

Characterization of Growth and Bacteriocin Production by *Lactococcus lactis* sub sp. *lactis* R10 Isolated from Fermented Radish

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In the present study, the kinetics of growth and bacteriocin production by a Lactic acid bacterial (LAB) strain obtained from fermented radish was studied. The growth of the strain showing a short lag phase of 2 h and steady increase up to 24 h, attained maximum growth at stationary phase. The LAB strain produced maximum bacteriocin activity of 644.00 AU ml⁻¹ at stationary phase of growth. The bacteriocin production of the strain under varied physiological conditions, such as pH, temperature and NaCl were evaluated. The maximum growth and bacteriocin production was observed at 6.5 pH and 37°C. Slight improvement in bacteriocin production was noted, when MRS broth was supplemented with 2% NaCl. The potential bacteriocin producing LAB strain was identified as *Lactococcus lactis* subsp. *lactis* R10 using 16S rRNA gene sequence.

Key words: Bacteriocin, Physiological characterization, *Lactococcus lactis* subsp. *lactis* R10.

Lactic acid bacteria (LAB) are a group of Gram positive, non spore forming, cocci or rod shaped, catalase negative and fastidious organisms, considered as Generally Recognized as Safe (GRAS) organisms. Mankind has exploited these bacteria for thousands of years for the production of fermented foods because of their ability to produce desirable changes in taste, flavour and texture¹. The antimicrobial activity of LAB may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins, of which bacteriocins are the most promising as they can be used as natural food preservation.

Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptide or peptide complexes which have a bactericidal or bacteriostatic effect on other species. Bacteriocins from food-isolated LAB are antimicrobial agents against many Gram-positive (e.g., *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Clostridium perfringens*) and Gram-negative (e.g., *Pseudomonas aureginosa*, *Escherichia coli*, *Salmonella* sp., *Shigella* sp.) bacteria associated with food-borne illnesses and food spoilage². Moreover, bacteriocins retain their properties after heat treatment. They are also degraded by the proteolytic enzymes of the gastrointestinal tract and seem to be nontoxic and non-antigenic to animals and humans³. For these reasons, bacteriocins have received much attention for use as natural or so-called 'biopreservatives' in food⁴. This preservation potential could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as food additive.

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Nisin is the only bacteriocin approved for food applications being considered to be safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969. Nisin is produced and is used as a food additive in at least 48 countries, particularly in processed cheese, dairy products and canned foods⁵. As a result, the field has developed increasingly, resulting in the discovery and detailed characterization of a great number of bacteriocins from LAB in the last few decades⁶. Bacteriocin producing species have now been identified among all the genera that comprise the LAB including *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Carnobacterium* as well as several *Enterococcus* species.

Exports of fruits and vegetables from the country have risen by more than 72% while fresh vegetables and processed fruits and vegetables have increased by 50% in 2009-10 as compared to the previous year (www.apeda.gov.in). The health benefits of processed fruits and vegetables without addition of chemical preservatives are becoming more attractive. The physiological and molecular characterization of bacteriocinogenic LAB strain was carried out.

MATERIALS AND METHODS

Bacterial strains, culture media and Growth conditions

The bacteriocinogenic LAB strain was isolated from fermented radish (*Raphanus sativus*). The strain was propagated in de Man Rogosa Sharpe (MRS) broth⁷ at 35°C. *Bacillus subtilis* was used as a test organism and grown in Nutrient agar media at 30°C.

Bacteriocin assay

Bacteriocin activity was determined by using agar well diffusion assay⁸. The supernatant of 36 h grown culture (from MRS broth) was centrifuged at 12,000 x g for 15 min at 4°C, then supernatant was neutralized with sterile 5M NaOH. Aliquots (50 µl) of culture supernatant was applied to disks on agar plates previously inoculated with a cell suspension of *B. subtilis* (10⁶ cfu ml⁻¹) and plates were incubated for 24 to 48 h and the diameter of inhibition zone around the wells was measured with a calliper. Bacteriocin activity was expressed

as arbitrary units (AU ml⁻¹) and defined as the reciprocal of the highest two fold serial dilution showing a distinct zone of inhibition.

Bacteriocin activity (AU ml⁻¹) = 2ⁿ x 100
n- Highest dilution showing growth inhibition zone

Kinetics of growth, acidification and bacteriocin production by LAB strain

The kinetics of growth and bacteriocin production were carried out in a series of 250 ml Erlenmeyer flask containing 100 ml MRS broth at pH 6.5 were inoculated with 1 ml (10⁷ cfu ml⁻¹) of LAB strain and incubated statically at 35°C. At different incubation periods, cultures were examined for changes in viable cell count (log cfu ml⁻¹), pH and bacteriocin activity (AU ml⁻¹) against *Bacillus subtilis*.

Effect of physiological parameters on growth and bacteriocin production of LAB strain

To determine the effect of initial medium pH (4.5, 6.5 and 7.5), incubation temperature (15°C, 37°C and 45°C) and NaCl (2.0%, 4.0% and 6.5% (w/v) on stationary phase grown cells (36 h) of LAB strain was inoculated into 100 ml MRS broth. Initial cell density was ca. 7.0 cfu ml⁻¹ was determined by adjusting optical density (OD) value to 0.4 (@ 600 nm). Final growth (log cfu ml⁻¹), acidification and bacteriocin activity (AU ml⁻¹) against *B. subtilis* were determined.

Molecular identification of potential bacteriocinogenic LAB strain

Genomic DNA isolation from LAB strain

LAB strain (LABR 10) was grown in MRS broth for 12-16 h at 37°C. DNA isolation was followed as per the standard protocol of hexadecyltrimethyl ammonium bromide (CTAB) method⁹ with slight modifications.

PCR amplification of 16S rRNA gene

Extracted genomic DNA from LAB strain was used for direct amplification of 16S rRNA gene portions. Universal primers were used to amplify the full length 16S rRNA gene from ribosomal RNA (rrn) operon. The universal primers were used for 16S rDNA amplification had the following sequence: The forward primer FD1 (5' AGA GTT TGA TCC TGG CTC AG-3') and reverse primer RP2 (5' ACG GCT ACC TTG TTA CGA CTT-3') were used for amplification. PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany)

using conditions: initial denaturation at 95°C for 1 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 5 min.

The amplification product was analysed by electrophoresis in 1.0 % (w/v) agarose gels electrophoresis with 1µg ml⁻¹ ethidium bromide and visualized with Alpha imager TM1200 documentation and analysis system. The 16S rRNA amplicon in the gel was excised and purified from agarose using spin columns (Bangalore Genei, India) according to manufacturer's instructions.

DNA sequencing

The nucleotide sequence was determined by sequencing reactions performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer by Chromous Biotech Company (Bangalore). The sequence comparison was done using BLAST program by performing a similarity search against the GenBank database (website: <http://www.ncbi.nih.gov/BLAST>) for species level identification. A phylogenetic tree was constructed by neighbour-joining method of Saitou and Nei¹⁰ using MEGA 5.0 and the tree file was analyzed using tree view¹¹.

RESULTS AND DISCUSSION

The kinetic of growth and bacteriocin production by LAB strain with initial medium pH of 6.5 at 35°C was determined (Fig.1). The isolate showed a short lag phase of 2 h and steady increase

up to 24 h, attained maximum growth at stationary phase. The production of bacteriocin started at 4 h, during onset of the exponential phase showing its primary metabolite kinetics. The bacteriocin production was increased from 52.00 AU ml⁻¹ to 570.33 AU ml⁻¹ during exponential growth phase and reaches maximum bacteriocin production (644.00 AU ml⁻¹) at the end of stationary phase of growth (36 h). This result was supported by Todorov and Dicks¹² who recorded a detectable level of bacteriocin after 3 and 5 h of growth. A similar trend of maximum bacteriocin accumulation during the stationary phase of growth has previously been reported¹³. The data also implies that production of bacteriocin is associated with growth. Most authors have noted that good cell growth frequently goes hand in hand with bacteriocin production¹⁴⁻¹⁵. High correlation between biomass production and bacteriocin biosynthesis were reported for *Brevibacterium linens*¹⁶ and *L. lactis*¹⁷.

After 36 h, reduction in bacteriocin activity was observed. This loss in bacteriocin activity may be due to proteolytic degradation, protein aggregation, adsorption to cell surfaces, and feedback regulation¹⁸. Zamfir et al¹⁹ also related the reduction of cell growth and bacteriocin production of *L. acidophilus* IBB 801 to the lactic acid accumulation and hence low pH or an exhausted energy source at the end of fermentation. Wolf-Hall et al²⁰ also claimed that the shortage of certain nutrients critical for bacteriocin production could be responsible for this phenomenon during the latter stages of fermentation.

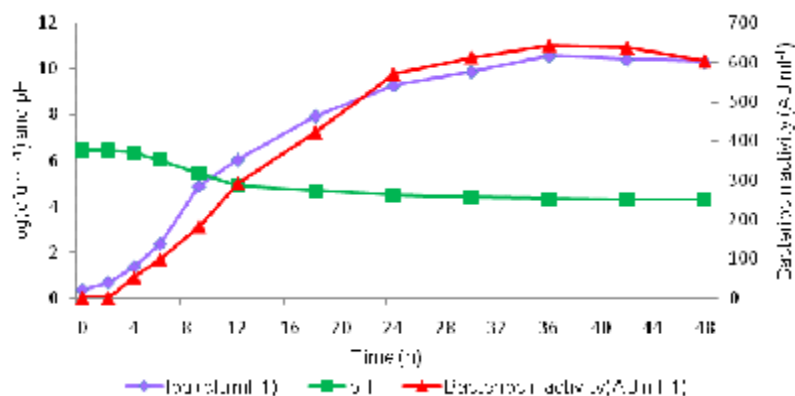
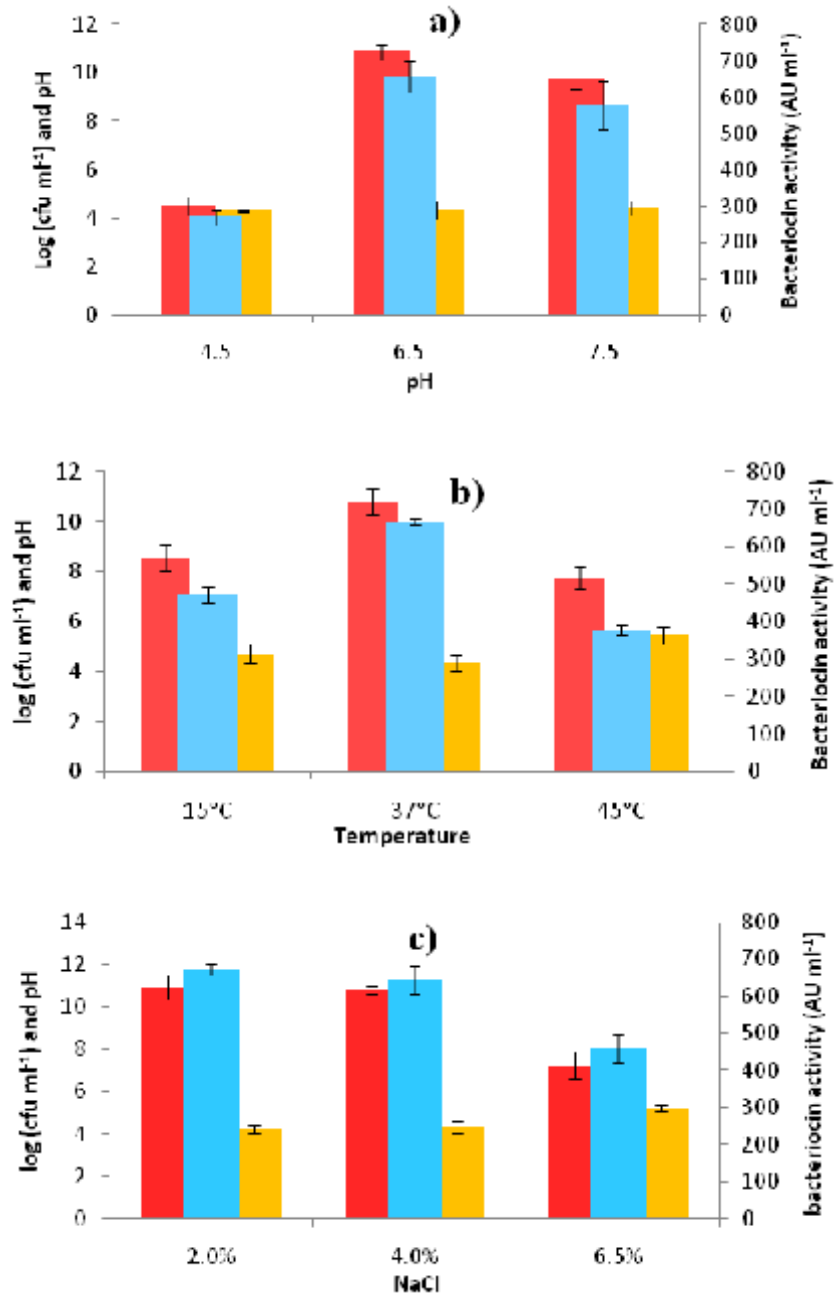


Fig. 1. Kinetics of growth and bacteriocin production by LAB strain in MRS broth at 35°C

The pH of the growth medium decreased from an initial pH of 6.6 to 4.5, indicating the production of lactic acid. Earlier reports by Yang and Ray²¹ revealed the need for a low final pH for

an efficient post-translational processing of both prebacteriocins to produce active bacteriocins. van Niel and Hahn-Hagerdal²² also pointed out that higher pH drops enhance both nisin and pediocin



RED COLOUR- LOG (cfu ml⁻¹), BLUE COLOUR - pH, YELLOW COLOUR - BACTERIOCIN ACTIVITY (AU ML⁻¹)

Fig. 2. Effect of pH (a), cultivation temperature (b) and NaCl (c) on pH, growth and bacteriocin production by LAB strain grown for 36 h. Data are means of three replicates and error bars represent the standard deviation of the mean

production until a final pH inappropriate for survivability and cell growth of *L. lactis* and *Pediococcus acidilactici* was reached. From these observations, it can be pointed out that the increase in the acidification rate of the MRS medium enhances the bacteriocin production, before a final pH value unsuitable for cell growth of LAB strain.

Maximum bacteriocin activity (658.00 AU ml⁻¹) and maximum growth (10.89 log cfu ml⁻¹) was recorded in MRS broth with an initial pH of 6.5 (Fig.2.a). Reduction in the bacteriocin production approximately 12.15% (578.00 AU ml⁻¹) and 58.86% (270.67 AU ml⁻¹) were recorded in the same medium when adjusted to an initial pH of 7.5 and 4.5 respectively. Lower growth was recorded for bacteriocinogenic LAB strain at pH 7.5 and 4.5, corresponding to a lower production of bacteriocin. This result demonstrates that the initial pH of the medium plays an important role in the production of bacteriocin. Thus, bacteriocin production was stimulated at an initial medium pH of 6.5.

It was reported that optimal bacteriocin production by *L. lactis* subsp. *lactis* occurred at pH 6.0-7.0 in MRS and M17 broth at 30°C²³. The dependence of bacteriocin production on pH suggested that the expression of the biosynthetic genes may be regulated by pH, as has been observed previously for several classes of genes²⁴. Similar results were reported for nisin production by *L. lactis* subsp. *lactis*²⁵. In contrast to the results, Mitra et al²⁶ observed a distinct behavior for *L. lactis* CM1 and claimed that under pH 11.0

the activity of the nisin production was more significant than the initial pH of 6.5. Moreover, the antilisterial activity of *E. faecium* RZS C5 was lower at pH ranging from 7.5 to 8.0 than pH in the range 5.5-6.5²⁷. Low levels of bacteriocin activity with pH of broth under 5.0 were also observed for bacteriocin from *L. plantarum*²⁸.

The influence of different temperatures on growth and bacteriocin production was investigated using MRS broth at an initial pH of 6.5 (Fig.2.b). Maximum activity (664.53 AU ml⁻¹) of bacteriocin and growth (10.80 log cfu ml⁻¹) obtained at 37°C was greater than that at other temperatures. Significant reduction in the growth and bacteriocin production of LAB strain was noticed at a low temperature of 15°C as 8.55 log cfu ml⁻¹ and 471.34 AU ml⁻¹ respectively and also at a high temperature of 45°C as 7.73 log cfu ml⁻¹ and 378.00 AU ml⁻¹. According to the results, the growth and bacteriocin production are correlated, as observed for lacticin A, enterocin 1146, lactocin S and nisin Z²⁹. Previous results demonstrated that the maximum growth and bacteriocin production of *L. lactis* subsp. *lactis*³⁰ and *Lactobacillus salivarius*³¹ was obtained at 37°C.

Effect of NaCl on growth and bacteriocin production of LAB strain has been studied in order to evaluate the food aptitude of our strain for future biopreservation strategies. The highest bacteriocin activity (672.00 AU ml⁻¹) was seen in bacterial cell growth in MRS broth supplemented with 2.0% NaCl and highest pH decrease was also recorded (4.23)

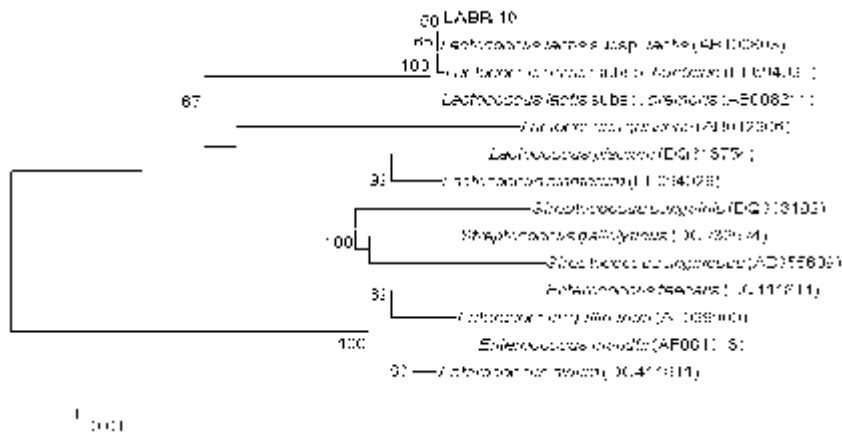


Fig. 3. Phylogenetic tree showing the relative positions of bacteriocinogenic LAB isolate LABR 10 as inferred by the neighbour-joining method of complete 16S rRNA gene sequences. Bootstrap values for 500 replicates are shown at the nodes of the tree. The bar indicates 1% sequence divergence.

at this concentration, however 2.0% NaCl produced 4.2% and 68.75% more bacteriocin than 4.0% and 6.5% NaCl respectively (Fig.2.C). Lower growth was recorded at 6.5% NaCl corresponding to a lower production of bacteriocin. Thus, bacteriocin production was slightly stimulated at a 2.0% NaCl. Earlier reports by Kayalvizhi and Gunasekaran³² reported that salt concentration plays an important role in the release of bacteriocin from bacteriocin producing strains during the growth by influencing absorption of the bacteriocins to the cell envelope. In contrast to our results, Rajaram et al³³ noticed that highest bacteriocin production from *L. lactis* was at 1.5% NaCl concentration. *S. phocae* PI80³⁴ and *E. faecium* MC13³⁵ produced higher amount bacteriocin and viable cells in the presence of low concentration of NaCl from 1 to 1.5%.

To identify the bacteriocinogenic LAB strain (LABR 10), the full length DNA amplification and sequencing of 16S rRNA gene was performed. The gene sequences of LABR 10 (1430 bp) and their similarity search revealed 100% sequence homology with reported *Lactococcus lactis* subsp. *lactis* (AB775185). It is known that sequence similarity $\approx 97\%$ is acceptable level for microbial identification and the microbial strain shall be considered as same species³⁶. The isolated LAB strain was also identified as *Lactococcus lactis* subsp. *lactis* in phylogenetic tree analysis (Fig.3). The phylogenetic tree based on the 16S rDNA sequence displayed high consistency regarding the relationships between the organisms included. All the nodes leading to LABR 10 clusters are supported by high bootstrap values and should be considered significant. Further, based on microscopic, biochemical and 16S rRNA gene sequence LAB strain was named as *Lactococcus lactis* subsp. *lactis* R10. Previously Yildirim and Johnson³⁷ isolated *L. lactis* subsp. *cremoris* R from radish and their bacteriocin was designated as lactococcin R.

CONCLUSION

Bacteriocinogenic LAB strain was previously isolated from fermented radish. The kinetic of growth and bacteriocin production by LAB strain shows that maximum growth and bacteriocin production occur at stationary phase of growth. The conditions for maximum growth

and bacteriocin production of LAB strain was evaluated in MRS as growth medium, pH 6.5 and incubation temperature 37°C. NaCl was slightly stimulating the bacteriocin production at 2.0% concentration. The potential bacteriocin producing LAB strain was identified by 16S rRNA sequencing and blast homology search as *L. lactis* subsp. *lactis*. In future bacteriocin producing *L. lactis* subsp. *lactis* R10 can be used as starter culture for vegetable fermentation.

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