Strain improvement of newly isolated
*Lactobacillus acidophilus* MS1 for enhanced bacteriocin production

Geliştirilmiş bakteriyosin üretimi için yeni izole edilen *Lactobacillus acidophilus* MS1’in şuğunun iyileştirilmesi

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Abstract

**Background:** Lactic acid bacteria (LAB) are considered as GRAS (generally recognized as safe) and being used extensively as bio-preservatives. Bacteriocins, the metabolites of LAB, belong to a diverse family of naturally synthesized antimicrobial peptides.

**Objective:** Strain improvement for enhanced bacteriocin production by physical and chemical mutagenesis.

**Methods:** The bacterial strain was identified by 16S rDNA sequence analysis and improved by ultraviolet and ethidium bromide mutation. The resultant bacteriocin was biochemically characterized, purified and analyzed for mass determination.

**Results:** Among mutants of identified *Lactobacillus acidophilus* MS1, the UV$_6$ (ultraviolet mutant) revealed 3400 AU bacteriocin activity with 42% survival rate and EB$_5$ (ethidium bromide mutant) exhibited 4020 AU with 28% survival rate. Bacteriocin of 6.5 kDa was purified by cation exchange and gel exclusion chromatography. It was found to be thermally stable at 100°C for 30 min and maintained the stability up to 121°C. The activity was monitored in a wide range of pH (4–9).

**Conclusion:** Being resistant to several biochemical parameters, the bacteriocins have an effective incorporation in food, forage and pharmacy. There is a need to engage more efforts to explore novel bacteriocins and multifarious applications.

**Keywords:** *Lactobacillus acidophilus*; Mozzarella cheese; Mutagenesis; Bacteriocin.

**Özet**

**Giriş ve amaç:** Laktik asit bakterileri GRAS (genellikle güvenli) olarak kabul edilir ve biyo-koruyucular olarak yaygın olarak kullanılmaktadır. Bakteriyosinler, laktik asit bakterilerinin metabolitleri, doğal olarak sentezlenmiş antimikrobiyal peptit ailesine aittir. Amacımız fiziksel ve kimyasal mutajenez ile bakteriyosin üretimi arttırmış yeni suşların üretmektir.

**Yöntem ve gereçler:** Bakteri türü 16S rDNA sekans analizi ile tanımlandı; ultraviyole ve etidyum bromür mutasyonu ile geliştirildi. Amacımız fiziksel ve kimyasal mutajenez ile bakteriyosin üretimi artırması yeni suşlar üretmektir.

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**Bulgular:** Tespit edilen *Lactobacillus acidophilus* MS1 mutantları arasında UV6 (ultraviyole mutant) % 42 canlılık oranı ile 3400 AU bakteriyosin aktivitesi ve EB5 (etidyum bromür mutantı), % 28 canlılık oranı ile 4020 AU göstermiştir. 6.5 kDa’lık bakteriyosin, katyon değişimi
ve jel dışlama kromatografisi ile saflaştırıldı. 30 dakika süreyle 100°C'de termal olarak kararlı olduğu bulundu ve 121°C'ye kadar stabilite muhafaza edildi. Etkinlik geniş bir pH aralığında (4–9) izlendi.

**Tartışma ve sonuç:** Birkaç biyokimyasal parametreye karşı dirençli olan bakteriyosinler gidai, yem ve eczacılıkta etkin bir şekilde kullanılmaktadır. Yeni bakteriyosinler ve çok yönlü uygulamaları keşfetmek için daha fazla çaba harcamaya ihtiyaç vardır.

**Anahtar Kelimeler:** Lactobacillus acidophilus; Mozzarella peyniri; Mutagenez; Bakteriyosin.

**Introduction**

Nowadays, biotechnology in food processing is improving food quality and safety by incorporating natural antimicrobial substances like metabolites of food fermentative bacteria instead of synthetic and chemical preservatives. The metabolites of lactic acid producing bacteria have been traditionally used as starter cultures in food fermentation and considered as safe regarding health [1].

Various genera of lactic acid bacteria (LAB) like Lactococcus, Lactobacillus, Leuconostoc, Streptococcus, Pediococcus and Oenococcus are generally used as food additives because of their non-conflicting nature in gastrointestinal tract. Some strains of LAB have health regarding benefits by providing antibacterial potential against many pathogenic bacteria [2]. The advancement of LAB as probiotics, nutraceuticals and vaccines brought considerable attention for those interested in food safety and promoting health. These bacteria can survive and express their function in digestive tract even under stress or less favorable conditions such as low pH, bile salts and starvation [3, 4].

Bacteriocins of LAB are small proteinaceous antimicrobial substances exhibiting antagonistic activity against closely related and even unrelated bacteria [5, 6]. These small peptides are ribosomally synthesized and extra-cellularly released having specific immunity mechanism against different bacteria [7, 8]. Bacteriocin production often takes place during late exponential or early stationary phase of bacterial growth as secondary metabolites depending on stress signaling and quorum sensing [9, 10]. These peptides were first discovered by Gratia in 1925 from Escherichia coli, and named as colicin [5, 11]. Comparatively, bacteriocins are considered more natural than therapeutic antibiotics because these peptides are rapidly digested by proteolytic enzymes in gastrointestinal tract and do not illicitly cause an allergic effect. According to recent classification, bacteriocins isolated from LAB are divided into three main classes: the class I comprises of unusual amino acid lanthionine and these are named as lantibiotics, class II are non-lantibiotics having small thermostable peptides and class III consists of large thermostable peptides [10, 12].

The low molecular mass bacteriocins produced by Lactobacillus species are non-lantibiotics. Various strains of L. acidophilus are beneficial members of normal intestinal microflora and have been extensively used in fermented dairy products. Moreover, these beneficial strains are widely distributed in market as probiotics and in functional food like supplements. The majority of class II bacteriocins are heat stable low molecular mass non-lantibiotic peptides [13, 14].

The aim of the current study was to improve strain by physical and chemical mutagenesis for hyper production of bacteriocin from newly isolated L. acidophilus MS1. We also investigated the characterization and purification of the resultant bacteriocin.

**Materials and methods**

**Selection of bacterial culture and media**

*Lactobacillus acidophilus* MS1 was isolated from mozzarella cheese by pour plate method and then purified by streak plate method aseptically. The culture media used were MRS agar/broth (Oxoid, England) for *L. acidophilus* MS1 and nutrient agar/broth (Oxoid, England) for test strain, Bacillus cereus ATCC 14579. A sample of 1:10 dilution was subsequently made using 0.85% (m/v) sterile saline solution. This sample was serially diluted with a dilution factor 10⁻¹–10⁻⁶ of which 0.1 mL diluents from each dilution was poured evenly on duplicate MRS agar plates. The plates were then incubated aerobically at 37°C for 48 h [15]. Bacterial colonies that grew on agar plates were randomly picked and purified by re-plating on MRS agar plates using streak plate method. The above procedure was repeated until pure and morphologically distinct colonies were obtained. The purified colonies were transferred to MRS broth and ultimately maintained at 4°C for further use.

**Identification of bacterial culture**

The preliminary identification of *L. acidophilus* MS1 isolated from mozzarella cheese was based on morphological and physiological characteristics according to Bergey’s manual. The biochemical characterization of isolated strain was done by observing carbohydrate fermentation profile using API 50 CHL system (Bio Merieux, France) and further confirmation was done by 16S rDNA sequence
Strain improvement by mutagenesis

UV mutagenesis

The effect of mutation on strain improvement was studied for bacteriocin production as described by Gawel et al. [16]. To achieve this, aliquot of growth culture in its logarithmic phase (14 h) was maintained to an optical density (OD 600) of 0.2–0.3. Cell suspension was centrifuged at 8000 x g and re-suspended in half the volume of ice-cold 0.9% NaCl. Ten milliliter quantity of aliquot was aseptically pipetted out in sterile Petri plates and exposed to UV radiations at a distance of 30 cm from an electric germicidal bulb 20 W (Phillips) for 5–50 min with 5 min intervals. Each irradiated sample was re-inoculated in fresh culture medium and incubated for 18 h. A 100 μL aliquot of 100 folds serial dilution was aseptically spread on MRS agar plates to give 30 colonies or less per plate. A control was included having same dilution but no exposure to UV radiations. All culture plates were kept in complete darkness by wrapping in aluminum foil for 48 h [16]. The bacterial colonies having 1% survival rate was confirmed again by biochemical tests. The survival rate (%) of mutants were calculated and compared with wild strain for bacteriocin activity.

Chemical mutagenesis by ethidium bromide

For chemical mutagenesis, a modified Sobrun et al. [17] procedure was used. The bacterial culture in logarithmic growth phase (18 h culture) was harvested by centrifugation and washed twice with cold sterile 0.9% NaCl solution. To 20 mL cell suspension, 0.5 mg/mL (w/v) concentration of ethidium bromide was added and aerated on orbital shaker at 200 rpm and 37°C. One milliliter sample was collected after each 30 min, washed once with 0.9% NaCl and twice with MRS broth. The culture was then diluted 100 times and 0.1 mL of each diluent was placed on MRS agar plates. The plates were incubated at 37°C for 48 h. A control was included with 0 min exposure to ethidium bromide [17]. The survival rate (%) of mutants were calculated and compared with wild strain for bacteriocin activity.

Colony growth restriction

In a dark room, the colony dilutions (0.1 mL) from UV and chemically treated mutants were spread onto MRS media containing 2% triton X-100 as colony restrictor. Untreated colonies were also plated as control. All processes were carried out in strict aseptic conditions in laminar air flow (Dalton, Japan). The plates were covered with aluminum foil and placed in an incubator at 37°C for 48–60 h [18].

Screening of mutants

Selection of mutants was done by colony morphological characterization and catalase test of colony isolates. Further screening was done by plate screening method using well test. To achieve specific selection, the colonies verified on triton X-100 were then exposed to test strain to obtain potential colonies. In this test, 2% agar plates were prepared having 0.3 mm wells. Then colonies were placed in these wells along 0.1 mL of phosphate buffer. After incubation at above mentioned conditions, the colonies forming greater diameter of the zone having higher activity titre in comparison with wild strain and were further studied for the bacteriocin production [19].

Growth curve and time course of bacteriocin production of wild and mutated strains

Five hundred milliliters of culture media was inoculated with 1.5% (v/v) seeded culture of wild and mutated strains of L. acidophilus MS1. The seeded culture was incubated aerobically without agitation at 37°C to monitor bacteriocin production during the time course of 24 h. The aliquots of 10 mL were aseptically taken after each 4 h and centrifuged at 4°C for 10 min at 8000 x g. The growth of the strains was observed by measuring optical density of aliquots at 600 nm during the 24 h period [15].

Bacteriocin activity assay

To achieve activity titre of bacteriocin, the culture supernatant was filtered through cellulose acetate membrane filter (pore size 0.45 μm) and assayed by agar well diffusion method [2]. The nutrient agar plates were aseptically inoculated with 10^5 CFU/mL seeded culture of test strain. The 8 mm diameter wells were punched in hardened agar plates and filled with 200 μL of culture filtrate, and incubated at 37°C for overnight. Similar method was used for measuring bacteriocin activity for mutated isolates. Each bacteriocin suspension was assayed against test strain. The antibacterial activity of bacteriocin was expressed in arbitrary units (AU). According to Usmiati and Marwati [20] and Hata et al.
[21], the activity was determined by the area of inhibition zone (mm²). One arbitrary unit is the unit area of inhibition zone per unit volume (mm²/mL) [20, 21]. The bacteriocin activity was calculated using the following formula:

\[ \text{Bacteriocin activity} = \frac{Lz - Ls}{V} \]

where \( Lz \) is the clear zone area (mm²); \( Ls \) is the well area (mm²); \( V \) is the volume of the sample (mL).

**Inhibition of biofilm producing bacterium**

The effect of bacteriocin on inhibition of bacterial biofilm was assayed for wild and mutated isolates, according to the method of Srdjan et al. [22]. Seventy-five, 50 and 25% (v/v) of bacteriocin suspensions from UV and ethidium bromide mutants of *L. acidophilus* MS1 were added in total 1 mL of nutrient broth in 24 well flat bottomed micro plate and inoculated with 100 μL broth culture of *B. cereus*. Negative control well contained broth and bacterial culture only while positive control contained rifampicin instead of bacteriocin suspension, and incubated aerobically for 24 h at 37°C. After incubation, the contents of each well were rinsed twice with 250 μL of sterile 0.9% NaCl and vigorously shaken in order to remove all non-adherent bacteria. The remaining adherent bacteria were fixed with 200 μL of 99% methanol per well, and after 15 min they were emptied and left to dry. The plate was further stained for 5 min with 0.2 mL of 2% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water and air dried. The dye bound to the adherent cells in well were re-solubilized with 160 μL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 630 nm using ELISA reader. The bacterial growth inhibition (INH%) was calculated by using the formula:

\[ \text{INH} = 100 - \left( \frac{\text{OD}_{630 \text{ sample}}}{\text{OD}_{630 \text{ control}}} \times 100 \right) \]

The experiment was conducted in triplicates [23].

**Bacteriocin stability under different stress conditions**

The bacteriocin activity of cell free supernatant of wild and mutated strains was analyzed after treatment with different enzymes, heat conditions, pH values and various surfactants. Solution of bacteriocin was prepared with 1 mg/mL of following enzymes as: amylase, protease K, trypsin and pepsin (Sigma Aldrich, USA) in different batch of experiments. The homogenates were incubated for 30 min at 30°C and then heated for 5 min at 95–97°C, while the sensitivity of catalase (Sigma Aldrich, USA) was tested for 6 h at 37°C. The untreated partially purified cell free supernatant was used as control. A separate batch of experiments were conducted in which the pH of supernatant was adjusted to 2, 4, 6, 8, 10 and 12 and incubated for 1 h at 30°C. The MRS broth pH ranges from 2 to 12 was used as control. Another batch was conducted to determine the effect of temperature by incubating culture supernatant at 30, 37, 45, 60, 80 and 100°C, for 30 min, and at 121°C for 15 min; the untreated supernatant was used as control. All treated samples were neutralized to pH 6 before measuring bacteriocin titre against test strain [6, 24].

**Bacteriocin purification to homogeneity**

The culture supernatant of bacteriocin was concentrated by 60% salt precipitation using ammonium sulfate at 4°C for 4 h [6]. After an additional centrifugation of 14,000×g at 4°C for 30 min, the collected precipitates were re-suspended in 50 mM phosphate buffer of pH 7. The homogenized purification of bacteriocin was performed by using cation exchange column of carboxy methyl (CM) cellulose. The column was equilibrated with 50 mM sodium acetate buffer of pH 5. The column was washed subsequently with buffers A (50 mM sodium acetate buffer of pH 5, 200 mM NaCl); B (50 mM sodium acetate buffer of pH 5, 400 mM NaCl); C (50 mM sodium acetate buffer of pH 5, 600 mM NaCl); D (50 mM sodium acetate buffer of pH 5, 800 mM NaCl) and E (50 mM sodium acetate buffer of pH 5, 1000 mM NaCl), respectively. Proteins were collected in 1.5 mL fractions with a flow rate of 1 mL/min [14]. After determining bacteriocin activity, all active fractions from ion exchange chromatography containing bacteriocin peptide were pooled together and loaded on Sephadex G-200 column, equilibrated with 30 mM phosphate buffer of pH 7.5 [25]. The eluted fractions were collected at a flow rate of 0.5 mL/min and again passed through bacteriocin activity assay.

**SDS-PAGE**

Tris-glycine sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) was done for molecular mass estimation of bacteriocin. Twelve percent resolving gel and 5% stacking gels were prepared. An 8 μL aliquot of 2X protein loading buffer was mixed with 8 μL of bacteriocin sample and heated at 90°C in water bath for 2 min. Electrophoresis was run at constant voltage of 135 V. A molecular protein standard of SeeBlue® Plus2 (3–188 kDa) was
run along crude extract, ion exchanged and gel filtrated samples of bacteriocin [26].

Results

Characteristics of isolated strain

The isolated strain was determined as catalase negative, Gram positive rods and therefore morphologically classified in the genus, *Lactobacillus*. The biochemical characterization was done by observing carbohydrate fermentation profile using API 50 CHL system (Bio Merieux, France) and found to have 99.9% similar profile index with reported *L. acidophilus* in apiweb data bases (V5.1). Moreover, the sequenced 16S rRNA gene also has close similarity with reported *L. acidophilus* and the sequence was submitted in NCBI (GenBank: KP987308). The microscopic and physiological characteristics of isolated strains are given in Table 1.

Strain improvement by mutagenesis

The locally identified *L. acidophilus* MS1 was subjected to strain improvement by UV radiations and ethidium bromide to achieve resistant strain and to maximize bacteriocin activity. The selected UV mutants were coded as UV<sub>0</sub>–UV<sub>10</sub> and ethidium bromide mutants as EB<sub>0</sub>–EB<sub>10</sub>. The resulted mutants were tested for their bacteriocin activity, as described earlier. The control was the wild strain of *L. acidophilus* with no exposure to UV radiations and ethidium bromide and with 100% survival rate. The activity results indicated that among UV mutants, UV<sub>6</sub> exhibited high bacteriocin titre (3400 AU) at 30 min exposure to UV radiations with 42% survival rate. Further exposure to UV radiations decreased activity of bacteriocin with decreasing survival rate. While, in ethidium bromide exposure, EB<sub>5</sub> was considered as highest bacteriocin producing mutant (4020 AU) with 28% survival rate at 150 min of exposure (Figure 1). The resulted mutant strains have an effective response in enhanced bacteriocin production by enhancing appropriate exposure to mutagens. The physical mutant UV<sub>6</sub> and chemical mutant EB<sub>5</sub> were considered

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Table 1: Microscopic and physiological characteristics of isolated bacteria.

| Characteristics of isolated bacteria | Interpretations |
|-------------------------------------|-----------------|
| Catalase test                       | –               |
| Gram staining                       | +               |
| Lactose fermentation test           | +               |
| Gas production                      | –               |
| Bacterial growth at: 10°C           | –               |
| Bacterial growth at: 15°C           | –               |
| Bacterial growth at: 37°C           | +               |
| Bacterial growth at: 45°C           | +               |
| Bacterial growth at: pH 4.5          | +               |
| Bacterial growth at: pH 6.5          | +               |
| Bacterial growth in the presence of 2% NaCl | + |
| Bacterial growth in the presence of 3% NaCl | – |
| Bacterial growth in the presence of 4% NaCl | – |
| Bacterial growth in the presence of 6.5% NaCl | – |

Positive test (+), negative test (–).

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Figure 1: Effect of mutation on bacteriocin activity relative to survival rate of *L. acidophilus*. (A) Exposure to UV revealed 3400 AU bacteriocin activity with 42% survival rate at 30 min (UV<sub>6</sub>). (B) Exposure to ethidium bromide exposed bacteriocin activity of 4020 AU with 28% survival rate at 150 min (EB<sub>5</sub>).
as most resistant mutants of *L. acidophilus* with highest bacteriocin production. The more resistant mutant strains revealed a dramatic increase in bacteriocin activity. Moreover, overdose of both mutants employed an adverse effect on activity titre and survival rate.

**Plate screening method**

All the isolated colonies of mutants having creamy gray colonies were found to be Gram positive rods with catalase negative activity. The plate screening for both UV and chemical mutants UV<sub>0</sub>–UV<sub>10</sub> and EB<sub>0</sub>–EB<sub>10</sub>, respectively, the mutated colonies having greater zone of inhibition against test strain as compared to other mutants were considered as more resistant mutants with higher activity titre. The mutated colonies of UV<sub>6</sub> and EB<sub>5</sub> were considered as more potent mutants and therefore selected for further studies.

**Growth characteristics and bacteriocin production**

After 4 h of incubation, the production of bacteriocin started increasing in both wild and mutated strains. The maximum bacteriocin titre of 2800 AU was observed for wild strain at 2.5 OD and 16 h of growth against test strain. Moreover its activity titre declined slowly with the passage of time, as shown in Figure 2A. The growth characteristics of mutants vary from wild strain. The UV<sub>6</sub> physical mutant has maximum activity titre of 3500 AU at 0.75 OD and 14 h time period, whereas the EB<sub>5</sub> chemical mutant has maximum activity titre of 4200 AU at 0.5 OD and 12 h incubation period. But both have rapid decline in activity as incubation time increases, as shown in Figure 2B. The results revealed that the chemical mutant is more potent and express high activity titre even at less survival rate, and the physical mutant express less activity titre at high survival rate, as described above.

**Biofilm inhibition assay**

The physical mutant UV<sub>6</sub> and chemical mutant EB<sub>5</sub> were considered as most effective upon enhanced bacteriocin production. Therefore, only these mutants were selected for antibiofilm inhibition assay. A 75% (v/v), 50% (v/v) and 25% (v/v) of bacteriocin suspension was used in comparison with negative control without bacteriocin suspension and positive control with rifampicin. There is a systemic increase in biofilm inhibition by these mutants. That is the 75% v/v treatment has highest inhibition activity as compared to 50% v/v and 25% v/v. The bacterial growth inhibition (INH%) was calculated by measuring OD at 630 nm in flat bottomed micro plate, given in Table 2.

**Biochemical characterization**

The activity of bacteriocin of both wild and mutated strains was destroyed after treating with proteolytic enzymes which confirms that the resultant bacteriocin was protein in nature. Moreover, its activity was not affected by amylase, lipase and catalase enzymes.

The bacteriocin activity of wild strain remains unchanged at different pH but slightly disturbed at a pH <4 and >8. Similarly, heat stress to culture supernatant of wild strain did not alter the activity of bacteriocin up to 100°C for 30 min but a slight decrease was observed.
Table 2: Biofilm inhibition by bacteriocin.

| Treatment       | INH% by UV mutant | INH% by ethidium bromide mutant |
|-----------------|-------------------|---------------------------------|
| 25% v/v         | 45 ± 0.7          | 58 ± 0.5                        |
| 50% v/v         | 68 ± 0.3          | 73 ± 0.7                        |
| 75% v/v         | 80 ± 0.8          | 90 ± 0.6                        |
| Positive control|                   | 94 ± 0.2                        |
| Negative control|                   | 0                               |

INH%, bacterial growth inhibition; positive control, rifampicin; negative control, bacterial growth inhibition in the absence of bacteriocin. Data are presented as mean ± SD (n = 3); the mean difference is statistically significant at 95% confidence level (p ≤ 0.001).

Table 3: Biochemical characterization of bacteriocin.

| Treatment       | Activity titre (AU) | Activity titre of UV mutant (AU) | Activity titre of EB5 mutant (AU) | Heat (°C) |
|-----------------|---------------------|----------------------------------|----------------------------------|-----------|
| Enzymes         |                     |                                  |                                  |           |
| Amylase         | 2900 ± 1.2          | 3600 ± 0.6                       | 4100 ± 0.8                       | 37, 30 min| 2900 ± 1.6 | 3600 ± 2.9 | 4100 ± 1.3 |
| Lipase          | 2900 ± 0.8          | 3600 ± 1.4                       | 4100 ± 1.3                       | 60, 30 min| 2900 ± 2.7 | 3600 ± 2.4 | 4100 ± 1.5 |
| Catalase        | 2900 ± 0.6          | 3600 ± 0.7                       | 4100 ± 1.9                       | 80, 30 min| 2900 ± 1.5 | 3600 ± 0.8 | 4100 ± 2.4 |
| Protease K      | 0                   | 0                                | 0                                | 90, 30 min| 2878 ± 0.8 | 3250 ± 1.3 | 3940 ± 2.9 |
| Trypsin         | 0                   | 0                                | 0                                | 100, 30 min| 2797 ± 2.5 | 3000 ± 2.5 | 3500 ± 3.1 |
| Pepsin          | 0                   | 0                                | 0                                | 121, 30 min| 2638 ± 2.9 | 2960 ± 1.7 | 3250 ± 1.4 |
| pH              | 2                   | 1480 ± 1.7                       | 2040 ± 0.7                       | 2560 ± 1.5| 2500 ± 0.9 | 3000 ± 0.6 |
| 4               | 2862 ± 0.6          | 2500 ± 0.9                       | 2800 ± 1.8                       | 2258 ± 1.7| 2220 ± 1.2 | 2500 ± 1.7 |
| 6               | 2900 ± 0.8          | 3200 ± 2.1                       | 3500 ± 2.4                       | 2638 ± 2.9| 2960 ± 1.7 | 3250 ± 1.4 |
| 8               | 2797 ± 0.5          | 3000 ± 1.6                       | 3300 ± 1.4                       | 121, 30 min| 2638 ± 2.9 | 2960 ± 1.7 | 3250 ± 1.4 |
| 10              | 2185 ± 0.9          | 2400 ± 2.5                       | 2600 ± 2.2                       | 121, 30 min| 2638 ± 2.9 | 2960 ± 1.7 | 3250 ± 1.4 |
| 12              | 1246 ± 1.5          | 2200 ± 2.1                       | 2300 ± 2.0                       | 121, 30 min| 2638 ± 2.9 | 2960 ± 1.7 | 3250 ± 1.4 |

Control: *The untreated bacteriocin sample from wild strain having 2900 AU activity titre. †The untreated bacteriocin sample of UV mutant having 3600 AU activity titre. ‡The untreated bacteriocin sample of EB5 mutant having 4100 AU activity titre. Data are presented as mean ± SD (n = 3); the mean difference is statistically significant at 95% confidence level (p ≤ 0.001).

Purification of bacteriocin

The bacteriocin was purified by three step purification protocol to apparent homogeneity. After first step of salt precipitation, the bacteriocin was concentrated to 4404 AU. In the subsequent purification step, the cation exchanger (CM cellulose) yielded maximum activity in a range of 0.4–0.5 M NaCl concentration. The active fractions yielded 1.6% bacteriocin with 15.9 purification fold from culture supernatant, and considered for further purification. Meanwhile, this purification step resulted in a greatest loss of bacteriocin activity. In the last step, the gel filtration column (Sephadex G-200) resulted in purification.

Table 4: Purification folds and recovery values of bacteriocin during purification steps.

| Purification fractions | Volume (mL) | Protein concentration (mg/mL) | Activity titre (AU) | Activity titre of UV mutant (AU) | Activity titre of EB5 mutant (AU) | Total activity (AU/mg) | Specific activity (AU/mg) | Purification fold: specific activity of subsequent step/specific activity of crude preparation | Yield (%): total activity of subsequent step × 100/total activity of crude preparation |
|------------------------|-------------|------------------------------|---------------------|----------------------------------|----------------------------------|------------------------|--------------------------|---------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| Culture supernatant    | 500         | 11.6                         | 2799                | 1,399,530                        | 214.3                            | 1                      | 100                      | 100                                                             | 100                                                               |
| a. Salt precipitation  | 100         | 4.4                          | 4404                | 440,425                          | 1000.9                           | 4.7                    | 31.5                     | 100                                                             | 100                                                               |
| b. CM cellulose (cation exchanger) | 6       | 1.1                          | 3741                | 22,448.5                         | 3401.3                           | 15.9                   | 1.6                      | 100                                                             | 100                                                               |
| c. Sephadex G-200 (Gel filtration) | 3        | 0.44                         | 4805                | 14,413.8                         | 10,919.6                         | 51                     | 1.1                      | 100                                                             | 100                                                               |

*Volume, †protein concentration, ‡purification fold. The concentration of protein was calculated at 595 nm absorbance using Bradford reagent. Arbitrary unit (AU): a unit area of inhibition zone per unit volume (mm²/mL); Specific activity (AU/mg): total activity of the subsequent purification step/total protein of the same step; yield (%): total activity of subsequent step × 100/total activity of crude preparation; purification fold: specific activity of subsequent step/specific activity of crude preparation.
The strain improvement strategies, including UV irradiation and ethidium bromide, resulted a considerable mutation leading to enhanced or reduced growth and the production of bacteriocin. In present study, ethidium bromide treated mutant displayed high bacteriocin production as compared to UV irradiations and considered as potent mutagen. Ultraviolet irradiation is the most common physical mutagen. Although these radiations are considered as weak but they can alter DNA structure by breaking its strand; intra-intermolecular cross linking in nucleic acids and proteins; cytosine and uracil hydration; formation of pyrimidine dimmers [37, 38]. Irradiation induced mutations mainly affects thymine dimmers altering the biological activity of DNA and ultimately proteins, leading to enhanced or reduced growth and the production of bacteriocin. In this study a bacteriocin of 6.5 kDa by both wild and mutated strain of *L. acidophilus* MS1 has been isolated and biochemically characterized. The treatment of bacteriocins with proteolytic enzymes completely destroyed their activity and confirmed that the resultant peptide was protein in nature. Its further treatment with amylase and lipase suggested that the bacteriocin activity is independent of glycosylation and the resulted antimicrobial activity was not related to lipid moieties. In addition, its activity was also not affected by catalase which demonstrated that the inhibition activity was not due to the presence of H$_2$O$_2$, and catalase excluded the effect of H$_2$O$_2$. Similar finding of 6.6 kDa and 6.5 kDa has been reported for acidocin D20079 and acidophilin 801, bacteriocin produced by *L. acidophilus* IBB801 and *L. acidophilus* DSM 20079, respectively [14, 32].

The resultant bacteriocin was found to be stable at a wide range of temperature and pH.

The heat stability of bacteriocin may result due to formation of small globular structures, the occurrence of strongly hydrophobic regions and high glycine contents or due to stable cross linkages [33]. In addition, heat stability as a foremost characteristic of low molecular mass bacteriocins, may arise from complex pattern of disulfide intramolecular bonds which stabilize secondary structures by reducing the number of possible unfolded structures [14, 34]. These characteristics conflict a significant preservative role of such bacteriocins in food industries as many processes in food preservation involve heating steps [35]. Similarly, the pH stability seems to be relatively common for strains of *L. acidophilus*. As previously reported for bacteriocin acidocin A by *L. acidophilus* TK9201 and acidocin D20079 by *L. acidophilus* DSM 20079 [14, 36]. These biochemical characterizations revealed that the resultant bacteriocin was a stable peptide. Moreover, the heat and pH stability increases its value to be used in various industrial sectors working for the benefits of mankind.

**Discussion**

*Lactobacillus acidophilus* has been anciently used in food processing as bio-preservative along with other beneficial LAB namely; *L. gasseri, L. delbrueckii* ssp. *bulgaricus, L. plantarum* and *S. thermophilus* [27, 28]. Various metabolites, including bacteriocins, are being used in various sectors like food, forage and pharmacy against harmful pathogens [29, 30].

Currently, one of the major issues regarding food processing, preservation and storage, is the biofilm formation of food borne bacteria. Biofilm is a causative pollutant of food industry sectors including dairy, red meat and poultry processing. Being biofilm forming microbe, *Bacillus cereus* causes food borne illness such as diarrheal or emetic syndromes [31]. The current study was planned to evaluate the effect of resultant antibacterial peptides against biofilm forming food borne pathogen. Moreover, our goal was to achieve an improved strain of *L. acidophilus* for enhanced production of bacteriocin against biofilms of *B. cereus*.
and causing bacterial mutation [39]. Similarly, ethidium bromide has been considered as replication inhibitor because it interferes with both DNA and RNA in several organisms [40] and also causes frameshift mutations in bacteria [41]. In addition, a long exposure to UV radiations and ethidium bromide may cause a loss of DNA fragments which can induce cell death and may cause cross linked DNA unopened or prevent DNA replication and transcription. Moreover, death rate of bacterium depends in a directly proportional way on mutagen amount. Indeed, survival cells may have fewer positive mutants and more negative mutants; and only few positive mutants are considered as high efficiency strains [42].

There is a need to conduct more research for enhanced production of such peptides as food preservatives in vegetables, canned foods, meat and beverages to combat against spoilage bacteria. Its applications in pharmacy can provide a good alternative to antibiotics and synthetic chemical compounds. We are further working to characterize the type and structure of bacteriocin and a comparative study of full amino acid sequence of both wild and mutated strains is underway.

Conclusions

The present study highlighted the presence of *L. acidophilus* in mozzarella cheese micro biota and effect of mutagenesis on its bacteriocin production. A highly resistant mutant of *L. acidophilus* MS1 displayed an increased bacteriocin production potential as compared to wild strain. Moreover, ethidium bromide was proved as a more effective mutagen as compared to ultraviolet radiations.

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