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RESEARCH ARTICLE

Bacteriocin production by lactic acid bacteria and their antioxidant property

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Abstract: Lactic acid bacteria (LAB) are well known for their health benefits and hence they have wide application in food industries. These lactic acid bacteria produce bacteriocin, low molecular weight protein, which inhibit the growth of several closely related bacteria. In present study, out of 25 lactic acid bacteria 9 isolates produce higher lactic acid were evaluated for their ability to produce bacteriocin. Their growth pattern suggested that L10 showed highest growth compared to other. These LAB were examined for their antimicrobial activity against pathogens and their results revealed that all the isolates inhibit the growth of *Staphyloccus*, *Enterococcus, Serratia, Micrococcus*, however, unable to inhibit growth of *E. coli, Salmonella, Proteus,* and *Yersinia*. Isolates L1 to L4 shows inhibition of *Listeria monocytogens* while rest of bacteria were unable to show antimicrobial activity against it. Antioxidant activity measured using FRAP assay suggested that isolated L6 (0.236 mM/g) showed highest antioxidant followed L3 and L11 which showed similar antioxidant activity (0.207 mM/g). Bacteriocin was partially purified using ammonium precipitation. Quantification of partially purified bacteriocin revealed that highest production by isolate L3 (32.2 IU) followed by L6 (30.45 IU) and L1 (30.38 IU). Among all the isolates L3 showed higher antioxidant and higher bacteriocin production and hence it was identified as *Enterococcus faecium.* Thus, potential culture can be used for inhibition of pathogenic microbes by its antimicrobial bacteriocin production.

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Introduction

Many lactic acid bacteria are extensively used as starter cultures for fermentation processes by the food industry. They provide many health benefits like maintaining a healthy gastrointestinal system, antioxidant effect, anti-inflammatory effect, protection against pathogens, immunomodulatory effect, etc. (George et al. 2018; Saadat et al. 2019; Ayivi et al. 2020). Many LABs have the ability to produce antimicrobial substances like bacteriocin, which hampered the growth of several pathogenic bacteria in the gut and thus maintained a healthy environment in the gastrointestine. Due to their abundant health benefits, many LABs like *Lactococcus lactis, Lactiplantibacillus pentosus, Lactiplantibacillus plantarum, Levilactobacillus brevis,* and *Leuconostoc mesenteroides* are widely used for fermentation in the food industry (Vyas et al. 2017; Mokoena, 2017). They are also used in the preparation of yogurt, cheeses, sauerkraut, fermented milk, fermented meat, fermented cereals, etc. (Choi et al. 2013; Kim et al. 2016).

Bacteriocins are antimicrobial peptides produced by bacteria, notably lactic acid bacteria (LAB), and they impede the growth of closely related bacterial strains. These chemicals are important in microbial ecology because they help bacteria compete for resources and keep their habitats balanced. Bacteriocins differ in structure and mechanism; however, they are broadly classified into classes depending on their properties. Broadly, they are classified into four different classes, viz., lantibiotic, non-lantibiotic, large peptide, and lipid-containing. Lantibiotics comprise unusual amino acids, i.e., lanthionine or methyllanthionine, and nisin is a typical and well-studied example of this class. Non-lantibiotics are smaller, heat-stable peptides, and pediocin and lactocin belong to this class. Class three comprised a larger peptide, which is heat-labile, while class four comprised peptide and lipid. Bacteriocins typically disrupt the bacterial cell membrane, leading to cell lysis. They may bind to specific receptors or interfere with essential cellular processes.

They have important applications in food preservation, where they act as natural preservatives, and in health, where they may contribute to combating infections and promoting gut health. Their efficacy, combined with their generally recognized as safe (GRAS) status, makes them a viable option to chemical preservatives in a variety of industries. Bacteriocins represent a promising area of research with applications in food safety, healthcare, and agriculture. Continued exploration into their mechanisms, production, and applications will likely yield significant benefits in combating microbial resistance and ensuring food safety. Hence, the present study aims on bacteriocin production and its antimicrobial and antioxidant activity by isolated lactic acid bacteria.

Materials and Methods

Lactic acid bacterial culture and their temporal growth

Lactic acid bacteria were isolated from the fermented batter samples of idali, khaman and handavo (Gujarti fermented food) as described previously (Vyas et al. 2017). Out of 25 isolated bacteria, 9 potential lactic acid producing bacteria (culture designated as L1, L2, L3, L4, L5, L6, L8, L10 and L11) were used for bacteriocin production. These bacteria were morphologically characterized by Gram's reaction and found to be Gram positive. For growth pattern, cells were inoculated in De Man–Rogosa–Sharpe (MRS) broth and optical density was monitored upto 96 hrs at 600 nm. Experiment was conducted in triplicate and standard deviation was calculated and expressed as error bar in figure.

Antimicrobial activity

Antimicrobial activity was carried out on Mueller Hinton Agar (MHA) by well diffusion method. The test organisms viz. *Staphylococcus aureus* (ATCC 11632), *Enterococcus faecalis* (ATCC 14506), *Serratia marcescens* (ATCC 14756), *Micrococcus luteus* (ATCC 10240), *Listeria monocytogenes* (ATCC 13932), *E. coli* (ATCC 10536), *Yersinia enterocolitica* (ATCC 23715), *Proteus vulgaris* (ATCC 33420) and *Salmonella poona* (ATCC 4840) were procured from HiMedia, Laboratories Private Limited, Mumbai. All the isolates were grown in nutrient broth for 18 hrs. All these test cultures were spread on Mueller Hinton Agar. A well (7mm diameter) was prepared with sterilized cup borer and 100 µl of supernatant was place in well. Plates were incubated and zone of inhibition was measure after 24 – 48 hrs of incubation.

Antioxidant activity

Antioxidant activity was measured by FRAP assay as method described by Benzie and Strain et al. (1996). Briefly, 5 ml of sample was mixed with 1.5 ml of FRAP reagent and optical density was recorded at 593 nm. After 4 min of incubation optical density was again measured at 593 nm. Change in the optical density after 4 min incubation from initial reading was calculated from the standard curve prepared by $FeSO_4 \cdot 7H_2O$. Experiment was conducted in triplicate and standard deviation was calculated and expressed as error bar in figure.

Protein content

Protein content was measured by Folin and Lowry's method (Lowry et al. 1951). Protein concentration from the sample was extrapolated from the standard graph prepared using bovine serum albumin.

Partial purification of bacteriocin

Partial purification of bacteriocin was carried out by ammonium sulphate precipitation method described by Srinivasan and co-workers (2013). Briefly, isolates were grown in MRS broth for 7 days. Cells were removed by centrifugation at 10,000 rpm for 20 min and supernatant was collected. For precipitation of protein from supernatant, 35 % solid ammonium sulfate was added. The solution was centrifuged, and the precipitate was discarded because it had low bacteriocin activity and the supernatant was used for further precipitation by 75 % ammonium saturation. The precipitates were collected by centrifugation and dissolved in sodium phosphate buffer (pH 6.8). Sample was dialyzed using phosphate buffer (pH 6.8) for 24 hrs. This was used for further bacateriocin estimation and designated as partially purified bacteriocin.

Bacteriocin production

Partially purified bacteriocin was further precipitated with ammonium sulphate and steam sterilized at 120 ºC at 15 psi as method described by Balogu et al. (2017). Quantification was done spectrophotometrically at 450 nm and value was extrapolated from the nisin standard graph and expressed as IU/ml as nisin equivalent. Standard graph of Nisin was prepared as method describe by Papagianni et al. (2006) by adding 0.1g of nisin to solution (10 ml 0.02 N HCl and 0.75% NaCl). Experiment was conducted in triplicate and standard deviation was calculated and expressed as error bar in figure.

Identification of isolate

Potential isolate was identified by morphological and biochemical characteristics. Isolated bacteria were morphologically characterized by Gram's reaction. For identification by biochemical testing, outsourcing service taken and identified using VITEK.

Results and Discussion

Growth of lactic acid bacteria

For growth pattern of LAB, cells were grown in MRS media and it was monitored for 96 hrs. Data revealed that isolated L10 showed higher growth compared to rest of the LAB. Isolated L2,

L6 and L10 showed higher growth at 48 hrs, while rest of the isolates showed higher growth at 72 hrs except isolate L8 which shoed higher growth at 96 hrs of incubation (Figure 1).

Antimicrobial activity

All the lactic acid producing bacteria were tested for their ability inhibit growth of other bacteria by producing antimicrobial agent. Antimicrobial activity was measured by well diffusion assay. Isolates were tested for their antimicrobial activity against *E. coli, Yersinia, Proteus, Salmonella, Staphylococcus, Enterococcus, Serratia, Micrococcus* and *Listeria*. Tested organisms were individually streaked on Mueller Hinton Agar (MHA). All the lactic acid bacteria inhibit the tested bacteria except they were unable to inhibit growth of *E. coli*, *Yersinia*, *Proteus* and *Salmonella* (Table 1, Figure 2). L1, L2, L3 and L4 inhibit the growth of all the tested isolates. L5, L6, L8 and L10 inhibits the growth of all tested organisms except *Listeria* sp. whereas, L11 inhibits three tested organisms except *Staphylococcus* and *Listeria* sp. Among the all tested samples which showed inhibition of tested bacteria, *Entrococcus* showed highest inhibition followed by *Serratia* and *Micrococcus* (Figure 2).

Antioxidant activity

Antioxidant activity of sample was measured using Ferric Reducing Antioxidant Power (FRAP) assay. Highest antioxidant activity was observed in L6 culture (0.236 mM/g), followed by in

Table 1 : Antimicrobial activity (zone of inhibition mm) of the crude bacteriocin by lactic acid bacteria

Tested Culture LAB	*S. aureus*	*E. faecalis*	*S. marcescens*	*M. luteus*	*L. monocytogenes*	*E. coli*	*Y. enterocolitica*	*P. vulgaris*	*S. poona*
L1	13	13	13	12	14	-	-	-	-
L2	14	15	14	13	13	-	-	-	-
L3	13	16	14	11	13	-	-	-	-
L4	14	14	14	12	15	-	-	-	-
L5	13	13	14	13	-	-	-	-	-
L6	12	16	15	12	-	-	-	-	-
L8	13	14	14	13	-	-	-	-	-
L10	14	13	14	13	-	-	-	-	-
L11	-	12	12	12	-	-	-	-	-

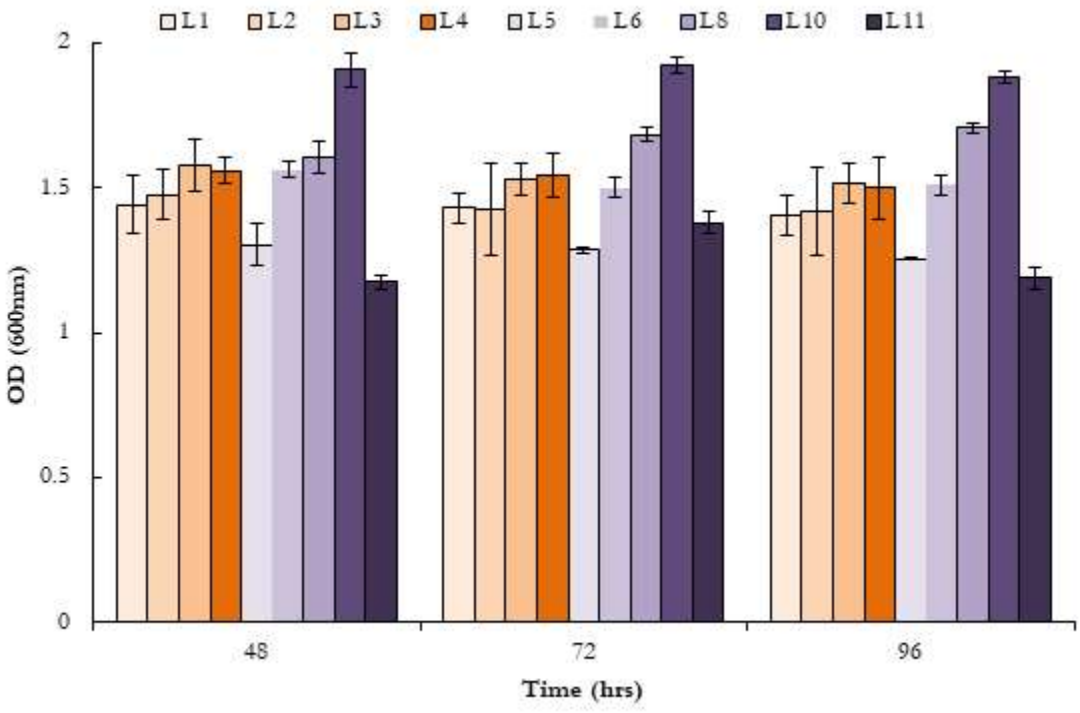

Fig. 1 : Growth of isolated LAB in MRS broth (*Error bar indicates ±Standard deviation

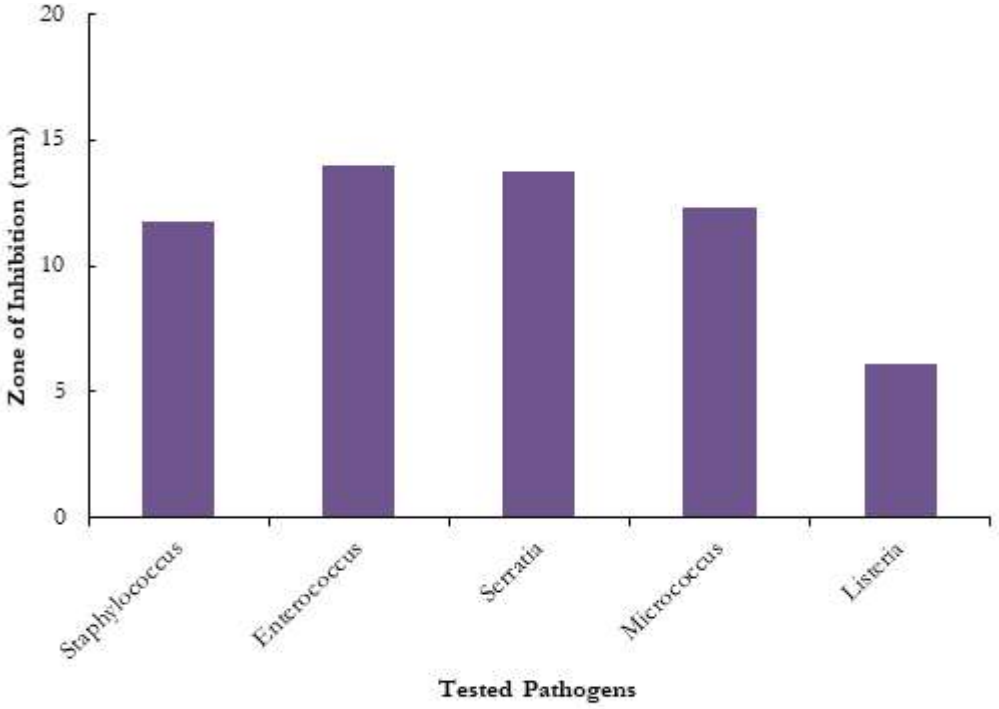

Fig. 2 : Zone of inhibition by lactic acid bacteria against pathogens

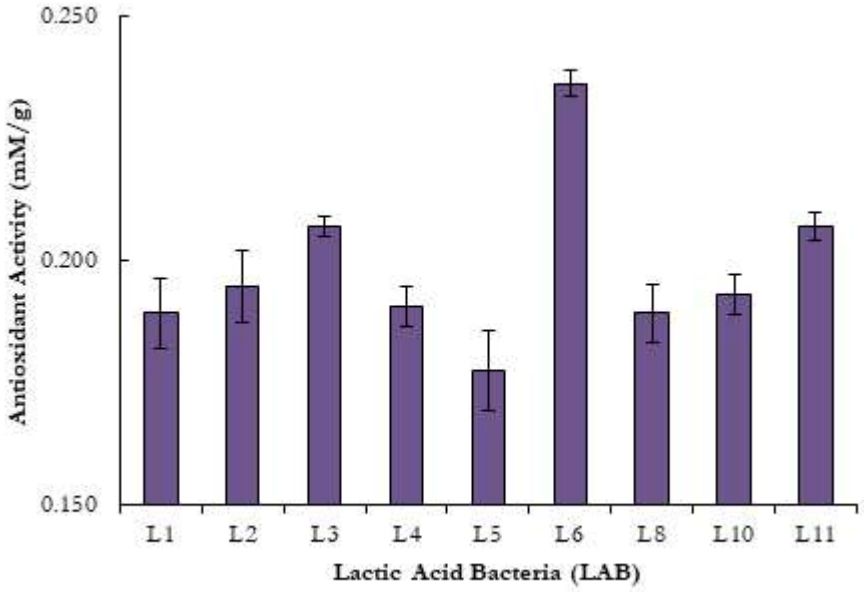

Fig. 3 : Antioxidant activity of crude bacteriocin by FRAP assay (*Error bar indicates ±Standard deviation)

L3 and L11 culture which showed same antioxidant activity (0.207 mM/g) (Figure 3).

Protein content

Lactic acid producing bacteria produced antimicrobial compound, a bacteriocin, which is protein in nature. Hence, protein content was measure by Folin and Lowry's method. L3 showed higher protein content (57.8 µg/ml) followed by L2 (48.2 µg/ml) and L11 (38 µg/ml) (Table 2).

Partial purification of bacteriocin

For extraction of bacteriocin produced by bacterial isolates, protein form the broth was precipitated with 60 - 80 % ammonium sulfate. Precipitated protein was collected and this partially purified protein was used for quantification of bacteriocin.

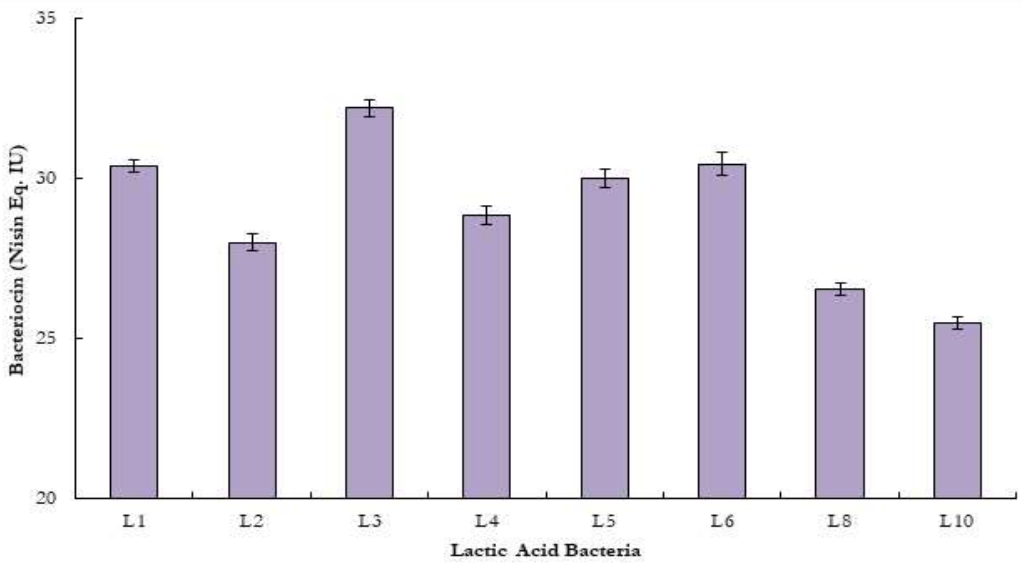

Fig. 4 : Bacteriocin (Nisin Equivalent) production by lactic acid bacteria (*Error bar indicates ±Standard deviation)

Table 2 : Protein content of the crude bacteriocin & partial purified bacteriocin

Bacterial isolates	Crude bacteriocin (µg/ml)	Partially Purified Bacteriocin (µg/ml)
L1	37.4	2.97
L2	48.2	3.19
L3	57.8	3.37
L4	20.3	3.41
L5	24.8	3.75
L6	20.9	3.14
L8	24.3	3.14
L10	17.7	2.17
L11	38.0	3.37

Bacteriocin production

Bacteriocin production was quantified as method described by Balogu et al. (2017) using nisin as standard. All the isolates were found positive Highest production of bacteriocin was observed in culture L3 (32.2 IU) followed by L6 (30.45 IU) and L1 (30.38 IU)(Figure 4).

Identification of isolates

L3 which showed higher protein content and higher nisin production compared to other lactic acid bacteria. Hence, it was morphologically and biochemically characterized. Isolate L3 was identified as *Enterococcus faecium* using VITEK (outsourcing service).

Bacteriocins are antimicrobial peptides produced by bacteria, particularly lactic acid bacteria (LAB), that inhibit the growth of closely related bacterial strains. These substances play a crucial role in microbial ecology by helping bacteria compete for resources and maintain a balance in their environments. Bacteriocins vary in structure and mechanism but are generally categorized into classes based on their characteristics. They have significant applications in food preservation, where they act as natural preservatives, and in health, where they may contribute to combating infections and promoting gut health. Their effectiveness, combined with a generally recognized as safe (GRAS) status, makes them an appealing alternative to chemical preservatives in various industries. The amount of bacteriocin produced by lactic acid bacteria (LAB) can vary widely based on several factors like temperature, pH, nutrient availability, and also on type of strain. Optimization of environmental conditions often enhances yields. *Lactococcus lactis* F44 strain genetically engineered by introducing 17 acid-tolerant genes and 6 lactic acid synthetic genes showed enhanced nisin titers from 2810 IU/ml to 3850, 3979, and 4377 IU/ml by overexpression of hdeAB, ldh, and murG, respectively (Zhang et al. 2016).

In laboratory settings, bacteriocin production is often quantified in terms of activity units (AU) per milliliter or as a concentration measured in micrograms per milliliter (µg/mL). In the present study, higher nisin equivalent production was reported in culture L3 (32.2 IU), followed by L6 (30.45 IU), and L1 (30.38 IU). In

Lactococcus lactis ssp. *lactis* 32 nisin production started in the second week and reached 97 µg/g after four weeks (Hassan et al. 2021). Shimizu and co-worker (1999) reported that *L. lactis,* when grown under anaerobic conditions, produces 7.4 mg/liter of nisin.

Out of the four lactic acid bacteria tested for their antioxidant activity of the DPPH assay, the highest antioxidant activity was reported in *L. brevis* (94.47%), followed by *L. gasseri* (91.29%) at 210 min, while the antioxidant activity of *L. rhamnosus* and *L. plantarum* was 83.41% and 77.53%, respectively, at 210 min (Vougiouklaki et al. 2023). Bacteriocin purified from *Lactococcus lactis* strain CH3 isolated from fermented dairy products showed radical scavenging potential with an EC_{50} value of 12.5 ìg/mL by DPPH assay (Krishnamoorthi et al. 2022). *Enterococcus faecium* GRD AA and *Paenibacillus polymyxa* isolated from toddy and milk showed 85% of DPPH and 85.5% of ABTS radical scavenging activity while 87.6% Fe^{2+} reduction potential (Krishna et al. 2021). Thus, present findings corroborate that *Enterococcus faecium* and other isolated lactic acid bacteria have potential antioxidant activity.

Antimicrobial activity of 28 isolated bacteria against *Escherichia coli*, *Pseudomonas fluorescens*, *L. innocua, Erwinia carotovora, Bacillus cereus*, and *Leuconostoc mesenteroides subsp. mesenteroides* using the agar diffusion bioassay and also against *Penicillium expansum*, *Botrytis cinerea,* and *Monilinia frucitcola* using the microdilution plate method revealed that isolated LABs strongly inhibit all microorganisms tested except *E. coli, Ent. faecium*, *Strep. thermophiles,* and *Lact. casei* (Yang et al. 2012). A similar study carried out by Thuy and co-worker (2024) reported that three strains of LAB *Weissella confusa* CYLB30, *Lactiplantibacillus plantarum* CYLB47, and *Limosilactobacillus fermentum* CYLB55 demonstrated a strong antibacterial effect against *Klebsiella pneumoniae*, *Salmonella enterica* serovar Choleraesuis, *Escherichia coli, Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* Thus, the antimicrobial activity of bacteriocin is well documented and supports the present findings.

Conclusion

In present study, 9 lactic acid bacteria were examined for their bacteriocin production. All the isolates showed antimicrobial effect against *Staphylococcus, Enterococcus, Serratia*, *Micrococcus and Listeri*a, while non-effective against *E. coli, Yersinia, Proteus* and *Salmonella*. They were also possessing antioxidant and have ability to scavenge reactive oxygen species. Isolate L3 showed higher protein content and bacteriocin production hence it was further identified as *Enterococcus faecium*. Thus, *Enterococcus faecium* can be used for inhibition of several plant pathogens by secretion of antimicrobial molecule i.e., bacteriocin.

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