7,8 and 9 using HCl and NaOH. The stability and its activity after heat treatment and pH change was checked and tabulated [19, 20].

Effect of storage temperature and period

The stability of crude bacteriocin over storage at different temperatures for a period of 30days was analysed by storing at three different temperatures: -20°C, 4°C and 25-30°C respectively. The activity was checked every 10 days by agar well diffusion method [21, 22].

Effect of surfactants and detergents on bacteriocin activity

Surfactants and detergents such as EDTA, SDS, Tween 20 and Tween 80 at concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% w/v were added individually into 10ml MRS broth. All tubes were inoculated with 1% overnight inoculum and incubated at 30°C for 24hours. The results were tabulated [23, 24].

Effect of metal ions on bacteriocin activity

Different metal ions were used to check the bacteriocin activity. Metal ions such as $CaCl_2$, $ZnSO_4$, $CuSO_4$ and $FeSO_4$ at concentrations of 0.05%, 0.1%, 0.15%. 0.2% and 0.25% respectively. These metal ions were added into individual tubes containing 10ml MRS broth and inoculated with 1% overnight inoculum of LAB. The tubes were incubated at 30°C for 24hours. After incubation the activity was checked by agar well diffusion method [4, 25, 26].

Bio preservation of sprouts

About 5ml of bacteriocin producing culture, crude bacteriocin and 70% saturated bacteriocin were lyophilised and used for bio preservation. Two varieties of sprouts, *Vigna radiate* (Green gram) and *Cicer arietinum* (Black chickpea) were checked for bio preservation. Both the varieties of were soaked overnight in water, strained and allowed to germinate. After 48hours, the sprouts were washed and surface sterilised using methanol and per chloric acid [27] and about 50g of sprout was mixed with 5ml lyophilised culture, 3.1g crude bacteriocin and 2.42g of 70% saturated bacteriocin in separate containers under sterile conditions. The samples were refrigerated at 4°C and checked for contamination periodically.

Results and discussions

Isolation and detection of bacteriocin production by Agar well diffusion Assay

Uniform colonies were obtained on MRS plates which was checked for bacteriocin production by agar well diffusion assay. The isolate gave a clear zone of inhibition against three test organisms i.e., *Pseudomonas aeruginosa* (Gram negative), *Staphylococcus aureus* (Gram positive) and *Proteus mirabilis* (Gram negative) with a zone of inhibition of 20mm, 21mm and 15mm respectively but could not inhibit *Klebsiella pneumoniae*. The bacteriocin produced by the isolate exhibited broad spectrum activity because it inhibited both Gram positive and Gram negative pathogens but could not inhibit the growth of *Klebsiella pneumonia*. The activity of some bacteriocins on pathogenic bacteria is blocked because the pathogen might be capsulated as in case of
Klebsiella pneumonia and this was also observed in case of subtilosin produced by *Bacillus subtilis* [28].

Todorov *et.al.,* and Shelburne *et.al.,*[6, 28] also obtained similar results in case of the bacteriocin produced by *L.sakei* ST22Ch which inhibited the growth of *Pseudomonas* sp and *Staphylococcus* sp. The production of broad spectrum bacteriocin by Lactic acid bacteria has also been reported by others. *Lactococcus lactis* subsp *lactis* produces Nisin, which is inhibitory against Gram positive and Gram negative bacteria when the target cells have been pre-treated with EDTA or when used in higher concentration[29, 30] .

Parada *et.al.,* [31] have reported that the frequency of bacteriocins against Gram negative bacteria is less because the outer membrane of these bacteria act as permeability barrier for these cells against antibiotics, detergents and dyes from reaching the cytoplasm but there have been reports were bacteriocins are inhibitory against Gram negative bacteria as well. Example *Lactobacillus paracasei* subsp *paracasei* was inhibitory to *Escherichia coli* [32].

Identification of LAB

The colony on MRS plates was Gram stained which showed the bacteria were Gram positive bacilli. Further, biochemical tests (Table 1) and carbohydrate fermentation profile (Table 2) showed that the isolate belonged to *Lactobacillus* genera according to Bergy's manual of bacteriology. The 16s rDNA sequencing revealed that the isolated LAB was *Lactobacillus delbrueckii* subsp *bulgaricus* which showed 96% sequence similarity with Lactobacillus and 100% similarity with the existing strain of *Lactobacillus delbrueckii* subsp *bulgaricus* ATCC 11842 (Fig 1).

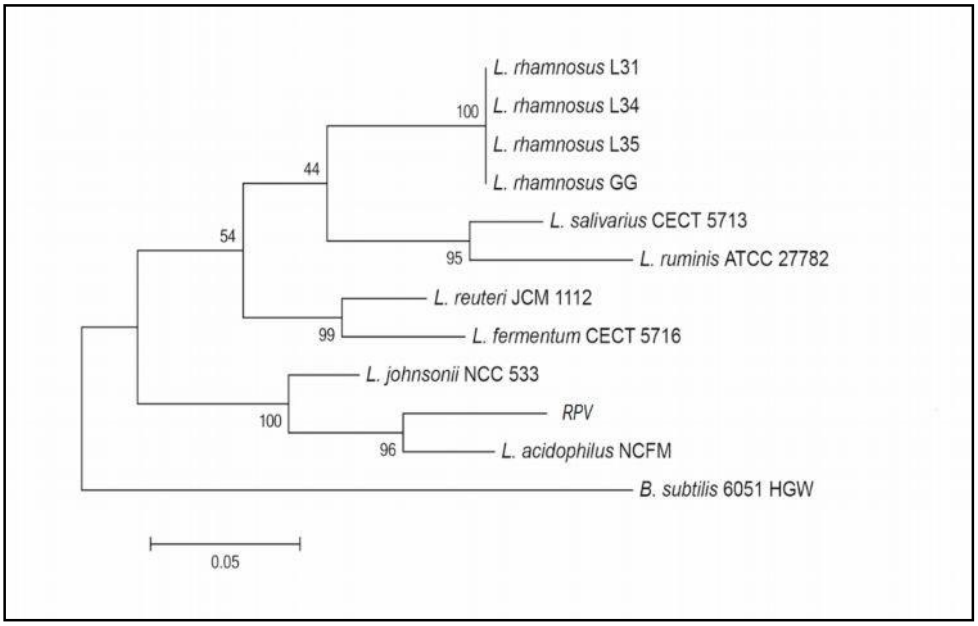


Figure 1. Phylogenetic tree of Lactobacillus isolate. The position of the isolate is shown as RPV

Table 1: Biochemical characterisation of LAB

Biochemical test	**Result**
Arginine test	-
Catalase test	-
Citrate test	+
Gelatine liquefaction	-
Indole production	-
Methyl red test	+
Vogues proskeur test	-
Nitrate reduction	-
Starch test	+
Cellulose degradation	-

Table 2. Carbohydrate fermentation test of LAB

Carbohydrate	Result	
	Acid	Gas
Dextrose	+	-
Lactose	+	-
Maltose	+	-
Fructose	+	-
Sucrose	+	-
Xylose	+	-

Purification of bacteriocin and molecular weight determination

During the three stages of purification, a significant increase in bacteriocin purity and yield was observed (Table 3). At 70% ammonium sulphate saturation the bacteriocin was active, this active bacteriocin was obtained in the form of pellet which was dissolved in 0.1M phosphate buffer (pH 7.0) and fractionated by gel chromatography using Sephadex G-25 column.

The fraction 7 showed activity against *P.aeruginosa*. This fractions was checked for its molecular weight by SDS-PAGE which showed a single band with an estimated molecular weight of 20KD in comparison with the marker protein (Fig 2). Hence, the purification showed homogeneity on SDS-PAGE and revealed its molecular weight as 20.0 KD. Therefore, based on the molecular weight, it can be concluded that the bacteriocin belonged to Class II i.e., a non lanthionine bacteriocin [33] .

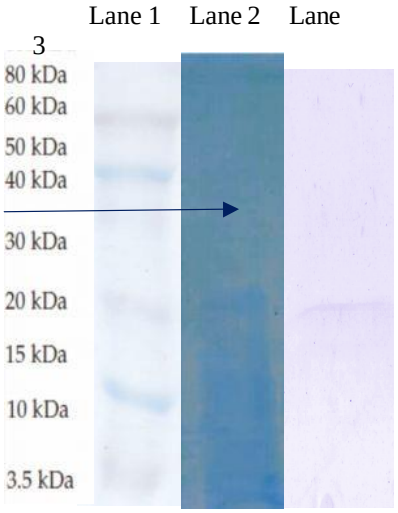


Figure 2. SDS PAGE of bacteriocin produced from *L.delbrueckii* subsp *bulgaricus*. Lane 1: Protein marker, Lane 2: Crude bacteriocin, Lane 3: Purified bacteriocin showing homogeneity

The electrophoresed gel was incubated on nutrient agar seeded with *P.aeruginosa* and an inhibition zone was observed which confirmed the inhibitory nature of the bacteriocin produced by *L.delbrueckii* subsp *bulgaricus.*

Yamamoto *et.al.,* [14] precipitated crude bactereriocin from culture supernatant of *E.faecalis* RJ-11 using ammonium sulphate and subjected it to gel chromatography using Sephadex G-50. The fractions were checked for activity against *L.monocytogenes* and the active fraction was pooled out to check its molecular weight by Tricine-SDS-PAGE which revealed that the bacteriocin had a mass of 5.0 KD which is homogenous. Similarly the bacteriocin G_2 produced by *L.plantarum* G_2 was purified by Seatovic *et.al.,* [4] using ultrafiltration membrane with a cut-off of 5KD, this was used as crude bacteriocin and its molecular weight was determined by SDS-PAGE and isoelectric focussing which revealed its molecular weight to be 2.2KD. The gel was overlaid with the test organism to check its activity and a zone was inhibition was seen.

The bacteriocin Weissellicin produced by *Weissella cibaria* 110 was partially purified using 40% ammonium sulphate salt saturation by Srionnual *et.al.,*[34]. The molecular weight of the bacteriocin was determined using SDS-PAGE which revealed its molecular weight to be 2.5KD which exhibited inhibition of *L.sakei* JCM 1157.

Table 3. Bacteriocin activity and concentration at three stages of purification

Purification stage	**Bacteriocin activity (AU/ml)**	**Protein concentration (µg/ml)**	**Specific activity (*)**	**Purification factor (+)**
Culture supernatant (crude)	800	310	2.5	1
Ammonium sulphate saturation (70%)	1400	280	5	2
Gel filtration	2733	210	13.0	2.6

			1	

*Specific activity is the activity unit divided by the protein concentration

+Purification factor is the increase in the initial specific activity

Estimation of protein by Lowry's method

The amount of protein present in crude bacteriocin was estimated as 620µl/ml, 70% ammonium sulphate saturated bacteriocin was 970µg/ml and gel purified bacteriocin was 860µg/ml respectively. The bacteriocin activity at all three purification stages is as tabulated in the Table 3. From Table 3 it can be said that the activity of bacteriocin increased at every stage of purification from 800AU/ml to 1400AU/ml in saturated bacteriocin i.e., 80% increase in activity and in gel filtered bacteriocin the activity was 2733AU/ml i.e., almost 99% increase in activity. The protein concentration of crude bacteriocin was 310µg/ml, salt saturated bacteriocin 280µg/ml but in the gel filtered bacteriocin, there was a decrease in protein concentration i.e., 210µg/ml.

Effect of pH, temperature and storage on bacteriocin activity

If a bacteriocin is considered as a bio preservative it should be stable at a wide range of pH to overcome the effects of acids and alkalis in food and also heat stable which is an important criteria since it should withstand the effect of heat during pasteurisation, it should also retain its activity for a longer period which in turn will increase the shelf life of the to be preserved food. Therefore, the bacteriocin produced from *L.delbrueckii subsp bulgaricus* was checked for all these parameters. The results obtained are as in Fig 3, 4 and Table 4.

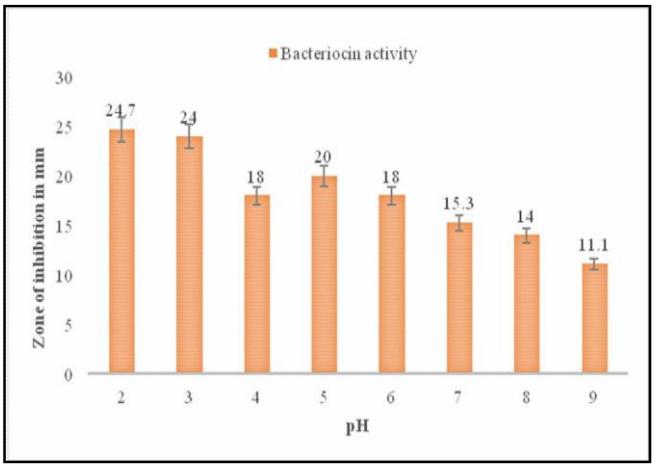


Figure 3. Stability of bacteriocin at different pH

Table 4. Stability of bacteriocin at different temperature and varying time intervals

Temperature in degree Celsius/minutes	15	20	45	60
	Zone of inhibition in mm			
30	14	15	15	15
40	16	15	15	13
60	15.05	16.25	12	12
80	11	12.25	12	13.05
100	--	--	--	--

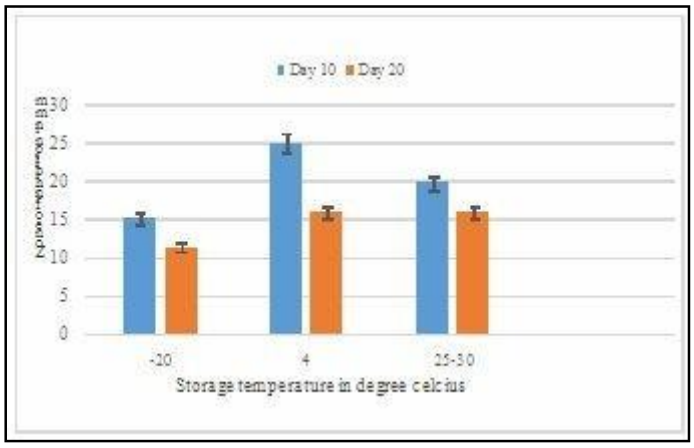


Figure 4: Stability of bacteriocin at different storage temperature

The pH of the crude bacteriocin was adjusted from pH 2 to 9 using 1N HCl and NaOH (about 5ml bacteriocin was used in each treatment) and checked for its stability by agar well diffusion method. The bacteriocin was highly stable at acidic pH when compared to alkaline pH with pH 2 showing the highest inhibition zone of 24.7mm followed by pH 3 with a zone of 24mm, the zone diameter decreased with increase in pH but the bacteriocin exhibited stability (Fig 4). It can be noted that there was a partial loss of activity when there was pH shift from acidic to basic.

The bacteriocin was stored at different temperatures i.e., at -20°C, 4°C and 25-30°C to check its optimum storage temperature and its stability with respect to storage temperature and duration of storage. The stability was checked for a period of 30days with 10days of interval, at day 10 it was noticed that the stability of bacteriocin stored at 4°C was stable in comparison with storage at -20°C and 25-30°C with a zone of 25mm (Fig 4). At day 20, similar results were obtained where stored at 4°C which gave a zone of

16mm and also storage at 25-30°C gave a zone of 16mm when compared to storage at -20°C with a zone of 11.3mm. Thus, storage at 4°C was found to be the optimum storage temperature for optimum stability and activity of bacteriocin produced by *L.dulbrueckii subsp bulgaricus*.

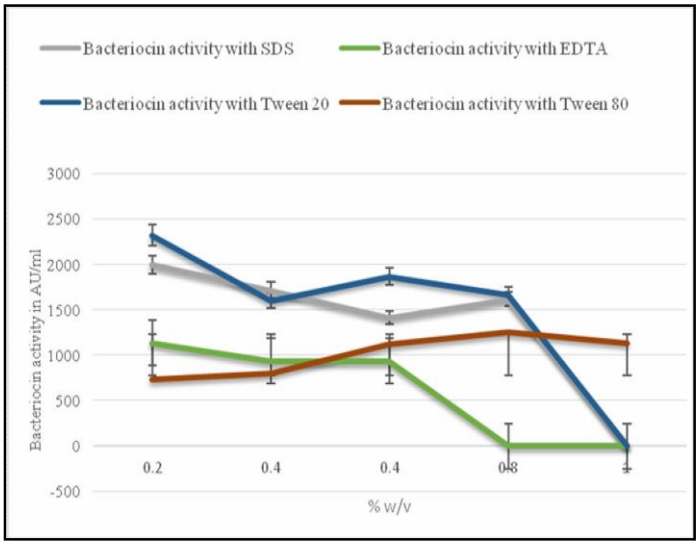
Different heat treatments were given to the bacteriocin to check its stability. The stability was assessed by noting the diameter of zone of inhibition after heat treatment. From Table 4 it can be noted that the bacteriocin was stable at temperatures ranging between 30°C to 80°C for a period of 60min which shows that the bacteriocin is thermo tolerant. Highest activity was observed when it was heated at 60°C for 30min and retained its activity up to 80°C for 60min which shows it is able to withstand pasteurisation which is related to its molecular structure composed of small peptides with no tertiary structure[31] which is an important characteristic of a bio preservative.

The bacteriocin produced from *L. plantarum* Lp6SH was found to be stable from pH 2.0 to 10 and its activity was observed up to 8 months when stored at 4°C and could withstand temperatures up to 121°C for 30min which is in agreement with the present study of bacteriocin stability[9], there are reports where the bacteriocin produced by *L. plantarum* lost activity at acidic pH and was active/ stable at neutral pH which is a limiting factor if to be used as a bio preservative, in case of thermal stability a decrease in activity was observed with increase in temperature from 30°C to 121°C with partial loss in activity[16]. The inhibitory activity of LAB isolated from mango pulp was investigated by Ravi *et.al.*,[12] and reported that the bacteriocin was stable only at near neutral to neutral pH of 4 to 8 and thermo stable up to 70°C with a partial loss in activity at 80°C [35] have reported that the bacteriocin produced by *L.brevis* MTCC 7539 was active at wide range of temperature from 50°C to 121°C for up to 20min till 100°C and up to 10min in case of 121°C, in case of pH it was found to be active from 3-10, with a maximum activity at pH 6 and concluded that it can be used as a food preservative. Alam *et.al.*, [36] and Xie *et.al.*,[37] have obtained similar results in case of heat stability where the bacteriocin was stable up to 80°C for 30min and pH 4-8 showed higher stability and thereafter partial loss in activity was reported in *Bacillus subtilis* BS 15 and *B.subtilis* LFB112, pH stability 4-8. Malini and Savita [22] and Nivedita *et.al.*, [38] studied the effect of storage temperature and storage period on bacteriocin activity. Malini and Savita [22] have reported that the bacteriocin produced by *L.paracasei* subsp *tolerens* showed activity up to 30 days when stored at a temperature of 4°C, similarly Nivedita *et.al.*, [38] reported that there was no loss in activity of bacteriocin produced by *B.subtilis* R75 when stored at 4°C for up to 75 days of storage in comparison with other storage temperature of -20°C and these reports are in favour of the present study.

Effect of surfactants and detergents

Surfactants and detergents at different concentrations were used to study the bacteriocin activity

profile. The MRS broth was supplemented with SDS, EDTA, Tween 20 and Tween 80 at concentrations 0.2 to 1.0% w/v. From Fig 5, it is noted that Tween 20 and 80 improved bacteriocin activity and similar results with SDS and EDTA decreased bacteriocin activity in comparison with. Among Tween 20 and 80,



Tween 20 supported the maximum bacteriocin activity of 2316.6AU/ml at a concentration of 0.2% w/v.

Figure 5. Effect of surfactants and detergents on bacteriocin activity.

Studies by Sahar *et.al.*, [39] have revealed that exposure of bacteriocins to surfactants (Tween) increases its activity due to unfolding of proteins which directly has an effect on bacteriocin activity which is in favour of the present study where bacteriocin activity was maximum in case of Tween 20.

Ogunbanwo *et.al.*, [21] have also reported similar findings in case of *L.plantarum* F1 where there was a threefold increase in bacteriocin activity in comparison with control without Tween 20. In case of *L.lactis* subsp *lactis* B14, Ivanova *et.al.*, [8] has reported that addition of SDS increased bacteriocin activity due to solubilisation of insoluble protein aggregates whereas with the present study where SDS did not exhibit bacteriocin activity to a considerable extent.

Effect of metal ions on bacteriocin activity

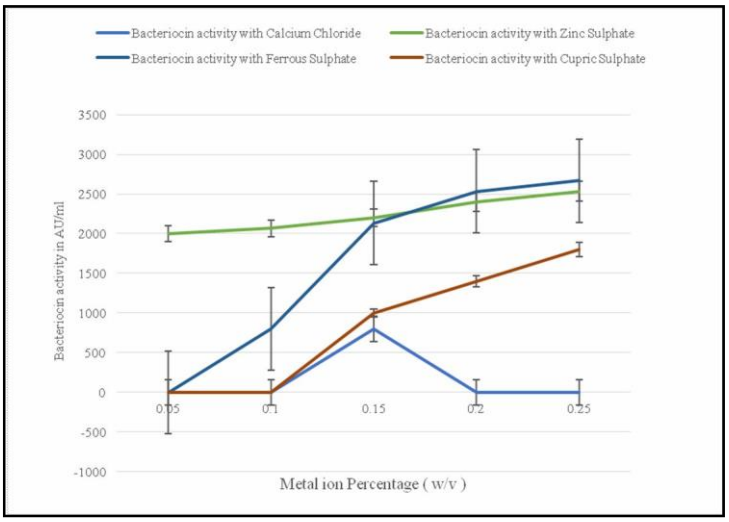


Figure 6: Effect of metal ions on bacteriocin activity

Four different metal ions $FeSO_4$, $CuSO_4$, $ZnSO_4$ and $CaCl_2$ at different concentrations of 0.05%. 0.1%, 0.15%, 0.2% and 0.25% w/v were selected to study the effect of metal ions on bacteriocin activity. The MRS media was supplemented with these ions and it was observed that $ZnSO_4$ and $FeSO_4$ increased bacteriocin activity at 0.05% concentration, at subsequent concentrations all four metal ions supported bacteriocin activity with an exception of $CaCl_2$ which supported activity only at a concentration of 0.15%. The optimum activity of 2666.6AU/ml was observed with $FeSO_4$ at a concentration of 0.25% (Fig 6).

In the present study it was observed that the activity of bacteriocin increased with the increase in concentration of the metal ion which is contradictory with the findings of Graciela *et.al.,* [40] where they observed a decrease in activity with increase in concentration. Kabore *et.al.,* [26], has reported that the activity of bacteriocin produced from *Bacillus subtilis* was completely lost when metal ions such as Fe^{2+}, Mg^{2+} or $Mn^{2+}$was added to growth media. But, according to the reports of Von Mollendorff *et.al.,* [7] addition of $MgSO_4$ increased bacteriocin activity in case of *L.fermentum* JW11BZ which is in agreement with the present study.

Bio preservation of sprouts using bacteriocin

A preliminary check was carried out using lyophilised *L.delbrueckii subsp bulgaricus culture*, crude

bacteriocin and 70% saturated bacteriocin were tested for its bio preservation characteristics. Two varieties of sprouts *Vigna radiate* (Green gram) and *Cicer arietinum* (Black chickpea) were tested for its shelf life. All three samples were mixed with 50g of both the varieties of sprouts separately in sterile containers under sterile conditions and stored at 4°C and checked periodically for contamination. The results obtained were as in Plates 1, 2 and 3.

Plate 1. Bio preservation of sprouts: day 5

1a. Control **1b. Day 5**

***Cicer arietinum* (Black chickpea)**

1c. Control **1d. Day 5**

***Vigna radiate* (Green gram)**

1a and 1c Control sample of *Cicer arietinum* (Black chickpea) and *Vigna radiate*(Green gram) at day 5, 1b and 1d showing *Cicer arietinum* (Black chickpea) and *Vigna radiate*(Green gram) preserved for 5days using culture, crude and 70% saturated bacteriocin.

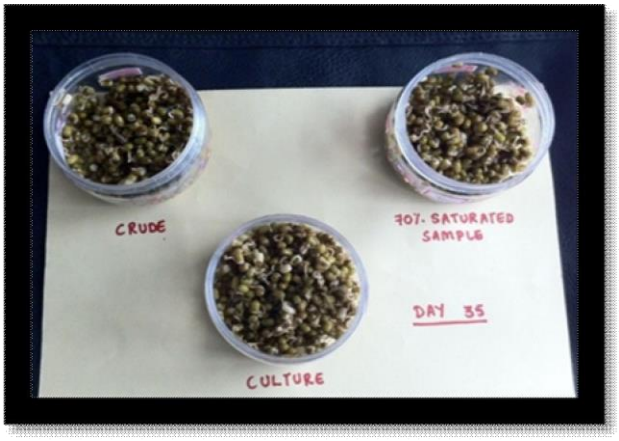
Plate 2. Bio preservation of sprouts: day 12.

2a. Day 12 **2b. Day 12**

***Vigna radiate* (Green gram)** ***Cicer arietinum* (Blackchickpea)**

2a, b; Biopreserved *Vigna radiate* (Green gram) and *Cicer arietinum* (Black chickpea) on day 12.

Plate 3. Biopreserved *Vigna radiate* (Green gram): day 35



In plate 1 both the varieties of sprouts showed no contamination on day 5 of preservation at 4°C using culture, crude and saturated sample except for the control where contamination occurred in both sets (Plate 1a, 1c). Fungal contamination was observed in case of black chickpea and a slimy bacterial growth was observed in green gram. Therefore, the bio preservative was found to be effective at day 5.

In green gram (Plate 2a) there was no sign of contamination hence bio preserved but contamination was observed in case of *Cicer arietinum* (Black chickpea) (Plate 2b) in all three bio preservatives i.e., the sample mixed with culture, crude bacteriocin and the 70% saturated bacteriocin. Hence, the shelf life of *Cicer arietinum* (Black chickpea) using bacteriocin as a preservative was found to be 10 days approximately when stored at 4°C.

Green gram was found to have a prolonged shelf life of about 35 days when compared to black chickpea. At day 35 there was no contamination observed which clearly indicates that green gram can be bio preserved for more than 35days at 4° using *L.delbrueckii subsp bulgaricus* culture and its crude and 70% saturated bacteriocin in comparison with black chickpea which could to bio preserved only for a period of 10days.

Kumar *et.al.,* [41] used a combination of pediocin and sodium nitrate at concentrations of 1000AU/ml and 100µg/ml to check the reduction in number of *Listeria* count in black gram and mung bean sprouts. A significant reduction was observed by 4 to 5 log units in *Listeria* count in comparison with the black gram sprouts control which showed an increase in *Listerial* count from 7.5 to 9.35 log units when stored at 4°C for 7 days. Mundticin produced by *Enterococcus mundtii* was evaluated for its bio preservative properties to control growth of *L.monocytogenes* in modified atmosphere mung bean sprouts by Bennik *et.al.,* [42]. They observed that the growth was not inhibited in the modified atmosphere of 1.5% O_2, 20% CO_2, and 78.5% N_2 at 8°C where the fresh mung bean sprouts were stored but inhibition was observed when *L. monocytogenes* was inoculated onto sterile vegetable media. But, the activity of mundticin was observed when used in processing steps such as washing and coating.

Conclusion

Lactobacillus sp have been explored for their bio preservation characteristic because they synthesize bacteriocins and come under GRAS status. In the present study, the obtained results were satisfactory to conclude that the bacteria, *Lactobacillus delbrueckii subsp bulgaricus* is a potent bacteriocin producer which inhibited the growth of both Gram positive and Gram negative pathogens thus exhibiting broad spectrum inhibition. The produced bacteriocin could withstand varied temperature and pH treatments which is a novel characteristic of a protein to be explored as a food preservative (bio preservative). It was found to be stable at higher temperatures of up to 80°C which makes it feasible to be used in liquid and solid foods which needs to be pasteurised before consumption, for example ready to eat foods. The bacteriocin can be used as a bio preservative in different foods from highly acidic foods such as fruit juices to vegetables which are alkaline. It can also be used as a preservative in cosmetic industry as it can tolerate the effects of metal ions such as $FeSO_4$ which is highly used in the manufacturing of cosmetics. Using this bacteriocin, the two variety of sprouts namely *Vigna radiate* (Green gram) and *Cicer arietinum* (Black chickpea) could be bio preserved for a period of about 35days and approximately 10days at 4°C. Therefore, the bacteriocin produced from *Lactobacillus delbrueckii subsp bulgaricus* can be treated as a potential bio preservative for replacing chemical food preservatives which have side effects on the foods which are to be preserved as well as on the health of the consumer.

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